Orchard and bin treatment with entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) for the control of the codling moth (*Cydia pomonella*)

by
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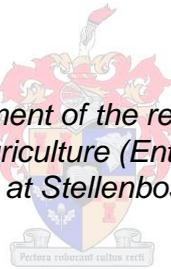
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Declaration

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Aan mamma en pappa

IF

If you can keep your head when all about you

Are losing theirs and blaming it on you,

If you can trust yourself when all men doubt you,

But make allowance for their doubting too;

If you can wait and not be tired by waiting,

Or being lied about, don't deal in lies,

Or being hated, don't give way to hating,

And yet don't look too good, nor talk too wise:

If you can make one heap of all your winnings

And risk it on one turn of pitch-and-toss,

And lose, and start again at your beginnings

And never breathe a word about your loss;

If you can force your heart and nerve and sinew

To serve your turn long after they are gone,

And so hold on when there is nothing in you

Except the Will which says to them: 'Hold on!'

If you can dream—and not make dreams your master;

If you can think—and not make thoughts your aim;

If you can meet with Triumph and Disaster

And treat those two impostors just the same;

If you can bear to hear the truth you've spoken

Twisted by knaves to make a trap for fools,

Or watch the things you gave your life to, broken,

And stoop and build 'em up with worn-out tools:

If you can talk with crowds and keep your virtue,

Or walk with Kings—nor lose the common touch,

If neither foes nor loving friends can hurt you,

If all men count with you, but none too much;

If you can fill the unforgiving minute

With sixty seconds' worth of distance run,

Yours is the Earth and everything that's in it,

And—which is more—you'll be a Man, my son!

BY RUDYARD KIPLING

Abstract

The codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), is the key pest of apples and pears worldwide. The withdrawal of certain fundamental chemicals from codling moth management spray programmes, due to concerns about human, environmental and ecosystem health, has resulted in the search for softer, more environmentally friendly, and safer control measures. Entomopathogenic nematodes (EPNs), naturally occur in the soil, and actively search for hosts. The interest in using EPNs from the families Heterorhabditidae and Steinernematidae as a control measure was sparked in 1953, when an EPN was discovered in an insect. The aim is to incorporate EPNs in an integrated pest management (IPM) programme, to ensure minimal residue and eventually residue-free pome fruit production in South Africa. In order to ensure EPN success, both the environmental and technical factors influencing their efficacy, were investigated in this study.

The biocontrol potential of three imported EPN isolates, being *Steinernema feltiae* and two isolates of *Heterorhabditis bacteriophora* (Hb1, Hb2), as well as a local isolate, *Steinernema yirgalemense*, were evaluated for the control of the codling moth under local conditions. All concentrations of *S. yirgalemense,* applied by immersion in a suspension of nematodes, gave > 98% control. The two formulated isolates of *H. bacteriophora*, Hb1-f and Hb2-f, gave < 30% control. When using the same nematode isolates, produced *in vivo*, *S. yirgalemense* still resulted in a higher codling moth control of > 90%, compared to 54% and 31% control of the *H. bacteriophora* Hb1 and Hb2 isolates, respectively. In follow up field trials, *S. feltiae* resulted in ≥ 80% control, and was thus more effective than both *S. yirgalemense* and the *H. bacteriophora* (Hb1) isolates, with 66% and 24%, and 24% and 9% control, for two separate trials, respectively. To validate the data obtained from the field trials, subsequent laboratory bioassays were conducted evaluating temperature regimes, following the same cycle as under natural conditions, with a constant humidity of 100%. *Steinernema feltiae* proved to be most effective, causing > 90% mortality, followed by *S. yirgalemense*, with 78% mortality. The two *H. bacteriophora* isolates (Hb1, Hb2) under the above-mentioned laboratory conditions, resulted in 73% and 59% control, respectively. Humidity thus seems to be the most important factor affecting EPN efficacy during above-ground applications. *Steinernema feltiae* proved to be a better candidate than *S. yirgalemense* for the control of the codling moth.

The efficacy of different EPN isolates in controlling diapausing codling moth larvae at different temperatures was also evaluated, under local conditions, using spray application. *Steinernema feltiae* and two isolates of *H. bacteriophora* Hb1 and Hb2, including two local isolates, *S. yirgalemense* and *Steinernema jeffreyense*, were evaluated. The use of *S. jeffreyense* resulted in the most effective control, with 67% mortality, followed by *H. bacteriophora* (Hb1) with 42%, and then by *S. yirgalemense* with 41%. Laboratory bioassays simulating field conditions revealed that *S. feltiae* was most virulent to codling moth larvae, with 67% mortality by infection, followed by *S. yirgalemense* with 58%, the *H. bacteriophora* strain Hb1 with 48%, and the Hb2 strain with 24%. A comparison of the infection and penetration rate of two isolates of *H. bacteriophora* (Hb1, Hb2), *S. feltiae and S. yirgalemense*, which was carried out in multiwell plates at 14°C and 25°C, respectively, confirms the dramatic effect of temperature on EPN efficacy. At 14°C, all treatments with EPN species resulted in slower codling moth mortality than they did at 25°C, as after 48 h, < 15% mortality was recorded for all species, whereas at the warmer temperature, > 98% mortality was recorded for all species. After the exposure of washed, cool-treated larvae to 25°C for 24 h, the application of both *S. feltiae* and *S. yirgalemense* resulted in 100% mortality, whereas the application of the two *H. bacteriophora* isolates, Hb1 and Hb2, resulted in 68% and 54% control, respectively, over the same time period. At 14°C, *S. feltiae* had the highest average penetration rate of 20 IJs/insect, followed by *S. yirgalemense* with 14 IJs/insect, whereas *S. yirgalemense* had the highest penetration rate at 25°C, with 39 IJs/insect, followed by *S. feltiae*, with 9 IJs/insect. The two *H. bacteriophora* isolates had higher average penetration rates at the higher temperature. This study has highlighted the biocontrol potential of *S. jeffreyense*, as well as showing that *S. feltiae* is a cold-active nematode, whereas the other three EPN isolates prefer warmer temperatures.

Stacked wooden fruit bins are regarded as preferred overwintering sites for codling moth diapausing larvae. Control strategies against the codling moth in South Africa have been hampered by the reinfestation of orchards by nearby stacked infested fruit bins or by the movement of bins between orchards. Worldwide, wooden fruit bins are systematically being replaced with plastic bins, which, in South Africa, will only be phased out over a few years. The objective of this study was to evaluate the potential of *H. bacteriophora*, *S. feltiae*, and *S. yirgalemense,* to disinfest miniature wooden fruit bins under controlled conditions in the laboratory. After dipping minibins in a suspension

of 25 IJs/ml of all three EPN species, under optimum conditions of temperature and humidity, the highest percentage of control was obtained using *S. feltiae* (75%) followed by *S. yirgalemense* (57%), and then by *H. bacteriophora* (Hb1) (27%). The addition of adjuvants significantly increased ($p < 0.001$) *S. feltiae* infectivity to > 95%, whereas it did not result in a significant increase in *H. bacteriophor*a or *S. yirgalemense* infectivity. The results indicated that *H. bacteriophora* would not be a suitable candidate to use for the control of the codling moth larvae in wooden fruit bins. The current preferred candidate for control would be *S. feltiae*, whose efficacy could be increased by means of the addition of an adjuvant.

During winter, when the whole codling moth population are larvae and in diapause, no control measures are applied in orchards. This study has shown that EPNs can be sprayed in orchards to lower the codling moth cohort emerging after winter, as well as be included in an IPM programme. EPNs can act as a second line of defence, through supplementary control, and ensure effective control of the codling moth larvae which survived chemical spray applications, to safeguard against resistant codling moth populations in the next season.

Opsomming

Kodlingmot, *Cydia pomonella* (Lepidoptera: Tortricidae), is 'n belangrike plaag van appels en pere wêreldwyd. Die onttrekking van sekere fundamentele chemikalieë vanuit die kodlingmot beheerprogram weens die kommer oor menslike, omgewings en ekosisteemgesondheid, het gelei tot die soektog na sagter, meer omgewingsvriendelike en veiliger beheermaatreëls. Entomopatogeniese nematodes (EPNs) kom natuurlik in die grond voor en soek aktief na gashere. Die belangstelling in die gebruik van EPNs van die families Heterorhabditidae en Steinernematidae as 'n beheermaatreël is te danke aan die ontdekking van 'n EPN in 'n insek in 1953. Die doel is om EPNs in 'n geïntegreerde plaagbeheerprogram (GPB) te inkorporeer om sodoende minimale residue te verseker en uiteindelik residu vrye produksie van kernvrugte in Suid-Afrika. Ten einde die sukses van EPNs te verseker, is beide die omgewings- en tegniese faktore wat hul doeltreffendheid beïnvloed in die studie ondersoek.

Die biologiese beheer potensiaal van drie ingevoerde EPN isolate, *Steinernema feltiae* en twee *Heterorhabditis bacteriophora* (Hb1, Hb2) isolate, sowel as 'n plaaslike isolaat, *Steinernema yirgalemense*, is vir die beheer van kodlingmot onder plaaslike toestande geëvalueer. Alle konsentrasies van *S. yirgalemense*, wat deur indompeling in 'n suspensie van nematodes toegedien is, het > 98% beheer tot gevolg gehad. Die twee geformuleerde isolate van *H. bacteriophora*, Hb1-f en Hb2-f, het < 30% beheer gegee. Met die gebruik van dieselfde nematode isolate, wat *in vivo* geproduseer is, het *S. yirgalemense* nog steeds 'n hoër kodlingmot beheer van > 90% opgelewer, in vergelyking met die 54% en 31% beheer van die *H. bacteriophora* Hb1 en Hb2 isolate, onderskeidelik. *Steinernema feltiae* het in opvolg veldproewe ≥ 80% beheer tot gevolg gehad en was dus meer effektief as beide *S. yirgalemense* en die *H. bacteriophora* (Hb1) isolate, met 66% en 24% en 24% en 9% beheer onderskeidelik in twee afsonderlike veldproewe. Om die resultate van die veldproewe te bevestig, is daaropvolgende laboratorium biotoetse uitgevoer en temperatuur regimes is geëvalueer deur die selfde siklus as onder natuurlike toestande te volg, met 'n konstante humiditeit van 100%. Die studie het bewys dat *S. feltiae* die mees doeltreffende isolate was met > 90% mortaliteit, *S. yirgalemense* het gevolg met 78% mortaliteit. Die twee *H. bacteriophora* isolate (Hb1, Hb2) het onderskeidelik onder bogenoemde laboratorium toestande 73% en 59% beheer tot gevolg gehad. Humiditeit blyk dus die belangrikste faktor te wees wat EPN se doeltreffendheid tydens

bogrondse toediening affekteer. Die studie het bewys dat *S. feltiae* 'n beter kandidaat as *S. yirgalemense* vir die beheer van kodlingmot is.

Die doeltreffendheid van verskillende EPN isolate vir die beheer van diapause kodlingmot larwes sowel as EPN se aktiwiteit by verskillende temperature is ook onder plaaslike toestande, deur bogrondse bespuitings, geëvalueer. *Steinernema feltiae* en twee isolate van *H. bacteriophora* (Hb1, Hb2), *S. yirgalemense* en 'n ander plaaslike isolaat, *Steinernema jeffreyense*, is geëvalueer. Die gebruik van *S. jeffreyense*, het tot die mees effektiewe beheer gelei, met 67% mortaliteit, gevolg deur *H. bacteriophora* (Hb1) met 42%, en dan *S. yirgalemense* met 41%. Laboratorium biotoetse wat veldtoestande simuleer, het bewys dat *S. feltiae* die mees doeltreffend teen kodlingmot larwes is, met 67% mortaliteit tydens infeksie, gevolg deur *S. yirgalemense* met 58%, die *H. bacteriophora* Hb1 isolaat met 48%, en die Hb2 isolaat met 24%. 'n Vergelyking van die infeksie- en penetrasie tempo van twee isolate van *H. bacteriophora* (Hb1, Hb2), *S. feltiae* en *S. yirgalemense* wat in 12-put plate teen 14°C en 25°C uitgevoer is, het die dramatiese effek van temperatuur op EPN doeltreffendheid bevestig. By 14°C het alle EPN spesies behandelings stadiger kodlingmot mortaliteit as by 25°C na 48h tot gevolg gehad. 'n Mortaliteit van < 15% is vir alle spesies aangeteken terwyl by die warmer temperature is > 98% mortaliteit vir alle spesies aangeteken. Na die blootstelling van afgespoelde, koel behandelde larwes aan 25°C vir 24 uur, het die toediening van beide *S. feltiae* en *S. yirgalemense*, 100% mortaliteit van larwes tot gevolg gehad terwyl die toediening van die twee *H. bacteriophora* isolate, Hb1 en Hb2, onderskeidelik 68% en 54% beheer tot gevolg gehad, oor dieselfde tydperk. By 14°C, het *S. feltiae* die hoogste gemiddelde penetrasie tempo van 20 ILs/ larwe, gevolg deur *S. yirgalemense* met 14 ILs/ larwe tot gevolg gehad, terwyl *S. yirgalemense* die hoogste penetrasie tempo getoon het by 25°C met 39 ILs/ insek, gevolg deur *S. feltiae* met 9 ILs/ insek. Die twee *H. bacteriophora* isolate (Hb1 en Hb2) het ook hoër gemiddelde penetrasie tempo by die hoër temperatuur getoon. Hierdie studie het die biobeheer potensiaal van *S. jeffreyense* beklemtoon, asook weereens bevestig dat *S. feltiae* 'n koue-aktiewe nematode is, terwyl die ander drie EPN isolate warmer temperature verkies.

Hout vrugtekratstapels, word beskou as 'n ideale oorwintering skuiling vir kodlingmot diapause larwes. In Suid-Afrika word beheerstrategieë teen kodlingmot in die wiele gery deur die herbesmetting van boorde deur nabygeleë besmette hout vrugtekratte of deur die beweging van

kratte tussen boorde. Hout vrugtekratte word wêreldwyd stelselmatig vervang met plastiek kratte. Dit sal egter eers oor 'n aantal jare in Suid-Afrika uitgefaseer word. Die doel van hierdie studie was om die potensiaal van *H. bacteriophora*, *S. feltiae*, en *S. yirgalemense* te evalueer deur miniatuur hout vrugtekratte onder gekontroleerde toestande in die laboratorium te disinfekteer. Na die onderdompeling van die mini vrugtekratte in 'n nematode suspensie van 25 ILs/ml van al drie EPN spesies, onder optimale toestande van temperatuur en humiditeit, is die hoogste persentasie van beheer met die gebruik van *S. feltiae* (74,85% ± 3.64%) verkry. Die byvoeging van toevoegings middels het *S. feltiae* se vermoë om te infekteer betekenisvol (p <0,001) tot > 95% verhoog, maar dit het nie tot 'n betekenisvolle toename in die infektiwiteit van *H. bacteriophora* of *S. yirgalemense* gelei nie. Die resultate dui daarop dat *H. bacteriophora* nie 'n geskikte kandidaat is om te gebruik vir die beheer van kodlingmot larwes in besmette hout kratte nie. Die voorkeurkandidaat tans vir beheer is *S. feltiae*, waarvan die doeltreffendheid verhoog kan word deur middel van die byvoeging van 'n bymiddel.

Gedurende die winter wanneer die hele kodlingmot populasie as larwes in diapause is, word geen beheer in boorde toegepas nie. Hierdie studie het getoon dat EPNs in boorde gespuit kan word om sodoende die opkomende kodlingmot populasie na die winter te verlaag en kan ook ingesluit word in 'n GPB program. Die EPNs kan as 'n tweede verdedigingslinie optree en doeltreffende beheer van kodlingmot larwes verseker wat chemiese bespuitings oorleef het, en sodoende beskerming teen weerstandige kodlingmot populasies in die volgende seisoen bied.

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CHAPTER 1

Control of the codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) in South Africa with special emphasis on using entomopathogenic nematodes

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Abstract

In the Western Cape province of South Africa, the codling moth (*Cydia pomonella*) is the most important lepidopteran pest of apples and pears. Currently an integrated pest management (IPM) strategy is followed. However, chemical control still plays an important role in the control of this pest. Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae have been successfully utilised as biological control agents in classical, conservation, and augmentative insect pest management programmes. In this review different biological control options for control of the codling moth are considered, with special emphasis on research being done on the biological control of the codling moth using EPNs. To integrate nematodes into an IPM system, it is important to conduct research under local environmental conditions for a specific crop. Application of EPNs against the codling moth will target the diapausing larval overwintering population above ground. Especially for commercial application, the unique environmental conditions in the various production areas need to be assessed to allow for the effective use of various EPN species. Orchard application, onto trees poses its own unique challenges with regard to the inundative application of EPNs. Research in the use of EPNs to control the codling moth and obstacles encountered in the success of codling moth control will be discussed.

Introduction

The codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) is one of the most studied tortricids in the family. In South Africa it was first found in Graaff-Reinet around 1885 (Lounsbury, 1898). The latter author speculated that the initial infestation may have originated from infested fruit that had been brought in from Madeira by a tourist. Its presence was reported in Stellenbosch in 1898 (Lounsbury, 1898). In 1918, the codling moth was regarded as an established pest in South Africa (Lounsbury, 1918). The spread of the codling moth was attributed to the cultivation of its hosts and the transport of infested fruit and wooden fruit bins (Giliomee & Riedl, 1998).

South Africa is one of the biggest deciduous fruit producers in the southern hemisphere, with apple (26 631 ha) and pear (12 034 ha) production forming a significant part of this industry (Addison, 2005; Hortgro, 2013). Ceres (Western Cape), Groenland (Western Cape) and Langkloof East (Eastern Cape) are the main apple- and pear-producing areas in South Africa. The Western Cape Province alone produces 75% of the country's apples, and 85% of its pears (Hortgro, 2013). In South Africa, Golden Delicious, Granny Smith, Royal Gala and Topred/Starking are the main apple cultivars produced, with Packham's Triumph, Forelle and Williams Bon Chretien being the most popular pear cultivars (Hortgro, 2013). In 2012, South Africa ranked sixteenth in world apple production and ninth in world pear production, making it one of the top ten fresh apple and pear exporters in the world.

It is presumed that the codling moth originated from Eurasia, as not only was the first definite report of the pest made in the Netherlands around 1635, long before the large-scale production of fruits first began, but it is also thought that apples and pears, which are the main hosts of the moth, originated from Europe and Central Asia (Barnes, 1991). The moth has since then managed to spread to all major apple- and pear- producing areas around the world, and is now a key pest in orchards where these fruits are produced (Barnes, 1991; Giliomee & Riedl, 1998; Riedl *et al*., 1998).

Depending on the climate and the host plant, the codling moth can have one to four generations per year, with the number varying in different parts of the world (Barnes, 1991; Lacey & Unruh, 1998; Welter, 2008). For example, due to the favourable conditions existing in South Africa, up to four generations have been recorded per growing season in the country (Blomefield, 2003; Pringle *et al*., 2003).

Fig. 1.1. A codling moth caught on a sticky trap.

Adult female moths emerge in spring and within a day deposit an average of 137 (spring) to 159 (summer) eggs, which are individually laid on the young fruit, or on the foliage of their host (Blomefield & Giliomee, 2012). Depending on the prevailing temperature, eggs hatch in the space of 4 to 19 days, and within 24 hours of hatching, neonate larvae start boring into the fruit (Blomefield & Giliomee, 2009). For the first few days, the larvae feed near the surface of the fruit, eventually moving to the fruit core, where they then feed on the seeds and on the surrounding flesh (Welter, 2008).

Fig.1.2. A codling moth larva in its silken cocoon.

The larvae pass through five instars within the fruit, and then exit as mature 5th instar larvae in search of a place to pupate in silken cocoons. Suitable pupating sites include pruning wounds and loose bark on trees, detritus at the base of trees (Blomefield & Giliomee, 2012; Cranshaw & Hammon,

2014), woodpiles that are in close proximity to the orchard and fruit bins that are stacked near, or in orchards (Blomefield, 2003). All the final instars of the last generation moths spend the winter in a state of diapause (Blomefield, 2003). In spring when the temperature increases, the larvae pupate in their silken cocoons, and shortly thereafter emerge as adult moths.

Fig. 1.3. The red ring and frass that is characteristic of the exit hole of the larvae and of codling moth damage.

Current codling moth control strategies

Insecticides

The codling moth has predominantly, and traditionally, been controlled with insecticides (Lacey & Unruh, 1998; Riedl *et al.*, 1998; Lacey & Chauvin, 1999), but due to the rise in awareness of the dangers of chemical pesticides (Gaugler, 1988; Grewal *et al.*, 2001) and the development of resistance (Stubbings, 1948; Myburgh, 1958; Riedl *et al.*, 1998). There is increased pressure for the agricultural sector to shift to the use of biological control agents.

The focus of the strategy for controlling the codling moth, has thus shifted from a chemicals-only approach, to a more multifaceted one, in which the minimal use of pesticides is emphasised (Blomefield, 2003). Although chemicals are still used for such control purposes, they tend to be used in combination with pheromone mating disruption, sterile insect release, and other biological control measures.

Use of pheromones

Monitoring

Before the development of synthetic codling moth pheromone baited traps as monitoring devices, growers relied on intensive spray programmes to ensure effective codling moth control (Myburgh *et al.,* 1974). As well as having enabled growers to monitor infestation levels in orchards, the use of pheromone traps has aided in determining the intensity of spray programmes, in terms of the timing of, and the number of sprays required, for effective control (Myburgh *et al.*, 1974). The use of pheromone traps can thus be regarded as the first successful attempt to move away from calendar-based insecticide spray programmes (Myburgh *et al.*, 1974, 1975).

The economic threshold for male codling moths caught in these pheromone traps was shown to be 2 moths/trap/week (Madsen & Vakenti, 1972; Madsen *et al.*, 1974). At this population level 1 trap/1.8 or 2 ha is sufficient for effective monitoring (Myburgh *et al.*, 1974; Myburgh & Madsen, 1976).

Mating disruption

The technique of mating disruption utilises a synthetic female sex pheromone, released by artificial emitters, to disrupt codling moth mating (Lacey & Chauvin, 1999; Unruh & Lacey, 2001). Rather than killing off the moths, the use of this method interferes with the male moth's ability to locate females (Welter, 2008), resulting in females laying unfertilised eggs. The effectiveness of mating disruption is, however, reduced when an orchard has an uneven topography, in windy conditions, when there are outside sources of infestation, and when the codling moth population densities are high (Cardé & Minks, 1995; Pringle *et al.*, 2003). Use of this tactic has contributed to the decline in conventional insecticide use, as well as aiding in the successful reduction of economic injury to fruit by the codling moth (Unruh & Lacey, 2001; Pringle *et al.,* 2003). However, the effectiveness of monitoring using pheromone traps is reduced when using mating disruption (Pringle et al., 2003).

Sterile insect technique (SIT)

The SIT involves area-wide release of large numbers of sterile male moths in orchards, which then compete with wild male moths for females. If females mate with a sterile male, the eggs are infertile, thus reducing the codling moth population in the next generation. Although the use of SIT alone is not effective at high population levels, when it is used in combination with other control measures, it

results in a reduction of pest numbers, reduced fruit damage and decreases in insecticide use (Addison, 2005).

Unfortunately, the use of SIT on a semi-commercial scale in South Africa for the control of the codling moth was officially terminated in October 2014. The high cost of the control measure, together with its sophisticated infrastructure and intensive management, combined with clear evidence of the success of the application of insecticides, resulted in the discouragement of growers and lack of support. Currently modern, more acceptable insecticides that are on the market, such as methoxyfenozide, the codling moth granulosis virus, spinetoram and novaluron are seen as relatively convenient to use and are currently more cost-effective when combined with mating disruption and the SIT.

Biological control

Although the biological control of insect pests as a stand-alone measure cannot be effective against pests that are damaging at low population levels, it can form an important component of an integrated pest management (IPM) programme (Brunner, 1993; Cranshaw & Hammon, 2014), as it provides important supplemental control of the pest. In addition to eliminating those insects escaping chemical control, biological control also serves to reduce the development of resistance to insecticides. Various biological control measures have been implemented in orchards, of which the *Cydia pomonella* granulovirus (CpGV) is well-known (Cross *et al*., 1999). However, both the development and the adoption of the CpGV have been limited, as growers have expressed concerns over the need for multiple applications, due to the short period of residual activity, the expense involved, as well as the slow rate of kill. In addition, the use of CpGV tends to be less effective in orchards with high codling moth populations (Arthurs & Lacey, 2004).

Biological control agents currently receiving much attention are the egg parasitoids in the genus *Trichogramma*, of which there are over 100 species (Hassan, 1994). These minute wasps predominantly parasitises eggs of a wide range of Lepidoptera, by laying eggs, and completing development, within the eggs of the host (Hassan, 1994; McDougall & Mills, 1997; Cossentine & Jensen, 2000; Mills *et al*., 2000). *Trichogramma lutea* (Girault) (Hymenoptera: Trichogrammatidae), which was discovered in 1909 by Lounsbury and Mally, is indigenous to South Africa (Pettey, 1919).

This parasitoid parasitized over 50% of the codling moth eggs (Pettey, 1919). A single female *T. lutea* was able to parasitise as many as 18 codling moth eggs and up to six *T. lutea* emerged from a single codling moth egg (Pettey, 1919; Nel, 1942). This parasite flourished in orchards where no insecticides were used (Pettey, 1919).

Although a method was developed to mass-produce *Trichogramma* in the 1930s (Theron, 1944; Hassan, 1994), its use has only recently received renewed interest as a potential biological control measure. Wahner *et al.* (2008) developed a protocol using DNA sequence analysis of the internal transcribed spacer 2 (ITS 2) for the identification of two indigenous wasps, *T. lutea* and *T. cryptophlebiae* (Nagaraja). This technique has enabled the simple and fast identification of the wasps, without the need for morphological knowledge, and it has confirmed them to be distinct species.

Pimpla heliophila (syn. *P. albipalpis*) Cameron (Hymenoptera: Ichneumonidae), was also studied as a potential biological control measure by Lounsbury in 1906 (Nel, 1942). The species, which is endoparasitic in the codling moth larvae and pupae, has been shown to be able to persist in moderate to heavily sprayed orchards (Nel, 1942). *Ascogaster quadridentus* Wesm. (Hymenoptera: Braconidae) has also been recorded as a predominating parasite of the codling moth in orchards (Nel, 1942). A few exotic species have been imported, including a Spanish parasite, *Calliephilates messor* Grav. (Hymenoptera: Ichneumonidae). This parasite, after being imported from California in 1907, was propagated and inundatively released in local orchards, only to succumb to the local environment (Crusman, 1913; Pettey, 1919).

Cultural control

Cultural control involves simple, low-cost alterations to the environment of the pest insect. Such alterations are achieved by, among other methods, fruit thinning and removal, orchard sanitation, trunk banding, fruit bagging (labour intensive and high cost) and the planting of different apple varieties, and smooth bark apple trees. Fruit thinning and removal involves the pruning, picking and removal of infested fruits, including fruits that remain after harvesting. The method concerned reduces the potential of infestation, as codling moths prefer to infest fruits that are protected by foliage and other fruits. Pruning improves the effectiveness of spray programmes, as it allows for improved spray coverage (Blomefield, 1991; Cranshaw & Hammon, 2014).

Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are the most extensively studied, due to their potential as inundatively applied biopesticides, for the fast short-term control of certain insect pests, with the potential of offering long-term control when applied to the soil (Grewal *et al.*, 2001; Lacey *et al.*, 2001; Hunt, 2007). EPNs possess a number of unique and favourable attributes, such as high virulence, and the ability to seek out hosts actively, which makes them promising alternatives to chemicals. The successful control of a number of soilborne insect pests has, been achieved through the use of EPNs (Lello *et al.*, 1996).

