Entomopathogenic nematodes: characterization of a new species, long–term storage and control of obscure mealybug, *Pseudococcus viburni* (Hemiptera: Pseudococcidae) under laboratory conditions

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Declaration

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Abstract

The obscure mealybug, *Pseudococcus viburni* (Signoret) (Pseudococcidae), is one of the common and serious pests of apples and pears in South Africa. The management of this pest in South Africa is dominated by the use of insecticides, while research into using natural enemies for biological control of mealybugs is still ongoing. Increasing concern over the environmental impact, pesticide residues in fruits, resistance, and expense associated with frequent use of insecticides make it necessary to investigate alternative biological control methods, such as the use of entomopathogenic nematodes, for the control of mealybugs. Entomopathogenic nematodes have proven comparable or even superior to chemicals in controlling certain insect pests, without residue problems or a harmful effect on the environment.

An important aspect of using endemic nematodes includes the identification of species of nematodes and their symbiotic bacterial cells. A study was carried out to describe a new species of *Steinernema,* which was recovered during a previous survey in citrus orchards in three provinces of South Africa. Morphometrics, morphology, crossbreeding, drawings, light microscopy and Scanning Electron Microscopy (SEM) photographs were used to describe the new species.

A cryopreservation method has been simplified and optimised for the long-term storage of *Steinernema khoisanae* (SF87) and *Heterorhabditis zealandica* (J34). Different cryoprotectants used included 15% glycerol, 8% ethylene glycol and 8% dimethyl sulfoxide (DMSO), in which *S. khoisanae* was incubated at room temperature for periods of two, three, four and five days, followed by a methanol wash. An optimum survival rate of 69% was obtained for *S. khoisanae* after a four-day incubation period in 15% glycerol. This technique has been used for the cryopreservation of *H. zealandica,* with a 78% survival rate. The thawed nematodes of both species were able to infect *Galleria mellonella* larvae after 42 days of cryopreservation (-196ºC) and were able to complete their life cycles.

A fast screening was done to identify isolates of entomopathogenic nematodes with a high percentage mortality against *P. viburni*. Five out of the 16 tested isolates were selected as promising candidates for the control of *P. viburni*. From the five isolates, *H. zealandica* (J34) was selected as the most promising isolate to be used for further studies. The biological development of a steinernematid and a heterorhabditid inside adult *P. viburni* females was followed. The number of nematodes that penetrated; the development of the different generations; and the time and number of IJ that emerged, were noted. *Heterorhabditis zealandica* (J34) and *S. yirgalemense* (157–C) successfully reproduced in *P. viburni*.

The effect that mealybug size has on infectivity was assessed. Adults and intermediates were more susceptible to nematode infection than crawlers. Nematodes were tested for their ability to locate and infect mealybugs on the surface, ovary and calyx of *P. viburni* field-infested apples. Results from the study indicated that the nematodes are capable of locating and infecting mealybugs, even in the ovaries of infested apples. To determine the lethal time and dose, mealybugs were exposed to 52, 73, 102, 143, and 200 IJ/insect for 12, 24, 36 and 48 hours. The LD_{50} and LD_{90} values were calculated as 54 and 330 nematodes per insect, respectively and the LT_{50} and LT_{90} values were 30 and 62.5 hours, respectively, were calculated.

The overall aims of this study were to describe a new *Steinernema* species*;* to develop a technique for long-term storage of nematodes; and to determine the potential of nematodes to control *P. viburni* under laboratory conditions. The study showed good potential use of entomopathogenic nematodes for the control of mealybugs. Further studies for the successful future use of entomopathogenic nematodes for the control of *P. viburni* in glasshouses and in the field are still needed. Innovative ways to overcome obstacles such as humidity, ultraviolet rays and temperature in the field should be overcome.

Opsomming

Die ligrooswitluis, *Pseudococcus viburni* (Signoret) (Pseudococcidae), is een van die algemene en ernstige peste van appels en pere in Suid-Afrika. Die bestuur van hierdie pes word tans in Suid-Afrika deur die gebruik van insekdoders gedomineer terwyl navorsing oor die gebruik van natuurlike vyande vir die beheer van *P. viburni* nog aan die gang is. Die verhoogde kommer oor die omgewing, residue in vrugte, weerstand, en die koste verbonde aan die gereelde gebruik van chemiese middels maak dit nodig om alternatiewe biologiese metodes van beheer, soos die gebruik van entomopatogeniese nematodes vir die beheer van witluis, te ondersoek. In ander lande is reeds aangetoon dat entomopatogeniese nematodes onder sekere omstandighede en vir sekere insekte gelykwaardige of selfs beter beheer kan gee as chemiese middels.

'n Belangrike aspek van die gebruik van endemiese nematodes vir die beheer van insekte sluit die korrekte identifikasie van die spesies met hul geassosieerde bakteriese simbionte in. 'n Nuwe spesie van *Steinernema* is uit 'n vorige opname van entomopatogeniese nematodes in sitrusboorde in drie provinsies van Suid-Afrika geïsoleer. Morfometrie, morfologie, kruisteling, ligmikroskoop en SEM fotografie is gebruik om 'n nuwe spesies te beskryf.

'n Kriopreserveringsmetode is ontwikkel en ge-optimaliseer vir die langtermyn bewaring van *Steinernema khoisanae* (SF87) en *Heterorhabditis zealandica* (J34). Verskillende kriobeskermingsmiddels insluitend 15% gliserol, 8% dimetiel sulfokied (DMSO) en 8% etileen glikol, waarin *S. khoisanae* vir periodes van twee, drie, vier, en vyf dae geïnkubeer is, is teen kamertemperatuur, getoets, gevolg deur 'n metanolbad. Optimum oorlewing van 69% is verkry vir *S. khoisanae* nadat die infektiewe larwes (IJ) vir vier dae in 15% gliserol gehou is. Hierdie tegniek is ook toegepas op *H. zealandica,* met 78% oorlewing van die IJ. Die ontvriesde nematodes van beide spesies was in staat om *Galleria mellonella* larwes suksesvol te infekteer en hulle lewensiklus te voltooi nadat hulle vir 45 dae onder kriopreservering gehou is teen -196ºC.

'n Vinnige siftingsproses is uitgevoer om vas te stel watter endemiese nematode-isolate die hoogste persentasie mortaliteit van volwasse *P. viburni* wyfies veroorsaak. 'n Totaal van 16 nematode-isolate is getoets, waarvan vyf as belowend geselekteer is. Vanuit hierdie vyf isolate is

die *H. zealandica*-isolaat J34 as die mees belowende kandidaat gekies en in verdere studies gebruik. Die biologiese ontwikkeling van 'n heterorhaditid en 'n steinernematid is in geïnfekteerde *P. viburni* wyfies gevolg. Die aantal nematodes wat die insek penetreer, die ontwikkeling deur die verskillende generasies en die tydsduur en getal IJ wat ontwikkel, is waargeneem. Beide *H. zealandica* (J34) en *S. yirgalemense* (157-C) het suksesvol in *P. viburni* gereproduseer.

Die effek van drie verskillende groottes van *P. viburni* op infeksie met *H. zealandica* is ondersoek. Volwassenes en intermediêre grootte witluise was meer vatbaar vir nematode-infeksie as kruipers. Die nematodes is ook getoets vir hulle vermoë om *P. viburni* op die oppervlakte van appels, onder die kelkblare en op die vrug ovarium te infekteer. Daar is aangetoon dat nematodes wel witluise onder die kelkblare en op die die ovarium kan infekteer. Om 'n aanduiding te kry van die dodingstyd en dosis van nematodes, is witluise blootgestel aan 52, 73, 102, 143 en 200 IJ/insek vir periodes van 12, 24, 36 en 48 uur. Die LD_{50} en die LD_{90} waardes bereken as 54 en 330 nematodes per insek, respektiewelik, en die LT_{50} en LT_{90} waardes as 30 en 62.5 uur.

Die oorhoofse doel van die studie was om 'n nuwe *Steinernema* spesies vir Suid-Afrika te beskryf; om 'n tegniek vir die suksesvolle langtermyn opberging van nematodes te optimaliseer; en om die potensiaal van nematodes as 'n biologiese beheermiddel vir *P. viburni* in laboratoriumstudies te ondersoek. Goeie potensiaal vir die gebruik van entomopatogeniese nematodes vir die beheer van witluis is aangetoon. Verdere studies in die gebruik van nematodes teen witluis in glashuise en onder veldtoestande word benodig. Die toekomstige suksevolle gebruik van entomopatogeniese nematodes vir die beheer van *P. viburni* berus op die ontwikkeling van innoverende tegnieke om die probleme geassosieer met ultraviolet skade en uiterste temperature in die veld te oorkom.