Life cycle

The Steinernematidae and Heterorhabditidae have similar life strategies, which starts with a special third-stage infective juvenile (IJ), also known as a dauer juvenile (Griffin *et al.*, 2005). As IJs are free-living and non-feeding, they are able to survive long periods within the soil environment, due to certain morphological and physiological adaptations (Ehlers, 1996; Grewal *et al.*, 2001). These IJs are mobile and possess chemoreceptors (amphids) which enable them to seek out hosts in cryptic habitats, using host carbon dioxide release and vibrations (Gaugler, 1988; Lacey *et al.*, 2001). Once an IJ locates a host, it infects it through natural openings (mouth, spiracles, and the anus) or, in the case of heterorhabditids using their dorsal tooth to penetrate thin areas of the host cuticle (Grewal *et al.*, 2001; Griffin *et al.*, 2005).
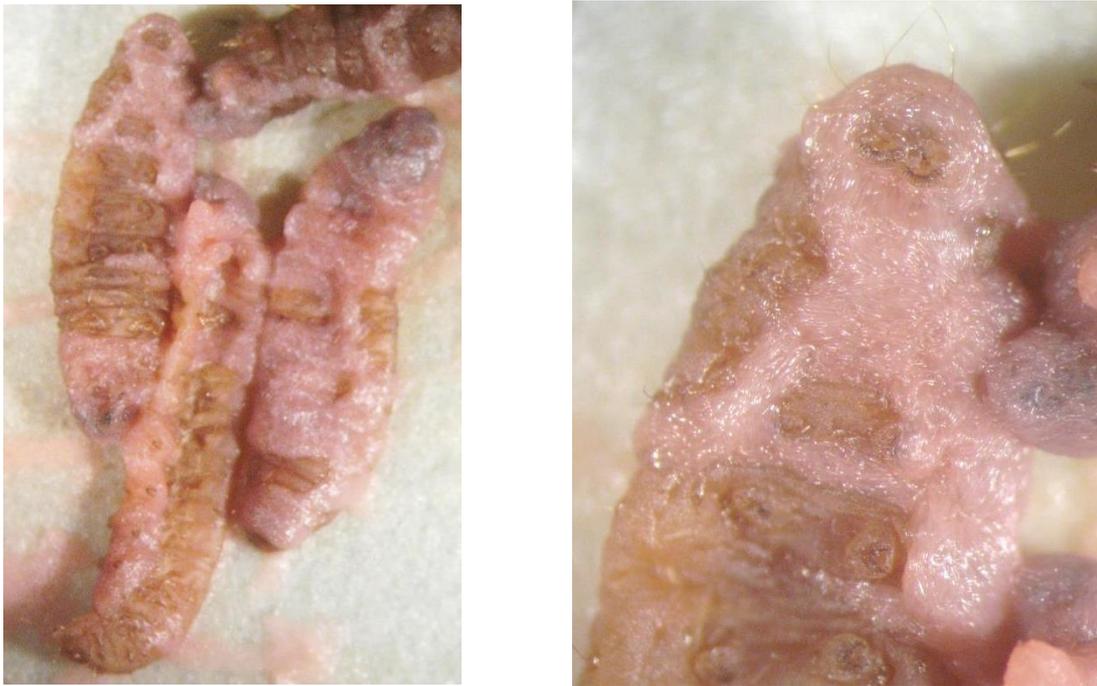
Fig. 1.4. Infective juveniles emerging from codling moth larvae cadavers.

These nematodes have a mutual relationship with a specific genus of bacteria belonging to the family Enterobacteriaceae (Griffin *et al.*, 2005; Koppenhöfer, 2007). Whereas steinernematids are associated with bacteria in the genus *Xenorhabdus* Thomas & Poinar, 1979*,* heterorhabditids are associated with *Photorhabdus* Boemare, Akhurst & Mourant, 1993 (Forst & Clarke, 2002; Griffin *et al.*, 2005). Although a nematode species has a specific relationship with one specific bacterium, the same bacterium may be associated with more than one species of nematode (Grewal *et al.*, 2001; Forst & Clarke, 2002).

After infection, and upon penetrating the haemocoel of the host, the IJ release their bacterial symbionts from their intestine. The bacterium then propagates exponentially, killing the host within 48 h. The nematodes then feed on the bacterial cells and the liquefying host tissues (Adams & Nguyen, 2002; Griffin *et al.*, 2005). The symbiotic bacteria excrete antimicrobial substances and bacteriocins, which protect the host cadaver from colonisation by other microorganisms, thus preventing competition for food resources during nematode development and reproduction (Boemare *et al.*, 1996). Depending on the size of the insect host, nematodes complete one to three generations within a host cadaver and as soon as the food resources are depleted, a new cohort of IJs is produced

(Grewal *et al.*, 2001; Adams & Nguyen, 2002). These IJs then emerge from the host cadaver, in search of new hosts in the soil environment (Griffin *et al.*, 2005).

Distribution of EPNs and their associated bacteria in South Africa

EPNs are wide-spread, with soil being their natural habitat (Campbell *et al.*, 1995; Grewal *et al.*, 2001). Soil provides protection and acts as a buffer from environmental extremes such as ultraviolet (UV) radiation, variable temperatures and erratic moisture levels (Kung *et al.*, 1991). These organisms have been recovered from soils all over the world, ranging from cultivated soils to deserts (Grewal *et al.*, 2001). The only continent on which they have not been found is Antarctica (Griffin *et al.*, 1990).

The first parasitic nematode, found associated with the codling moth in South Africa, was reported as a Mermitidae, infecting 40% of cocooned larvae (Nel, 1943). The first record of the presence of EPNs in South Africa was that of Harington (1953), who discovered an EPN in a maize beetle *Heteronychus arator* (Fabricius) in Grahamstown, situated in the Eastern Cape Province. Since then, EPNs have been recovered from the Western Cape (Grenier *et al.*, 1996; Malan *et al.*, 2006; Hatting *et al.*, 2009; Malan *et al.*, 2011), the Eastern Cape (Malan *et al.*, 2006, 2011), KwaZulu-Natal (Spaull, 1988, 1990, 1991; Hatting *et al.*, 2009), the Free State (Hatting *et al.*, 2009), Mpumalanga (Hatting *et al.*, 2009; Malan *et al.*, 2011), Gauteng, and the North West Provinces (Molotsane *et al.*, 2007).

Of the 11 EPN species (consisting of four heterorhabditids, and seven steinernematids) found in South Africa (Table 1), seven are endemic. Within the EPN species concerned, three new symbiotic bacterial species have been discovered *viz. Xenorhabdus khoisanae* Ferreira, Van Reenen, Gozel, Malan & Dicks, 2013, which is associated with *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006 (Ferreira *et al.*, 2013b); *Photorhabdus luminescence* subsp. *noenieputensis* Ferreira, Van Reenen, Pagès, Tailiez, Malan & Dicks, 2013, which is associated with *H. noenieputensis* Malan, Knoetze & Tiedt, 2014 (Ferreira *et al.*, 2013a); and *Photorhabdus zealandica* Ferreira, Van Reenen, Endo, Tailiez, Pagès, Spröer, Malan & Dicks, 2014, which is associated with *Heterorhabditis zealandica* Poinar, 1990 (Ferreira *et al.*, 2014a). A new nematode-bacterial association was found with an already identified *Photorhabdus* species. *Heterorhabditis zealandica* which is associated with *P. zealandica* in South Africa and not with *Photorhabdus temperata* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare, 1999, which differs from the *H. zealandica* strain in New Zealand and Florida

(Ferreira *et al.*, 2014a) (Table 1). The associated bacteria of *S. yirgalemense* was identified as being *Xenorhabdus indica* Somvanshi, Lang, Ganguly, Swiderski, Saxena & Stakebrant, 2006 (Ferreira *et al.*, 2014b), previously described from *Steinernema abbasi* Elawad, Asmad & Reid, 1997 (syn. *S. termophylum*).

Table 1.1. Occurrence of entomopathogenic nematodes and their associated symbiotic bacteria in South Africa.

Nematode species	Reference	Associated bacteria	Reference
H. bacteriophora	Malan *et al.*, 2006; Hatting *et al.*, 2009; Malan *et al.*, 2011	Unknown	-
H. noenieputensis*	Malan *et al.*, 2014	*Photorhabdus luminescence* subsp. *noenieputensis**	Ferreira *et al.* 2013a
H. safricana*	Malan *et al.*, 2008	Unknown	-
H. zealandica	Malan *et al.*, 2011	*P. zealandica**	Ferreira *et al.* 2014a
S. citrae*	Stokwe *et al.*, 2011	Unknown	-
S. innovationi*	Çimen *et al.*, 2014a	Unknown	-
S. jeffreyense*	Malan *et al.,* 2015	Unknown	-
S. khoisanae*	Nguyen *et al.*, 2006	*X. khoisanae**	Ferreira *et al.* 2013b
S. sacchari*	Nthenga *et al.*, 2014	Unknown	-
S. tophus*	Çimen *et al.*, 2014b	Unknown	-
S. yirgalemense	Malan *et al.*, 2011	*X. indica*	Ferreira *et al.* 2014b

* Type specimen

Host range and safety

In the laboratory, EPNs have a broad host range, as conditions are optimal for host finding, penetration, and infection (Boemare *et al.*, 1996; Grewal *et al.*, 2001; Lacey *et al.*, 2001). The wide host range is due to the rapid host mortality that is caused by the nematode-bacterium complex, as the EPN is not in its host long enough to form a highly adapted and specific, host-parasite relationship (Boemare *et al.*, 1996; Grewal *et al.*, 2001). EPNs are thus able to exploit a much wider spectrum of hosts than many microbial control agents can (Grewal *et al.*, 2001).

The broad host range that has been displayed by EPNs in the laboratory is a major concern, as they might not only have a detrimental effect on target insects, but also on non-target, including beneficial, insects (Cross *et al.*, 1999). However, in the field, the EPNs host range is very limited

(Lacey & Unruh, 1998; Cross *et al.*, 1999). Such a restriction is regarded as being due to exposure to environmental extremes (temperature, UV radiation, and low moisture levels), ecological barriers and behavioural barriers (Cross *et al.*, 1999; Grewal *et al.*, 2001). In addition EPNs encounter a variety of challenges, such as uncertain host contact, limited movement due to their low mobility, and deactivation by environmental extremes, EPN thus have a much narrower and more restricted host range in the field, than they do in the laboratory, further restricting their host range in the field (Grewal *et al.*, 2001).

A few studies have shown that EPNs have no effect on either plants or mammals (Gaugler, 1988; Boemare *et al.*, 1996; Grewal *et al.*, 2001; Lacey *et al.*, 2001). In the laboratory, some cold-blooded species (tadpoles and lizards) have been recorded as being susceptible at high dosages, but the negative effects obtained under laboratory conditions could not be replicated in the field (Grewal *et al.*, 2001). All negative impacts are thus limited to treated areas, due to low EPN mobility, and due to their sensitivity to harsh environmental conditions (Grewal *et al.*, 2001). No negative effects of using EPNs as biological control agents have been recorded (Ehlers, 1996).

Regulation and registration

No universal protocol exists for the regulation of EPNs, thus their introduction, release, and commercialisation varies from country to country (Grewal *et al.*, 2001). In some countries, EPNs are treated as microorganisms, and they are thus regulated as such, but according to the conclusions and recommendations of a combined workshop that was held under the auspices of both the Organization for Economic Cooperation and Development (OECD) and European Cooperation in Science and Technology (COST), they should, in fact, be treated as macro-organisms, as they are multicellular (Ehlers & Hokkanen, 1996; Grewal *et al.*, 2001). In many countries, the registration of indigenous EPNs is not required (Ehlers, 1996).

The final recommendations and conclusions of the OECD and COST workshop was that: indigenous EPN species should not have to be regulated. Rather, the introduction of exotic EPN species must be regulated, and EPNs must not be regulated at the strain level, but at the species level (Ehlers & Hokkanen, 1996). The exchange of exotic EPNs between laboratories should also, according to the workshop, not have to be regulated if there is no intention of releasing the EPNs into

the environment. Instead, those laboratories participating in such research should make available proper documentation, if they are required to do so (Ehlers & Hokkanen, 1996).

Integration of EPNs in an IPM strategy

EPNs can fill a very important niche in an IPM strategy, as they have proven to be effective biological alternatives to chemicals (Lacey & Unruh, 2005). Studies have shown that EPNs have no effect on the environment, and that they conserve natural control agents, thus maintaining the biodiversity in orchard agro-ecosystems that broad-spectrum insecticides fail to do (Lacey & Unruh, 1998; Unruh & Lacey, 2001). The reduction of reliance on chemical pesticides through the use of EPNs results in reduced contact between humans and chemical residues, the conservation of biodiversity and less environmental degradation; thus the advantages of including EPNs in an IPM strategy cannot be measured in monetary terms.

Currently, there is no one control measure that can be applied to eradicate, or to successfully control the codling moth, as this pest is dynamically adapted to withstand such control measures (Giliomee & Riedl, 1998). Many soil-inhabiting insect pests have, by means of co-evolution, developed barriers to infection (Koppenhöfer, 2007). A major advantage of using EPNs against the codling moth is that it is an above-ground pest which, therefore, eliminates the possibility of co-evolutionary adaptations (Kaya & Hara, 1981; Koppenhöfer, 2007). Diapausing codling moth larvae are therefore, very susceptible to EPNs, as they possess no barriers to infection.

During autumn the entire codling moth population enters diapause in cocoons, in which they spend the winter, thus providing an ideal target for EPNs (Lacey & Unruh, 1998). At this stage, the diapausing codling moth larvae are very susceptible to EPNs, and if the latter is successfully applied, the entire codling moth population in an orchard can be controlled (Unruh & Lacey, 2001). No other control measures target this winter population, as they all target the summer population

Previous research using EPNs to control the codling moth

Many studies, using EPNs as biological control agents against the codling moth, have focused on determining the optimum conditions for nematode infection. These conditions include temperature, relative humidity (RH), different nematode species, isolates of the same species, nematode

concentrations, the addition of adjuvants and water activity (a$_w$-values, the amount of free water that is available for nematodes to move around in) required, and pre- and post-wetting, of which all are conducive to codling moth control, depending the extent and degree to which these factors are present.

Laboratory bioassays

EPN species

Of all EPN species tested, *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 has proved to be the most effective against the codling moth (Lacey & Unruh, 1998; Lacey & Chauvin, 1999; Unruh & Lacey, 2001; Cossentine *et al.*, 2002; Lacey *et al.*, 2005; Lacey *et al.*, 2006a). The species is regarded as being alien to South Africa, as it has not been recovered from local soils (Malan *et al.*, 2006; De Waal, 2008; Hatting *et al.*, 2009; Malan *et al.*, 2011). The only report of the occurrence of *S. feltiae* on the African continent is from in Algeria (Tarasco *et al.*, 2009).

The local South African EPN isolate that has shown most promise against the codling moth in South Africa is that of *H. zealandica* (De Waal, 2008; De Waal *et al.*, 2010, 2011a,b, 2013). This nematode has also been shown to be the most effective species for the control of the banded fruit weevil, *Phlyctinus callosus* Schönher (Ferreira & Malan, 2014), and the mealybug, *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) (Stokwe, 2009), whereas later research has shown *Steinernema yirgalemense* Nguyen, Tesfamarian, Gozel, Gaugler & Adams 2005 (Malan *et al.*, 2011) to be the more effective species against the false codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), the mealybugs *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) (Van Niekerk & Malan, 2012, 2013, 2014a, b) and *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) (Le Vieux & Malan, 2013, 2014). Using three local EPN species, including an undescribed *Steinernema* sp. (J194), against the codling moth, De Waal *et al.* (2011a) showed the latter to give comparable control to that of *H. zealandica* and *S. yirgalemense*.

An ongoing search for new species of EPNs is aimed at determining their occurrence and diversity in South Africa, with the hope of finding an isolate that is highly effective at controlling the codling moth, under the various climatic conditions that are found in the country. As the window of opportunity

for the application of EPNs for the control of the codling moth is during the winter months, the use of a low-temperature, active nematode, with desiccation tolerance, is of high importance.

EPN concentration

The LD_{50} for *H. zealandica* is 71 IJs/ml and the LD_{90} is 275 IJs/ml, under controlled conditions (De Waal, 2008). Lacey and Unruh (1998) found the LD_{50} to be 5, 5 and 6 IJs/cm^2 for *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, *Steinernema riobrave* Cabanillas, Poinar & Raulston 1994, and *Heterorhabditis bacteriophora* Poinar, 1976, respectively. The LD_{90} for *S. carpocapsae* was 16 IJs/cm^2, for *S. riobrave* 20 IJs/cm^2 and for *H. bacteriophora* 3 IJs/cm^2 (Lacey & Unruh, 1998). The differences identified cannot only be ascribed to the nematode species involved, but they are also, at least in part, due to the different laboratory conditions, such as experimental arena (Petri dishes, 24-well bioassay plates, cardboard strips, etc.) and the exposure time prior to the determination of infectivity.

Temperature

The niche breadth for temperature tolerance differs among EPN species (Kung *et al.*, 1991; Wright, 1992). A few species have been found to be cold- and heat-tolerant outside of the normal range, which has led to the species being tested at the specific temperatures to which they are adapted, as nematode survival is favoured by optimal in conditions representative of their natural climatic origin (Kung *et al.*, 1991). Increased EPN survival increases their effectiveness, thus encouraging the use of locally found cold- or warm-adapted strains over imported alien strains.

Most EPNs are known to become inactive at temperatures below 12°C, waiting for periods of optimum temperature before they can move and infect insects. *Steinernema feltiae* and *S. carpocapsae* infect hosts at temperatures below 10°C, suggesting that they have evolved as cool-temperature-active species (Wright, 1992; Grewal *et al.*, 1994). This characteristic has sparked interest in using *S. feltiae* as a possible biocontrol method for such insects as the codling moth, as the IJs are active during cold winter temperatures (Wright, 1992; Grewal *et al.*, 1994), which is the window period for targeting the diapausing codling moth population.

Lacey and Unruh (1998) showed that *S. carpocapsae* and *S. riobrave* can cause high codling moth mortality, with the former performing optimally at 25°C, whereas *S. riobrave* performs optimally at 30°C. *Steinernema carpocapsae* performed better at a broader temperature range than either *S. riobrave* or *H. bacteriophora* (Lacey & Unruh, 1998). Soil temperature had a significant effect on steinernematid survival, and pathogenicity (Kung *et al.*, 1991). *Steinernema carpocapsae* and *Steinernema glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982 were exposed to four soil temperatures. Survival for *S. carpocapsae* decreased with increasing temperatures, and *S. glaseri* survival was better at higher, than at lower, temperatures (Kung *et al.*, 1991). In the case of *S. carpocapsae*, pathogenicity was optimal at 25°C, while it was optimal at 35°C for *S. glaseri* (Kung *et al.*, 1991). Overall, the optimum temperature at which nematodes tend to be able to control the codling moth falls within the range of 20-25°C, with little, or no host mortality being recorded below 10-15°C, or above 35°C.

Humidity

EPNs require high RH in order to actively search for an insect host, as well as for their survival. The optimum RH was found to be > 95%, with lower humidity resulting in the EPN performing below their potential (Lacey & Unruh, 1998; De Waal *et al.*, 2010).

At different RH ranges, the survival of *S. carpocapsae* was unaffected for at least 2 days, whereas that of *S. glaseri* decreased, although it remained pathogenic for at least 4 h at all tested humidity ranges (Kung *et al.*, 1991). In addition, Navaneethan *et al.* (2010) found that the LC_{50} and LC_{90} of EPN increases with decreasing RH.

Water activity levels (aw)

The a_{w50}, measured using the Decagon Pawkit water activity meter (Decagon Devices, Inc., Pullman, WA, USA) at a constant temperature, of codling moth larvae for the isolates *S. citrae*, *S. khoisanae*, *S. yirgalemense*, *Steinernema* sp. (J194), and *H. zealandica* was 0.94, and that of *S. khoisanae* 0.97 (De Waal *et al.*, 2011a). Very low levels of mortality were recorded for *H. zealandica* at a_w levels of 0.80 to 0.93, after which the a_{w50} of this species was 0.94, with an a_{w90} of 0.96 (De Waal *et al.*, 2013). Navaneethan *et al.* (2010) showed *S. feltiae* caused no mortality at an a_w value of below 0.90, while the a_{w90} of this species was 0.99.

Heterorhabditis zealandica and *S. yirgalemense* were able to infect hosts at an a_w as low as 0.95 (Van Niekerk, 2011; Van Niekerk & Malan, 2012). *Steinernema yirgalemense* was more tolerant of low a_w than *H. zealandica* (Van Niekerk, 2011; Van Niekerk & Malan, 2012). There was a positive relationship between the insecticidal activity of EPNs and the a_w level, with nematode infectivity increasing with increasing a_w (De Waal *et al.*, 2011a; Van Niekerk, 2011; Van Niekerk & Malan, 2012; De Waal *et al.*, 2013). In general, the results obtained underline the importance of pre-wetting, post-wetting and high humidity for the aerial control of the codling moth during field application.

Adjuvants

The addition of adjuvants to nematode formulations reduces the negative impact of low humidity and low a_w on EPNs, thus increasing their efficacy (Lacey *et al.*, 2005; Navaneethan *et al.,* 2010). In a study by Navaneethan *et al.* (2010), using the surfactant-polymer formulation [0.3% Rimulgan® (surfactant) and 0.3% Xanthan® (anti-desiccant)], the infectivity of *S. feltiae* was improved.

Van Niekerk and Malan (2013) evaluated the effect of adding two polymers (Zeba® and Xanthan gum) to nematode suspensions in relation to *P. citri* mortality. The addition of Zeba® to *S. yirgalemense* and *H. zealandica* suspensions led to significantly higher mealybug mortality than did the addition of Xanthan gum (Van Niekerk & Malan, 2013). Adjuvants were also shown to improve nematode deposition (Van Niekerk & Malan, 2013). When added separately to nematode suspensions, neither Nu-Film-P®, nor Zeba® significantly increased the level of deposition. Only when they were combined, was the level of deposition significantly increased. *Steinernema yirgalemense* achieved higher mealybug mortality when combined with the adjuvants, Nu-Film-P® and Zeba® (Van Niekerk & Malan, 2013). De Waal *et al.* (2013) tested the effect of Zeba® on *H. zealandica* performance, at a RH of 60% and 80%, with a significant increase in the codling moth mortality at both percentages.

During a laboratory trial, De Waal *et al.* (2013) showed that at a RH of 60%, the a_w decreased less in the presence of Zeba® than it did in the absence of Zeba®. In contrast, at a RH of 80%, the a_w decreased both in the presence and in the absence of Zeba®, although the difference was not pronounced. Therefore, the addition of an adjuvant to nematode suspensions ensures a more constant a_w level on the bark (De Waal *et al.*, 2013).

Mulches

The mortality of the codling moth larvae, using *H. zealandica*, was significantly higher when pine shavings were used than when blackwood, pine wood, apple wood chips or straw were used (De Waal *et al.*, 2011b). In order to obtain 50% control, suitable humidity levels should be maintained for at least 9 hours in straw, and for 13 hours with apple wood chips. To obtain 90% control, suitable humidity levels should be maintained for at least 30 hours with straw, and for 50 hours with apple wood chips (De Waal *et al.*, 2011b). The conditions required for optimal codling moth control differed significantly between the laboratory and the field, due to the vast range of environmental variances (e.g. temperature, humidity, proximity to the codling moth larvae) outdoors.

Bin application

Aspects on the use of EPNs for the disinfestation of wooden fruit bins that have been investigated include the optimum conditions before and after nematode application, nematode concentration, the pre-wetting period of bins, the time of incubation, post-treatment bin tarping and incubation humidity. The latter was the most important factor influencing EPN efficacy.

De Waal *et al.* (2010) used *H. zealandica* in bin trials, whereas in other studies, *S. carpocapsae* (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005), *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 (Lacey & Chauvin, 1999), *Heterorhabditis marelatus* Liu & Berry, 1996 (Lacey & Chauvin, 1999), and *S. feltiae* (Lacey *et al.*, 2005) have been used. *Steinernema carpocapsae* was more effective in the corners of fruit bins than either *S. kraussei* or *H. marelatus* (Lacey & Chauvin, 1999). In the skids of fruit bins, *S. carpocapsae* and *S. kraussei* gave comparable results, while *H. marelatus* resulted in the lowest mortality (Lacey & Chauvin, 1999). Overall, both *S. carpocapsae* and *S. feltiae* appeared to have effective and comparable, activity against the codling moth (Lacey *et al.*, 2005).

An application rate of 100 IJs/ml *H. zealandica* ensured adequate control of fruit bins infested with codling moth (De Waal *et al.*, 2010). When using *S. carpocapsae*, a concentration of 50 IJs/ml ensured more than 80% mortality of the codling moth larvae (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005), the use of a higher concentration did not increase the codling moth mortality significantly.

The incubation time, humidity and temperature required for optimum codling moth control were at least 3 days, with a RH of 75-95%, and at temperatures of 20-25°C (Lacey & Chauvin, 1999; De Waal *et al.*, 2010). Such conditions can be achieved by storing bins indoors post-treatment, under controlled conditions, or covering the bins with plastic tarp (Cossentine *et al.*, 2002; De Waal *et al.*, 2010). Keeping bins in an area with little or no air flow, helps ensure more effective control of the codling moth, as this aids in maintaining a relatively high humidity, which slows down the desiccation of IJs, thus increasing their effectiveness (Lacey & Chauvin, 1999).

The wood hydrophobicity of fruit bins was lowered by pre-wetting bins for a minimum of 1 min pre-treatment, which increased nematode efficacy, due to the improved inoculum absorption of the bins (Unruh & Lacey, 2001; Cossentine *et al.*, 2002; De Waal *et al.*, 2010). The addition of adjuvants is beneficial, as they increase the spread and the absorption of inoculum, and they also slow down the desiccation of IJs, thus increasing their persistence and therefore their effectiveness (Unruh & Lacey, 2001; De Waal, 2008; De Waal *et al.*, 2010). In the case of *H. zealandica*, the addition of adjuvants increased the codling moth mortality (De Waal *et al.*, 2010).

Field trials

In general, relatively few field studies have focused on the control of the codling moth using EPNs. Previous studies have involved, among other aspects, determining the optimum environmental conditions (time of day for application, pre-wetting, the effect of wind, and sunlight, on IJ activity), the application technique (EPN species, the concentration of IJs, the addition of adjuvants, and the method of application), and the post-application conditions (post-wetting of fruit trees) required for successful codling moth control.

Concentrations of 5 million IJs/tree (Lacey & Unruh, 1998), 2 million IJs/tree (Unruh & Lacey, 2001; Lacey *et al.*, 2006a), 1 million IJs/tree (Lacey *et al.*, 2006a), and 0.25 million IJs/tree (De Waal *et al.*, 2011a) have been used in various field trials. A concentration of 1 million IJs/tree seems to provide satisfactory control of the codling moth, if the environmental conditions and the application technique used, are conducive to IJ survival (Unruh & Lacey, 2001; De Waal, 2008).

Overall, the ideal setting that is essential for successful codling moth control is an application either early in the morning, in the evening, or on cloudy days, when there is no or a minimal amount of

wind, and a few hours with temperatures above 20°C, during a 24 h exposure time. The best time for application was in the evening, with the degree of infectivity differing significantly between morning and evening application (Unruh & Lacey, 2001). Wetting was recommended, as doing so increased the amount of moisture that was available for nematode survival and movement, thus affecting EPN success (Lacey & Unruh, 1998; Unruh & Lacey, 2001; Lacey *et al.*, 2006a; De Waal, 2008).

Lacey *et al.* (2010) tested the effect of two adjuvants (wood flour foam and Barricade® II fire retardant gel) on *S. carpocapsae* and *S. feltiae* performance against the codling moth in the field. A significant improvement in larvicidal activity was observed, due to the addition of the adjuvants to the nematode suspensions (Lacey *et al.*, 2010). For both species of EPNs, the wood flour foam gave better results than the gel, with the use of *S. feltiae* resulting in significantly higher codling moth mortality than *S. carpocapsae*. Combined with pre- and post-wetting, the addition of adjuvants to the nematode formulation to improved EPN efficacy even further (Lacey *et al.*, 2006a, 2010; De Waal *et al.*, 2013).

Lacey *et al.* (2006b) compared the effect of different mulch types on the activity of *S. feltiae* and *S. carpocapsae*. There was no difference between EPN activity in mulched and non-mulched plots. In this study, mulch seemed to have a greater effect on the efficacy of *S. carpocapsae* than it did on that of *S. feltiae*. The latter appeared to be more affected by the concentration applied than by the addition of mulch (Lacey *et al.*, 2006b). *Steinernema carpocapsae* caused significantly higher mortality with mulches of paper, grass, hay, and the bare plots, compared with the mortality in wood chip plots (Lacey *et al.*, 2006b). Combining *S. carpocapsae* and *S. feltiae* with an adjuvant (wood flour foam) sprayed onto fine wood mulch, a pronounced effect on infectivity was observed, with *S. feltiae* causing significantly higher mortality than *S. carpocapsae* (Lacey *et al.*, 2010).

De Waal *et al.* (2011b) examined the effect of two types of readily available mulches (straw and apple wood chips) on the activity of *H. zealandica*, combined with Solitaire™. No significant difference was found between the two mulch types, although higher mortality was recorded in the apple wood chip plots than in the straw plots in both trials.

Mulches combined with the application of EPNs, might play a significant role in reducing the number of overwintering larvae in orchards with smooth bark trees (Lacey *et al.*, 2006b). The mulch would provide an alternative overwintering site, with resultant higher EPN efficacy than might

otherwise be possible, as it provides a site that is relatively easy to treat, and where moisture levels can easily be manipulated (Lacey *et al.*, 2006b).

Obstacles in the use of EPNs for codling moth control

As with all biocontrol agents, EPNs are not as predictable in their efficacy as are the chemicals that are more traditionally used, so erratic results might be obtained when the former are applied in the field (Gaugler, 1988; Cross *et al.*, 1999). Both De Waal *et al.* (2013) and Van Niekerk & Malan (2014b), diverted from using optimum conditions, especially with regard to temperature, which is considered to be the main environmental factor leading to poor codling moth control.

Nematodes, which occur naturally in the soil environment, have proved effective against the soil-dwelling stages of numerous insect pests (Campbell *et al.*, 1995; Grewal *et al.*, 2001). In the case of codling moth control, EPNs are applied above ground, where they are exposed to such environmental conditions as low humidity, from which they are buffered in the soil environment (Gaugler, 1988; Grewal *et al.*, 2001). The greatest hindrance to the above-ground application of EPNs is therefore, poor nematode persistence after application to exposed foliage or bare tree trunks (Gaugler, 1988; Lello *et al.*, 1996), which in turn, adversely affected their host-finding ability, and thus their infectivity (Campbell *et al.*, 1995).

Environmental challenges

Temperature is a major factor influencing nematode efficacy in the field, as it affects nematode development, reproduction, infectivity, and thus persistence (Kung *et al.*, 1991; Glazer, 1996; Lello *et al.*, 1996; Lacey & Unruh, 1998; Mason *et al.*, 1998). *Heterorhabditis zealandica* was most effective at temperatures ranging from 20-25°C (De Waal, 2008). A few heterorhabditid isolates from Israel were effective at higher temperatures (Glazer, 1996). At lower temperatures, *S. feltiae* (Glazer, 1996; Unruh & Lacey, 2001) and *S. kraussei* (Unruh & Lacey, 2001) remained active and maintained their infectivity.

Nematodes, with their hydrostatic skeleton, lack adequate defence against desiccation, thus moisture plays an important role in their survival (Kung *et al.*, 1991). In order to be able to move, nematodes require a film of water around soil particles, thus moisture affects nematode host-finding

ability (Glazer, 1996; Lacey & Unruh, 1998). In the soil, it is possible to maintain high humidity, and to ensure the availability of free water, which is much more challenging above ground (Lello *et al.*, 1996). The highest mortality rate of the codling moth has been achieved at a RH of 85-100% (Lello *et al.*, 1996; Unruh & Lacey, 2001). Tolerance to desiccation differs between species with some able to survive in drier conditions than can others (Lello *et al.*, 1996; Unruh & Lacey, 2001).

Strategies proposed to overcome moisture-limiting environments, thereby preventing desiccation and enhancing host infection, include the addition of humectants and anti-desiccants to EPN suspensions (Unruh & Lacey, 2001), the pre-wetting and post-wetting of orchards before and after EPN application (Unruh & Lacey, 2001), the installation of overhead mist irrigation in orchards (Cross *et al.*, 1999), and a more economical alternative, the application of nematodes on cloudy days with little to no wind, or in rainy weather (Unruh & Lacey, 2001). The movement of nematodes is enhanced, and the hydrophobicity of cryptic areas is decreased by pre-wetting and post-wetting, for at least 5 h after EPN application, this helps ensure that there is available water during the infection phase (Unruh & Lacey, 2001).

Application challenges

A multitude of methods are used for applying chemicals in orchards, many of the same methods have been used to apply EPN above ground (Lello *et al.*, 1996; Arthurs *et al.*, 2004; Shapiro-Ilan *et al.*, 2006). Not every technique is as effective, and just as the method of application affects pesticide effectiveness, so too does it influence EPN effectiveness (Gaugler, 1988; Unruh & Lacey, 2001; Shapiro-Ilan *et al.*, 2006). The level and successful control of above-ground pests, is thus highly dependent on the choice of application method (Lello *et al.*, 1996; Mason *et al.*, 1998; Shapiro-Ilan *et al.*, 2006).

Although many of the application methods used to apply EPNs are similar to those used to apply chemicals (Gaugler, 1988; Mason *et al.*, 1998; Fife, 2003; Shapiro-Ilan *et al.*, 2006), there are some differences. The reasons for these differences result from the many factors (pressure differentials, droplet size, pump recirculation, and hydrodynamic stress, i.e. nozzle type) that affect, and contribute to EPN efficacy (Fife, 2003; Fife *et al.*, 2003; Shapiro-Ilan *et al.*, 2006).

Considering droplet size and spray distribution when applying IJs in orchards is critical (Lello *et al.*, 1996). A wide range of droplet sizes is produced using conventional nozzles. The minimum required droplet size to carry an IJ is 178 µl. Therefore the type of nozzle used, plays an important role in successful nematode delivery onto foliage (Lello *et al.*, 1996). The overall goal is to find a technique that is effective, but which uses the lowest concentration of nematodes, and the lowest volume of water (Lello *et al.*, 1996).

Flow rates and pressure have also been found to be important factors in IJ delivery, with droplet size increasing with flow rate (Lello *et al.*, 1996). Fife *et al.* (2003) found that the effect of the pressure differential was species-specific, and different EPN species can handle different pressure differentials. For example, the maximum pressure that is required to maintain at least 85% viability of *S. carpocapsae* and *H. bacteriophora* is less than 2000 kPa (290 psi), whereas, for *H. megidis*, it is less than 1380 kPa (200 psi) (Fife *et al.*, 2003). In the same study, it was shown that pump recirculation has no effect on EPN viability, and that a reduction in EPN efficacy might be the result of the prevailing temperature. By increasing the volume of liquid in the spray tank, and by using diaphragm or roller pumps, instead of centrifugal pumps, the effect of high temperature can be moderated, and EPN viability can be maintained (Fife *et al.*, 2003).

Adjuvants are added to EPN formulations to enhance their performance (Mason *et al.*, 1998). They increase the spread of the formulation, ensuring greater coverage of fruit tree surfaces, and reduce surface tension of droplets; thus facilitating improved movement of IJs (Lello *et al.*, 1996). Adjuvants also slow down the rate of evaporation of the formulation, reducing the adverse effect of desiccation in IJs (Mason *et al.*, 1998), thus increasing their persistence and distribution on tree surfaces, and therefore their effectiveness (Unruh & Lacey, 2001).

A wide range of adjuvants is available, although none are suitable for addition to all nematode suspensions. Adjuvants need to be screened for toxicity against nematodes, as well as for the enhancement of their efficacy. A number of factors, such as method of application, host plant surface, and target insect characteristics, affect adjuvant performance (Mason *et al.*, 1998).

The above-mentioned obstacles could be overcome by means of further investment in the research into, and the development of, EPNs, as well as by means of the collaboration, and the cooperation, of nematologists around the world. Only when farmers, scientists and agrochemical

companies start to work together will fast and effective, progress be made in the field of biocontrol, using EPNs.

Aim of the study

To contribute to the development of a standardised protocol for effective EPN efficacy evaluation, by applying the knowledge obtained from previous and current research, aimed at controlling the diapausing population of the codling moth in orchards and wooden fruit bins.

Objectives of the study include the following:

- to determine a standard protocol to test for EPN efficacy in field trials;

- to determine the lowest effective IJ concentration for field application;

- to evaluate the potential of commercially available EPNs against the codling moth;

- to evaluate EPN performance under different climatic conditions in the Western Cape province; and

- to use mini-bins to determine the efficacy of commercially available nematodes.

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

Evaluation of the above-ground application of entomopathogenic nematodes for the control of diapausing codling moth (*Cydia pomonella* L.) under natural field conditions

Abstract

In South Africa, after harvest and prior to winter, when the entire codling moth population enters diapause, no control measures are applied in apple and pear orchards. The biocontrol potential of three imported entomopathogenic nematode (EPN) isolates, *Steinernema feltiae* and two isolates of *Heterorhabditis bacteriophora* (Hb1, Hb2), as well as a local isolate, *Steinernema yirgalemense*, were evaluated for the control of the codling moth under local field conditions. All concentrations of *S. yirgalemense,* applied by immersion in a suspension of nematodes, gave > 98% control. The two formulated isolates of *H. bacteriophora*, Hb1-f and Hb2-f, gave < 30% control. When using the same nematode isolates all as *in vivo* produced, *S. yirgalemense* still resulted in a higher codling moth control of > 90%, compared to 54% and 31% control by *H. bacteriophora* Hb1 and Hb2 isolates, respectively. In follow up field trials*, S. feltiae* resulted in ≥ 80% control, and was thus more effective than both the *S. yirgalemense* and the *H. bacteriophora* (Hb1) isolates, with 66% and 24%, and 24% and 9% control, for two separate trials, respectively. To validate the data obtained from the field trials, subsequent laboratory bioassays were conducted evaluating temperature regimes, following the same temperature cycle as under natural conditions, with a constant humidity of 100%. *Steinernema feltiae* proved to be most effective, causing > 90% mortality, followed by *S. yirgalemense*, with 78% mortality. The two *H. bacteriophora* isolates (Hb1, Hb2) under the above-mentioned laboratory conditions, resulted in 73% and 59% control, respectively. Humidity, thus seems to be the most important factor affecting EPN efficacy during above-ground applications. From the results obtained, it can be concluded that *H. bacteriophora* will not be suitable for the control of the codling moth, with *S. feltiae* proving to be a better candidate than *S. yirgalemense* for such control purposes.

Introduction

The codling moth, *Cydia pomonella* (L.), which is an economically significant pest of apples and pears, has spread around the world due to agricultural practices (Blomefield *et al.*, 1997). The distribution of this pest is attributed to its extraordinary ability to adapt to a variety of climatic, as well as trophic conditions (Audemard, 1991). South Africa has one of the highest codling moth infestation potentials in the world, due to the country's favourable climatic conditions as well as to the lack of effective natural enemies of this moth pest. In neglected orchards, up to four generations may occur per growing season, with infestation rates being as high as 80% (Myburgh, 1980; Blomefield, 2003; Pringle *et al.*, 2003).

In recent years, the primary method of codling moth control has been heavily reliant on the use of such broad-spectrum chemicals as organophosphorus and pyrethroid insecticides (Lacey & Unruh, 1998; Lacey & Chauvin, 1999). Due to the rise in awareness of the risks of non-selective chemical pesticides and the development of codling moth resistance to chemical applications (Giliomee & Riedl, 1998; Riedl *et al.*, 1998), restricted use of such products was implemented. This initiative has led to the control strategy shifting from a primarily chemical control approach to a more area-wide and multifaceted, integrated pest management (IPM) approach, where the minimal use of pesticides is emphasised (Blomefield, 2003; Addison, 2005). This has led to a search for more selective chemicals, with a softer environmental profile, as well as to the adoption of biological alternatives (Ehlers, 1996). In South Africa, IPM involves the combination of softer chemicals, and the use of such techniques as the sterile insect technique (SIT), and pheromone mating disruption. In addition, other biocontrol measures that are employed, are the use of the *Cydia pomonella* granulovirus (CpGV), egg parasitoids in the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), and entomopathogenic nematodes (EPNs), of which the latter two methods are still in an experimental phase when the current study was conducted.

EPNs of the families Steinernematidae and Heterorhabditidae have been extensively studied, due to their potential as biological control for not only the codling moth, but also for a vast array of other insect pests (Ehlers, 1996; Lacey & Georgis, 2012). With the exception of Antarctica, the organisms concerned are found worldwide, and occur naturally within the soil, where they are buffered from such environmental extremes such as high temperatures and varying moisture levels (Griffin *et al.*, 1990;

Kung *et al.*, 1991; Grewal *et al.*, 2001). Both of the families have a symbiotic relationship with a specific genus of bacterium; steinernematids are associated with the genus *Xenorhabdus* Thomas & Poinar, 1979, whereas heterorhabditids are associated with *Photorhabdus* Boemare, Akhurst & Mourant, 1993 (Forst & Clarke, 2002; Griffin *et al.*, 2005).

In 1955, Weiser, as well as Dutky and Hough, simultaneously discovered the unique relationship between the codling moth and EPNs in Czechoslovakia (Central Europe) and Virginia (Eastern Coast of North America), respectively. The interest in using EPNs as a biological control agent was ignited by this discovery (Dutky & Hough, 1955) leading to a multitude of studies being undertaken on the control potential of EPNs against the codling moth. The studies included, determining optimum environmental conditions (Kung *et al.*, 1991; Wright, 1992; Grewal *et al.*, 1994; Lacey & Unruh, 1998; Arthurs *et al.*, 2004; De Waal *et al.*, 2010; Navaneethan *et al.*, 2010; De Waal *et al.*, 2011a, 2013), on application technique (Lello *et al.*, 1996; Lacey & Unruh, 1998; Unruh & Lacey, 2001; Fife, 2003; Fife *et al.*, 2003; Lacey *et al.*, 2006a; De Waal *et al.*, 2011b), on bin trials (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005; De Waal *et al.*, 2010), on post-application conditions (Lacey & Unruh, 2005; Lacey *et al.*, 2006a), and on the use of mulches (Lacey *et al.* 2006b; De Waal *et al.*, 2011a) and adjuvants (Lacey *et al.*, 2005, 2006a, 2010; Navaneethan *et al.*, 2010; De Waal *et al.*, 2013) to improve the success of control.

In South Africa, no control options are available for application in apple and pear orchards during the period where the entire codling moth population enters diapause before the winter months. EPNs could fill this gap, as well as the important niche left by the withdrawal of certain chemicals, in terms of a comprehensive IPM strategy. New chemicals that are on the market might not be as effective, due to their softer chemical profile, with them acting more slowly, and being more expensive, than the latter. EPNs have proven, in many cases, to be effective biological alternatives to chemicals with regards to the codling moth, as not only do they have no negative effect on the environment but, in addition, they provide short-term control of the winter diapausing population.

Another advantage of using EPNs for the control of the codling moth is that resistance to these nematodes has never been reported. In an overwintering population, the insects that escaped chemical control during the previous season, due to resistance development, are likely to be eliminated with the use of these nematodes. By including the use of EPNs in the IPM strategy,

producers should be able to start the following growing season with a much reduced codling moth population, with lesser chemical resistance, as a benefit brought about through the use of an IPM strategy.

The objective of this study was to evaluate the control of overwintering diapausing codling moth larvae, 24 h post EPN application. Field trials were conducted by applying various nematode concentrations and isolates under different environmental conditions, mainly consisting of variable temperature and humidity levels. To validate data from the field trials, subsequent laboratory bioassays were conducted.

Materials and methods

Source of insects

The codling moth eggs and diet were obtained from Entomon Technologies (Pty) Ltd, a codling moth rearing facility established for the production and release, of sterile moths. Entomon is located on Welgevallen Experimental Farm in Stellenbosch, in the Western Cape province of South Africa. Diapausing larvae were reared from eggs on artificial diet, in a growth chamber under diapausing conditions [photoperiod 10:14 (L:D), 25°C, 60% RH]. Once they developed into fifth-instar larvae, they were stored in the diet, at 4°C until used.

Source of nematodes

Formulated products of *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 and two isolates of *Heterorhabditis bacteriophora* Poinar, 1976 (Hb1-f and Hb2-f) were obtained from River Bioscience, Eastern Cape province, imported from e-nema, Schwentinental, Germany. After the formulated products had been used in the first field trial, nematodes from the imported formulations were recycled in final instar *Cydia pomonella* (L.) larvae and were then used as such in subsequent experiments.

Infective juveniles (IJs) of the different imported nematode isolates, as well as of a native species, *Steinernema yirgalemense* Mráček, Tesfmariam, Gozel, Gaugler & Adams, 2005 (EU625295), were reared using codling moth larvae in a growth chamber at 25°C for a period of seven days, before

being transferred to a modified White trap (Kaya & Stock, 1997). The IJs were harvested within the first week of emergence, were stored in 150 ml filtered water, in horizontally placed, vented culture flasks at 14°C, which were shaken weekly for aeration. An hour before each trial, nematode concentrations were calculated, following the procedure described by Navon and Ascher (2000). Nematode isolates used in the study are indicated in Table 2.1 below.

Table 2.1. Source of heterorhabditids and steinernematids used in the study.

Species	Isolate	Origin
Heterorhabditis bacteriophora	Hb1-f	e-nema, Germany (formulated)
Heterorhabditis bacteriophora	Hb2-f	e-nema, Germany (formulated)
Heterorhabditis bacteriophora	Hb1	e-nema, Germany (recycled)
Heterorhabditis bacteriophora	Hb2	e-nema, Germany (recycled)
Steinernema feltiae	Sf	e-nema, Germany (recycled)
Steinernema yirgalemense	157-C	Friedenheim, Mpumalanga (recycled)

Field trial layout

Semi-field trials were conducted in a 16-year-old, 0.416 ha block, consisting of 11 rows of Pink Lady, Royal Gala and Granny Smith apple trees, at the Welgevallen Experimental Farm in Stellenbosch. The spacing was 1.5 m between trees and 4 m between rows, with an average of 44 trees per row.

The experimental design consisted of four rows, with eight treatment trees per row (i.e. 32 treatment trees). On either side of the block were two buffer rows, with one buffer row between each treatment row, and four buffer trees between treatment trees. Treatment trees were selected in a completely randomised design.

The iButtons® [Maxim Integrated iButton® temperature/humidity logger (DS1923)] were mounted on a scaffold branch (±1.5m above ground) of the tree located in the middle of each treated row. The temperature and humidity levels experienced during the 24 h exposure of the codling moth larvae to field conditions were recorded after nematode application.

Codling moth larval containment protocol

Cylindrical wire-mesh (40 mesh/425-μm aperture size) cages, based on the design by Duncan *et al.* (2003) and De Waal *et al.* (2011a,b), were constructed by rolling pieces of wire mesh (40-mesh) around a glass cylinder, and then removing the glass cylinder (Fig. 2.1). A plastic cap on the one end was glued shut, while the other end of the cylinder was left unglued, to grant access to the cage. A cage measured 7×3 cm in diameter. After being filled with apple tree bark and 20 codling moth last-instar larvae, the cages were closed with another plastic cap.
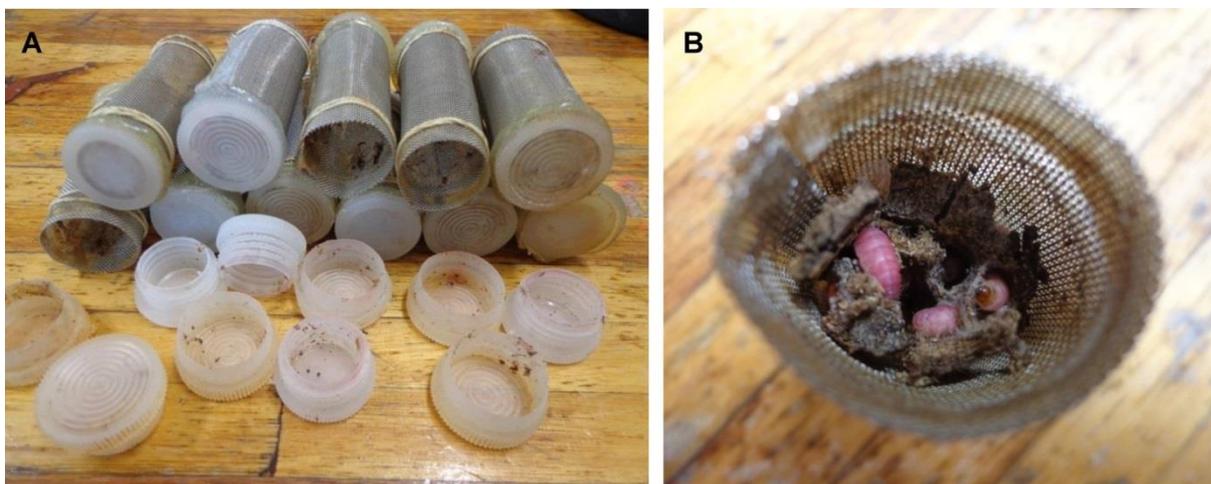
Fig. 2.1. A. Wire cages with plastic caps glued onto one side. **B.** Loaded cage, filled with apple tree bark and 20 last-instar diapausing codling moth larvae, used as a containment method in field trials.

Cages loaded with bark and codling moth larvae were placed in 1-L plastic containers for 24 h, to provide the larvae enough time to spin cocoons, and to ensure that the cages were secured in such a way as to prevent the larvae from escaping. Eight cages, each of which contained 20 last-instar diapausing codling moth larvae (*n* = 160), were used per treatment, with a total of 640 larvae being used for each trial.

EPN application protocol

The cages were thoroughly pre-wetted, by submerging them in a beaker of filtered water, prior to treatment applications (Fig. 2.2). EPN suspensions (200 ml) of a predetermined concentration were poured into 500-ml beakers, and the cages were dipped by completely submerging them in the nematode suspension. The cages were tilted onto the lip of the beaker, until all excess suspension

that collected in the plastic caps had been drained into the beaker. The cages were kept moist by misting them with filtered water contained in handheld, household sprayers every 2 h, for the first 6 h (till late afternoon) of the trial.

Fig. 2.2. A. Nematode suspension. **B.** Application method - dipping the cage in the suspension, and the prevention of dripping.

Evaluation protocol

After 24 h, on retrieval of the cages from the orchard, the bark was removed from each cage (Fig. 2.3), whereupon the codling moth larvae were recovered, and washed with filtered water, to remove any surface nematodes. Larvae from each cage ($n = 20$) were placed in separate Petri dishes (9 cm in diameter) on moist filter paper. Petri dishes from each treatment ($n = 8$) were enclosed in separate 2-L plastic containers lined with moist paper towelling and placed in a growth chamber at 25ºC for 72 h (3 days). Mortality by infection was assessed by means of the dissection of each larva.
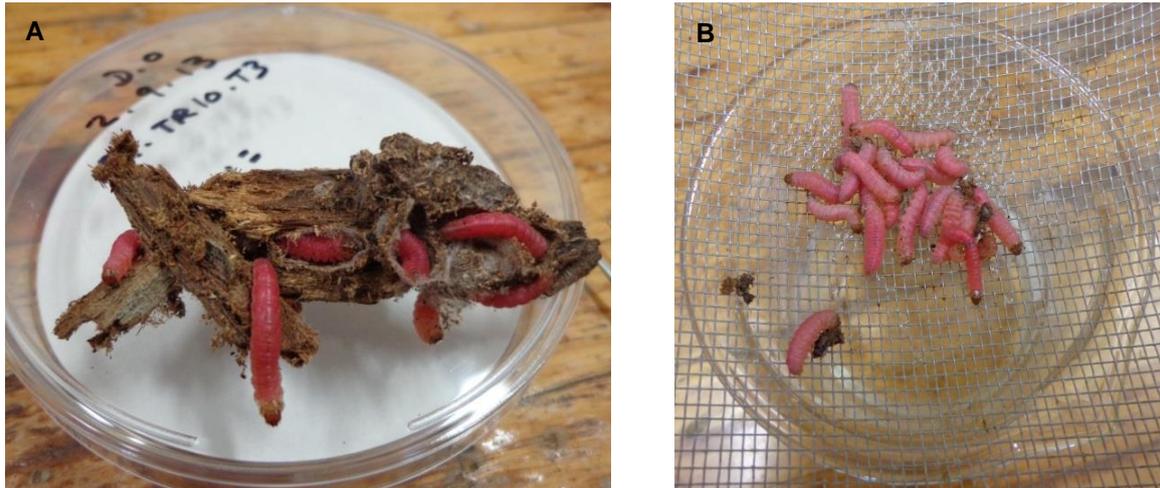
Fig. 2.3. A. Apple bark removed from the cages after exposure for 24 h in the field, after nematode application. **B.** Codling moth larvae being rinsed with distilled water to remove surface nematodes

Steinernema yirgalemense concentration field trials

The cages were loaded with apple bark and codling moth larvae, as described in the containment protocol. A cage was tied to a scaffold branch that was situated close to the main stem of each treatment tree. Two trials were conducted, using different nematode concentrations. In the first trial, the *S. yirgalemense* concentrations applied were 1 000, 2 000, 4 000 IJs/ml and water only was used as the control. In the second trial, *S. yirgalemense* was applied at concentrations of 250, 500, 1 000 IJs/ml, also with water only as the control.

Trials were conducted in the morning, between 10:00 and 11:00, after which the application protocol was followed. After having been exposed for 24 h, the cages were retrieved and the evaluation protocol was followed to determine the mortality caused by infection.

Field trials with different nematode species

Cages, loaded with the codling moth larvae and apple bark, were prepared according to the described containment method. In the first trial, EPN suspensions of the formulated product of the two *H. bacteriophora* strains (Hb1-f and Hb2-f) and *S. yirgalemense* (recycled), at a concentration of 1 000 IJ/ml, were applied to the cages set into the treatment trees, with water only as a control. After an exposure period of 24 h following the treatment, on retrieval from the field, the cages were evaluated as has previously been described.

In the second trial, the same nematode isolates and concentrations were used, but the formulated *H. bacteriophora* strains were also recycled using the codling moth as host. After an exposure period of 24 h under field conditions and retrieval of the codling moth larvae from the cages, the evaluation protocol was followed, to determine the mortality caused by infection. Both trials were conducted between 10:00 and 11:00 in the morning, and on two separate dates, with fresh batches of nematodes.

Field trial species comparison with *Steinernema feltiae*

The cages, on having been prepared according to the described containment method, were tied to a scaffold branch sited close to the main stem of each treatment tree. The performance of recycled *H. bacteriophora* (Hb1), *S. feltiae* and *S. yirgalemense* was evaluated under field conditions. EPN suspensions of three nematode species, at a concentration of 1 000 IJs/ml, were applied to the cages that were hung in the treatment trees, with water only being applied as a control. The trials were conducted early in the morning, between 10:00 and 11:00, after which the application protocol was followed. After the cages had been exposed for 24 h under field conditions, they were retrieved from the field and the evaluation protocol was followed to determine the mortality caused by infection. The trial was repeated on a different test date, using freshly recycled nematodes of the same nematode species.

Laboratory bioassay with different nematode isolates

The same containment method that was used for the field trials was also employed for the laboratory bioassays. The different pilot trials were conducted under laboratory conditions, with a delimiting concentration of 25 IJs/ml being chosen for the study. The species used included *H. bacteriophora* (Hb1 and Hb2), *S. feltiae* and *S. yirgalemense*. All nematode species were cultured using codling moth larvae as hosts. The cages containing the codling moth larvae were prepared according to the containment protocol. Suspensions of the different nematode species were prepared at a concentration of 25 IJs/ml, with water only being applied as a control. After each treatment, the cages were placed in separate 1-L containers that were closed with a lid and then incubated for 24 h, at a temperature regime of 22ºC for 4 h, at 14ºC for 12 h, and at 22ºC for 8 h. After 24 h, the codling

moth larvae, on being retrieved from the cages, were assessed according to the evaluation protocol. The trials were conducted on two separate dates, using fresh batches of nematodes.