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Table of contents

Chapter 1

Literature review

Pseudococcus viburni (Signoret) (obscure mealybug)

Occurrence and distribution

Mealybugs (Homiptera: Pseudococcidae) are major agricultural pests and pose serious problems when introduced into new areas of the world, in absence of their natural enemies (Miller *et al*., 2002). In South Africa alone, 50 mealybug genera have been reported, with about 50% occurring in the Western Cape Province (Millar, 2002). Thirteen of the 50 genera are endemic to the country. Wakgari and Giliomee (2004) documented the presence of three species of mealybugs on apple and pear orchards in the Western Cape Province. These were: *Pseudococcus viburni*, also called the obscure mealybug (on apples and pears): *Pseudococcus calceolariae* (Maskell), known as the citrophilus mealybug (which occurs mainly on apples): and *Pseudococcus longispinus* (Targioni-Tozzetti), the long-tailed mealybug (found mainly on pears). The most dominant mealybug species found on apples and pears was found to be *P. viburni* .

Originally described by Signoret as *Dactylopius viburni* and *D. indicus* in 1875 (Miller & Polavarapu, 1997), *P. viburni* was formerly known as *P. affinis* (Maskell) and *P. obscurus* (Essig). Its close resemblance to other mealybug species makes it difficult to identify *P. viburni* in the field, with the result that some authors in the past have regarded it as a synonym of *Planococcus citri* (Risso). *Pseudococcus viburni* has been verified as a pest in pear and apple orchards, as well as in coastal vineyards, especially in association with the honeydew-feeding Argentine ant, *Iridomyrmex humilis* (Mayr) (Hymenoptera: Formicidae) (Phillips & Sherk, 1991). It can be spread via plant material, mainly by humans and animals (Schoen & Martin, 1999).

Host plant range

Pseudococcus viburni has been recorded from 296 host plant species in 87 families in all zoogeographical regions (Ben-Dov *et al.*, 2002). Such hosts include herbaceous plants, woody

plants, and weeds such as wild carrot, pineapple, prickly pear, pawpaw, persimmon, cockspur coral tree, pomegranate, apple, boxthorn, yew, daphne and red ginger. In vineyards in the Western Cape Province of South Africa, *P. viburni* has been recorded on the roots of *Conyzia bonariensis* (L.) Cronq, *Bidens pilosa* L, *Datura stramonium* L., *Erodium moshantum* (L.) L' Herit ex Ait., and *Sonchus oleraceus* (L.) Hill (Walton & Pringle, 2004). In The Netherlands, *P. viburni* has been reported as a pest of tomatoes in greenhouses (Schoen & Martin, 1999) with it also being reported as an apple pest in New Zealand (Ward, 1966). It is a pest of grape vines in California where its control has been improved by eliminating the attending ant species (Phillips & Sherk, 1991). In Iran, *P. viburni* has been found in tea gardens (Abbasipour & Taghavi, 2007). In France it has been found to infest soilless tomatoes in greenhouses (Kreiter *et al.*, 2005).

Life cycle

With a good food source and mild temperatures, adult female mealybugs can lay as many as 500 eggs. The clutches of orange eggs are laid in a profusion of very fine wax threads which the females secrete to form a characteristic egg-sac in order to protect the eggs from predators and the environment. The eggs may hatch within a week in summer; however if environmental conditions are too cold, young nymphs will remain in the sac until temperatures rise (Sheard & Kaiser, 2001). Like all pseudococcids, *P. viburni* females undergo development through three nymphal instars, while males have four (Gullan, 2000). The development of the female and male is similar for the first two instars. Females complete an additional instar before they develop into adults, while males spin a cocoon after a number of instars in which they moult a number of times and from which they eventually emerge (Swart, 1977; Gullan, 2000). Male mealybugs are tiny, fragile insects with long antennae, and no functional mouth parts. They have a single pair of wings and very short life spans as they do not feed, having as their main purpose to mate with females. Young mealybug instars are known as crawlers, and it is during this stage that the mealybugs are most mobile. Adult female mealybugs are less mobile, but can intermittently move about. Very old individuals become totally immobile due to the degeneration of their legs (Swart, 1977).

The obscure mealybug overwinters in sheltered places such as under the bark of trees, spurs and canes, where they continue to breed slowly. In late spring and summer, the crawlers emerge and move considerable distances to shoots, leaves and fruits where they start feeding. In the case of fruits, the mealybugs concentrate and breed at the stalk and calyx ends and may penetrate into the ovary (Swart, 1977). By autumn, mealybugs return to the woody parts of the plant to overwinter.

Economic importance

In South Africa, there are about 20 species of Pseudococcidae, which are of economic importance on cultivated plants (Annecke & Moran, 1982). Among these is *P. viburni,* which is a well-known cosmopolitan pest. Though not registered as a quarantine pest for the USA markets, the presence of *P. viburni* may result in rejection of fruit. During 2002, growers sustained heavy financial losses when about 30% of apples and 9% of pears destined for the USA were rejected due to the presence of mealybug eggs and immature mealybugs. The sooty mould growth in the sticky honeydew secreted by mealybugs decreases the rate of photosynthesis and makes the fruit unmarketable (Abbasipour & Taghavi, 2007). Some fruit varieties are more susceptible to attack by mealybug than are others and the pest can potentially result in up to 60% of the crop beingunsuitable for export (Kriegler & Basson, 1962). Damage by mealybugs is of a secondary nature in that the fruits become fouled with the mealy bugs themselves, as well as with their wax secretions, egg-sacs and especially honeydew, on which black sooty mould grows. In cases where the mealybugs have penetrated the core at the calyx ends, honeydew and or sooty mould are usually the only external indications of infestation of the fruits by the insects. Mealybug-infested fruits cannot be marketed, although little or no damage is caused to apple and pear trees and their fruits (Swart, 1977).

Control measures

Mealybug has been a serious pest on pears since the 1930s, with it reaching epidemic proportions for the first time during the 1961/1962 season (Van der Merwe, 2000). The increase in

mealybug infestation could have been the result of certain pest management practices, including the use of DDT (Kriegler & Basson, 1962). In South Africa, the management of the pest is dominated by the use of insecticides (Swart, 1977). Mealybugs are difficult to control chemically, mainly due to the rapid development of resistance to insecticides, with their powdery hydrophobic wax repelling water-based insecticide solutions (Blumberg & Van Driesche, 2001; Derzelle *et al.*, 2004). They also often live deep inside cracks in the bark of the tree or inside the fruit where they are protected from contact with insecticides. Mealybugs have been known to develop resistance to organophosphates, especially parathion, in the USA and in South Africa (Myburgh & Siebert, 1964; Flaherty *et al.*, 1982). Although insecticides can be used to control populations of mealybugs when they increase to pest levels, only few insecticides can be applied on fresh fruits without exceeding the residue tolerance set by the export markets (Derzelle *et al.*, 2004).

In greenhouses, mealybugs can be successfully controlled by natural enemies such as *Leptomastix dactylopii* Howard and *Leptomastix epona* Walker (Blumberg & Van Driesche, 2001). Parasitoids of obscure mealybug are fended off by the presence of the Argentine ant, *Iridomyrmex humilis* Mayr, which tends the mealybugs. Phillips and Sherk (991) found that, by controlling such ants, mealybug infestation levels can be significantly reduced. Five primary parasitoids that were recovered from *P. viburni* are *Anagyrus* sp., *Acerophagus* sp., *Pseudaphycus maculipennis* (Mercet), *Pseudectroma* sp. and *Tetracnemoidea* sp. (Wakgari & Giliomee, 2004). *Pseudaphycus maculipennis* is the primary biological control agent used against *P. viburni* in New Zealand (Charles *et al.*, 2004), but in South Africa research into the species of natural enemies to be used as biological control agents of *P. viburni* is still ongoing. In France, *Pseudaphycus favidulus* (Brèthes) was identified and produced as a parasitoid of *P. viburni* (Siham & Kreiter, 2009). When it was released into fields infested with *P. viburni,* parasitism was very high at some sites, while, in some other sites, no parasitism was found.

Entomopathogenic nematodes

Introduction

Of all nematodes that have been studied for the biological control of insects, members of the Steinernematidae Filipjev, 1934 and Heterorhabditidae Poinar, 1976 have been found to be the most successful (Kaya & Gaugler, 1993). They are symbiotically associated with the entomopathogenic bacteria *Xenorhabdus* Thomas & Poinar, 1979 (for Steinernematidae) and *Photorhabdus* Boemare, Akhurst & Mourant, 1993 (for Heterorhabditidae) (Boemare *et al.*, 1993). The bacterial symbiont's role is to kill the insect host and to digest the host tissue, thereby providing suitable nutrient conditions for nematode growth and development (Fallon *et al.*, 2007). Entomopathogenic nematodes have an extraordinary host range and, together with their bacterial symbionts, kill insects within a short period of time. There is no intimate, highly adapted host–parasite relationship, as is the case with other parasites (Kaya & Gaugler, 1993).