Statistical analysis

All trials were conducted on separate test dates, with the data being analysed with the aid of STATISTICA12 software (StatSoft Inc. 2013). If no significant test date versus treatment interactions could be identified, the data from different test dates were pooled and analysed, using a one-way analysis of variance (ANOVA), with *post hoc* comparison of means. When the residuals were found not to be normally distributed, bootstrap multiple comparisons were performed (Efron & Tibshirani, 1993).

Results

Steinernema yirgalemense concentration trial

Trial 1:

High mortality of last-instar diapausing codling moth larvae was found with the three applied *S. yirgalemense* concentrations (1 000, 2 000, and 4 000 IJs/ml), ranging from 95-100%. Analysis of the results using a one-way ANOVA showed a significant effect of the treatments on the codling moth larvae mortality ($F_{(3, 28)}$ = 2264.9; $p < 0.001$). No significant differences were found between T1 (1 000 IJs/ml) and T2 (2 000 IJs/ml) ($p = 0.094$) and between T2 and T3 (4 000 IJs/ml) ($p = 1$). However, a significant difference was found between T1 and T3 ($p = 0.0112$) (Fig. 2.4 A). At a concentration of 4 000 IJs/ml, a 100% infection of the codling moth larvae was obtained, with 98.75% ± 0.82% at a concentration of 2 000 IJs/ml and 95% ± 1.89% at a concentration of 1 000 IJs/ml.
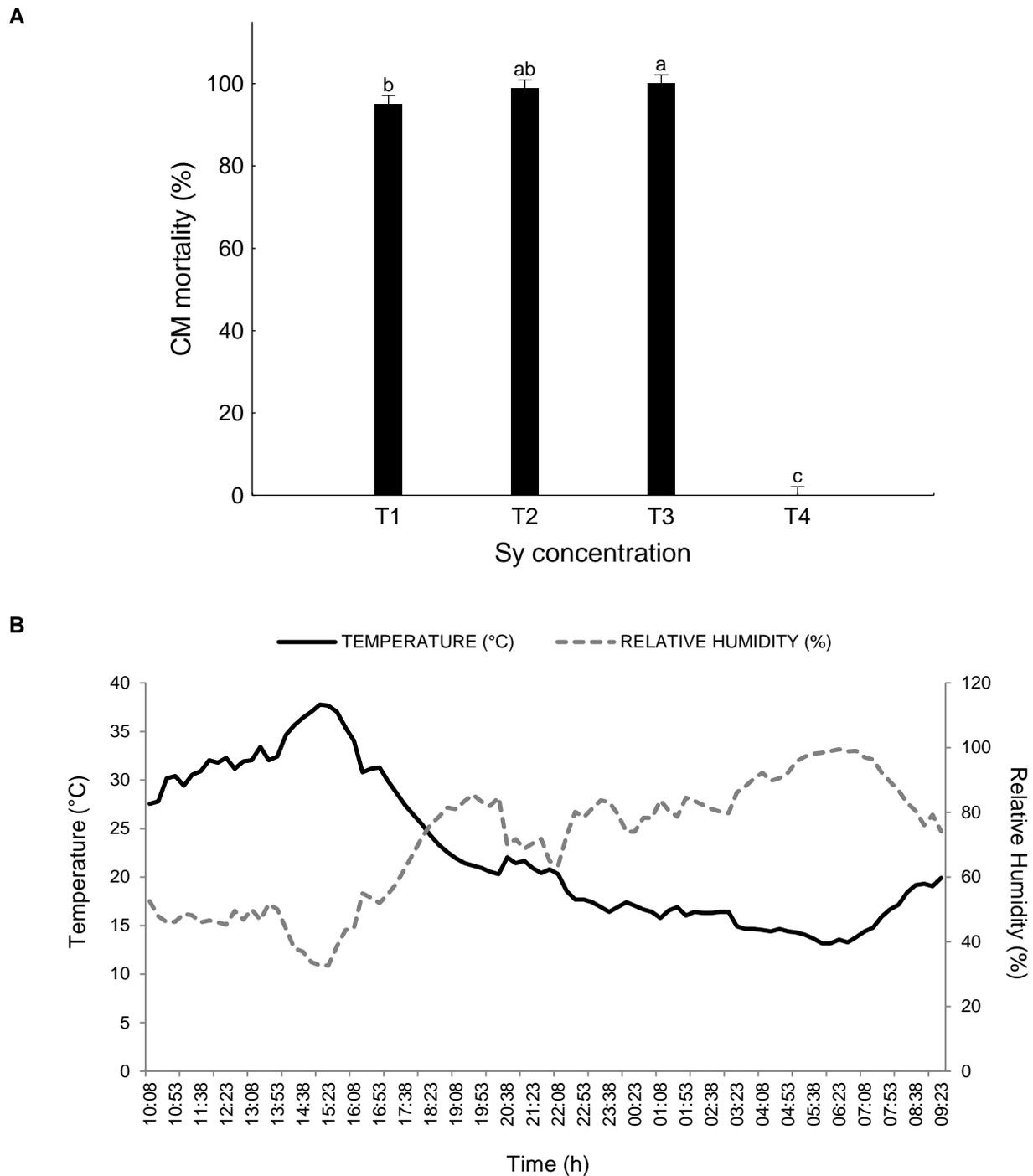
Fig. 2.4. A. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth (CM) larvae after treatment by dipping in three concentrations of *S. yirgalemense* (Sy). The treatments consisted of: T1 = 1 000 IJs/ml; T2 = 2 000 IJs/ml; T3 = 4 000 IJs/ml; T4 = water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 28)}$ = 2264.9; $p < 0.001$). **B**. Temperature and relative humidity data recorded during the 24 h trial period.

During the first trial, the minimum and maximum temperatures were 13ºC during the night and 38ºC just after midday, with an average of 23ºC. The minimum and maximum RH was 33% just after midday when the temperature was the highest, and it was 100% in the early morning, with an average of 71% (Fig. 2.4 B). The temperature increased from around 26°C to above 35°C within the first 5 h of the trial, and then it gradually decreased to below 15°C over the next 15 h. During the last 4 h of the trial, the temperature increased to above 20°C. At the start of the trial, the humidity was above 50%, with it gradually decreasing to below 40% over the first 5 h. After the next 3 h, as the temperature fell, the humidity gradually increased to above 80%. The relative humidity lingered between 60% and 80% for 9 h, after which it steadily increased to 100% for the next 3 h. During the last 4 h, the humidity decreased. No rainfall was experienced during the 24 h trial period.

Trial 2:

The three nematode concentrations of 250, 500 and 1 000 IJs/ml caused a high level of mortality of the codling moth larvae, ranging from 90-99%. A one-way ANOVA analysis of the results showed a significant effect of the different treatments ($F_{(3, 28)}$ = 405.82; p = 0.001). However, no significant differences were found between the three *S. yirgalemense* concentrations applied (Fig. 2.5 A). At a concentration of 1 000 IJs/ml, 98.75% ± 0.82% infection of the codling moth larvae was obtained, with the infection rate being 89.88% ± 4.53% at a concentration of 500 IJs/ml and 98.75% ± 1.25% at a concentration of 250 IJs/ml.

A

B
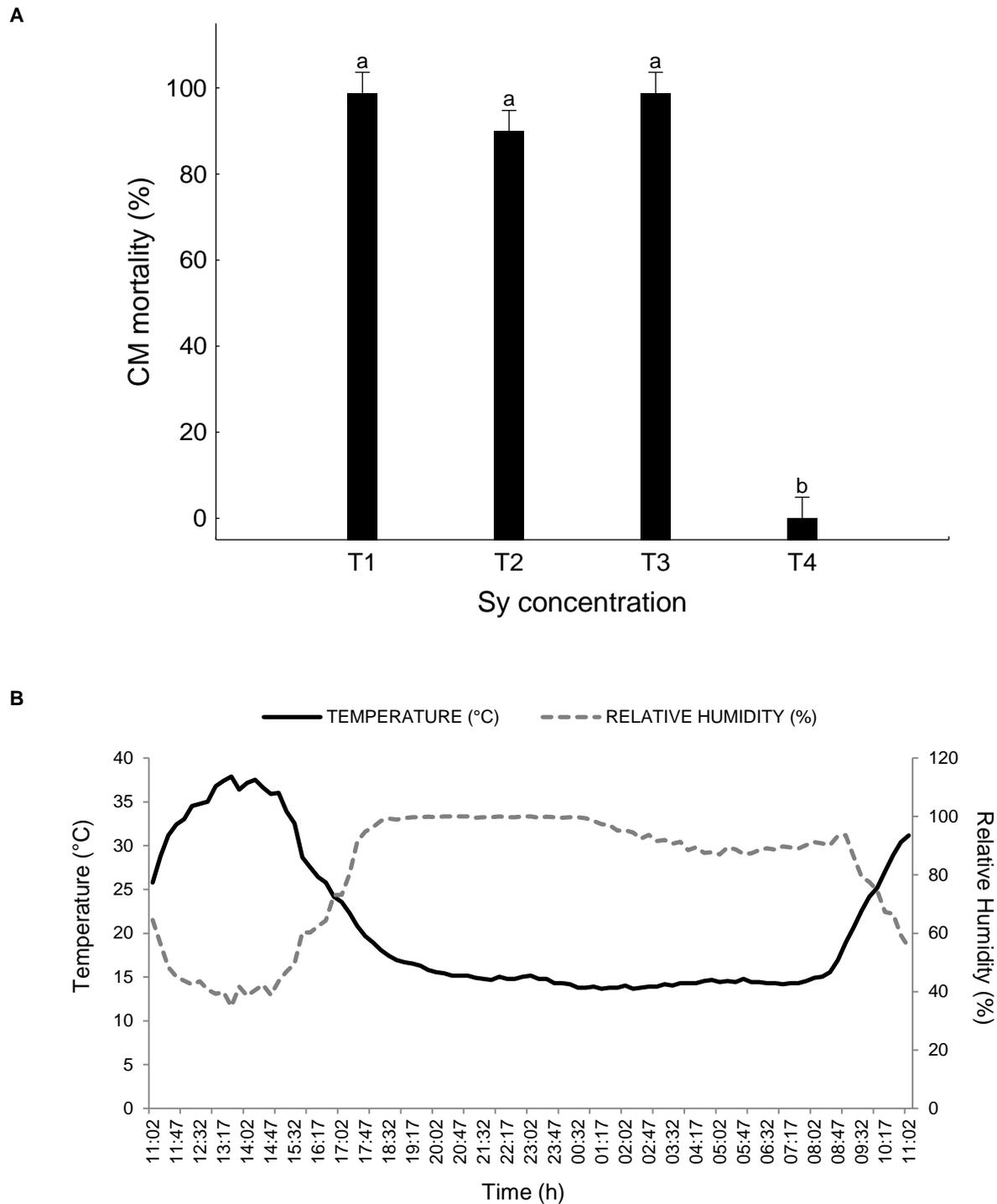
Fig. 2.5. A. Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after treatment by dipping containment cages in three concentrations of *S. yirgalemense* (Sy) during a field trial. The infective juvenile (IJ) treatments consisted of: (T1) 250 IJs/ml; (T2) 500 IJs/ml; (T3) 1 000 IJs/ml; (T4) water only. Different letters above bars indicate significant differences (one-way ANOVA; F $_{(3, 28)}$ = 405.82; p < 0.001). **B.** Temperature and relative humidity data recorded during the 24 h field trial.

During the second trial, the minimum and maximum temperatures were 14ºC and 38ºC respectively, with an average of 20ºC. The minimum and maximum RH was 35% and 100%, with the daily average being 82% (Fig. 2.5 B). The graph, depicting the conditions experienced during the second 24 h exposure period, follows more or less the same cycle as the first, with the humidity decreasing with increasing temperature, and *vice versa*. The trial was started at a temperature above 25°C, which then rose to over 35°C, over the first 2 h. The temperature then slowly decreased to about 15°C during the following 6 h, remaining constant for the next 12 h. During the last 4 h of the exposure period, temperatures gradually increased to over 30°C. The 60% humidity that was recorded at the start of the trial gradually decreased to just below 40%, within the following 3 h. During the following 5 h, the humidity increased to 100%, where it remained for the next 6 h, after which, over a period of 8 h, it gradually tapered off, to just below 90%. The humidity once again dropped to below 60% during the last 4 h of the trial. No rainfall was experienced during the 24 h exposure trial.

Field trials with different nematode species

Trial 1:

Analysis of the results showed a significant effect of the treatments ($F_{(3, 28)}$ = 296.90; $p < 0.001$). A significant difference was found between *S. yirgalemense* and the two *Heterorhabditis* strains, Hb1-f ($p < 0.005$) and Hb2-f ($p < 0.005$), respectively, with no difference ($p = 0.098$) being found between *H. bacteriophora* (Hb2-f) and the control treatment. At a concentration of 1 000 IJs/ml, *S. yirgalemense* caused the highest level of mortality of the codling moth larvae (89.75% ± 0.82%), followed, first, by *H. bacteriophora* (Hb1-f) (28.13% ± 4.72%), and then, by *H. bacteriophora* (Hb2-f) (9.38% ± 1.99%) (Fig.2.6 A).
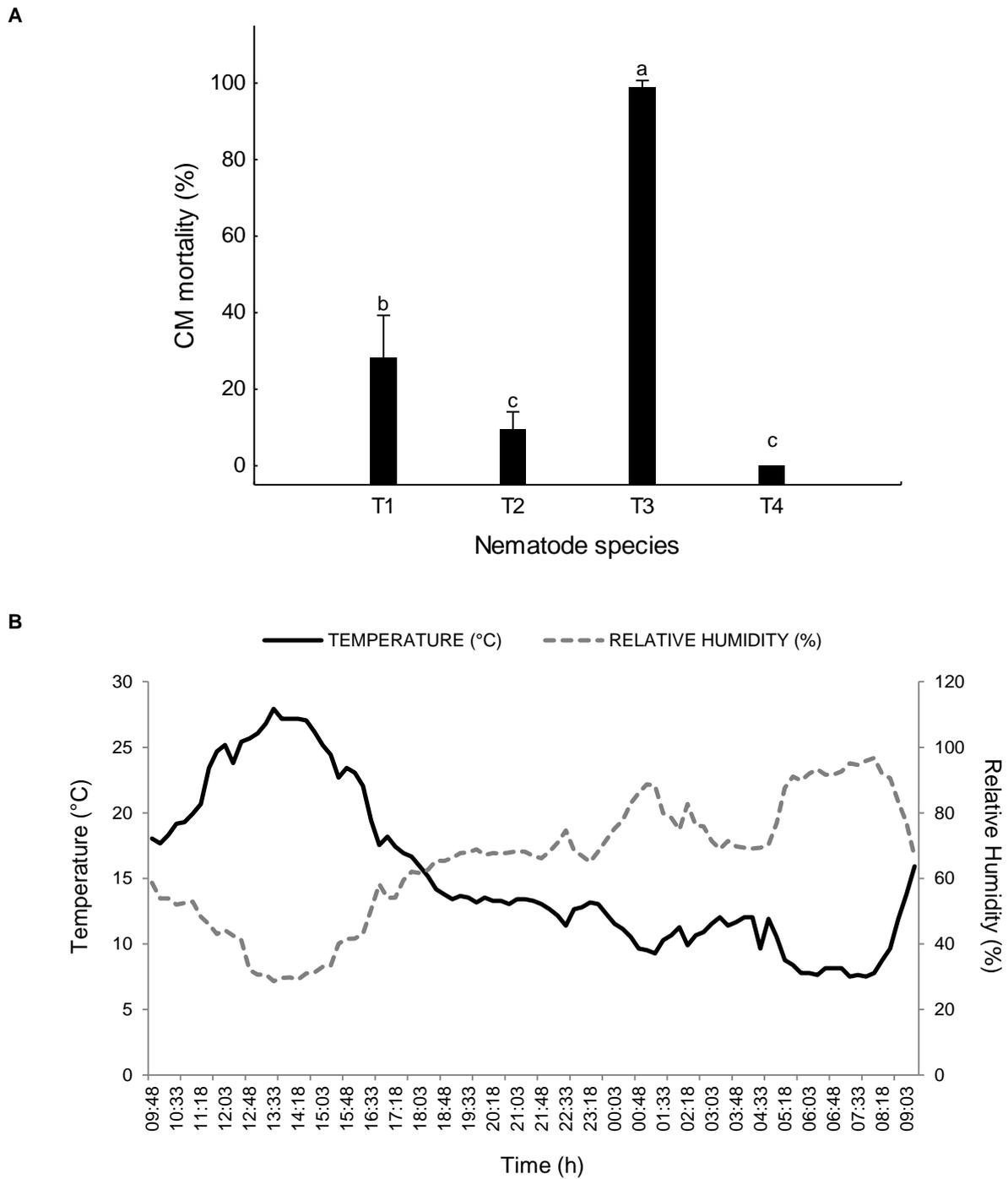
Fig. 2.6. A. Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after treatment by dipping the containment cages using three isolates of nematodes (1 000 IJs/ml) during a field trial. The treatments consisted of: (T1) *H. bacteriophora* (Hb1-f); (T2) *H. bacteriophora* (Hb2-f); (T3) *S. yirgalemense*; (T4) water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 28)}$ = 296.90; *p* < 0.001). **B.** Temperature and relative humidity data recorded during the 24 h trial period.

During the 24 h exposure period, the minimum temperature was 8⁰C during the night, with the maximum temperature being 28⁰C at midday, with an average of 15⁰C. The lowest humidity of 29% was found during the high midday temperatures, and a high humidity of 97% was recorded in the early morning, with an average of 65% (Fig. 2.6 B). During the first 4 h of the trial, the temperature increased from below 20⁰C to above 25⁰C. It then decreased rapidly over a period of 6 h to below 15⁰C, and continued to decrease, albeit more gradually, to below 10⁰C over the next 12 h. Over the next 2 h, the temperature rose to above 15⁰C. The humidity, once again, reflected the exact opposite trend to temperature, with the former decreasing when the latter increased, and vice versa. The humidity decreased from 60% to below 40% within the first 4 h, after which it gradually increased to above 90% over the next 12 h. The humidity then started to decrease to 70% over the next 3 h, after which it once more increased to over 90% over the following 3 h. For the remainder of the trial, the humidity decreased to below 70%. No rainfall was experienced during the 24 h trial period.

Trial 2:

Analysis of the results from the three recycled nematode strains showed a significant difference in effect between the different treatments ($F_{(3, 28)}$ = 52.497; $p < 0.01$), on the codling moth larvae mortality. *Steinernema yirgalemense* differed significantly from *H. bacteriophora* (Hb1) ($p < 0.005$) and from Hb2 ($p < 0.005$) respectively, with the two *H. bacteriophora* isolates also differing significantly from each other ($p = 0.0365$). The highest level of mortality of the codling moth larvae (93.75% ± 3.37%) was obtained for *S. yirgalemense*, followed by *H. bacteriophora* (Hb1) (53.50% ± 8.18%), and *H. bacteriophora* (Hb2) (30.63% ± 6.37%). A significant difference was found between the three nematode treatments; however, no difference was found between *H. bacteriophora* Hb2 and the control (Fig. 2.7 A).
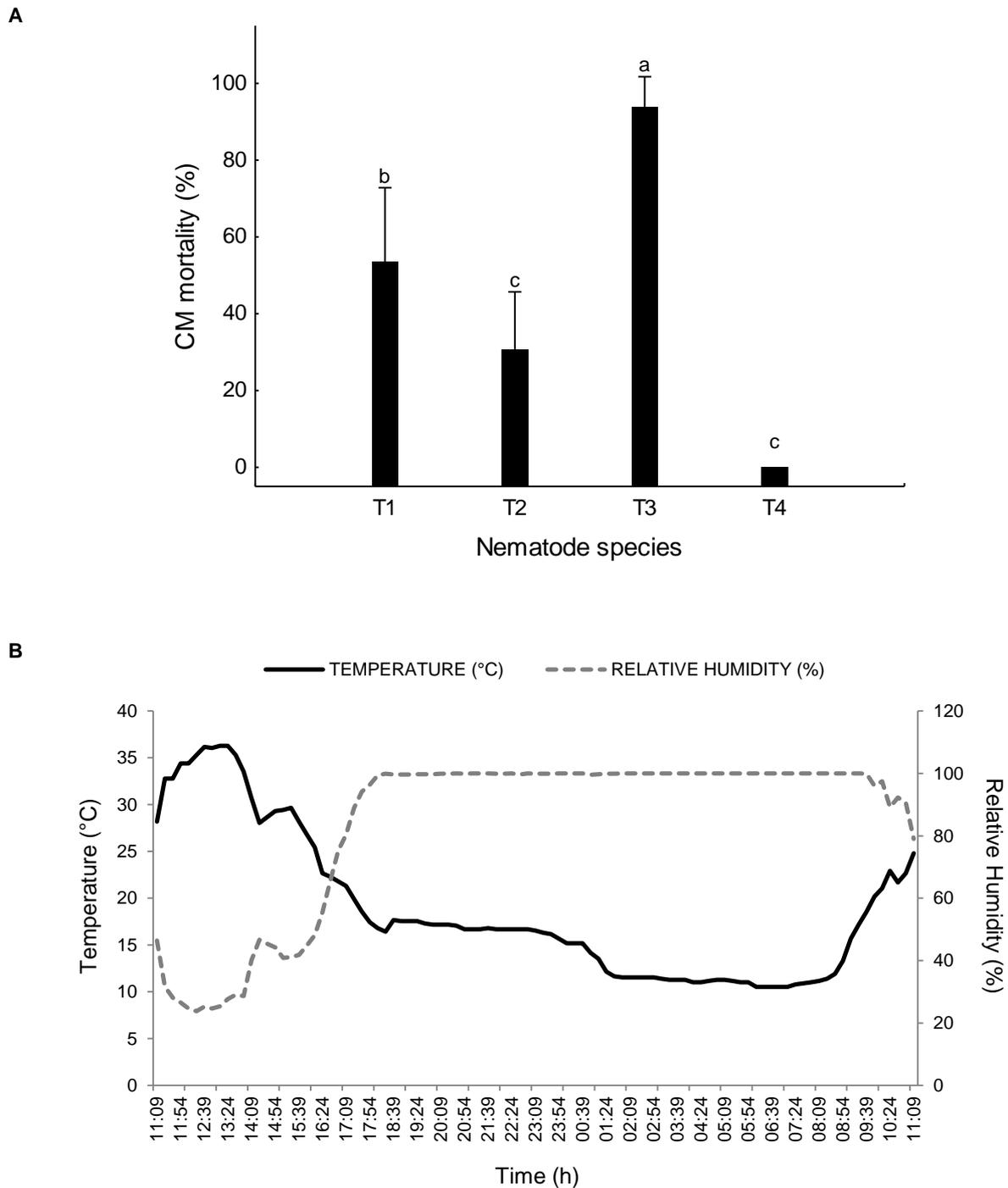
Fig. 2.7. A. Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after treatment by dipping containment cages in three recycled nematode isolates (1 000 IJs/ml) during a field trial. The treatments consisted of: (T1) *H. bacteriophora* (Hb1); (T2) *H. bacteriophora* (Hb2); (T3) *S. yirgalemense*; (T4) water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 28)}$ = 52.497; *p* < 0.01). **B.** Temperature and relative humidity data recorded during the 24 h trial period.

The minimum and maximum temperatures were 11ºC and 36ºC, respectively, with an average of 19ºC. The minimum and maximum RH was 24% and 100%, with an average RH of 84%, until the cages were retrieved from the field (Fig. 2.7 B). During the first few hours of the trial, the temperature rose to above 35ºC, and the humidity dropped to below 30%. As the temperature started gradually decreasing to around 16ºC over the next 3 h, the humidity increased to 100%. The temperature continued to decrease gradually to 11ºC in the following 15 h, whereas the humidity remained constant for the next 16 h. During the last few hours of the trial, the temperature rose to over 25ºC, and the humidity decreased. No rainfall was experienced during this 24 h trial period.

Field trial species comparison with *Steinernema feltiae*

Significant interactions were found between the test dates and the treatments of the two trials ($F_{(3, 65)}$ = 91.508, $p < 0.001$) and, for that reason, the data from the two field trials could not be pooled.

Trial 1:

The analysis of the results from the three nematode species showed a significant effect of the different treatments ($F_{(3, 28)}$ = 34.324, $p < 0.001$) on the codling moth larvae. *Steinernema feltiae* (T2) caused the highest level of infection of the codling moth larvae (79.55% ± 4.98%), followed by *S. yirgalemense* (T3) (65.97% ± 10.74%), and *H. bacteriophora* (Hb1) (24.14% ± 4.20%). Analysis of the results with a one-way ANOVA showed no significant difference (p = 0.827) between the *S. feltiae* and *S. yirgalemense* treatments, but both differed significantly ($p < 0.005$) from *H. bacteriophora* (Hb1) (Fig. 2.8 A).

A

B
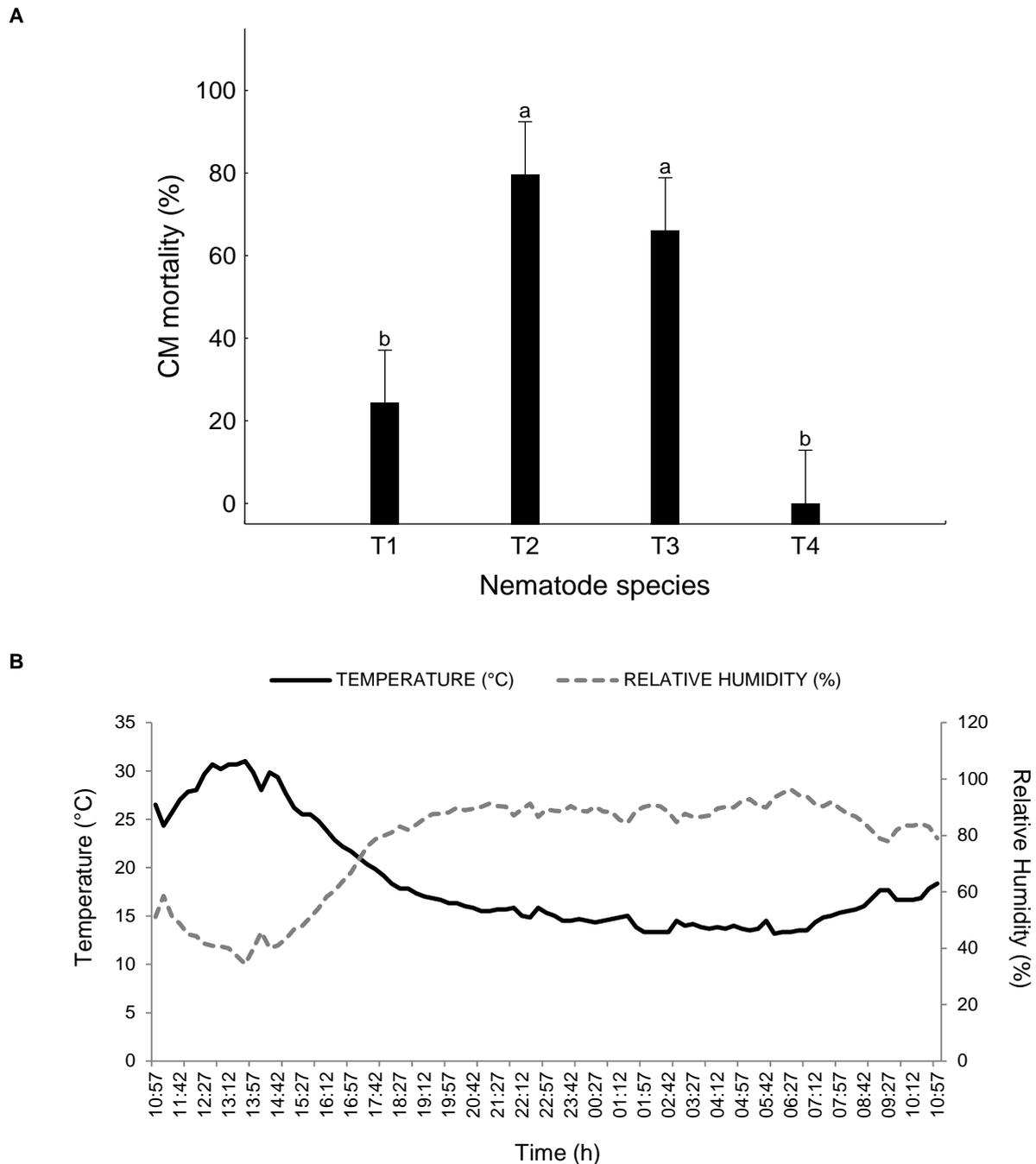
**Fig. 2 8. A**. Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after treatment by dipping cages in three species of recycled nematodes (1 000 IJs/ml) during a field trial. The treatments consisted of: (T1) *H. bacteriophora* (Hb1); (T2) *S. feltiae*; (T3) *S. yirgalemense*; (T4) water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 28)} = 34.324$, $p < 0.001$). **B**. Temperature and relative humidity data recorded during the 24 h trial period.

The minimum and maximum temperatures were 13ºC and 31ºC, respectively, with an average of 19ºC. The minimum and maximum RH was 34% and 96%, with the 24 h average being 77% (Fig. 2.8 B). The temperature increased to over 30ºC during the first 4 hours of the trial, whereas the humidity decreased to below 40%. Following the next 6 h, the temperature decreased to 15ºC, with the humidity increasing to over 90%. Both the humidity and the temperature remained more or less constant for the next 11 h, after which the humidity started to decrease, and the temperature to increase, for the remaining 3 h of the trial. No rainfall was experienced during the 24 h trial period.

Trial 2:

Analysis of the results from the three recycled nematode strains showed a significant difference in the effect between the treatments ($F_{(3, 28)}$ = 200.51, *p* = <0.01) on the codling moth larvae. *Steinernema feltiae* again caused the highest level of mortality of the codling moth larvae (82.37% ± 2.58%), followed by *S. yirgalemense* (23.92% ± 4.21%), and then by *H. bacteriophora* (Hb1) (8.75% ± 4.21%). A significant difference was found between all four treatments applied (Fig. 2.9 A). However, in the case of *H. bacteriophora* (Hb1), there was no difference between the treatment and the control.

A

B
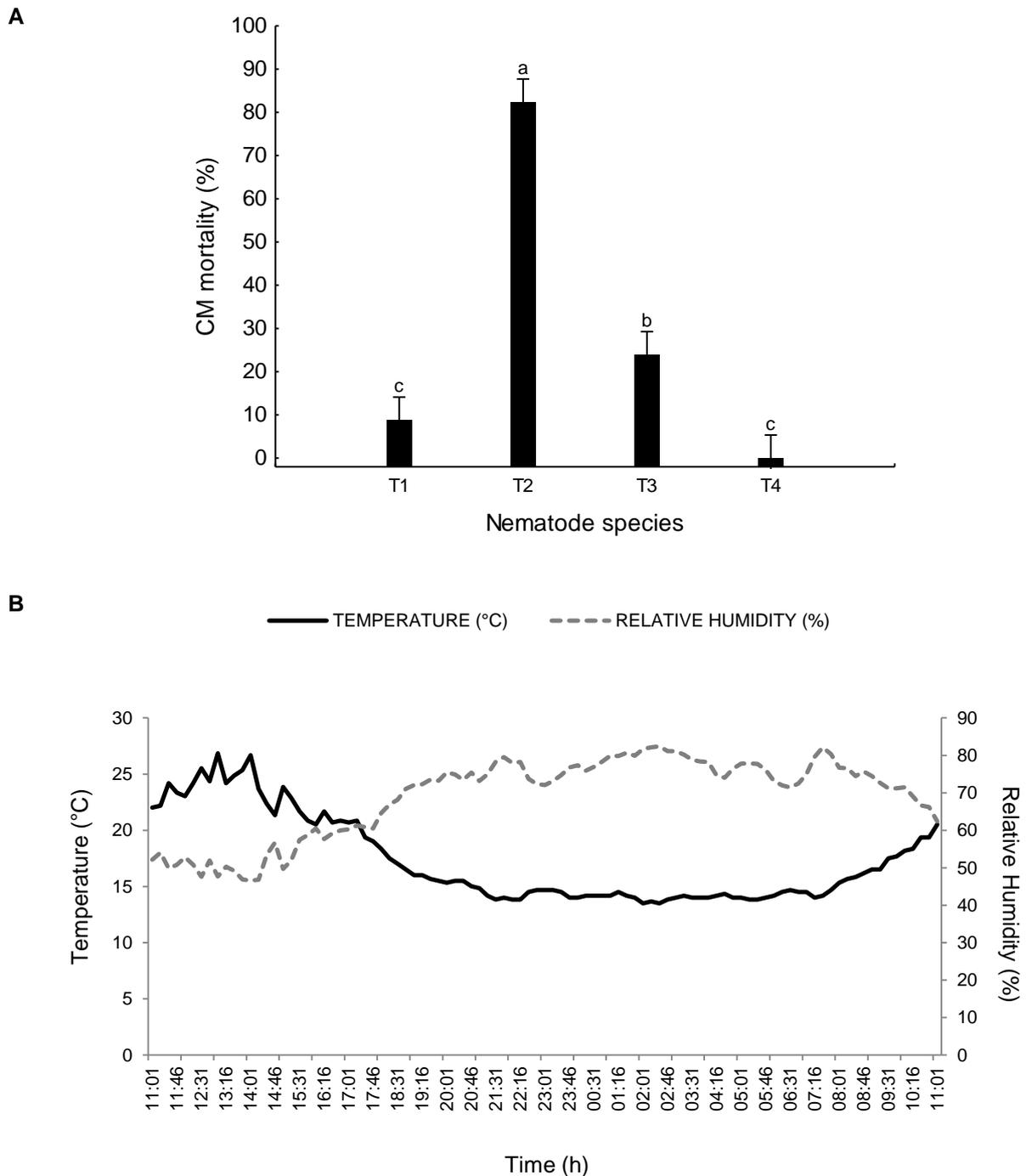
Fig. 2.9. A. Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after treatment by dipping containment cages with three species of nematodes (1 000 IJs/ml) during a field trial. The treatments consisted of: (T1) *H. bacteriophora* (Hb1); (T2) *S. feltiae*; (T3) *S. yirgalemense*; (T4) water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 28)}$ = 200.51, *p* = < 0.01). **B**. Temperature and relative humidity data recorded during the 24 h trial period.

For the 24 h trial conducted, the minimum and maximum temperatures were 14ºC and 27ºC, respectively, with an average of 17ºC. The minimum and maximum RH was 47% and 82%, with the daily average being 69% (Fig. 2.9 B). At the start of the trial, the temperature increased from 22ºC to about 27ºC, while the humidity decreased from 55% to 50%. The temperature then rapidly decreased, and the humidity rapidly increased, over the next 3 h. Both the temperature and the humidity levelled off, remaining constant at 15ºC and 75%, respectively, for the next 14 h. During the last 4 h of the exposure period, the temperature gradually increased to over 20ºC, whereas the humidity dropped to below 65%. No rainfall was experienced during the 24 h trial period.

Laboratory bioassay with different nematode isolates

As there were no significant interactions between the test dates and the treatments of the two trials, the data from the two trials were pooled, with a one-way ANOVA showing significant differences between the treatments ($F_{(4, 75)}$ = 187.02, $p < 0.001$). *Steinernema feltiae* caused the highest level of infectivity of the codling moth larvae (90.86% ± 2.42%), followed by *S. yirgalemense* (77.55% ± 2.94%), and then *H. bacteriophora* (Hb1) (72.45% ± 3.38%), with the lowest infectivity being obtained with *H. bacteriophora* (Hb2) (59.44% ± 2.76%). *Steinernema feltiae* performed significantly better than all the other treatments; however, no significant difference was found between *S. yirgalemense* and the two *H. bacteriophora* isolates applied ($p < 0.001$) (Fig. 2.10).
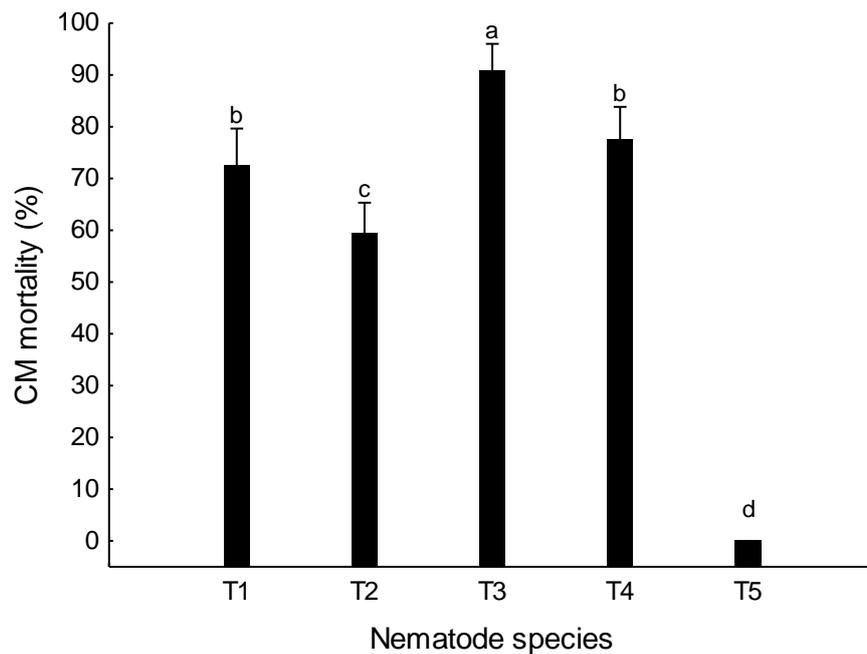
**Fig. 2.10.** Mean percentage mortality (95% confidence limits) recorded for codling moth (CM) larvae after dipping containment cages in suspensions (25 IJs/ml) of nematodes during a laboratory trial. The treatments consisted of: (T1) *H. bacteriophora* (Hb1); *H. bacteriophora* (Hb2); (T3) *S. feltiae*; (T4) S*. yirgalemense*; (T5) water only. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(4, 75)}$ = 187.02, *p* < 0.001).

Discussion

Soil, which is the natural habitat of EPNs, buffers the nematodes against the main environmental variables, humidity and temperature. As the total codling moth population overwinters in cryptic habitats on the tree itself and in debris on the soil around the tree trunk, nematodes would need to be applied as an aerial application. In the Western Cape Province, one of the main environmental factors to be overcome in using EPNs against the codling moth is the cycle of humidity and temperature, as was observed in the current study. In Stellenbosch, in the late autumn and winter months of May to August, the temperature increases significantly during the day, while the humidity decreases, with the opposite occurring during the night. For effective EPN control, application must occur on days when the temperature ranges between 20°C and 25°C, when the humidity is above 85%, and when there is no wind (Kung *et al.*, 1991; Lacey & Unruh, 1998; Unruh & Lacey, 2001; De Waal *et al.*, 2010). The window of opportunity for EPNs to be able to infect the codling moth larvae on a tree in the Western Cape Province only lasts 24 h after application, taking into consideration manual pre- and post-

wetting to compensate for the low daily humidity. Under normal conditions, the trees would be completely dry during the day. As the weather varies from day to day in the field, the results obtained from the trials also tend to vary greatly. In order to explain the variances concerned, field trials with EPNs to control the codling moth were repeated as laboratory bioassays, to explain what the main biological and environmental factors, causing the difference in EPN efficacy are.

The containment method that was used in this study differs from the methods used in previous research with EPNs and codling moth. Other methods include the use of perforated cardboard strips (Lacey & Unruh, 1998); mesh cages (filled with different mulches) (De Waal *et al.*, 2011a); old wooden bin planks (De Waal *et al.*, 2010); apple wood logs (Lacey & Unruh, 1998; De Waal *et al.*, 2011b); and cardboard tree bands (Kaya *et al.*, 1984). In this study, mesh cages filled with apple bark were used as a containment method as they represent a more natural environment, possessing a more realistic water-holding capacity than previous methods used, due to the more typical natural drying process of the apple bark. De Waal *et al.* (2011b) employed three different containment methods during their field experimentation, including the use of wooden planks cut from old fruit bins; the use of pear logs, with holes drilled into them to allow the codling moth larvae to spin cocoons within the logs; and the use of cages filled with pinewood shavings. Significant differences in containment methods were found, with the highest mortality being recorded in mesh cages filled with pine wood shavings, followed by wooden planks, and then by the pear tree logs. De Waal *et al.* (2011b) have raised concerns regarding the host containment methods used during field trials, as they are not representative of such natural cryptic habitats as pruning wounds, and tree bark. The conclusion was drawn from this study that these artificial containment methods were sufficient for purposes of inter-experimental analysis, and for the related conclusions to be drawn, due to the higher levels of control that were obtained using such containment methods, in relation to others.

In the most recent field trials, nematodes were applied using handheld sprayers, or backpack sprayers (De Waal *et al.*, 2011b). In this study, the application technique selected was that of dipping cages into a suspension of IJs of the different species. This method was chosen to allow for a comparison of the efficacy of *S. feltiae* with endemic species, without the risk of adding to biological pollution in orchards.

Field trials were first directed towards the evaluation of an effective *S. yirgalemense* concentration to be applied in the field, in order to obtain optimum codling moth control. Initially, although the nematode treatment concentrations of *S. yirgalemense* used were high, a significant difference was still found between the highest and the lowest concentration used. The environmental conditions experienced during this trial were also more conducive to control with nematodes, as the mean temperature during the 24 h period exposure time was 23°C, with an average humidity of 71%. During the first 6 h, while temperatures ranged between 26°C and 36°C, the humidity was kept high by means of manually spraying the cages every hour, until the humidity rose to 100% during the night. De Waal *et al.* (2013) specifically investigated the effect of humidity on the control of the codling moth, using *H. zealandica*. They concluded that ensuring at least 4 h of conditions that are conducive to nematode activity is necessary to ensure efficacy and subsequent infectivity.

In the second trial to test *S. yirgalemense* concentrations, lower nematode concentrations were used than in the first trial, resulting in a high mortality of > 90%, with no significant differences being found between the different concentration treatments. During this trial, the average temperature was lower than 20°C, and the humidity was higher than 82%, but both the temperature and the humidity basically followed the same pattern as presented in Trial 1. A laboratory bioassay that was performed using 24-well bioassay plates showed that *S. yirgalemense* outperformed five other local endemic species that were tested for codling moth mortality at a concentration of 50 IJs/ml (De Waal *et al.* 2013). However, *S. yirgalemense* had not previously been subjected to any field trials.

Field trials were undertaken to compare the performance of formulated strains of *H. bacteriophora* (Hb1-f, Hb2-f) to the performance of a local isolate*, S. yirgalemense*. The *in vivo* cultured *S. yirgalemense* resulted in a high codling moth larvae mortality of > 90%, whereas the formulated *H. bacteriophora* gave poor codling moth control (< 28%). It is, however, not the correct procedure to compare a formulated product with *in vivo* produced nematodes. In this study, *S. yirgalemense* should be regarded as a control, as no previous information on the efficacy of both *H. bacteriophora* and *S. yirgalemense* was available under South African field conditions. In addition, no locally formulated product was available on the local market to serve as a comparison. Ferreira *et al.* (2014) compared the virulence of the IJs of *H. zealandica* produced by means of *in vivo* methods with that of IJs produced using *in vitro* methods. The results indicated a significant difference in terms of virulence.

The imported formulations were subjected to stress during the commercialisation process (formulation, packaging, transportation, and storage), which might have led to decreased IJ survival or to loss of quality, thus reducing their efficacy. It is thus of major importance, that the correct nematode species, with high virulence, be used for codling moth control, since its efficacy is likely to be affected during formulation.

In the repetition of the previous trial, both the imported and the local isolates were recycled, using codling moth larvae as the *in vivo* host, to enable the making of a fair comparison of the efficacy of the three isolates against codling moth mortality. Significant differences were found between the three species, with both the imported and the recycled *H. bacteriophora* (Hb1, 54%, and Hb2, 31%) performing better than did the formulated product (Hb1-f, 28%, and Hb2-f, 9%). Again, however, *H. bacteriophora* isolates performed significantly more poorly than did the local *S. yirgalemense*.

The bulk of the research that has been conducted using EPNs for codling moth control has been undertaken using *S. feltiae* and *S. carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, as they have been proven to be the most effective EPN species, in terms of codling moth control (Lacey & Unruh, 1998; Lacey & Chauvin, 1999; Unruh & Lacey, 2001; Cossentine *et al.*, 2002; Lacey *et al.*, 2005, 2006a). However, *S. feltiae* has not, as yet, been recovered from South African soils (Grenier *et al.*, 1996; Malan *et al.*, 2006; Molotsane *et al.*, 2007; Hatting *et al.*, 2009; Malan *et al.*, 2011). The natural occurrence of *S. feltiae* on the African continent has only been reported during a survey in Algeria (Tarasco *et al.*, 2009). Previous work with endemic South African species indicates *Heterorhabditis zealandica* Poinar, 1990, *S. yirgalemense* and a new *Steinernema* sp. (J194) to be the most virulent species against diapausing codling moth larvae (De Waal, 2011a).

The local *S. yirgalemense* isolate was compared to recycled batches of the imported *S. feltiae* and *H. bacteriophora* (Hb1). Both *S. feltiae* (80%) and *S. yirgalemense* (66%) resulted in good control of the codling moth, whereas *H. bacteriophora* (Hb1) performed poorly (< 24%), and did not differ significantly from the control. During a follow-up trial, *S. feltiae* (82%) proved to be the most effective of the isolates used, while both *S. yirgalemense* (23%) and *H. bacteriophora* (Hb1) (9%) performed poorly. During the 24 h trial period, the mean ambient temperature was generally low, being 19°C in the first trial, and 17°C in the second trial. Humidity averaged of 77%, which might have contributed to the poor performance of *S.yirgalemense*. In contrast, the good performance of *S. feltiae* that was

obtained during the trial might be attributed to the fact that it is known to be a cold-active nematode (Wright, 1992; Grewal *et al.*, 1994). EPNs are adapted to conditions that are representative of their natural climatic origin (Kung *et al.*, 1991). Therefore, the lesser efficacy of *S. yirgalemense* might be due to it not being adapted to the lower temperature ranges to which it was exposed to during the trial period.

In the simulated laboratory trial, using the same cages filled with bark as a containment method, the humidity was kept at an optimum, and only the temperature was changed to a suboptimal cycle, as would generally be encountered under field conditions in the Western Cape province, Stellenbosch. *Steinernema feltiae* (91%) proved to be significantly more effective against diapausing codling moth larvae than was *S. yirgalemense* (78%). Under the conditions mentioned, *H. bacteriophora* (Hb1) (72%) also gave effective, and comparable control, with the *H. bacteriophora* (Hb2) isolate (60%) resulting in the lowest control of the codling moth. Thus, although the temperature regime followed the same cycle as was likely to have existed in the field, all three isolates resulted in > 59% codling moth mortality under laboratory conditions in 100% humidity. Therefore, the main factor affecting the performance of the different EPN species in the field might be ascribed to the prevailing temperature, and to the generally better performance of all species under conditions of constant high humidity during the 24 h period covered in this study. The importance of maintaining high humidity was shown in research that was previously conducted by Navaneethan *et al.* (2010) and De Waal *et al.* (2011b).

The use of EPNs to control the codling moth in South Africa is currently in an experimental phase, and additional research needs to be done, to determine the successful application of EPNs and to evaluate EPN efficacy. Due to the low daily humidity and low night temperatures, the environmental conditions in the Western Cape Province are the main challenge to using EPNs for the control of the codling moth. This study has shown *S. feltiae* to be the more effective candidate, in comparison to the local *S. yirgalemense*, for outdoor field application against diapausing codling moth populations, especially with regard to lower temperature regimes. As *H. bacteriophora* cannot be regarded as an effective option, it should not be used for codling moth control in the Western Cape Province. Significant differences found in the various imported recycled batches of *H. bacteriophora* for field application are also a concern which requires to be addressed.

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CHAPTER 3

Evaluation of spray application efficacy of entomopathogenic nematodes for the control of the codling moth (*Cydia pomonella* L.) in field and laboratory conditions

Abstract

The efficacy of different entomopathogenic nematodes (EPNs) in controlling diapausing codling moth (*Cydia pomonella*) larvae at different temperatures was evaluated, under local conditions using spray application. Three imported isolates, *Steinernema feltiae* and two isolates of *Heterorhabditis bacteriophora* Hb1 and Hb2, including two local isolates, *Steinernema jeffreyense* and *Steinernema yirgalemense*, were evaluated. The use of *S. jeffreyense* resulted in the most effective control, with 67% mortality, followed by *H. bacteriophora* (Hb1) with 42%, and then by *S. yirgalemense* with 41%. Laboratory bioassays simulating field conditions revealed that *S. feltiae* was most virulent to codling moth larvae, resulting in 67% mortality, followed by *S. yirgalemense* with 58%, the *H. bacteriophora* strain Hb1 with 48%, and the Hb2 strain with 24%. A comparison of the infection and penetration rate of two isolates of *H. bacteriophora* (Hb1, Hb2), *S. feltiae and S. yirgalemense*, which was carried out in multiwell plates at 14°C and 25°C, confirms the pronounced effect of temperature on EPN efficacy. At 14°C, all EPN species resulted in slower codling moth mortality than they did at 25°C, as after 48 h, < 15% mortality was recorded for all species, whereas at the warmer temperature, > 98% mortality was recorded for all species. After the exposure of washed, cool-treated larvae to 25°C for 24 h, the application of both *S. feltiae* and *S. yirgalemense* resulted in 100% mortality, whereas the application of the two *H. bacteriophora* isolates, Hb1 and Hb2, resulted in 68% and 54% control, respectively, over the same time period. At 14°C, *S. feltiae* had the highest average penetration rate of 20 IJs/insect, followed by *S. yirgalemense* with 14 IJs/insect, whereas *S. yirgalemense* had the highest penetration rate at 25°C, with 39 IJs/insect, followed by *S. feltiae*, with 9 IJs/insect. The two *H. bacteriophora* isolates had higher average penetration rates at the higher temperature. This study has

highlighted the biocontrol potential of *S. jeffreyense*, as well as showing that *S. feltiae* is a cold-active nematode, whereas the other three EPN isolates prefer warmer temperatures.

Introduction

Entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae are known worldwide to be lethal and obligate pathogens of a wide range of insects, due to the unique nematode-bacterium complex. Each EPN species has its own set of unique characteristics such as foraging strategy and environmental tolerances, with some performing better under certain conditions than do others (Campbell *et al.*, 1995; Lacey & Unruh, 1998; Unruh & Lacey, 2001). One EPN species is not effective at controlling all insect pests, and some species are more effective against certain insect groups (Lacey *et al.*, 2001). To ensure successful control, it is important to match the appropriate nematode species against a particular insect pest (Lacey *et al.*, 2001; Shapiro-Ilan *et al.*, 2004). Since 1955, when EPNs were first reported (Dutky & Hough, 1955; Weiser, 1955), many studies have been done on their pathogenicity to numerous insect pests around the world (Ehlers, 1996; Koppenhöfer, 2000; Lacey & Georgis, 2012).

The first report of EPNs in South Africa was made by Harington (1953), who discovered an EPN in Grahamstown, Eastern Cape province, affecting all life stages of the maize beetle *Heteronychus arator* (Fabricius). Since this first discovery, EPNs have been recovered in eight of the nine provinces of South Africa (Spaull, 1988; 1990; 1991; Grenier *et al.*, 1996; Malan *et al.*, 2006; Molotsane *et al.*, 2007; Hatting *et al.*, 2009; Malan *et al.*, 2011; 2014).

The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), was first reported from South Africa around 1885, and by 1918, it had gained established pest status within the country (Giliomee & Riedl, 1998). Originating in Eurasia, this moth has managed to become the key pest of apples and pears around the world, due to its successful dispersal and adaptation strategies (Audemard, 1991; Barnes, 1991). South Africa is the third largest deciduous fruit producer in the southern hemisphere, with apples and pears making up the bulk of the production (Hortgro, 2013). With the stricter quality control measures concerning the chemicals used for codling moth control, and with reduced minimum residues, it has become a challenge to manage codling moth populations in orchards. The withdrawal

of primary chemicals that were used for codling moth control, and increased demand for more sustainable production, has led to the search for biological alternatives.

Most research on EPNs has been done under optimum laboratory conditions that are required for the control of the codling moth. In the laboratory, studies have been conducted on the efficacy of codling moth control using different species (Lacey & Unruh, 1998; Lacey & Chauvin, 1999; Unruh & Lacey, 2001; Cossentine *et al.*, 2002; Lacey *et al.*, 2005; 2006a; De Waal *et al.*, 2010; 2011a,b; 2013); concentrations to provide optimal control (Lacey and Unruh, 1998; De Waal, 2008); optimum temperature (Kung *et al.*, 1991; Wright, 1992; Grewal *et al.*, 1994b; Lacey & Unruh, 1998); humidity (Kung *et al.*, 1991; Lacey & Unruh, 1998; De Waal *et al.*, 2010; Navaneethan *et al.*, 2010); and water activity levels (Navaneethan *et al.*, 2010; De Waal *et al.*, 2011a; 2013). Techniques such as the addition of adjuvants (Lacey *et al.*, 2005; Navaneethan *et al.*, 2010; De Waal *et al.*, 2013) and mulches (De Waal *et al.*, 2011b) to improve EPN efficacy have also been studied. Results obtained under laboratory conditions, as well as from the field, have in many cases displayed significant differences (Lacey & Unruh, 1998; Cross *et al.*, 1999). These inconsistencies have been attributed to the variety of behavioural, ecological and environmental challenges encountered by the nematodes in the field, including inactivation by environmental extremes, indefinite host contact, and limited movement (Cross *et al.*, 1999; Grewal *et al.*, 2001).

This variability between results from the laboratory and field trials has led to a move to the use of laboratory conditions that are more realistic and representative of the local climate. Overall, relatively few studies have focused on the control of the codling moth with EPNs under field conditions in comparison with the plethora of laboratory studies. Previous field studies include the effect of environmental conditions, application technique and post-application conditions required for successful codling moth control (Lacey & Unruh, 1998; Unruh & Lacey, 2001; Lacey *et al.*, 2006a,b; 2010; De Waal, 2008; De Waal *et al.*, 2011a,b; 2013).

EPNs qualify as an environmentally friendly control measure, as they have a narrow, and thus selective host range, with limited to no effect on non-target organisms, low mobility and no detrimental effects on the environment around them (Lacey & Unruh, 1998; Ehlers & Hokkanen, 1996; Cross *et al.*, 1999; Grewal *et al.*, 2001; Unruh & Lacey, 2001). A major advantage of using EPNs to control the codling moth is that these organisms are capable of locating insect pests in cryptic habitats.

Furthermore, with above-ground pests not yet having developed coevolutionary barriers towards these nematodes, EPNs can provide effective supplementary control as part of an IPM strategy (Lacey *et al.*, 2001; Koppenhöfer, 2007). Up until now, no evidence has been found with regards to a naturally acquired resistance to the symbiotic bacteria that are associated with nematodes (Divya & Sanker, 2009).

The objectives of this study were to assess the efficacy of different EPN isolates on the mortality of diapausing codling moth larvae under local field conditions, using spray application. Field spray trial results were compared to simulated field conditions in the laboratory. Additionally, temperature as a main factor in the outdoor effectiveness of nematodes was evaluated, to ascertain their efficacy at lower temperatures under laboratory conditions.

Materials and methods

Source of insects

The codling moth eggs, and the artificial diet on which they are reared in the laboratory, were obtained from Entomon Technologies (Pty) Ltd situated on Welgevallen Experimental Farm in Stellenbosch, Western Cape province, South Africa. Diapausing larvae were reared in a growth chamber under diapausing conditions [photoperiod 10:14 (L:D), 25°C, 60% relative humidity (RH)]. Once they had developed into late instar larvae, they were stored directly in the diet, covered in brown bags and kept at 4°C until they were needed for trials.