Life cycle

The infective juvenile (IJ), which is the only free-living stage within the life cycle of entomopathogenic nematodes, enters the insect host through the mouth, anus, spiracles, or by direct penetration through the cuticle. Nematodes use carbon dioxide and perhaps other chemicals produced in the waste products of insects as cues to find their host. In the haemocoel of the host, the IJ releases the symbiotic bacterial cells which multiply and digest the host tissue, thereby providing suitable nutrient conditions for nematode growth and development. Within 24 to 48 hours, the insect host is killed by toxic metabolites released by the nematode bacterium complex (Dowds & Peters, 2002). Though the bacterium is primarily responsible for the mortality of most insect hosts, the nematode also produces a toxin that is lethal to the insect (Burman, 1982). The dead insect generally maintains its original shape and does not decay in a normal manner because its body is filled with such specialised bacteria. Associated colour changes may occur. The nematodes feed on the bacteria, and reproduce for several generations inside the cadaver. The first generation inside the host consists of both males and females for

steinernematids and hermaphrodites for heterorhabditids (Kaya & Gaugler, 1993). When food resources in the host become scarce, the adults produce new IJ adapted to withstand the outside environment. The body wall of the host insect disintegrates and hundreds to thousands of IJ emerge and leaving the insect host in search of other hosts, carrying with them an inoculation of mutualistic bacteria, received from the internal host environment (Boemare, 2002). The cycle, from the entry of the IJ into a host, to its emergence from the host, depends on the temperature and amount of food available, and varies for different species and isolates.

Though the IJ do not feed, they can survive for weeks on storage as active juveniles, and for months by entering a near-anhydrobiotic state. The length of time that the IJ survive in the soil in the absence of an insect host is dependent on factors such as temperature, humidity, natural enemies and soil type. Their survival is better in a sandy soil or sandy loam soil, at low moisture and at temperatures between 15 and 25ºC, than in clay soils and at lower or higher temperatures (Kung *et al.*, 1991).

The relationship between the nematode and the bacterium is mutualistic because the nematode is dependent on the bacterium for quickly killing the insect, transforming the host tissue into a food source, and creating a suitable environment for its development by producing antibiotics that suppress competing secondary microorganisms. The bacterium needs the nematode for protection from the external environment and for penetration into the host haemocoel (Kaya & Gaugler, 1993).

Surveys and description of new species

An important aspect of using endemic nematodes includes the identification of species of nematodes and their symbiotic bacterial cells. In South Africa, the first record of nematodes was from the maize beetle *Heteronychus arator* (Fabricius) (=*H. sanctae-helenae* Blanch). Harington (1953) observed large numbers of *Steinernema (Neoaplectana*) sp. in third instar larvae, pupae and adults of the beetle from a farm in Grahamstown, Eastern Cape Province. Three isolates of *Steinernema* and a *Heterorhabditis* were evaluated in KwaZulu-Natal against the African

sugarcane stalk borer, *Eldana saccharina* Walker, in laboratory and field tests (Spaull, 1988; 1990). A further survey was conducted in 1991 to obtain nematode isolates more virulent against *E. saccharina*, during which seven *Heterorhabditis* and 15 *Steinernema* isolates were found (Spaull, 1991), but were not identified to species level. A new species of *Steinernema* for South Africa, *Steinernema khoisanae*, was described by Nguyen *et al.* (2006) and in a survey by Malan *et al.* (2006), *Heterorhabditis* was the dominant genus, with *H. bacteriophora* Poinar 1975 the most common species for the Western Cape Province, while *Steinernema* species were rarely detected. A new *Heterorhabditis* species was also found during this survey and subsequently described by Malan et al. (2008) as *Heterorhabditis safricana* Malan, Nguyen, De Waal & Tiedt, 2008. In a survey by De Waal *et al.* (2009) aimed at obtaining South African isolates of nematodes for the control of codling moth, eight *Steinernema* spp. (three isolates of *S. khoisanae* and five undescribed *Steinernema* spp.) and 12 *Heterorhabditis* spp. (six isolates of *H. bacteriophora*, five isolates of *H. zealandica* and one isolate of *H. safricana*) were recovered.

Advantages and disadvantages of using nematodes for the control of mealybugs

As previously mentioned, mealybugs are difficult to control with insecticides due to their cryptic lifestyle. The powdery wax excreted by the mealybugs acts as a repellent to insecticides. Nematodes are ideal for control in cryptic habitats (Gaugler, 1981), because the IJ is mobile and can therefore seek out its insect host. As the nematodes are susceptible to drying and ultraviolet light, they are most effective against insects that occur in moist, dark locations. Other positive attributes of such nematodes as biological control agents are that they have a broad host range, and are safe for vertebrates, plants and other non-target organisms (Gaugler, 1981). They have no known negative effect on the environment, are easy to mass produce *in vivo* and *in vitro*, and are easily applied using standard spray equipment. They kill their hosts rapidly, and have the potential to recycle in the environment. Nematodes are compatible with many chemical and other biological pesticides, are amenable to genetic selection for desirable traits, and are exempt from registration in many countries (Kaya, 1993; Kaya & Gaugler, 1993). Negative attributes include their narrow tolerance of environmental conditions, their poor long-term storage, thir poor field

persistence, and their relatively high cost in comparison to that of chemical pesticides (Kaya, 1993).

Long-term storage of entomopathogenic nematodes

Cryopreservation is the short- or long-term storage of living cells using liquid nitrogen as a coolant (Chao & Liao, 2001) at –196ºC. For any cryopreservation method to be successful, cells must survive freezing and thawing. The simple, less expensive and most widely used cryopreservation protocol is vitrification, which is described as the formation of glasslike, noncrystalline solids at temperatures at or below the freezing point of an aqueous solution. This technique relies on treatment of the samples with a concentrated vitrification solution (cryoprotectant) for variable periods of time, followed by a direct plunge into liquid nitrogen (Panis & Lambardi, 2005).

The growing interest in nematodes as biological control agents has made it necessary to develop a successful technique for their long-term storage. Cryopreservation has many benefits over the normal maintenance of cultures, because it is less expensive, as it does not require the same amount of labour and space, or the maintenance of controlled environmental conditions (Sayre & Hwang, 1975; Bridge & Ham, 1985). It can save time in respect of the continuous culturing of organisms; eliminate the recurring problems of loss of lines through infection or cross–contamination; and ensure the availability and uniformity of material or lines for ongoing research. The survival of nematodes following cryopreservation is highly dependent on the age and strength of the nematodes prior to freezing; the preincubation period of nematodes in the cryoprotectant; the nematode concentration (before and after freezing); and the rates of freezing and thawing (Sayre & Hwang, 1975; Bridge & Ham 1985).

Many parasitic and free-living nematodes have been cryopreserved with varying success, using cryoprotective agents or partial dehydration approaches, which are believed to induce a cryoprotectable state in nematodes (Triantaphyllou & McCabe, 1989; Popiel & Vasquez, 1991; Curran *et al.*, 1992). Popiel and Vasquez (1991) reported a protocol for the cryopreservation of

Steinernema carpocapsae (Weiseri, 1955) Wouts, Mrácek, Gerdin & Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976, using a two-stage procedure of incubation of infective stage juveniles in a glycerol solution for 24 hours followed by incubation in 70% methanol at 0 ºC to 1ºC for 10 min before immersion in liquid nitrogen. When Curran *et al.* (1992) modified this protocol, they obtained a mean survival rate of 69% for isolates of *Steinernema* and 68% for isolates of *Heterorhabditis.* A study by Nugent *et al.* (1996) showed that while *S. carpocapsae* could be successfully cryopreserved in 0.5 ml volumes of 70% methanol, post-cryopreservation survival was considerably higher for *H. bacteriophora* when the pre-treated IJ were frozen on filter paper strips rather than in liquid suspension.

Previous work with entomopathogenic nematodes to control mealybugs

Only two studies have been conducted on the susceptibility of Homoptera to nematodes. Stuart *et al.* (1997) looked at the susceptibility of *Dysmicoccus vaccinii* Miller & Polavarapu to infection by various species and strains of nematodes. In a sand-dish assay they tested four nematode species, *S. carpocapsae*, *S. glaseri*, *H. bacteriophora* and *S. feltiae* at doses of 10, 50, 100 and 500 IJ. Results showed that none of the species produced significant levels of mealybug mortality compared to the control after a 48-hr exposure period. However, after 120 hours, significant levels of mortality were found for *H. bacteriophora* (HP88 strain) at doses of 50, 100 and 500 IJ with 63.6% mortality at the higher dosage. They investigated whether the wax layer served as a defence against nematode infection and showed that removal of the waxy coating of the mealybugs had no influence on the degree of susceptibility to *H. bacteriophora*. In another experiment they compared the effectiveness of various heterorhabditid species and strains against *D. vaccinii*. They also tested the effectiveness of *H. bacteriophora* and *H. indicus* (EMS-13 strain) in a sand–column assay. This required the nematodes to move through a column of sand, in order to locate and infect a single mealybug. Results from the sand-column assay showed that both *H. bacteriophora* and *H. indicus* are effective against *D. vaccinii*. *Heterorhabditis bacteriophora* did not induce any significant level of mortality at a dose of 100 IJ per mealybug, though it did at a

dose of 500 IJ per mealybug (93.8% mortality). *Heterorhabditis indica* induced significant levels of mortality for both doses (56.3% and 100.0%, respectively), and produced a significant dose response. When dead mealybugs were placed in White traps (White, 1927) to assess whether nematode reproduction would occur, three *Heterorhabditis* species (*H. bacteriophora*, *H. hawaiiensis*, and *H. indica)* successfully reproduced in and emerged from the mealybug cadavers.