Source of nematodes

Infective juveniles (IJs) of the different nematode isolates (Table 3.1), *Steinernema jeffreyense* Malan, Knoetze & Tiedt, 2015 (KC897093) (Malan *et al.*, 2015)*, Steinernema yirgalemense* Mráček, Sturhan & Reid, 2003 (EU625295), *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, and two isolates of *Heterorhabditis bacteriophora* Poinar, 1976 (Hb1 and Hb2) were reared in codling moth larvae in a growth chamber at 25°C. After 7 days, the infected codling moth cadavers were placed on a modified White trap (Kaya & Stock, 1997). On emergence, the IJs were harvested during the first week and stored at 4°C in 150 ml of filtered water, in 500-ml horizontally

positioned, vented culture flasks. Flasks were shaken weekly for aeration, and the nematode suspensions were used within four weeks of harvesting. Following the procedure described by Navon and Ascher (2000), nematode concentrations were quantified an hour before each trial.

Table 3.1. Source of entomopathogenic nematode isolates used in the study.

Species	Isolate	Origin
Heterorhabditis bacteriophora	Hb1	e-nema, Germany
Heterorhabditis bacteriophora	Hb2	e-nema, Germany
Steinernema jeffreyense	J194	Jeffrey's Bay, South Africa
Steinernema feltiae	Sf	e-nema, Germany
Steinernema yirgalemense	157-C	Friedenheim, Mpumalanga

Field trial layout

The semi-field trials were conducted at Welgevallen Experimental Farm in Stellenbosch. Trials were run in a 16-year-old, 0.416 ha block, comprised of Granny Smith, Royal Gala and Pink Lady apple trees. The trial block consisted of 11 rows with an average of 44 trees per row, with 1.5 m spacing between the trees and 4 m between the rows.

The experimental design consisted of four treatment rows, with eight treatment trees per row ($n = 32$ treatment trees). There were two buffer rows on the edges of the block, one buffer row between treatment rows, and four buffer trees between treatment trees. A complete randomised design was used for the experimental layout.

The temperature and humidity for each row was monitored by iButton[®] [Maxim Integrated iButton[®] temperature/humidity logger (DS1923)] data loggers, mounted on a scaffold branch of the tree located in the middle of each of the four treatment rows. These data loggers recorded the conditions experienced by the EPN during the 24 h exposure period.

Codling moth larval containment protocol

Cages, 7 × 3 cm, were constructed by rolling wire mesh (40 mesh / 425-µm aperture size) around a glass cylinder, and sticking the edges together using a glue gun. Both sides of the cylinder were

fitted with plastic caps, of which the one was glued shut, while the other was left unglued, to allow access to the cage (De Waal *et al.* 2011a, b).

Each cage was filled with apple tree bark and 20 late instar codling moth larvae 24 h before a trial. Cages were placed in 1-L plastic containers, to provide the larvae sufficient time to spin themselves into cocoons, and to ensure that the cages are secure, and that no larvae escape. One cage was hung per treatment tree ($n = 32$), with a total of 640 larvae being used for each trial.

EPN application protocol

Before treatment applications, cages were pre-wet by being completely submerged into a beaker filled with filtered water. EPN suspensions of a predetermined concentration were dispensed into a handheld sprayer. Every cage was then sprayed, at a distance of around 10 cm, until they were so wet that they started dripping. The handheld sprayer was shaken before every spray, to avoid sedimentation of nematodes. The cages were post-wet by means of being sprayed with filtered water every 2 h for the first 6 h of the trial.

Evaluation protocol

After 24 h in the orchard, the cages were recovered and their contents (apple tree bark and codling moth larvae) removed. The codling moth larvae were rinsed with filtered water to remove any remaining surface nematodes, and they were then placed in a Petri dish (9 cm in diameter) on moist filter paper. Petri dishes from each treatment ($n = 8$) were placed in separate plastic containers lined with moist paper towels, closed and placed in a growth chamber at 25°C for 72 h (3 days). On day 4 (96 h after the onset of trials), mortality that resulted from EPN infection was assessed and confirmed by dissection of the insect larvae.

Spray field trials using different nematode species

Cages were prepared for the trial as described in the containment protocol, and tied to a scaffold branch close to the main stem of each treatment tree. The treatments consisted of *H. bacteriophora* (Hb1)*, S. yirgalemense* and *S. jeffreyense*, with water only being used as the control treatment. *Steinernema feltiae* was replaced by *S. jeffreyense* to avoid biological pollution of orchards with an exotic species. *Heterorhabditis bacteriophora* (Hb2) was not used because of previous low infection in

field trials (Odendaal *et al.* 2015). The cages were treated as described in the EPN application protocol between 11:00 and 13:00. After the 24 h exposure period, the cages were retrieved from the field, and handled as laid out in the evaluation protocol. The trial was repeated on a different test date, with freshly reared batches of nematodes.

Laboratory bioassays with sprayed field cages

The containment method and loading of cages, with apple tree bark and codling moth larvae, remained unchanged in the laboratory study, and was carried out as indicated in the containment protocol. In order to determine a delimiting concentration, pilot trials were done (data not shown), and a concentration of 25 IJs/ml was selected for this study. The nematode species used were *H. bacteriophora* (Hb1 and Hb2), *S. feltiae* and *S. yirgalemense*, with water only being used as the control treatment.

The cages were pre-wet and nematode isolates applied according to the application protocol used for the field trials, after which cages of each treatment (*n* = 8) were placed in separate 1-L plastic containers, lined with moist paper towels, and closed. The treated cages were then incubated in a growth chamber for 24 h at a temperature regime of 22ºC for 4 h, 14ºC for 12 h, and 22ºC for 8 h (simulating field temperatures). After 24 h in the growth chamber, the cages were removed from the containers, and handled as stated in the evaluation protocol. Laboratory trials were done on separate dates with fresh batches of recycled nematodes.

Laboratory bioassays at different temperatures using multiwell plates

A comparison of the infection and penetration rate of two isolates of *H. bacteriophora* (Hb1, Hb2), *S. feltiae and S. yirgalemense* was carried out at 14°C and 25°C, in multiwell plates (24 wells, volume 3.8 cm^2 per well, flat bottom, Nunc^{TM}, cat no. 144530). Filter paper (13 mm diam.) was placed in each of the ten wells per plate. One well was left open between treatment wells, to avoid movement of nematodes from one well to another. Five multiwell plates were used per treatment (*n* = 50). The nematode concentration used was 50 IJs/50 µl of filtered water per codling moth larvae, with water only being used as the control treatment.

The nematode suspensions and bioassay plates were placed at the required temperatures 1 h before commencing the trial, to allow the inoculum to reach that specific temperature. The nematode suspension was then applied onto the filter paper discs, and one last instar codling moth larvae was added to each of the 10 wells. The bioassay plate lids were fitted with a 3-mm glass pane, to ensure that the larvae did not escape from their wells. Bioassay plates of each treatment (n = 5) were enclosed in separate 2-L plastic containers, lined with moist paper towels, to ensure a high humidity. The containers were left at the required temperatures for 48 h, after which the larvae were removed from the bioassay plates, rinsed and placed in Petri dishes lined with moist filter paper. The Petri dishes were then placed in a growth chamber at 25°C for two more days, with the larval mortality being assessed on day 4 (96 h after inoculation).

Cadavers were then inspected every day to allow the first-generation nematodes to develop. Once their full development had taken place, the Petri dishes from all treatments were placed in a freezer, to cease any further nematode development. The cadavers were dissected, and the number of first-generation nematodes was counted.

Statistical analysis

The data were evaluated using STATISTICA 12 software (StatSoft Inc. 2013). In the absence of any significant test date versus treatment differences, the data from the different test dates were pooled and analysed, using a one-way analysis of variance (ANOVA), with *post hoc* comparison of means. If the residuals were not normally distributed, bootstrap multiple comparisons were performed (Efron & Tibshirani, 1993).

Results

Spray field trials with different nematode species

Analysis of the results of the two field spray trials using a two-way ANOVA showed no difference between the main effects of test dates and the treatments conducted ($F_{(3,56)}$ = 0.964, p = 0.461), and data from the two field trials were pooled. Analysis of the combined data using a one-way ANOVA showed a significant effect of treatments on the mortality of codling moth ($F_{(3,60)}$ = 23.648, p < 0.005).

Steinernema jeffreyense caused the highest level of mortality of codling moth larvae (66.81% ± 5.46%), followed by *H. bacteriophora* (Hb1) (41.81% ± 6.40%) and *S. yirgalemense* (41.18% ± 7.67%). There was a significant difference between *S. jeffreyense* and *H. bacteriophora* (Hb1) (p = 0.027) and *S. yirgalemense* (p = 0.045), with no difference being found between *S. yirgalemense* and *H. bacteriophora* (Hb1) (p = 1) (Fig. 3.1).
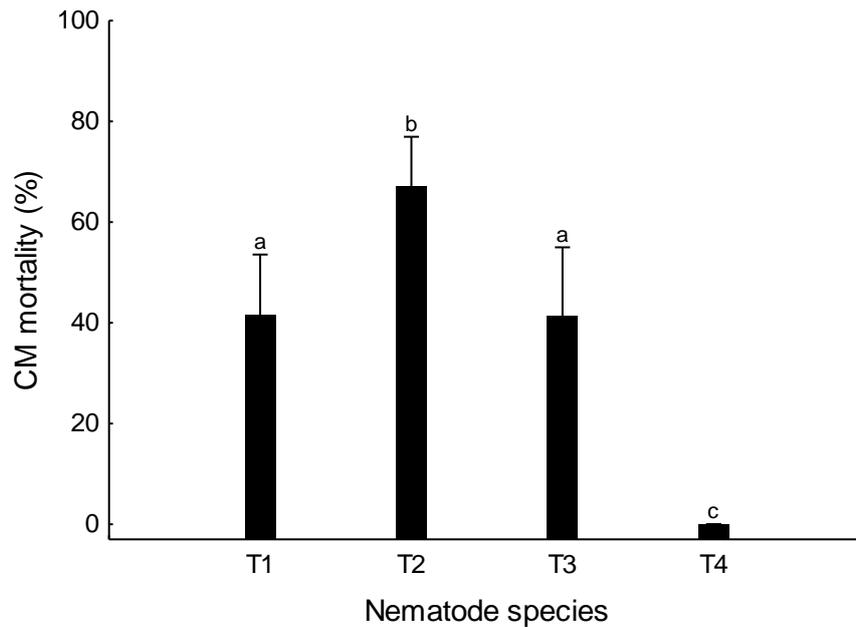
Fig. 3.1. Mean percentage mortality (95% confidence limits) recorded for codling moth larvae after spraying with three nematode isolates, *Heterorhabditis bacteriophora* (Hb1), *Steinernema jeffreyense* (Sj), *S. yirgalemense* (Sy), and water only (control), at a concentration of 1 000 IJs/ml, during a field trial. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 60)}$ = 23.648, $p < 0.005$).

During the 24 h exposure time of the first spray trial, the minimum and maximum temperatures were 15ºC and 35ºC respectively, with an average of 20ºC. The minimum and maximum RH was 42% during the day and 100% during the night, with the average for the 24 h exposure period being 84% (Fig. 3.2 A). Temperature and humidity were recorded at 15- min intervals over the 24 h exposure period. For the first 3 h of the trial, the temperature was above 30°C, after which it decreased to a temperature of around 15°C over the next 3 h. It then remained constant for the next 14 h, until the next morning. Over the next 4 h, the temperatures gradually increased again to around 30°C at the end of the trial. As the temperature increased, the humidity decreased, and vice versa. The humidity tapered below 40% for the first 4 h of the trial, and then increased to 100% over the next 3 h. The

humidity remained at 100% for the next 15 h, and then started decreasing, as soon as the temperatures started to rise in the morning, to around 40% again. No rainfall was experienced during this trial.

A

B
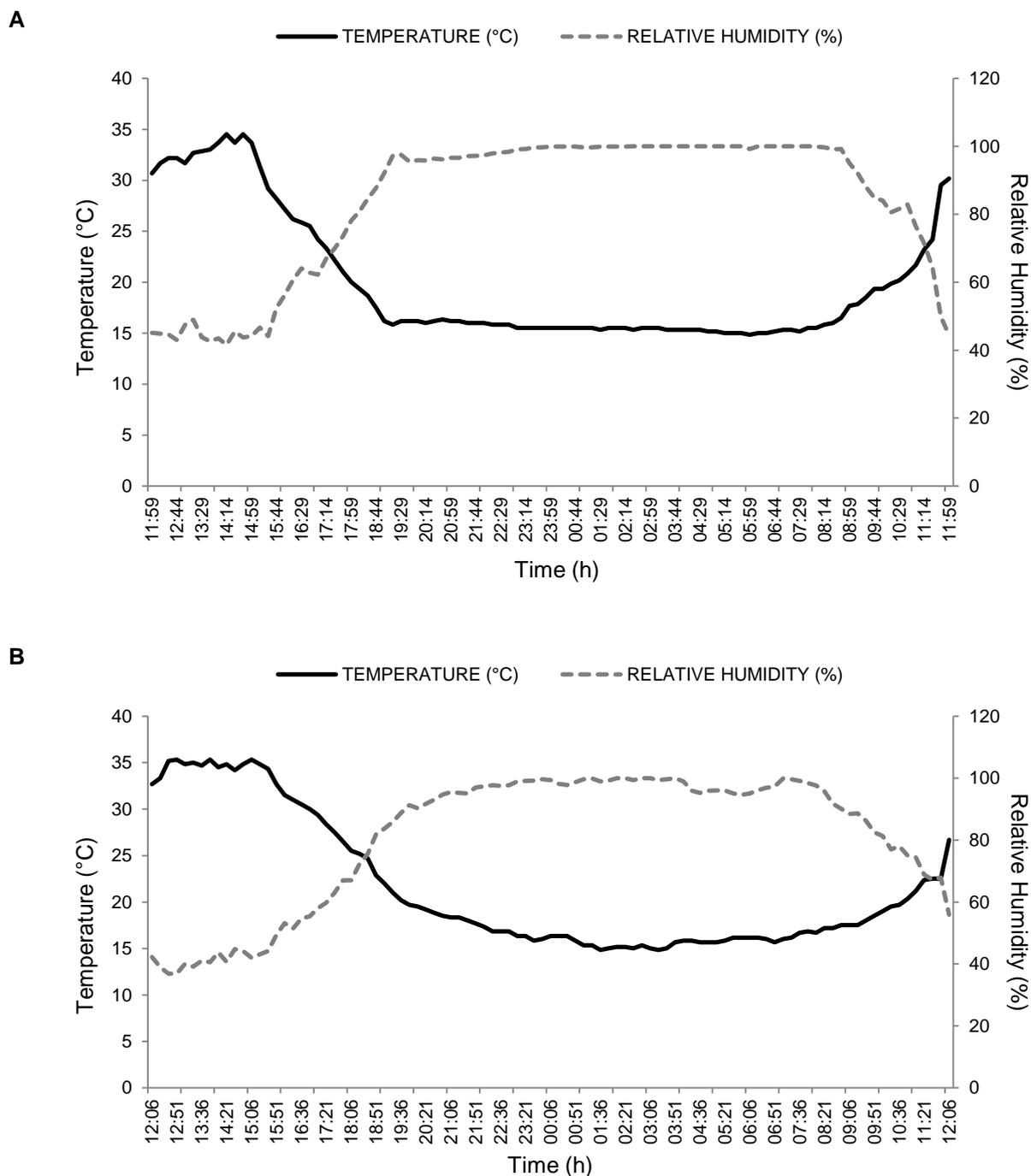
Fig. 3.2. Temperature and relative humidity data recorded during the 24 hour trial period of the **A**. first spray trial and, **B**. second spray trial.

On the day on which the spray trial was repeated, the minimum and maximum temperatures were 15ºC and 35ºC, being the same as for the first trial, but with a lower average temperature of 22ºC. The minimum and maximum RH was even lower, at 37% during the day, and once again, 100% during the night and with the 24 h exposure period averaging at 81% (Fig. 3.2 B). The trial was commenced in temperatures tapering around 35°C for the first 3 h. Temperatures then started to decline over the next 9 h to around 16°C, remaining constant for 6 h. The temperature then started to steadily increase over the next 6 h, reaching above 25°C at the end of the trial. Again, no rainfall was experienced during the trial period. The temperature and humidity followed the same cycle as in the previous trial, with humidity decreasing as temperature increased and vice versa. Humidity gradually increased from below 40% to above 90% over the first 8 h of the trial, after which it tapered between 90% and 100% for around 11 h. Over the next remaining 5 h of the trial, the humidity dropped below 60%.

Laboratory bioassays with sprayed field cages

Analysis of the results of the two laboratory trials with a two-way ANOVA showed no difference between the test dates and the treatments ($F_{(4, 70)}$ = 1.8321, p = 0.134) and the data from the two laboratory trials were pooled. A significant effect ($F_{(4, 75)}$ = 90.946, p = 0.0000) of the treatments on codling moth larval mortality was obtained after analysis of the data using a one-way ANOVA. No significant differences were found between *S. feltiae* and *S. yirgalemense* (p = 0.369). *Steinernema feltiae* differed significantly from both isolates of *H. bacteriophora,* Hb1 (p < 0.005) and Hb2 (p < 0.005). There was also a significant difference between *S. yirgalemense* and the Hb1 and Hb2 isolates of *H. bacteriophora*, Hb2 (p < 0.005). Overall, *S. feltiae* gave the highest mortality of codling moth larvae by infection (67.06% ± 4.18%), followed by *S. yirgalemense* (58.44% ± 2.02%) and then the two *H. bacteriophora* strains Hb1 (48.25% ± 2.98%) and Hb2 (23.88% ± 3.29%), respectively.
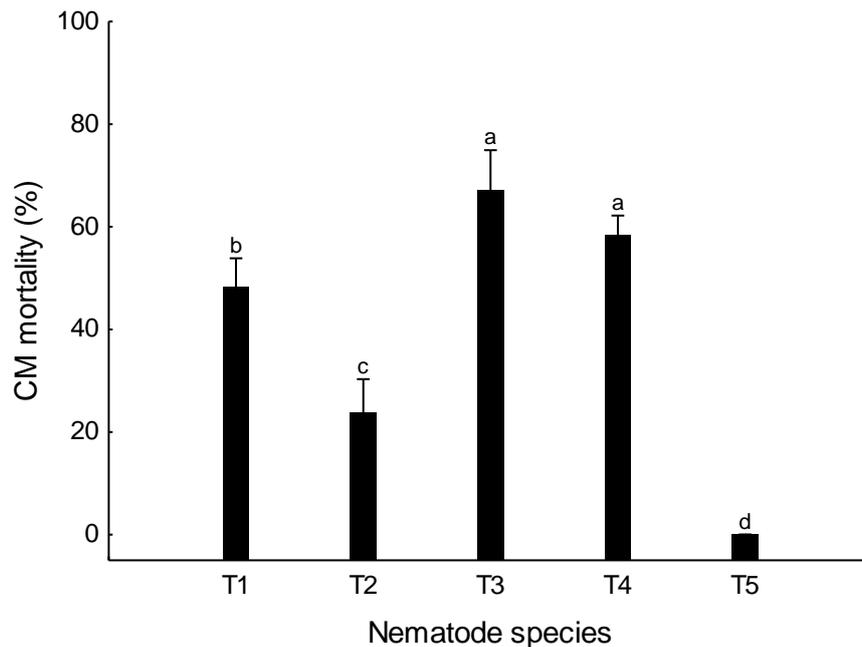
Fig. 3.3. Mean percentage mortality (95% confidence limits) recorded for codling moth larvae confined in cages filled with apple bark, after treatment by spraying cages with four nematode species, *H. bacteriophora* (Hb1), *H. bacteriophora* (Hb2), *S. feltiae*, *S. yirgalemense* and water only, at a concentration of 25 IJs/ml during a laboratory trial. Different letters above bars indicate significant differences (one-way ANOVA; $F_{(4, 75)} = 90.946$, *p* < 0.001).

Laboratory bioassays at different temperatures using multiwell plates

At 25°C, after an exposure period of 48 h in 24-well bioassay plates, all four nematode isolates caused high codling moth mortalities of between 98% and 100% (Fig. 3.4 A). After 48 h exposure to the four nematode isolates, at a temperature of 14°C, the codling moth mortality was lower than 15% for all isolates (Fig. 3.4 B). However, when freed from surface nematodes by washing (after 48 h) and left for another 24 h in a growth chamber at 25°C, both *S. feltiae* and *S. yirgalemense* caused 100% codling moth mortality, followed by *H. bacteriophora* (Hb1), with mortality of 45%, and *H. bacteriophora* (Hb2) with 42% mortality, indicating latent infection (Fig. 3.4 B). After 96 h, *H. bacteriophora* (Hb1) resulted in a mortality of 68%, and *H. bacteriophora* (Hb2) in 54%. No mortality was found using water only as the control treatment, at either temperature, and was thus not included in the graphs below.
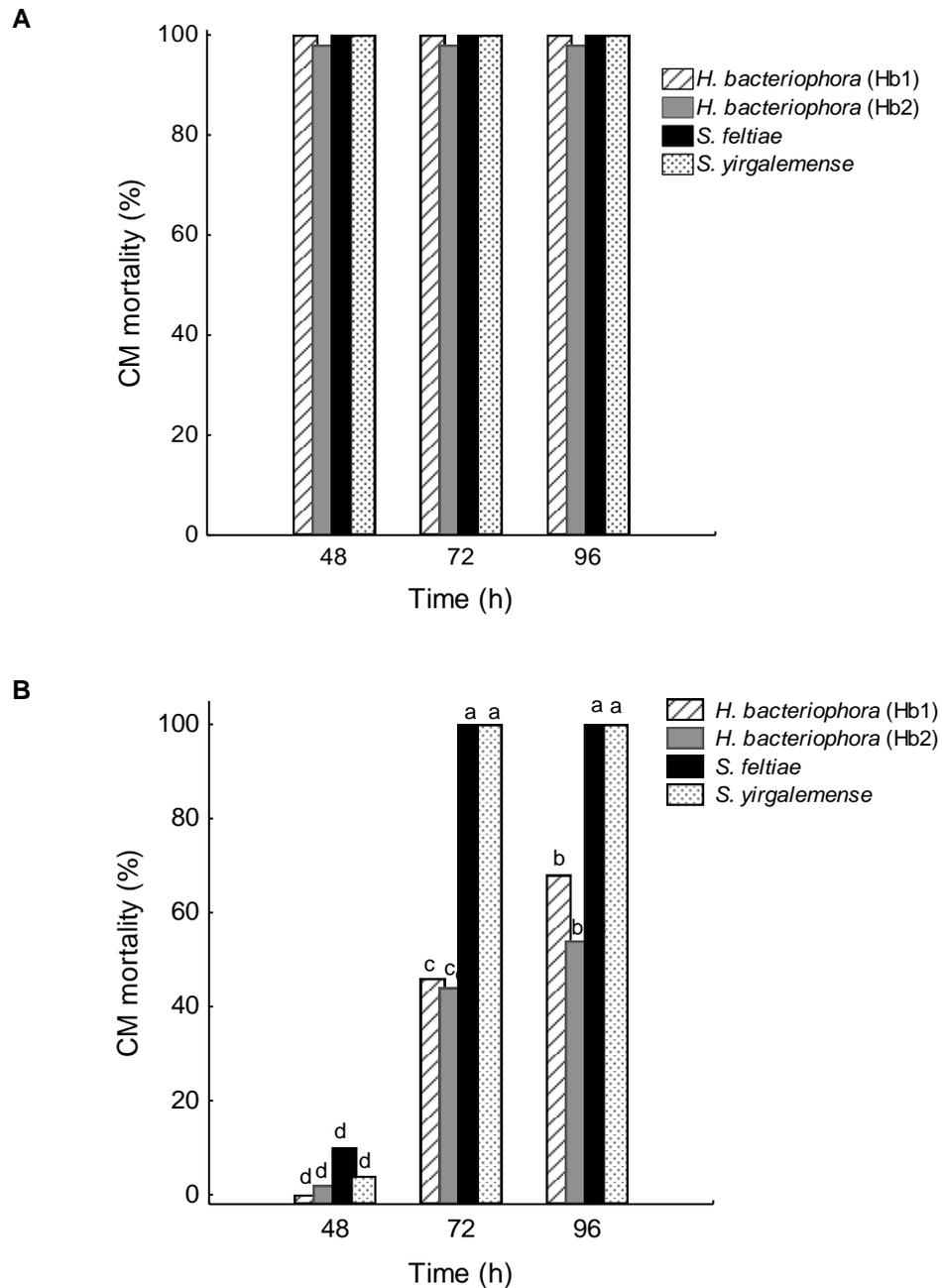
Fig. 3.4. Percentage diapausing codling moth (CM) larvae mortality, recorded after inoculation with *H. bacteriophora* (Hb1), *H. bacteriophora* (Hb2), S*. feltiae*, and *S. yirgalemense* at a concentration of 50 IJs/insect, using 24-well bioassay plates at **A**. 25°C, where there were no significant differences between species; and **B**. at 14°C, where different letters above bars indicate significant differences (one-way ANOVA; $F_{(6, 32)}$ = 14.615, *p* < 0.001).

At 25°C, *S. yirgalemense* had the highest average penetration rate of 39 IJs per insect (Fig. 3.5 A). All three of the other nematode isolates had a very low average penetration, with *S. feltiae* at 9 IJs, *H. bacteriophora* isolate Hb1 with a penetration rate at 7 IJs, and isolate Hb2 with the lowest penetration rate of 5 IJs per insect. The codling moth larvae, treated with the three different nematode species, at a temperature of 14°C, were dissected to determine the average IJ penetration rate (Fig. 3.5 B). *Steinernema feltiae* had the highest average penetration rate of 20 IJs per codling moth larva, followed by *S. yirgalemense*, with an average penetration rate of 14 IJs. The two *H. bacteriophora* isolates had the lowest penetration rate of only one IJ per insect.
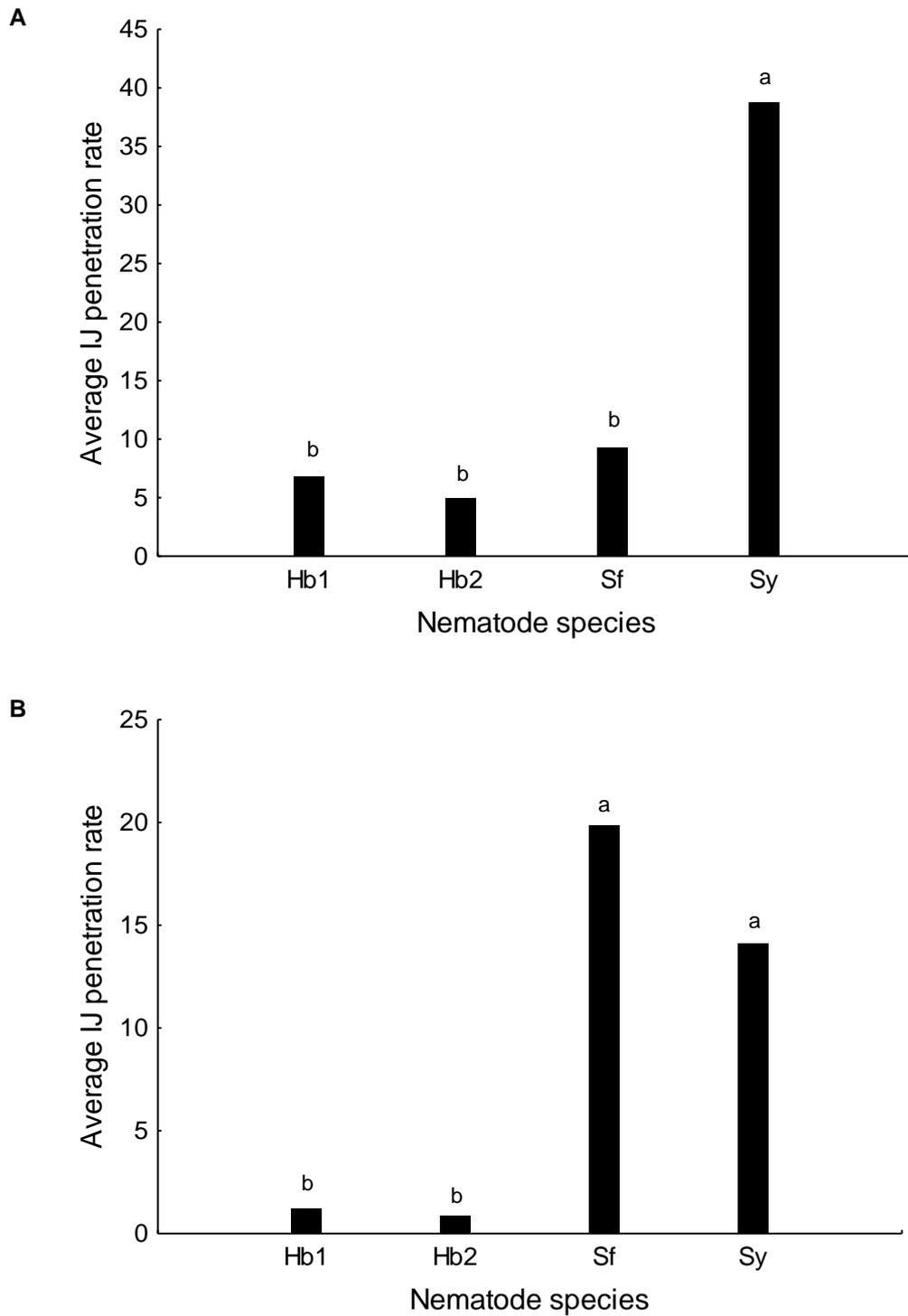
Fig. 3.5. Average penetration rate of infective juveniles (IJs) of *H. bacteriophora* (Hb1), *H. bacteriophora* (Hb2), S*. feltiae* and *S. yirgalemense* per codling moth larva, 48 h after inoculation with a concentration of 50 IJs/ml using 24-well bioassay plates at **A**. 25°C, where different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 16)}$ = 139.41, p < 0.001), and **B**. at 14°C, where different letters above bars indicate significant differences (one-way ANOVA; $F_{(3, 16)}$ = 32.943, p < 0.001).