De Waal *et al.* (2009) tested three South African species (*H. zealandica*, *H. bacteriophora* and *S. khoisanae*) for their potential to infect the adult female stage of *Planococcus ficus* (Signoret). In their measurement of the possible entry points they observed that though the ostioles, vulva and anus of the mealybugs were wide enough for all three species to enter, spiracles were too small. Screening results indicated that *P. ficus* was more susceptible to *Heterorhabditids* (with mortality ranging from 70 to 100%), compared to *S. khoisanae* with 54% mortality. They also noted that, during the infection process, the mealybugs released a yellowish liquid as a possible defence response, and mealybug colour changed and became dark after infection. The infected mealybugs also became very soft and fragile. These results, together with those obtained from the sand-column bioassay show that nematodes have a good potential for use against *P. ficus*.

lissues such as pesticide resistance; increasing concerns over pesticide residues on products; toxicity; the expense associated with frequent insecticide use, the restrictions set on the current use of pesticides, and the lack of effective biological control agents, make an alternative method of reducing *P. viburni* desirable. One of the alternatives to the use of chemicals is the use of insect pathogens, such as entomopathogenic nematodes, which have proven superior to chemicals in controlling certain target insects (Gaugler, 1981). The IJ of these nematodes possess attributes of both insect parasitoids and predators and microbial pathogens. Their chemoreceptors and motility are very important as *P. viburni* is known to occupy cryptic habitats in fruit trees. This lifestyle of *P. viburni* is, thus, an advantage to the nematodes because they are protected from UV light at such sites. Such nematodes have high virulence, kill their host quickly and have shown considerable potential as biological control agents of many insect pests such as sugar beet beetle

(Saleh *et al.*, 2009), pickleworm (Shannag *et al.*, 1994), white grubs (Grewal *et al.*, 2002) and dampwood termite (Wilson-Rich *et al.*, 2007).

Aims and objectives

The objectives of the current study were to:

1) use morphology and morphometrics to describe a new *Steinernema* species;

2) develop a protocol for the long-term storage of *S. khoisanae* and *H. zealandica;*

3) determine the potential of nematodes for the control of *P. viburni* under laboratory conditions.

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Chapter 2

Insect and nematode production

Source of insects

Mealybugs

A laboratory culture of mealybugs was essential to provide a constant and reliable supply of insects for the laboratory studies. According to Meyerdirk *et al.* (2002), a fruit or vegetable can be substituted as the host plant substrate for the mass production of insects. The culture of the mealybug *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae) was established in the laboratory from individuals originally collected from the field, which were identified and maintained on butternuts *Cucurbita moschata* (Duchesne ex Poir). Prior to use, each butternut was washed using a water and household bleach solution and then thoroughly rinsed with tap water and open-air dried. Cages (650 × 350 × 590) with a large viewing area and substantial ventilation to allow air circulation were used. To increase the size of the culture, uninfested butternuts were covered with a layer of infested butternuts. The colony increased as mealybugs dispersed naturally to fresh butternuts. This procedure was repeated once a fortnight and any rotting butternuts were discarded. Only adult female mealybugs were selected for most of the experiments.

Potatoes were also used to rear *P. viburni.* However such potatoes were only used to support a maintenance culture because developing a culture capable of being maintained on potato sprouts is a lengthy process. Sprouted white potato varieties purchased from a local supermarket were used (Karamaouna & Copland, 2000; Charles *et al.*, 2004). The potatoes were kept in a dark place for a few weeks to encourage sprouting. Daane (1998) indicated that the presence of large sprouts is important for the growth of *P. viburni*. The mealybug colony was kept in a 2-litre plastic container at 25ºC on a single layer of sprouting potatoes. The top of the plastic container was covered with nylon organza fabric to minimise crawler and adult male escape and to allow air circulation, so that the quality of the tubers was prolonged. Every two weeks a new container was established, with a single layer of sprouting potatoes, plus 2 to 3 potatoes which

were heavily infested with *P. viburni* (Sandanayaka *et al.*, 2009). The colony increased as mealybugs moved naturally from the infested to the fresh potatoes. In general, only small numbers of mealybugs were produced with the use of potato sprouts.

Wax moth larvae

The larvae of the Greater wax moth *Galleria mellonella* (Pyralidae, Lepidoptera) were reared in the laboratory by first collecting eggs. The eggs were collected by means of folding a piece of waxed paper in tight little accordion folds, like a fan, with which the eggs were lifted and placed in a glass container with adult moths. The adult wax moth laid their eggs on the wax paper and on the walls of the container. The eggs were then transferred to a container with a fresh culture medium (Büyükgüzel & Kalender, 2009) (consisting of baby cereal, brown bread flour, yeast, wheat germ, beeswax, glycerin and honey) and kept at 25ºC in a growth chamber. Wax moth eggs hatched to the larval stage in five to eight days.

Mealworm (yellow)

The larvae of the beetle *Tenebrio molitor (L)* were raised on wheat bran in a plastic lidded container that was kept closed. Slices of apple, potato, or carrots were laid over the surface of the colony to provide humidity for the mealworms. The bran was replaced whenever necessary. To maintain a supply of mealworms in a state of dormancy, a container with mealworms was kept at 4ºC in the refrigerator. At this temperature, the larvae became inactive, and no breeding or growth activity took place. Over time, a build-up of powdery residue called frass, appeared in the container. The frass which consisted of mealworm waste was regularly cleaned out.

Preparation of nematode inoculum

Nematodes were obtained from the Stellenbosch University collection and reared *in vivo* in either the last instar larvae of *Galleria Mellonella* (L.) or in the mealworm, *Tenebrio molitor* (L.), using standard procedures (Woodring & Kaya, 1988). Groups of 10 to 15 *G. mellonella* or *T.*

molitor larvae were placed in 9-cm Petri dishes, on filter paper. Nematode infections were induced by adding infective juveniles (IJ) in drops of tap water to the filter paper. After several days, a White trap (Woodring & Kaya, 1988) was created by placing the base of the Petri dish with the infected cadavers inside a 15-cm Petri dish that was half filled with tap water. Young IJ subsequently emerged from the host cadavers and migrated into the water of the White trap. Nematodes were harvested daily from White traps for one week by replacing water in the large Petri dish, and they were stored horizontally in a culture flask at 14ºC, being used in experiments within one to four weeks of harvesting.

The nematode concentration was calculated using the formula of Navon and Ascher (2000), $[(i/c)-1] \times V = Va$, where i = initial concentration, c = final concentration, V = volume of the suspension (ml), Va = the amount of water (ml) to be added (if positive) or to be removed (if negative) from the suspension. To determine the initial concentration, five drops of 10 µl using an Eppendorf micropipette were placed on a glass slide. The number of IJ in each drop was counted under a microscope with their sum being used to determine the concentration.

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Chapter 3

Steinernema n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa

Abstract

During a survey for the occurrence and distribution of entomopathogenic nematodes in citrus orchards throughout South Africa, a new species of *Steinernema* was isolated from a citrus orchard on the farm Rietkloof near the town of Piketberg in the Western Cape Province of South Africa. The species is herein morphologically described as *Steinernema* n. sp. The nematode was trapped from the soil using the *Galleria*-baiting technique. The new species is characterised by the following morphological characters: (third stage) infective juvenile (IJ) with a body length of 754 (623–849) µm, distance from head to excretory pore of 56 (49–63) µm, tail length of 71 (63–81) µm, ratio E value of 110 (85–132) µm. *Steinernema* n. sp. is closely related to species in the *feltiae* group. The body length of the IJ is close to that of *S. texanum* and *S. weiseri*, but it differs in body width, the length of the pharynx and the E%. The male of *Steinernema* n. sp, differs from *S. feltiae* in the length of the spicule and the body width. *Steinernema* n. sp differs from all species in the feltiae group in the morphology of the vulva as it has a single flapped low epiptygmata. It also differs from most closely related species of S. *feltiae* because there is no interbreeding between the two species.