Discussion

Soil is the natural habitat of EPNs, which protects them from such environmental extremes as high temperatures and low moisture levels (Kung *et al.*, 1991; Grewal *et al.*, 2001). The above-ground application of EPNs removes them from this safe environment, and exposes them to harsher conditions from which they need to be protected. The failure to do so results in desiccation, and thus, ineffective control. Suitable time for application is in the evening, early in the morning, or on cloudy days (Unruh & Lacey, 2001; De Waal, 2008), to minimise EPN exposure to ultraviolet (UV) radiation and high temperatures. Pre- and post-wetting is highly recommended, as it increases the amount of moisture that is available for EPN survival and movement (Lacey & Unruh, 1998; Unruh & Lacey, 2001; Lacey *et al.*, 2006a; De Waal, 2008).

In a previous study (Chapter 2), the choice of application was dipping the container with codling moth in nematode suspension to enable a comparison of the efficacy of *S. feltiae* under local field conditions in an apple orchard, without biological pollution. However, this method is not practical for field application of above-ground pests, which should be similar to those used for applying chemicals in orchards (Gaugler, 1988; Mason *et al.*, 1998; Fife, 2003; Shapiro-Ilan *et al.*, 2006).

Steinernema feltiae was replaced with a promising new isolate, *S. jeffreyense*, in the field spray trials. In general, it was found that by spaying nematodes onto the cages in the field, codling moth mortality was much lower than when using the technique of dipping the cage into a nematode suspension. *Steinernema jeffreyense* caused the highest codling moth larval mortality (67%), whereas both *H. bacteriophora* (Hb1) and *S. yirgalemense* resulted in poor (± 40%) control, with no significant difference being found between them. The cause for the low mortality caused by the latter two species is uncertain, as the average temperature for both spray trials during the 24 h exposure time was above 20°C, and the average humidity above 80%. Such conditions have been reported by other researchers (Unruh & Lacey, 2001; De Waal *et al.*, 2010) to be optimum for effective EPN application, as well as by the results obtained in Chapter 2.

In order to clarify the results obtained from the field trials, bioassays simulating the field trials with regard to the temperature and method of containment were conducted at a constant humidity of 100%. The same method of applying the nematodes by means of spraying was used, as had been used in the field trial. As this trial was done under controlled conditions, *S. jeffreyense* was replaced

by *S. feltiae* as the information regarding the effectivity of S. feltiae was regarded as more important. In general, it was found that, by means of the application of nematodes using the spray technique, lower control of the codling moth was obtained. *Steinernema feltiae* caused a higher percentage (67%) codling moth control, followed by *S. yirgalemense* (58%), with no significant difference being present between the two species, followed by *H. bacteriophora* (Hb1) (48%), which also differed significantly from *S. feltiae*. The *H. bacteriophora* (Hb2) was least effective, resulting in < 25% control.

In the laboratory, the performance of *S. yirgalemense* improved compared to that in the field. This may be due to the more constant and higher humidity to which the nematode in question was exposed in the laboratory. In the field *S. yirgalemense* might be more sensitive to the lower humidity, and thus, lower water activity levels (a_w), than are the EPN isolates that proved to be more effective. *Steinernema feltiae* resulted in the same efficacy that *S. jeffreyense* achieved under field conditions. Future studies should compare the efficacy of *S. feltiae* and *S. jeffreyense* in laboratory bioassays.

In the field trials, because of practical considerations and logistics, nematodes were applied midday, at a time when the temperatures were generally at a maximum, and humidity at a minimum. This was compensated for by means of wetting the cages every 2 h for a period of 6 h. With the dipping of the cages into the nematode suspension, more nematodes and moisture could have been retained within the cages, in comparison to when the cages were sprayed with the EPN suspensions. With spray applications, the likely lower nematode numbers applied, including the initial high temperature, and the possible drying out of the cage contents during the first 6 h post EPN application, could have led to the general lower EPN efficacy. Improvement of the efficacy though the spraying of nematodes could be obtained through applying the nematodes in the late afternoon, to avoid conditions of high temperatures and low humidity. In future studies, the addition of adjuvants should be used as standard practice, as it has been shown in previous studies, to slow down desiccation, and thus, to increase EPN efficacy (Lacey *et al.*, 2005; Navaneethan *et al.*, 2010; De Waal *et al.*, 2013; Van Niekerk & Malan, 2013).

Four EPN isolates were exposed to a constant temperature of 14°C and 25°C, to determine the effect of temperature on EPN efficacy and activity. At a concentration of 50 IJs/insect and a temperature of 14°C, <15% codling moth larvae mortality was recorded for all EPN species after a 48 h exposure period. After removing surface nematodes, by rinsing the codling moth larvae with distilled

water, and incubating treated larvae at 25°C in a growth chamber for 24 h, both *S. feltiae* and *S. yirgalemense* resulted in 100% mortality. Of the two *H. bacteriophora* isolates, (Hb1) (68%) resulted in more efficacious codling moth control than did the *H. bacteriophora* (Hb2) isolate (54%).

An important difference with this temperature study, in comparison to previous studies (De Waal, 2011a), is that EPN suspensions were acclimatised to 14°C before inoculation. Van Niekerk and Malan (2014) showed that IJs were able to penetrate mealybugs within half an hour of exposure to nematodes. If the experiment was done at room temperature (25°C), and the bioassay plates were only then transferred to the lower temperature of 14°C, higher infection rates could have been recorded. *Steinernema feltiae* is known as a low temperature nematode (Wright, 1992; Grewal *et al.*, 1994b). However, in this study, when mortality was recorded after 48 h, all species performed poorly, including *S. feltiae*. When these nematodes, including their controls, were washed to remove surface nematodes and kept under optimum conditions for another 24 h, 100% codling moth larvae mortality was recorded. Thus, even though the codling moth larvae were not dead after 48 h at 14°C, they were all infected with nematodes, and destined to be killed over time.

After 48 h, during which they were exposed to 25°C using the same concentration of nematodes, 98-100% codling moth mortality was recorded for all the EPN strains. This trial has revealed the drastic effect that temperature has on EPN activity, and thus, on efficacy. All four EPN species caused faster codling moth larval mortality at the higher temperature, resulting in 100% mortality within 48 h, excluding the *H. bacteriophora* (Hb2) isolate.

Steinernema feltiae had a much higher average penetration rate at 14°C (20 IJs/insect) than at 25°C (9 IJs/insect), providing even more evidence that *S. feltiae* is a cold-active nematode. This EPN species had the highest average penetration rate at 14°C, followed by *S. yirgalemense*, with 14 IJs/insect, and then the two *H. bacteriophora* strains (Hb1, Hb2), each with only one IJ/insect. At 25°C, *S. yirgalemense* had the highest average penetration rate of 39 IJs/insect, followed by *S. feltiae* (9 IJs/insect), *H. bacteriophora* (Hb1) (7 IJs/insect) and then *H. bacteriophora* (Hb2) (5 IJs/insect). *Steinernema yirgalemense* and the two *H. bacteriophora* isolates (Hb1, Hb2) are more active, and thus efficient, at warmer temperatures.

Steinernema feltiae has thus far, proved to be the better candidate for the control of diapausing codling moth larvae in the Western Cape Province. However, *S. jeffreyense* is a promising biocontrol

agent for diapausing codling moth larval populations in apple and pear orchards. A recommendation would be that more studies be done on this species, to determine an effective application protocol, its temperature range, its efficacy when combined with an adjuvant, and its sensitivity to agrochemicals. Different application techniques need to be studied to determine the efficacy of each technique for the application of each species, as well as for the addition of adjuvants.

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CHAPTER 4

Control of diapausing codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), in wooden fruit bins using entomopathogenic nematodes

Abstract

Stacked wooden fruit bins are regarded as preferred overwintering sites for diapausing codling moth (*Cydia pomonella*) larvae. Control strategies against the codling moth in South Africa have been hampered by the reinfestation of orchards by fruit bins, infested with this insect pest, stacked in close proximity to treated orchards or by the movement of bins between orchards. Worldwide, wooden fruit bins are systematically being replaced with plastic bins, which, in South Africa, will only be phased out over a number years. The objective of this study was to evaluate the potential of two recycled imported species of entomopathogenic nematode (EPN), *Heterorhabditis bacteriophora* and *Steinernema feltiae*, as well as of a local species, *Steinernema yirgalemense,* to disinfest miniature wooden fruit bins under controlled conditions in the laboratory. After dipping minibins in a suspension of 25 IJs/ml of all three EPN species, under optimum conditions of temperature and humidity, the highest percentage of control was obtained using *S. feltiae* (75%) followed by *S. yirgalemense* (57%), and then by *H. bacteriophora* (Hb1) (28%). The addition of adjuvants significantly increased (p < 0.001) *S. feltiae* infectivity to > 95%, whereas it did not result in a significant increase in *H. bacteriophor*a or *S. yirgalemense* infectivity. The results indicated that *H. bacteriophora* would not be a suitable candidate to use for the control of the codling moth larvae in wooden fruit bins. The current preferred candidate for control would be *S. feltiae*, whose efficacy could be increased by means of the addition of an adjuvant.

Introduction

The spread of the codling moth, which can be attributed to the cultivation of its hosts, was further facilitated by the transport of infested fruit and wooden bins (Giliomee & Riedl, 1998). This insect has succeeded in spreading its range to all the main apple and pear growing areas around the world, and has gained key pest status within orchards producing these fruits (Barnes, 1991; Giliomee & Riedl,

1998; Riedl *et al.*, 1998). South Africa is one of the top ten fresh apple and pear producers in the world (Hortgro, 2013), and due to the country's favourable climate, up to four generations of the codling moth have been recorded per season in untreated orchards (Blomefield, 2003; Pringle *et al.*, 2003). The Western Cape Province, which is the country's most important apple and pear growing area (Hortgro, 2013), has one of the highest codling moth infestation potentials in the world, with infestation rates as high as 80% being recorded in derelict orchards (Pringle *et al.*, 2003).

After passing through five larval instars within the fruit, last instar codling moth larvae emerge just before winter and search for cryptic overwintering sites in which to spin their cocoons (Blomefield & Giliomee 2012). Suitable sites include pruning wounds, loose bark, litter at the base of trees, woodpiles and stacked wooden fruit bins surrounding orchards (Blomefield, 2003; Cranshaw & Hammon, 2014). These overwintering sites can be markedly reduced through orchard sanitation, but they can be reinfested by means of the moths overwintering in wooden fruit bins that are stacked in close proximity to orchards, and by the movement of bins between orchards (Cranshaw & Hammon, 2014).

Over the years, many countries have started using plastic fruit bins, as they have the advantage of not absorbing moisture or chemicals, as well as being easy to sanitise, having an interlocking system, and lasting longer than wooden fruit bins (Waelti, 1992). The use of wooden bins in South Africa is not likely to change soon, as, unlike in the case of plastic bins, the former bins are cheap, easy to handle, and superior in strength, as well as being able to be produced and assembled locally (Waelti, 1992). At the time of writing, the advantages of wooden fruit bins outweigh those of plastic bins, thus the only alternative to replacing wooden bins with plastic bins is to implement an effective codling moth disinfestation technique for wooden bins.

An integrated pest management (IPM) strategy is utilised in South African apple and pear orchards. This strategy involves the use of density-dependent biological control techniques, such as mating disruption and the sterile insect technique (Pringle *et al.*, 2003; Addison, 2005), in combination with softer, more environmentally friendly chemicals such as methoxyfenozide, spinetoram, and novaluron. Although this strategy may provide effective codling moth control within orchards, it is likely to be compromised if the bin stacks surrounding the orchards are left untreated, as they are known to be sources of reinfestation (Higbee *et al.*, 2001; Lacey *et al.*, 2005).

The broad spectrum fumigant, methyl bromide, was the most popular method of bin disinfestation. Due to this chemical being classified as an ozone-depleting substance and as a result of stricter chemical registration guidelines, it was phased out in 2005 (Hansen, 2007). Various other tactics have been used to control diapausing codling moth larvae in fruit bins, ranging from heat treatments (Higbee *et al.*, 2001), the fumigation of bins with carbon dioxide (Cossentine *et al.*, 2004), the treatment of bins by submergence in hot water (Hansen *et al.*, 2006), and biofumigation with the fungus *Muscodor albus* Worapong, Strobel & Hess (Ascomycota: Xylariales) (Lacey *et al.*, 2009).

With the ever-changing and stricter regulations regarding the use of agrochemicals, growers are encouraged to reduce the use of chemical control by incorporating more sustainable and environmentally friendly control measures in an IPM programme for deciduous fruit. The two main tactics on which growers currently rely is mating disruption and the sterile insect technique. Unfortunately, both of these tactics have reduced efficacy in windy conditions, in orchards with uneven topography and high codling moth population densities, and in orchards struggling with outside sources of infestation (Cardé & Minks, 1995; Pringle *et al.*, 2003). The answer is thus to combine these two techniques with other control measures, to warrant effective control of the codling moth population (Addison, 2005). One of these measures is the treatment of wooden fruit bins and surrounding orchards, which act as sources of reinfestation for the codling moth.

Lacey and Chauvin (1999) were the first to treat bins that were infested with diapausing codling moth larvae by submerging them into suspensions of entomopathogenic nematodes (EPNs). They found that miniature replicates of wooden fruit bins that were treated with suspensions of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, resulted in up to 100% larval mortality (Lacey & Chauvin, 1999). Larval mortalities as high as 93% were obtained by Cossentine *et al.* (2002), when full-scale commercial fruit bins were submerged in a bin drencher containing a similar treatment. Lacey *et al.* (2005) inserted infested miniature wooden fruit bins into commercial scale plastic bins, which were then dipped into a drop tank filled with different concentrations of *S. carpocapsae* and *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, combined with a wetting agent. Both EPN species were effective and resulted in a significant mortality of the codling moth larvae of up to 80% (Lacey *et al.*, 2005). De Waal *et al.* (2010) dipped miniature replicates of fruit bins into suspensions of nematodes of a South African isolate of *Heterorhabditis*

zealandica (Poinar, 1990). In this study, over 80% larval mortality was obtained with a concentration of 50 infective juveniles (IJs) /ml.

The objective of this study was to determine the potential of three EPN species to control diapausing codling moth larvae in wooden fruit bins. All nematodes were recycled, including two steinernematid and one heterorhabditid species, with one local strain and two that were imported. The selection of the most effective species should prove to be the first step in the potential use of nematodes on a commercial scale for the control of the codling moth in wooden fruit bins.

Materials and methods

Source of insects

The codling moth eggs and the artificial diet on which the larvae were reared were sourced from Entomon Technologies (Pty) Ltd, located on the Welgevallen Experimental Farm in Stellenbosch, Western Cape province. Larvae were reared in a growth chamber under diapausing conditions [photoperiod 10:14 (L: D), 25°C, 60% RH]. Once the codling moth had become last instar larvae, they were stored at 4°C, until utilised in trials.

Source of nematodes

Recycled IJs of the imported species, *Heterorhabditis bacteriophora* Poinar, 1976 (Hb1) and *S. feltiae*, as well as of the indigenous isolate, *Steinernema yirgalemense* Mráček, Sturhan & Reid, 2003 (Genbank accession number: EU625295), were reared in final instar *Cydia pomonella* (L.) larvae in a growth chamber kept at 25°C for seven days (Table 4.1). The larval cadavers were then transferred to a modified White trap (Kaya & Stock, 1997). Upon emergence from the host cadaver, the IJs were harvested and stored in horizontally placed, vented culture flasks at 14°C, which were shaken weekly for aeration. Using the method described by Navon and Ascher (2000), fresh nematode concentrations were quantified an hour before each trial.

Table 4.1. Source of recycled entomopathogenic nematodes used in the study.

Species	Isolate	Origin
Heterorhabditis bacteriophora	Hb1	e-nema, Germany
Steinernema feltiae	Sf	e-nema, Germany
Steinernema yirgalemense	157-C	Friedenheim, Mpumalanga

Codling moth larval containment protocol

Planks

Wooden planks were constructed from the same wood that had been used to build commercial wooden fruit bins (De Waal *et al.*, 2010). The planks, which measured 17.7 cm × 6.5 cm, were each sawn into three sections, after which they were bolted back together again. Twenty holes (5 mm in diameter, with 2 cm spacing between holes) were then drilled into the planks along the groove of the saw lines (Fig. 4.1). Use of this method facilitates the easy removal of the codling moth larvae after treatment.

Fig. 4.1. Wooden planks used for the containment of codling moth larva during treatments.

Each plank was put into a separate closed 2-L plastic container, together with 20 codling moth larvae. The larvae were given 24 h in which to spin themselves into cocoons within the holes. Before the trial, each plank was checked, to ensure that the larvae had entered the drill holes. If a larva had not entered a hole, it was inserted in one, which was then closed off with tape in which small holes had been made to allow access to the IJs.

Minibins

Mini wooden fruit bins (≈ 1/10 the size of commercial wooden fruit bins) were constructed from old wood (2.2 cm thick) that had been used to construct commercial wooden fruit bins (De Waal *et al.*, 2010) (Fig. 4.2). A supporting corner cove (9 × 9 × 14 cm) was fixed in each corner, leaving a space large enough in which to insert the wooden planks containing the codling moth larvae. One plank, containing 20 diapausing codling moth larvae, was slotted into each corner of a minibin (planks: $n = 4$; codling moth larvae: $n = 80$), with each plank being considered as a replicate.

Fig. 4.2. Minibins and wooden planks loaded with 20 codling moth larvae slotted into each corner to allow for evaluation of the efficacy of entomopathogenic nematodes.

Effect of EPN species using minibins

A concentration of 25 IJs/ml was used for the minibin trials. Concentrations were quantified an hour before commencement of the trials. The minibins were pre-wet by submerging them for 2 min in a plastic container containing filtered water, after which they were drip-dried on a drying rack for 2 min. The bins were then dipped into another container [36 cm (width) × 38 cm (length) × 29 cm (height)] containing the 36-L nematode suspension, and once again drip-dried for 2 min on a drying rack. After treatment, the bins were placed in black plastic bags, lined with moist paper towels, and tied closed. The bins were then placed in a growth chamber at 25°C for three days (72 h).

The temperature and the humidity to which the bins were exposed was monitored throughout the trial period by iButtons® [Maxim Integrated iButton® temperature/humidity logger (DS1923)], which

were placed within the bins, before being enclosed within the black plastic bags to validate temperature and humidity.

After the incubation period, the minibins were removed from the growth chamber and the wooden planks disassembled. The recovered codling moth larvae were then assessed, and the mortality by infection determined by means of dissection. The minibins and planks used were then rinsed with bleach water and dried in an oven at 60°C for 24 h, before the next trial took place. Fresh batches of recycled IJs were used for every treatment.

Effect of adjuvants on EPN efficacy (using only wooden planks)

To test the effect of the adjuvants on the infectivity of *H. bacteriophora* (Hb1), *S. feltiae*, and *S. yirgalemense*, a superabsorbent polymer known as Zeba® [starch-g-poly (2-propenamideco-2-propenoic acid) potassium salt, Tongaat Hulett Starch, Germiston, South Africa] and a spreader/sticker known as Nu-Film-P® (poly-1-p-menthene, Hygrotech, Pretoria, South Africa) were used. A concentration of 3 g/L Zeba® and 0.6 ml/L Nu-Film-P® was used. For this trial, a concentration of 25 IJs/ml was used, with the treatments consisting of (1) nematodes, (2) nematodes + Zeba®, and (3) nematodes + Zeba® + Nu-Film-P®, with (4) water only, being used as a control. Concentrations were quantified an hour before the trial took place and the adjuvants were added to the nematode suspensions approximately 5 min before the treatment was applied.

Eight planks were used per treatment, with there being 20 diapausing codling moth larvae loaded per plank (*n* = 640 larvae). The planks were loaded with larvae 24 h before the trial, to allow the larvae to spin their cocoons, and then each plank was placed separately into a 2-L plastic container, which was closed with a lid.

The planks were pre-wet for 2 min and drip-dried for another 2 min, prior to treatment. The planks were then dipped into the treatment suspension (which was stirred mechanically prior to the dipping of each plank), drip-dried for a further 2 min, and then placed into separate 2-L plastic containers lined with moist paper towels, and closed with a lid. The containers were then placed in a growth chamber at 25°C for 3 days (72 h).

Following the incubation period, the planks were disassembled after removal from the growth chamber. The codling moth larvae were recovered and assessed for mortality by infection by means

of dissection. The planks were then placed into an oven at 60°C for 24 h before the next trial. A fresh batch of nematodes was used for each trial.

Statistical analysis

All trials were conducted on separate dates, with STATISTICA 12 software (StatSoft Inc. 2013) being used to perform all statistical analyses. Data obtained from the bin trials were analysed using analysis of variance (ANOVA), with post hoc comparison of means if there were no significant trial date-treatment interactions.

Results

Effect of nematode species using minibins

A two-way ANOVA of the results obtained, with the treatment and date as the main effects, showed no significant difference ($F_{(3, 56)} = 1.3599$, $p = 0.26445$) and the results from the two test dates were pooled. A one-way ANOVA of the results showed a significant effect of all treatments applied ($F_{(3, 56)} = 82.128$, $p < 0.005$) on the codling moth larvae mortality. *Steinernema feltiae* was found to differ significantly from *H. bacteriophora* (Hb1) ($p < 0.005$) and *S. yirgalemense* ($p = 0.027$), with the latter, *S. yirgalemense*, differing significantly from the former, *H. bacteriophora* (Hb1) ($p < 0.005$). *Steinernema feltiae* gave the highest mortality by infection (74.85% ± 3.64%), followed by *S. yirgalemense* (57.03% ± 3.64%), and then by *H. bacteriophora* (Hb1) (27.33% ± 3.64%) (Fig. 4.3).
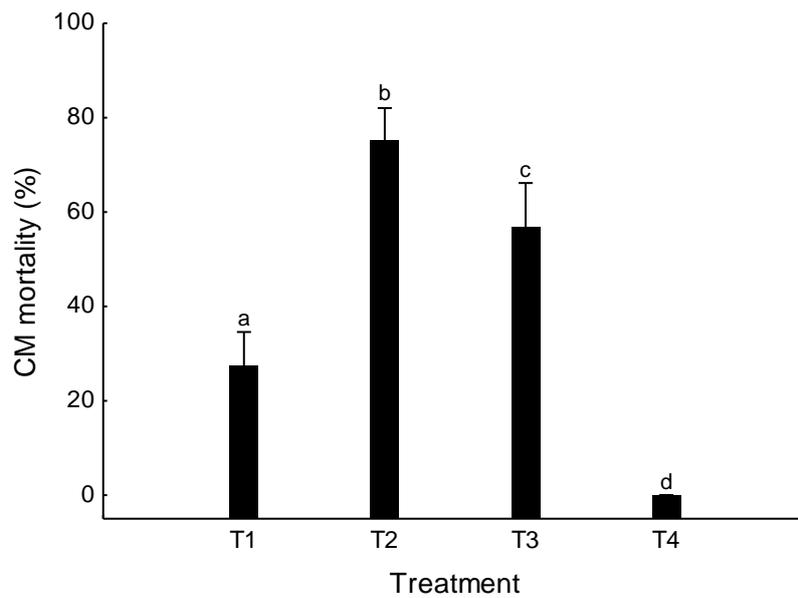
Fig. 4.3. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth (CM) larvae after submerging minibins in suspensions of three EPN isolates at 25 IJs/ml. Treatments were: *H. bacteriophora* (Hb1) (T1), *S. feltiae* (Sf) (T2), *S. yirgalemense* (Sy) (T3), and water only (T4). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(3, 56)}$ = 82.128, p < 0.001).

Effect of adjuvants on EPN efficacy (using only wooden planks)

H. bacteriophora

All three nematode treatments resulted in > 60% codling moth larval infectivity, *H. bacteriophora* (Hb1) alone resulted in 62.13% ± 8.7% infectivity, with the addition of Zeba® + Nu-Film-P® to the *H. bacteriophora* suspension increasing infectivity to 80.88% ± 4.7%, and the addition of Zeba® resulting in the highest infectivity of 86.25% ± 5.7%. A one-way ANOVA of the results showed a significant effect of all treatments applied ($F_{(3, 28)}$ = 48.030, p < 0.001) in relation to the codling moth larvae mortality. Although certain treatments resulted in a higher percentage of codling moth infectivity, no significant differences were found between the three *H. bacteriophora* (Hb1) suspensions (Fig. 4.4).
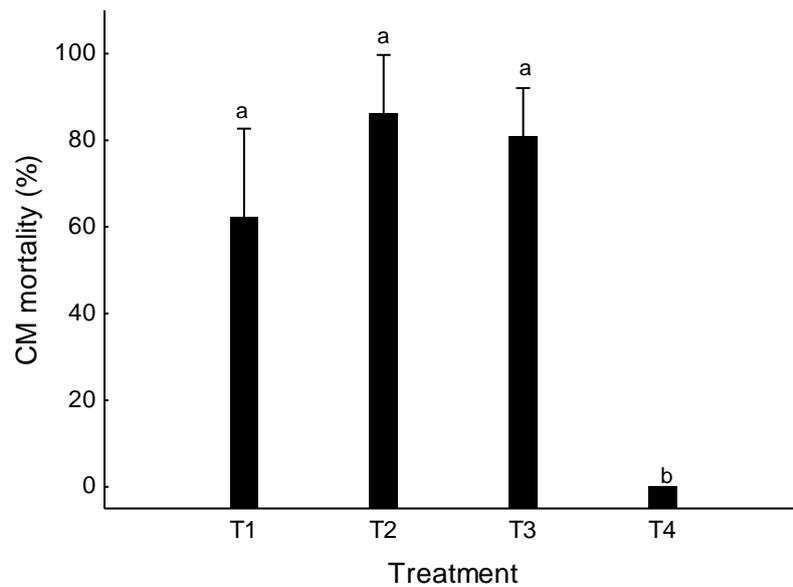
Fig. 4.4. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth (CM) larvae after submerging planks in three suspensions of the *H. bacteriophora* (Hb1) isolate at 25 IJs/ml. Treatments were: Hb1 + water (T1), Hb1 + Zeba® (T2), Hb1 + Zeba® + Nu-Film-P® (T3), and water only (T4). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(3, 28)}$ = 48.030, $p < 0.001$).