Introduction

Members of the Steinernematidae Filipjev, 1934, and Heterorhabditidae Poinar, 1976 are symbiotically associated with the entomopathogenic bacteria, *Xenorhabdus* Thomas & Poinar, 1979 and *Photorhabdus* Boemare, Arkhust & Mourant, 1993, respectively. They have been shown to be effective biological control agents of several economically important pest insects (Klein, 1990; Shapiro-Ilan *et al.*, 2002; Shapiro-Ilan & Gaugler, 2002). So far, 56 species of *Steinernema*

Travassos, 1927 and 12 species of *Heterorhabditis* Poinar, 1976 are reported (Nguyen *et al.*, 2006a) with new species being discovered by the year. Entomopathogenic nematodes have been known since 1923 (Nguyen *et al.*, 2007). However, interest in them was rekindled by the necessity of having to use more environmentally friendly alternatives to chemicals.

Although many surveys have been carried out worldwide (Hominick, 2002), on the African continent knowledge of the geographical distribution of entomopathogenic nematodes is still in its infancy. Isolates of steinernematids from different areas may exhibit variation in their host range, in their biological, physiological and environmental adaptation, and in their suitability as commercial products. Collection of indigenous nematodes may provide more suitable isolates and species for inundative release against local insect pests (Kaya & Gaugler, 1993). Due to this rationale, many surveys have been coordinated in regions where the nematodes are unknown or because of restrictions on the import of exotic organisms, as in the case of South Africa. Different species or strains of nematode may possess different biological, ecological, or physiological characteristics that affect the field efficacy of the nematode-based biopesticides, such as high host range, behaviour, and tolerances to high or low temperature. Therefore, accumulation and correct identification of entomopathogenic nematode species are critical for success in using them as biopesticides for controlling insect pests (Qiu *et al*., 2004).

In South Africa, the first record of entomopathogenic nematodes was from a maize field in Grahamstown, in the Eastern Cape Province, by Harington (1953), who found numerous *Steinernema* (=*Neoaplectana*) in the larvae, pupae and adults of *Heteronychus arator* (Fabricius) (*H. sanctae-helenae*), the maize beetle. In 1988, one *Heterorhabditis* and three *Steinernema* isolates were evaluated in KwaZulu-Natal for the control of the African sugarcane stalk borer, *Eldana saccharina* Walker, in laboratory and field tests (Spaull, 1988; 1990). During a survey to obtain more virulent isolates against *E. saccharina*, seven *Heterorhabditis* and 15 *Steinernema* were found (Spaull, 1991), though they were not identified to species level. The *Steinernema* species were found to be rare in the surveys conducted in South Africa (Malan *et al.*, 2006; De Waal *et al.*, 2009; Malan *et al.*, 2009). A new *Heterorhabditis* species that was found in one of the

surveys was described as *Heterorhabditis safricana* Malan, Nguyen, De Waal & Tiedt, 2008 by Malan et al. (2008). Only two *Steinernema* species have been identified for South Africa, viz *S. khoisanae* Nguyen, Malan & Gozel, 2006 and *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005. *Steinernema khoisanae* is a newly described species for South Africa (Nguyen *et al.*, 2006a) and *S. yirgalemense* a new record for South Africa and the second record for the African continent. The current description concerns a second *Steinernema* in South Africa.

A recent survey in citrus orchards throughout South Africa was carried out to determine the occurrence and distribution of entomopathogenic nematodes for their potential to control false codling moth (*Thaumatotibia leucotreta*, Meyrick). The survey resulted in the recovery of more than 30 isolates of insect-parasitic nematodes, including *S. yirgalemense,* and the isolate described in the current study as a new species of *Steinernema*. Species of *Steinernema* have been grouped on the basis of the length of the infective juveniles (IJ). This new species belongs to the feltiae-group of *Steinernema*, of which the IJ are between 700 and 1000 *µ*m. It differs from all other species of *Steinernema* in morphological, morphometrical and in cross hybridisation tests with the closely related *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 (Nguyen *et al.*, 2006b). The new nematode is described and illustrated in the current study as *Steinernema* n. sp.

Materials and methods

Nematodes source

The *Steinernema* n. sp. was isolated in the laboratory from soil samples by means of trapping with *Galleria mellonella* (L.) larvae (Bedding & Akhurst, 1975). The isolate 141-C was collected from a citrus orchard (latitude 32°35’285S, longitude E18°39’383E) on the farm Rietkloof near the town of Piketberg in the Western Cape Province of South Africa. The infective juveniles (IJ) were maintained by recycling through *G. mellonella* (Dutky *et al.*, 1964) every three to four months and were stored in 100 ml of water in 500-ml culture flasks at 14ºC.

Morphological observations

For taxonomic and life cycle studies, 10 *G. mellonella* larvae in each of three 8.5–cm Petri dishes which were lined with moistened filter paper were inoculated with 200 IJ per *G. mellonella*. *Galleria* larvae died two days after inoculation, and the first generation males and females were obtained five days after the *Galleria* died. Second-generation males and females were observed after five to seven days, by dissecting the cadavers in Ringer's solution. Third-stage IJ were obtained after 14 days and harvested during the first week of emergence. Nematodes used for measurements were fixed in hot TAF (85ºC). For measurements, fixed nematodes of different stages were transferred to a drop of lactophenol on a glass slide; with 20 nematodes of each stage being measured as soon is they cleared. For direct observations to confirm the morphology or variation of some structures, different stages were examined live or killed with gentle heat. Type specimens were fixed in TAF and processed to glycerin by the Seinhorst method (Seinhorst, 1959) and mounted in pure glycerin supported by glass rods to prevent flattening. Measurements and drawings were made by using a Nikon compound microscope with a drawing tube.

Scanning electron microscopy (SEM)

Males, females of the first and second generation and IJ were fixed in TAF, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold (Nguyen & Smart, 1995). Spicules and gubernacula were dissected from the males and mounted on glass slides and photographed using a Nikon digital camera through the eyepiece of a light microscope (Nguyen & Smart, 1995).

Cross hybridisation

IJ of *S. feltiae* (Russian strain) were imported from the Czech Republic, after obtaining a South African import permit. The tests were conducted under quarantine conditions in South Africa. The method used was suggested by Nguyen and Duncan (Nguyen & Duncan, 2002), using *G. mellonella* haemolymph. A *G. mellonella* larva was double folded and a sterile syringe needle used to prick the skin close to a leg, and a drop of haemolymph of *G. mellonella* was placed in a

sterile Petri dish (35 × 10 mm). Drops of water were placed on the side of the dish to keep humidity high. One IJ of *Steinernema* n. sp. and one of *S. feltiae* were added to the dish. As a control, crosses between IJ of the same species were conducted. The treatment was replicated 20 times. All the Petri dishes were kept in closed plastic containers with a moistened facial tissue to keep the haemolymph from drying. The development of the inoculated IJ into adults and the reproduction of the nematodes were observed and recorded during the experimental period. All nematodes were killed by heat under the supervision of a plant quarantine officer as *S. feltiae* is an exotic entomopathogenic nematode for South Africa.

***Steinernema* n. sp.**

Description

Males (first generation)

Body slender, J-shaped when heat killed (Fig. 2A). Cuticle smooth under the light microscope but striations visible with SEM. Lateral field present in midbody with one narrow ridge. Cephalic extremity truncate with four cephalic papillae and six labial papillae, with cephalic papillae larger than labial papillae. Amphidial apertures pore-like. Cephalic extremity without a perioral disk. Stoma shallow and narrow, posterior part of the stoma funnel-shaped, with pronounced cheilorhabdions. Pharynx with cylindrical procorpus, metacorpus slightly swollen, narrow isthmus, with the nerve ring just anterior of the basal bulb, basal bulb distinct containing a valve. Excretory pore anterior of nerve ring, excretory duct well cuticularised. Excretory gland not observed. Cardia prominent. Genital system monorchic, reflexed. Testis reflexed, comprising germinal zone, growth zone and vas deferens and paired. Spicules paired. Six pairs precloacal subventral papillae, single precloacal midventral papilla, one pair lateral, two pairs adcloacal (Fig. 3). Postanal include one pair lateral, two pairs subterminal and one pair subdorsal. Manubrium is longer than wide, short shaft present, blade moderately curved. In light microscopy photographs, spicule blade has three lobes, dorsal, lateral and ventral lobe. Gubernaculum boat shaped in lateral view, anterior end

round, arrow-shaped cuneus present (Fig. 2E). Tail conoid; tail terminus with a small mucron in 10% of males (Fig. 2C).

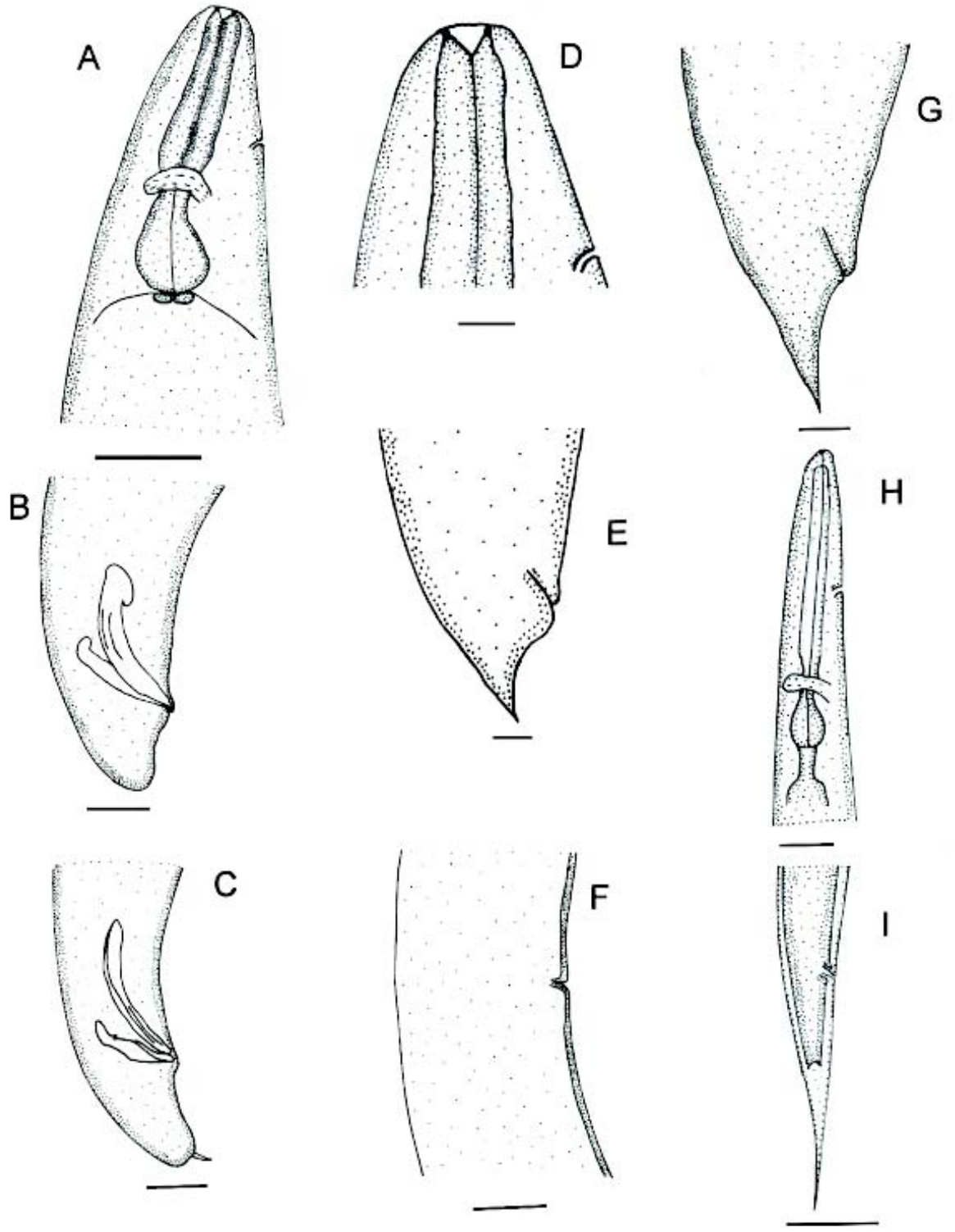

Fig. 1. *Steinernema* n. sp. A,B: Anterior region and tail of first generation male; C: Second generation male tail. D–F: First generation female head, tail and vulva. G: Second generation female tail. H, I: Infective juvenile anterior region and tail. (Scale bars: A= 50 µm, B–I= 20 µm).

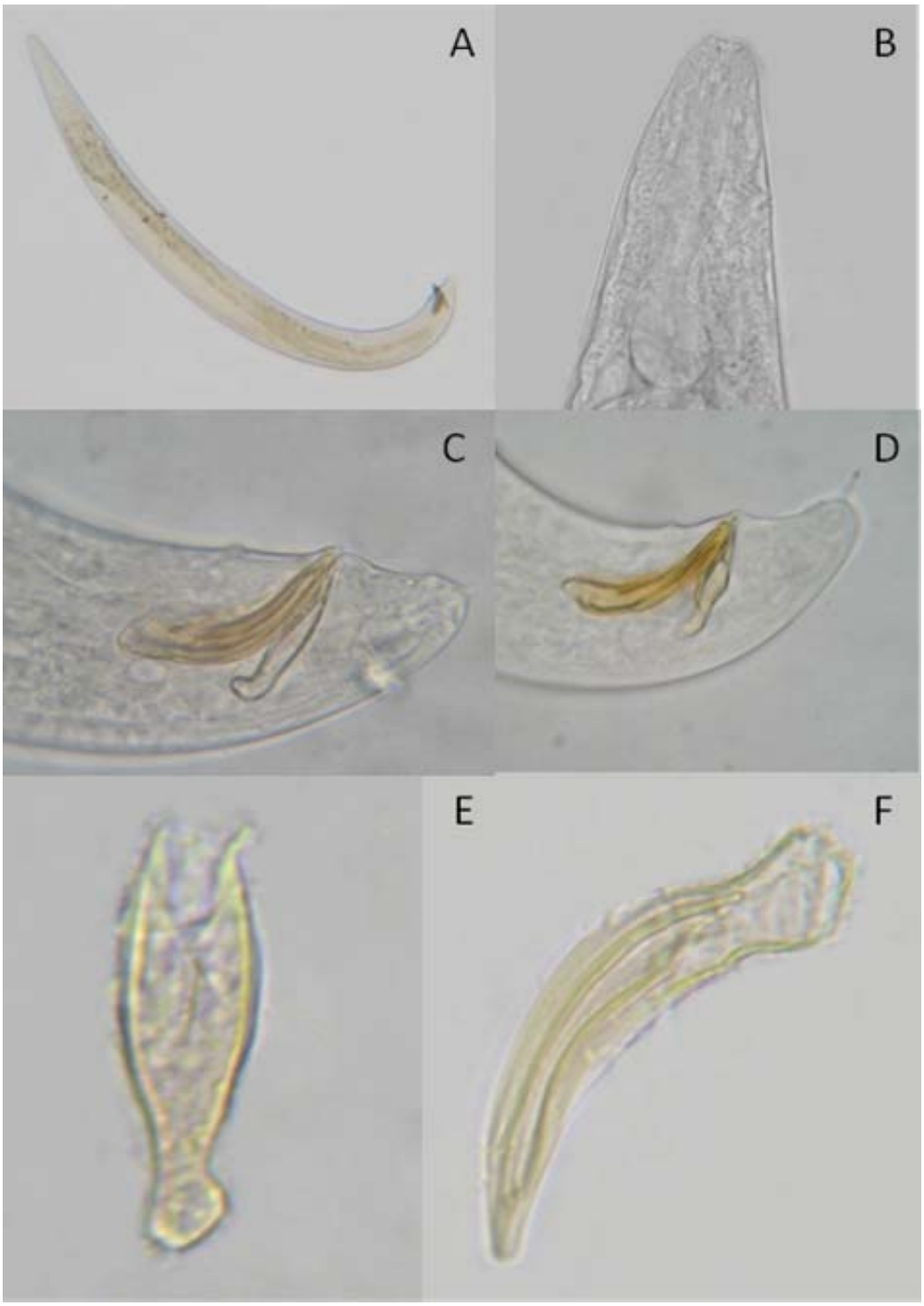

Fig. 2: *Steinernema* n. sp. A: First-generation male, entire body; B: First generation male anterior end; C: First generation male, posterior end with mucron, spicules and gubernaculum; D: Second generation male, posterior end with mucron, spicules and gubernaculum; E: First generation male gubernaculum; F: First generation male spicule.