S. feltiae

Analysis of the results showed a significant effect of all treatments applied ($F_{(3, 28)}$ = 214.55, $p < 0.001$). A significant difference was found between *S. feltiae* only and the two *S. feltiae* suspensions to which adjuvants were added, being *S. feltiae* + Zeba® (p < 0.001) and *S. feltiae* + Zeba® + Nu-Film-P® (p < 0.001). There was no significant difference between the *S. feltiae* suspensions to which adjuvants were added (p = 1). All three treatments resulted in > 70% infectivity of the codling moth larvae, with *S. feltiae* alone resulting in 76.13% ± 3.15% infectivity, followed by *S. feltiae* + Zeba® with 96.88% ± 3.15% infectivity, and then by *S. feltiae* + Zeba® + Nu-Film-P® with 97.63% ± 3.15% infectivity (Fig. 4.5).
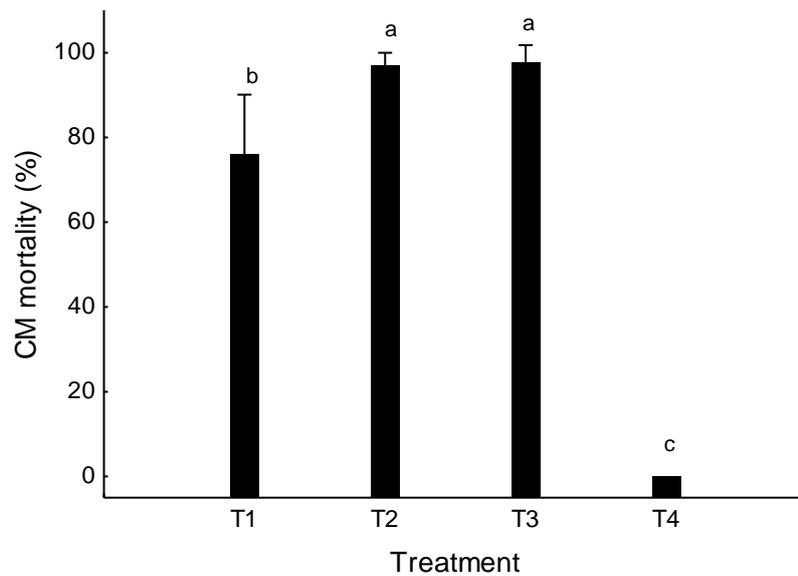
Fig. 4.5. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth larvae after submerging planks in three suspensions of the *S. feltiae* (Sf) isolate at 25 IJs/ml. Treatments were: Sf + water (T1), Sf + Zeba[®] (T2), Sf + Zeba[®] + Nu-Film-P[®] (T3), and water only (T4). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(3, 28)}$ = 214.55, $p < 0.001$).

S. yirgalemense

A one-way ANOVA of the results showed a significant effect of all treatments applied ($F_{(3, 28)}$ = 94.773, $p < 0.001$). There was no significant difference between any of the *S. yirgalemense* treatments ($p > 0.8$), with all three *S. yirgalemense* suspensions resulting in > 75% infectivity. *Steinernema yirgalemense* + Zeba[®] resulted in the highest infectivity (89.25% ± 4.27%), followed by *S. yirgalemense* (80.38% ± 4.27%), and then by *S. yirgalemense* + Zeba[®] + Nu-Film-P[®] (77.75% ± 4.27%) (Fig. 4.6).
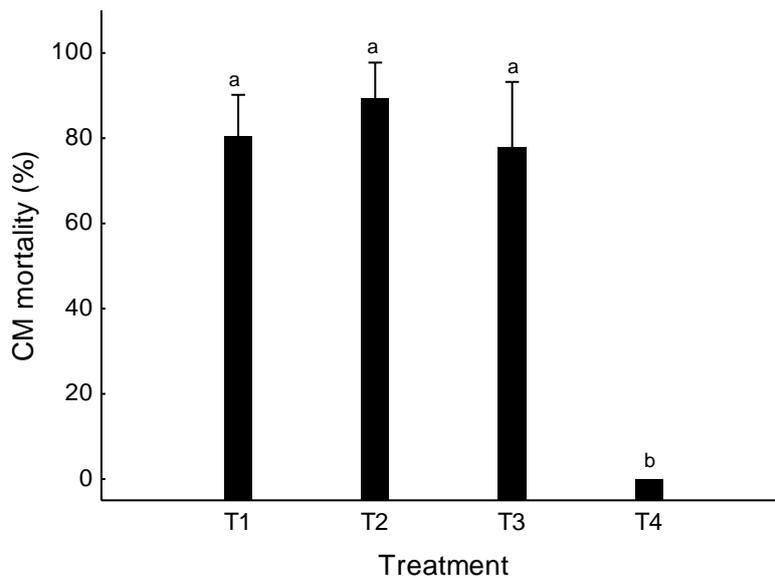
Fig. 4.6. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth larvae after submerging planks in three suspensions of the *S. yirgalemense* (Sy) isolate at 25 IJs/ml. Treatments were: Sy + water (T1), Sy + Zeba® (T2), Sy + Zeba® + Nu-Film-P® (T3), and water only (T4). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(3, 28)} = 94.773$, $p < 0.001$).

Combined effect of the addition of adjuvants

Analysis of the results showed a significant effect of all treatments concerned ($F_{(9, 86)} = 92.615$, $p < 0.001$). *Steinernema feltiae* resulted in the highest infectivity when combined with adjuvants, Zeba® + Nu-Film-P® (97.63% ± 4.45%) (T6), and Zeba® only (96.88% ± 4.45%) (T5), followed by *S. yirgalemense* + Zeba® (89.25% ± 4.45%) (T8), and *H. bacteriophora* + Zeba® (86.25 ± 4.45%) (T2). The *H. bacteriophora* treatment (T1) resulted in the lowest infectivity (62.13% ± 4.45%) and differed significantly from all nine EPN treatments (p < 0.03) (Fig. 4.7).
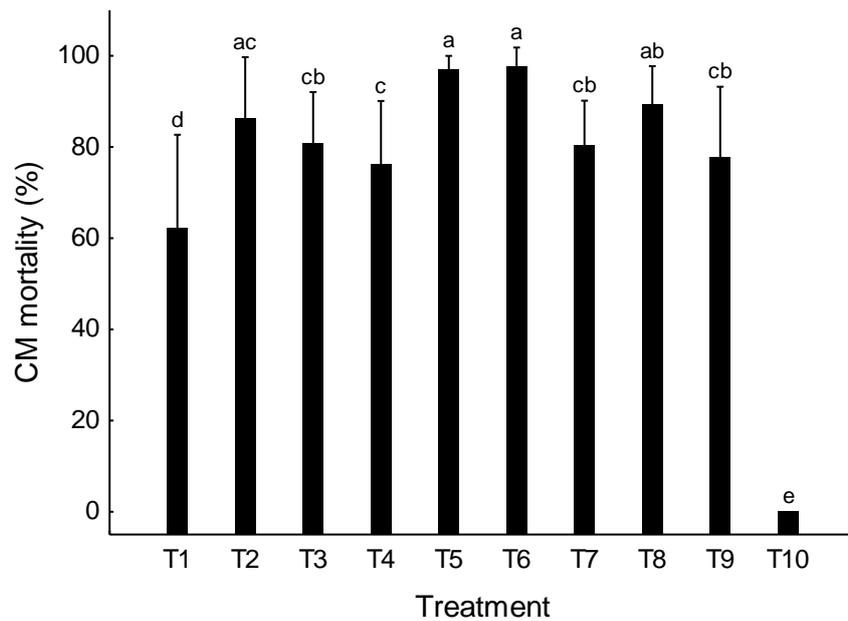
Fig. 4.7. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth larvae after submerging planks in three suspensions of the *H. bacteriophora* (Hb1), *S. feltiae* (Sf), and *S. yirgalemense* (Sy) isolates at 25 IJs/ml. Treatments were: Hb1 (T1), Hb1 + Zeba® (T2), Hb1 + Zeba® + Nu-Film-P® (T3), Sf (T4), Sf + Zeba® (T5), Sf + Zeba® + Nu-Film-P® (T6), Sy (T7), Sy + Zeba® (T8), Sy + Zeba® + Nu-Film-P® (T9) and water only (T10). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(9, 86)}$ = 92.615, *p* < 0.001).

Effect of containment method on EPN efficacy

The significant effect resulting from the containment method used was noted ($F_{(3, 88)}$ = 6.8896, p < 0.001) in respect of EPN efficacy in causing codling moth larval mortality. The containment method had no significant effect on the performance of *S. feltiae*, yet significantly influenced the efficacy of *H, bacteriophora* and *S. yirgalemense.* The planks increased *H. bacteriophora* efficacy from 27.33% ± 3.64% to 62.13% ± 8.7%, whereas they increased *S. yirgalemense* efficacy from 57.03% ± 3.64 to 80.30% ± 4.27% (Fig. 4.8).
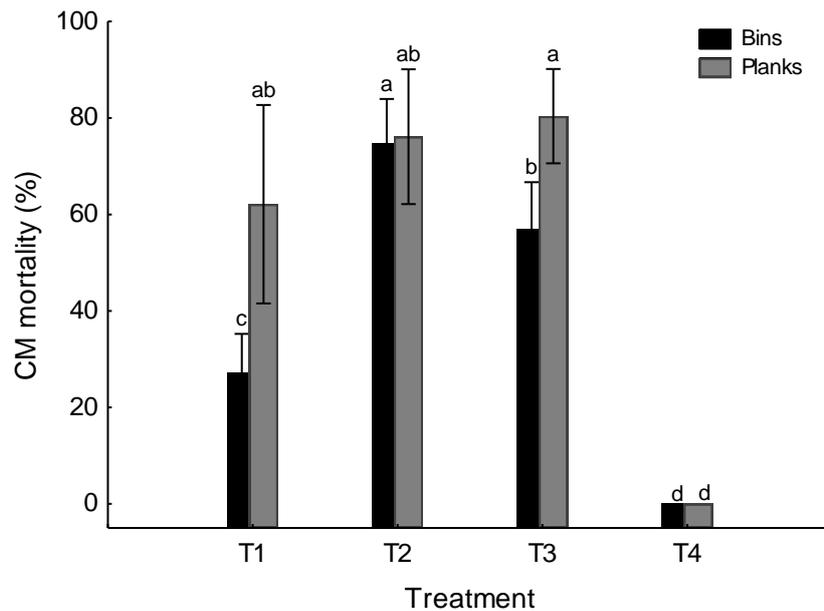
Fig. 4.8. Mean percentage mortality (95% confidence limits) recorded for diapausing codling moth larvae after submerging minibins and planks, and planks only, in suspensions of three EPN isolates at 25 IJs/ml. Treatments were: *H. bacteriophora* (Hb1) (T1), *S. feltiae* (T2), and *S. yirgalemense* (T3), and water only (T4). Different lettering above bars indicates significant differences (one-way ANOVA; $F_{(3, 88)} = 6.8896$, $p < 0.001$).

Discussion

Steinernema feltiae, at a concentration of 25 IJs/ml, resulted in the most effective disinfestation of the wooden fruit bins, with codling moth mortalities > 70%, followed by *S. yirgalemense* > 57% and *H. bacteriophora* < 30%. At the low concentration used, all three nematode species differed significantly from one another. De Waal *et al.* (2010) followed exactly the same procedure in testing the efficacy of a South African isolate of *H. zealandica*, attaining > 60% disinfestation of the wooden fruit bins used. The results obtained with *H. zealandica* are comparable with those obtained for *S. yirgalemense*.

The containment method used in the study was similar to the method that was used in studies by Cossentine *et al.* (2002), Lacey *et al.* (2005) and De Waal *et al.* (2010). The method used to retain the codling moth larvae seems to have had an effect on the efficacy of the EPN species involved (De Waal *et al.*, 2011). In this study, the planks that were used alone seemed to favour EPN efficacy, over the planks that were used together with bins. The containment method did not influence *S. feltiae* infectivity, as this EPN might be capable of acting faster than *H. bacteriophora* and *S. yirgalemense*. *Steinernema feltiae* thus does not seem to be influenced by the smaller surface area of the plank, or

by the consequently closer proximity of the codling moth larvae, as it is able to swiftly locate the larvae before moisture is lost on the surface. *Heterorhabditis bacteriophora* and *S. yirgalemense* act more slowly, and thus are favoured by the smaller surface area, and by the consequently closer proximity to the larvae.

Most of the other studies evaluating the potential of EPNs to disinfest wooden fruit bins have focused on *S. carpocapsae* (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005), *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 (Lacey & Chauvin, 1999), *Heterorhabditis marelatus* Liu & Berry, 1996 (Lacey & Chauvin, 1999) and *S. feltiae* (Lacey *et al.*, 2005). *Steinernema feltiae* and *S. carpocapsae* have shown comparable efficacy against wooden bins infested with codling moth larvae, resulting in > 80% control (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005).

In previous studies concerning wooden fruit bins, concentrations of 5 IJs/ml (Lacey & Chauvin, 1999; Lacey *et al.*, 2005), 6 IJs/ml (De Waal *et al.*, 2010), 10 IJs/ml (Lacey & Chauvin, 1999; Lacey *et al.*, 2005), 12 IJs/ml (De Waal *et al.*, 2010), 25 IJs/ml (Lacey *et al.*, 2005; De Waal *et al.*, 2010), 50 IJs/ml (Lacey & Chauvin, 1999; Cossentine *et al.*, 2002; Lacey *et al.*, 2005; De Waal *et al.*, 2010), 90 IJs/ml, 100 IJs/ml (Lacey *et al.*, 2005; De Waal *et al.*, 2010), and 153 IJs/ml (Cossentine *et al.*, 2002) were used to treat the bins. In the study by Lacey and Chauvin (1999), the use of 5 IJs/ml of *S. carpocapsae* resulted in effective control (bin corners: 68.1% ± 6.3%; bin skids: 34.9% ± 7.8%), and was thus, the concentration that they used throughout their study. Cossentine *et al.* (2002) found that *S. carpocapsae*, at 50 IJs/ml, ensured > 80% codling moth larval control, and that a higher IJ concentration was unnecessary, as it did not ensure more effective control in the fruit bins than might otherwise have occurred. In a concentration trial by Lacey *et al.* (2005), 50 IJs/ml of *S. feltiae* resulted in > 80% codling moth larval mortality, with further trials being conducted using a concentration of 10 IJs/ml that gave 50% control in the bins. *Steinernema feltiae* resulted in > 70% codling moth larval mortality at a concentration of 25 IJs/ml, whereas a higher concentration of *S. carpocapsae* (50 IJs/ml) was required to achieve comparative control (Lacey *et al.*, 2005). De Waal *et al.* (2010) used a concentration of 25 IJs/ml of *H. zealandica*, as this was the lethal dosage that ensured at least 50% (LD_{50}) codling moth larval mortality. The concentration of IJs used, can thus be seen to be influenced by the EPN species used.

The effect of tarping was not investigated, but has proven to increase codling moth larval mortality via EPN infection, due to an increased humidity within tarped bin stacks compared to within untarped bin stacks (Cossentine *et al.*, 2002; De Waal *et al.*, 2010). De Waal *et al.* (2010) recorded a humidity of 90% and 100% within tarped bin stacks, and a humidity of 25% and 40% within untarped bin stacks. Diapausing codling moth mortalities were much higher in pre-wet and tarped (63%, 88%) stacks than they were in pre-wet and untarped (42%, 44%) bin stacks (De Waal *et al.*, 2010). Cossentine *et al.* (2002) obtained similar results, and also proved that pre-wetting increases EPN efficacy.

The addition of adjuvants increased *S. feltiae* efficacy, resulting in > 95% codling moth larval control, although it had no significant effect on *H. bacteriophora* (Fig. 4.4) and *S. yirgalemense* (Fig. 4.6). Zeba® proved to be the most effective adjuvant in the case of *H. bacteriophora* and *S. yirgalemense*, while the mixture of Zeba® + Nu-Film-P®, seemed to decrease their infectivity. When all treatments were compared (Fig. 4.7), the addition of adjuvants increased *H. bacteriophora* and *S. feltiae* efficacy significantly, and resulted in higher codling moth larval control. The most effective EPN suspensions were found to be *S. feltiae* + Zeba®, followed by *S. feltiae* + Zeba® + Nu-Film-P®, then *S. yirgalemense* + Zeba®, and lastly, *H. bacteriophora* + Zeba®. EPN efficacy can thus be seen to be further improved by adding adjuvants to the suspension, as this slows down moisture loss, allowing for easier movement by the nematodes, as well as enabling longer survival times, and thus increased effectiveness (Lacey *et al.*, 2005; De Waal *et al.*, 2010).

This study has shed light on the efficacy of a local *S. yirgalemense* strain, as well as on that of the two imported strains, *S. feltiae* and *H. bacteriophora*, in terms of controlling the codling moth. It has also shown that the addition of adjuvants can increase EPN efficacy in this regard. In future studies, it would be interesting to investigate the effect of tarping and the addition of adjuvants and novel gelling agents to EPN suspensions in the disinfestation of commercial wooden bins under field conditions.

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CHAPTER 5

Conclusion

The codling moth is the key pest of apples and pears in South Africa, and their successful control with minimal chemical use is of key importance to the deciduous fruit industry, as far as ensuring the future export of the country's fresh fruit. The overall purpose of this study was to explore the use of entomopathogenic nematodes (EPNs) as a stand-alone, biological control agent for the control of the diapausing winter population of the codling moth in apple and pear orchards. This was achieved by containing artificially reared diapausing codling moth larvae in mesh cages, filled with apple bark, to evaluate the immediate effect of the nematodes 24 h after application in an apple orchard, without having to wait for moth emergence during the next growing season. For each field trial and simulated laboratory trial, a total of 640 diapausing codling moth larvae were used to indicate any significant differences between the treatments. All trials were repeated on different test dates.

The nematodes used in this study include commercially available, currently imported, formulated EPNs, as well as a local isolate proved to be highly virulent in previous studies, and which is in the process of being mass cultured and formulated. Different aspects of orchard application have been explored, including different nematode isolates, concentrations, application methods, and the effect of the temperature and humidity during the time of application. Conditions encountered in the orchards were simulated in the laboratory, to try and clarify the variability in the efficacy obtained during field application. The efficacy of available nematode isolates was tested in bin application trials, conducted in the laboratory, to obtain an indication of the different levels of efficacy among the different nematode isolates. Concurrently, the addition of adjuvants to improve the efficacy of EPNs in bin application was also investigated.

Care was taken in the outside use of *S. feltiae*, to avoid the biological pollution of the orchards with an exotic species. For this reason, the EPNs were applied by means of dipping the cages in nematode suspensions, rather than using spray applications, which would have been the norm. In a follow-up spray field trial, *S. feltiae* was replaced with a new local nematode isolate, *S. jeffreyense,* which incidentally gave the best results and warranted further investigation.

The first objective in evaluating an effective *S. yirgalemense* concentration to be applied under field conditions, to obtain effective codling moth control, showed that at even the lowest concentration used in the study (250 infective juveniles (IJs)/ml), >90% mortality was recorded. This concentration was thus used for all follow-up field trials. This was also the first field trial to be conducted with *S. yirgalemense*. In future studies, it will be important to determine the lowest effective concentration (LD_{50} and LD_{90}) of *S. yirgalemense* to be used in commercial application.

Due to the absence of prior data on the efficacy of both *H. bacteriophora* and *S. yirgalemense* under South African field conditions, and the unavailability of a local formulated product on the market for comparative purposes, field trials using *S. yirgalemense* as a control were conducted to assess the efficacy of two formulated strains of *H. bacteriophora* (Hb1-f, Hb2-f). *Steinernema yirgalemense* resulted in the highest codling moth mortality of >90%, whereas the formulated *H. bacteriophora* strains resulted in <28% control. The trial was repeated using recycled suspensions of freshly harvested IJs, resulting in a significant difference between all three isolates, and an increased efficacy of the two *H. bacteriophora* isolates (Hb1: 54%, Hb2: 31%), although they still performed poorly in comparison with *S. yirgalemense* (94%). This result might be due to *S. yirgalemense* being more adapted to the local climatic conditions and having high virulence against the codling moth. Future research could focus on the development of a formulated product of the local *S. yirgalemense* isolate, to allow for a fair comparison of this product to other imported, formulated products. The formulation and handling of EPNs during transportation plays a major role in their quality and in their efficacy. Investing in the research and development of a local formulated product might result in reliable high-quality EPNs being used in local orchards, as it reduces the cost as well as the risk of handling errors that may occur during transportation.

In all subsequent field and laboratory trials, *S. yirgalemense* was compared only to recycled suspensions of *S. feltiae* and *H. bacteriophora* (Hb1). *Steinernema feltiae* outperformed the other two isolates, with an infectivity of >75% being recorded for both field trials. The performance of *S. yirgalemense* (66% to 24%) and *H. bacteriophora* (Hb1) (24% to 9%) dramatically declined in the second field trial, while *S. feltiae* remained highly effective. This might be due to the lower average temperature and humidity during the 24 h exposure period, once again indicating the variance in susceptibility of different EPN isolates to certain temperatures and humidity. A prospective study,

examining the temperatures at which *S. yirgalemense* becomes inactive, could assist in the development of a protocol for the treatment of orchards with this EPN.

During a bioassay in the laboratory, in which four EPN isolates were compared, *S. feltiae*, followed by *S. yirgalemense*, once again resulted in higher codling moth larval infectivity. The *H. bacteriophora* isolates (Hb1 and Hb2) gave infectivity of 72% (Hb1) and 59% (Hb2), respectively. These findings indicate that *H. bacteriophora* is not an effective alternative option, and that it should not be used for codling moth control in the Western Cape Province, as in field conditions it is bound to result in an even lower infectivity than was obtained in the above-mentioned laboratory trials.

The first goal of identifying which EPN isolates effectively control diapausing codling moth was therefore achieved. Up to now, the local isolates, *S. yirgalemense*, *H. zealandic*a, and *S. jeffreyense*, have proven to be most virulent against diapausing codling moth in South African orchards. Future studies should focus on the field application of these three isolates, and concentrate on examining the effect of agrochemicals, adjuvants and application techniques on their efficacy. The most effective isolate must then be identified, with further research being pursued, focusing on the development of a local formulated product that will be effective under local climatic conditions.

The second objective was to assess the effect of spray application on the effectiveness of different EPN isolates to control the codling moth larvae in a local orchard. The data obtained from the field were then compared to those that were obtained in simulated laboratory trials, to examine the effect of temperature on the activity of these EPN isolates. For the field spray trials, *S. jeffreyense* (67%) resulted in the highest mortality of codling moth larvae, while *H. bacteriophora* (42%) and *S. yirgalemense* (41%) performed poorly. These are rather variable results for *S. yirgalemense*, as in previous trials, when EPNs were applied by dipping, this isolate resulted in >89% codling moth larval mortality during field trials. The inconsistency of these data might be due to the fact that the apple tree bark in the cages remained moist for longer when the cages were dipped, than when they were sprayed. This might have resulted in an environment that was more conducive to *S. yirgalemense*, leading to this isolate's higher efficacy against codling moth larvae. The variable results might also be attributed to the different behaviours and foraging strategies of the three EPN isolates concerned, and *S. jeffreyense* might have located the codling moth larvae faster (before the bark dried out) than did the other two isolates. A future study could examine the host seeking ability of the three EPN isolates,

and compare the speed ($LD_{50, 90}$) at which they find and infect host larvae. It would also be interesting to assess to what extent pre-wetting aids in the effectiveness of the three EPN isolates, when they are sprayed in an orchard.

In order to try and clarify this variance in the data, laboratory trials simulating the field spray trials were conducted. *Steinernema jeffreyense* was replaced by *S. feltiae*, due to there being no chance of contamination with this alien species in the laboratory. *Steinernema feltiae* resulted in the highest control of codling moth larvae (67%), followed by *S. yirgalemense* (58%). These results indicate that the method of application has an effect on the efficacy of the EPNs involved. Further research should therefore, be aimed at identifying a suitable EPN application technique for use in orchards, which will not only be compatible with the EPN isolate used, but which will also ensure good coverage of trees in the orchards. The coverage of trees can be improved through the addition of adjuvants to EPN suspensions, thus the effect of different commercially available adjuvants on EPNs, as well as whether adjuvants significantly increase EPN efficacy in real-time/large-scale trials is worth investigating.

Data from the temperature bioassays conducted in the laboratory confirmed that *S. feltiae* is a cold-active nematode, whereas *S. yirgalemense* and the two *H. bacteriophora* isolates (Hb1 and Hb2) are more efficient at warmer temperatures. In future trials, it would be interesting to compare the efficacy of *S. feltiae* and *S. jeffreyense* in laboratory bioassays, and to assess the efficacy of the *S. jeffreyense* isolate at cooler temperatures.

The second aim of assessing the effect of spray application on EPN efficacy was thus achieved; however, further research is pivotal in determining the most effective application technique to be employed. Not all EPNs have the same size IJs, thus the application technology used has to be compatible with the EPN isolate employed.

In the last objective, the potential of two imported, and one local, EPN species to disinfest wooden fruit bins of codling moth larvae was investigated. *Steinernema feltiae* resulted in the most successful disinfestation of bins (75%), followed by *S. yirgalemense* (57%) and then *H. bacteriophora* (Hb1) (27%). A future study could investigate the efficacy of *S. jeffreyense* in the laboratory, as well as assess the efficacy of the most promising isolates under local field conditions, without the control of either temperature or humidity, and using commercial wooden bins.

During the evaluation of the effect of adjuvants on the performance of the three EPN isolates mentioned, it was found that the adjuvants improved EPN performance significantly. The addition of Zeba® (3 g/L water) to EPN suspensions showed the most promising results (infectivity: Hb ↑ed from 62% to 86%; Sf ↑ed from 76% to 97%; and Sy ↑ed from 80% to 89%). The addition of adjuvants seems to permit wooden fruit bins to retain moisture for longer, thus extending the period during which EPNs can find and infect the codling moth larvae. Future studies could be done on the effect of adjuvants and novel gelling agents on EPNs (e.g. how long before EPN spraying the adjuvant should be added, negative effects on performance, etc.), and to determine the most effective, environmentally friendly, and cost-effective adjuvant to be used in field trials. Once these aspects have been determined, trials on commercial bins in bin stacks could be conducted, to develop a protocol for commercial bin disinfestation.

Results from this study were, in many ways, unexpected. From all the experiments conducted, it was concluded that *H. bacteriophora* would not be suitable for the biological control of the codling moth, despite it naturally occurring in many South African orchards. The significant differences between the different batches of the same *H. bacteriophora* species was also an unexpected result that should be further investigated.

The superiority of *S. feltiae* to control the codling moth was confirmed, with it also supporting other studies in being more effective at relatively low temperatures. It was also shown that *S. feltiae* was able to adapt better to environmental variation, especially in terms of temperature and humidity, whereas, in the case of the local *S. yirgalemense*, the outdoor environment added greatly to its variation in efficacy. Laboratory-based temperature studies showed that, over time, comparable results were obtained by all EPN species tested, but that the penetration rate of *S. feltiae* was higher at a lower temperature, compared to those of *S. yirgalemense,* while at higher temperatures, the reverse was found. In general, *H. bacteriophora,* apart from its low efficacy in controlling the codling moth, also showed a low penetration rate.

The fact that laboratory results cannot be directly extrapolated to field conditions has also been confirmed by numerous preceding studies. In this study, field trials were first performed, followed by simulated laboratory trials. Clear indication was given that the efficacy of EPNs to control the codling moth was compromised during the first 24 h of field conditions, especially with regard to the local

isolate *S. yirgalemense.* The results obtained also confirmed that most biological control agents cannot be used as a stand-alone control measure, but that they should be applied in combination with other control measures, in an integrated pest management system (IPM). Even 60% control of the overwintering codling moth population, would be sufficient to rid the next generation of those insect larvae escaping chemical control with possible resistance development. However, even though the results from this study clearly show that, under optimum field conditions, and with a highly virulent nematode isolate, good codling moth control can be obtained, combining EPNs with another biocontrol agent, such as entomopathogenic fungi, should be considered in future research.