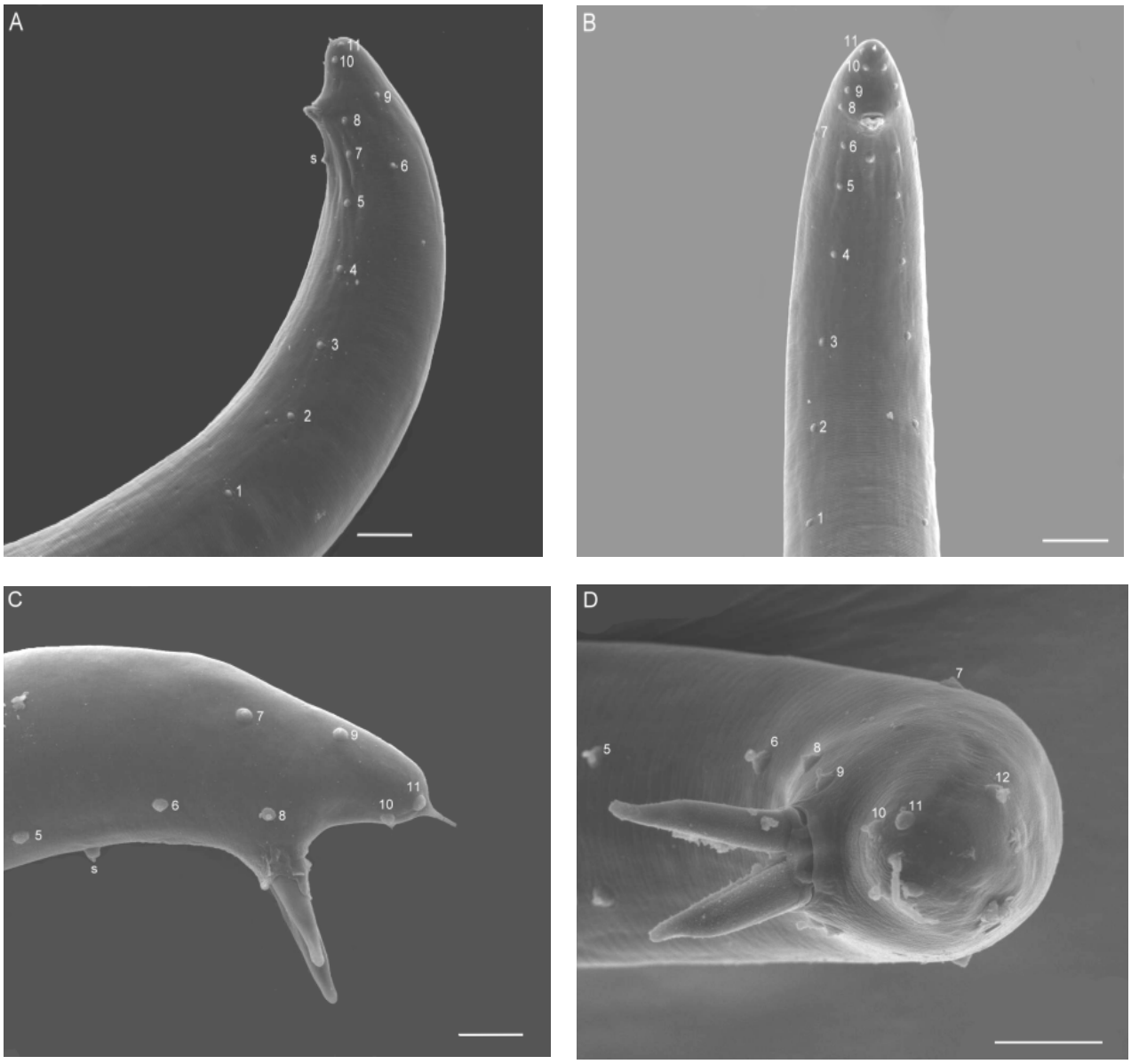

Fig. 3: Posterior region of the male *of Steinernema* n. sp. A-B First generation males, C-D Second generation males, showing the arrangement and position of 11 pairs of genital papillae and single papillae (s). (Scale bars: A = 10 µm, B = 25 µm, C = 20 µm, D = 10 µm),

Males (second generation)

Second-generation male similar to the first-generation male except body length shorter and body width less. The tail is conoid, always with a mucron (Fig. 2D).

Females (first generation)

Body long and robust, C-shaped when heat relaxed and fixed with TAF. Head rounded, continuous with body, with six labial papillae and four cephalic papillae (Fig. 4A). Cuticle smooth

or fine annulations visible with SEM. Cheilorhabdions prominent, well sclerotised, posterior part funnel-shaped. Pharynx procorpus cylindrical, muscular; metacorpus swollen; isthmus distinct; basal bulb enlarged, valvate. Nerve ring surrounding isthmus. Excretory pore anterior to nerve ring, well cuticularised; excretory gland not observed. Cardia prominent. Lateral field absent. Vulva a transverse slit on a slight protruding area. Low single-flapped epiptygmata present (Fig. 4B). Tail tapering to a pointed terminus. Slight preanal swelling in fully mature females. Tail shorter than anal body diam.. (Fig. 4D).

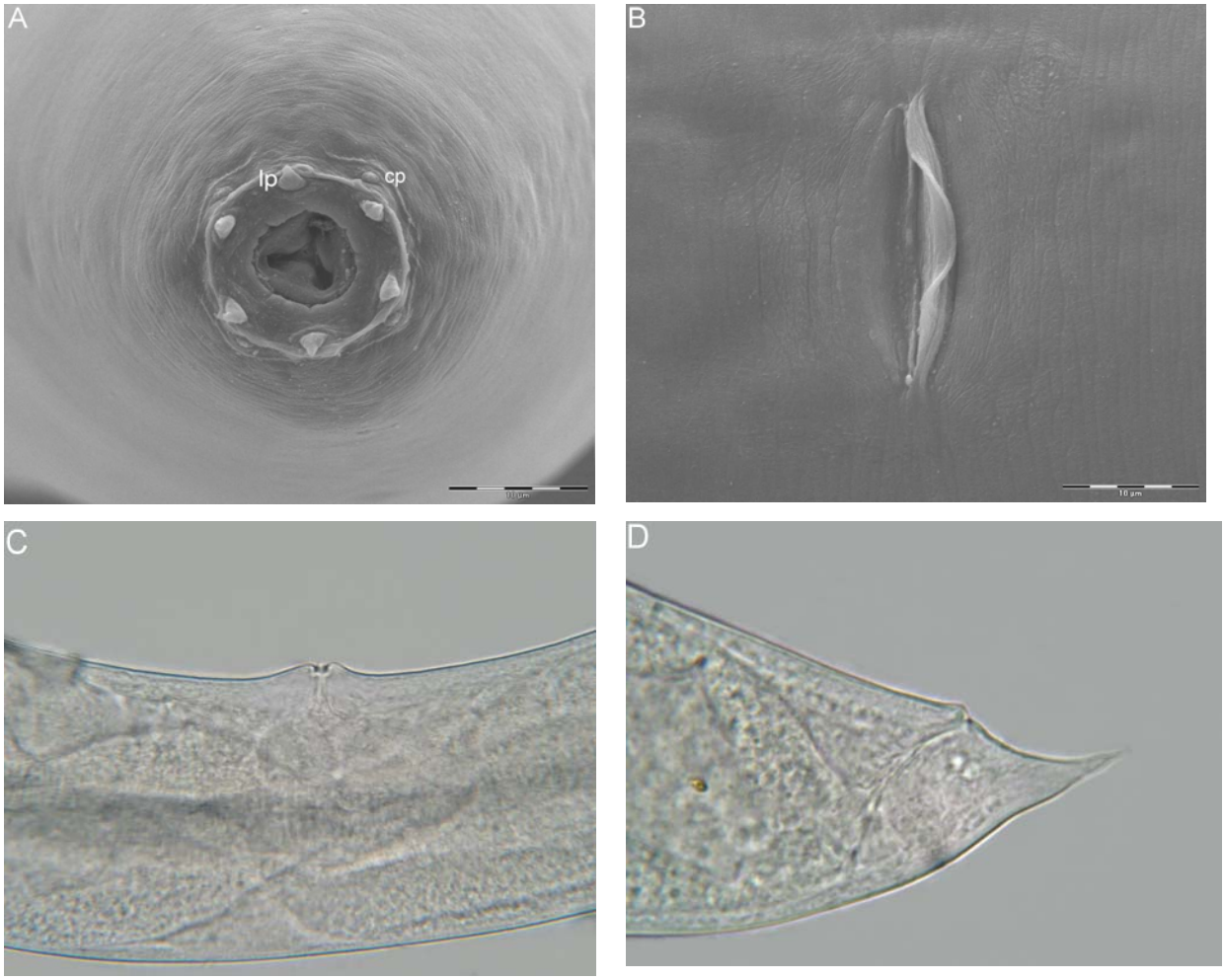

Fig. 4: *Steinernema* n. sp. first generation female A: Face view of anterior end of female showing six labial papillae (lp) and four cephalic papillae (cp); B:Single low flaped epiptygmata; C: Light micrograph of vulva, D: Tail region.

Female (second generation)

Similar to first-generation female but smaller and more slender. Body an open C when heat killed and fixed with 80°C TAF. Vulva on a slight protuberance. No postanal swelling present. Tail tapering gently to a sharp point as for the first-generation female.

Infective juvenile

Body of specimens killed by 80°C TAF elongate and slightly curved. Cuticle striated. Sheath (second-stage cuticle) present immediately after harvesting, but many IJ losing sheath in storage. Head smooth with four cephalic papillae and six labial papillae in ensheathed specimens. Cephalic region smooth, continuous with body, oral aperature closed. Amphidial apertures prominent. Cuticle marked with prominent transverse striations. Excretory pore anterior to nerve ring. Lateral field beginning anteriorly with one line at the seventh or eigth annule. A short distance posteriorly two additional lines appear to form two ridges and posterior to the excretory pore the number of ridges increases to eight. At anus level, only four ridges remaining and at about mid-tail, only two ridges remain. In the middle of the tail, two marginal lines in lateral field converging, central line disappearing to form one ridge, disappearing in the tail terminus. Pharynx with cylindrical narrow corpus, isthmus present, nerve ring in the middle of the isthmus, basal bulb elongate with visible valve. Cardia inconspicuous. Bacterial chamber prominent, located just posterior to the cardia. Hemizonion and hemizonid not observed. Tail three times as long as anal body diam., attenuate and tapering evenly. Hyaline portion 41 (30–54)% occupying a third of tail length.

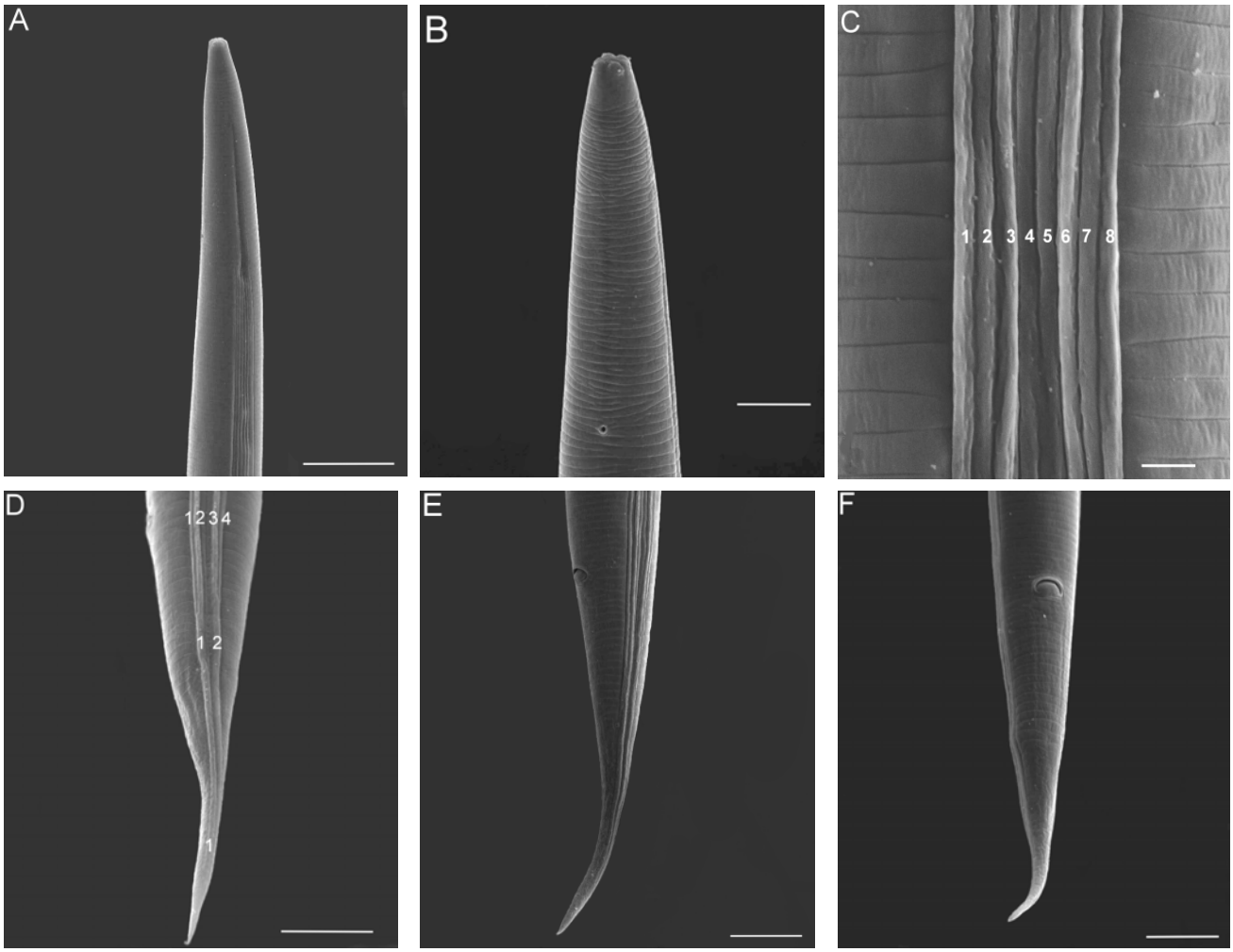

Fig. 5: SEM of *Steinernema* n. sp., third stage infective juvenile. A: Anterior region; B: Position of the excretory pore; C: Lateral field showing six ridges; D: Lateral field narrowing posteriorly, changing to two ridges; E–F: Posterior region showing lateral field and anus. (Scale bars: A = 20 µm, B = 10 µm, C = 2 µm, D–F = 10 µm).

Table 1: Morphometrics of different stages of *Steinernema* n. sp. Measurements are in µm and in the form mean ± s.d. (range).

Character	First generation			Second generation		Infective juvenile
	Male		Female	Male	Female	
	Holotype	Paratype	Paratypes			
N		20	20	20	20	25
L	1099	1154 ± 80 (1028-1402)	3087 ± 75 (2038-4019)	770 ± 64 (640-859)	1732 ± 291 (1232-2152)	754 ± 48 (623 - 849)
A						30 ± 2.2 (25-34)
B						6.0 ± 0.4 (5.1-7.1)
C						15 ± 1.3 (13-17)
V			54 ± 2.5 (50-59)		54 ± 2.1 (50-57)	
Body diam..	92	103 ± 7.2 (87.1-113.7)	175 ± 22 (137-212)	51 ± 3.4 (43-60)	121 ± 16 (90-140)	26 ± 1.5 (23 - 28)
Stoma length	4.2	6.7 ± 1.5 (4.7-9.7)	9.4 ± 0.9 (7.1-11.9)	5 ± 0.7 (4.2-6.6)	9 ± 2.1 (5.6-12.6)	
Stoma diam..	6.3	10.3±1.6 (7.1-12.2)	16.0±2.2 (11.9-19.1)	8 ± 0.9 (6.9-9.7)	16 ± 1.9 (12.6-18.3)	
Excretory pore (EP)	82	81 ± 7 (64-92)	75 ± 10 (54-90)	57 ± 5 (46-67)	71 ± 8 (59-84)	56 ± 4.6 (49 - 70)
Nerve ring (NR)	97	106 ± 7 (92-119)	151 ± 14 (130-179)	96 ± 6 (83-108)	115 ± 9 (101-130)	98 ± 5.9 (88- 110)
Pharynx length (ES)	126	139 ± 7 (123-155)	206 ± 16 (182-238)	130 ± 5 (122-141)	172 ± 11 (152-187)	125 ± 5.3 (118-137)
Testis reflex	67	91 ± 19 (59-130)		104 ± 24 (68-145)		
Tail length (T)	25	25 ± 4 (17-31)	44 ± 7 (33-60)	25 ± 5 (14-33)	48 ± 4 (41-55)	71± 4.3 (63-81)
Anal body diam.. (ABD)	34	33 ± 4 (28-43)	62 ± 11 (43-79)	27 ± 3 (22-33)	35 ± 4 (29-39)	14 ± 0.9 (13-17)
Mucron		8.5 ± 1.7 (7.3-9.7)		12.4 ± 3.4 (7.9-18.3)		
Hyaline (H)						20 ± 2.4 (15 - 24)
Spicule length (SL)	63	65 ± 6 (57-80)		49 ± 4 (42-56)		
Spicule width (SW)	15	14 ± 2.8 (9.5-19.4)		10 ± 1.7 (7.0-12.5)		
Sp. head length				12 ± 2.7 (6.9-18.3)		
Sp. head width				8 ± 3.8 (6.9-11.1)		
Gubernaculum length (GL)	44	44 ± 6.8 (32-59)		28 ± 5.2 (18-35)		
Gubernaculum width	11	10 ± 1.7 (7.1-12.1)		6.3 ± 1.4 (4.2-9.7)		
D% =EP/ES x 100	65	58 ± 5 (47-67)	37 ± 6.0 (27-46)	44 ± 4 (35-52)	41 ± 3.8 (34-48)	44±3.8 (39-58)
E% = EP/T x 100						110 ± 10.5 (85-132)
SW% =SL/ABD x 100	188	198 (156-233)		1.8 ± 0.23 (1.38-2.24)		
GS% = GL/SL x 100	70	68 ± 12 (48-89)		1.6 ± 0.1 (0.4-0.8)		
H% = hyaline/T x 100						41 ± 6.6 (30-54)
T/ABW			0.7 ± 0.10 (0.55-0.95)		1.4 ± 0.14 (1.18-1.71)	

Table 2: Comparative morphometrics of third-stage juveniles of *Steinernema* n. sp. and related *Steinernema* spp. (in descending order of body length). Measurements are in µm and in the form: mean (range).

	Morphometric Character[a] (range)												
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%	n	References
S. oregonense	980 (820-1110)	34 (28-38)	66 (60-72)	-	132 (116-148)	70 (64-78)	30 (24-37)	7.6 (6-8)	14.0 (12-16)	50 (40-60)	100 (90-110)	20	Liu & Berry, 1996
S. litorale	909 (834-988)	31 (28-33)	61 (54-69)	96 (89-104)	125 (114-133)	83 (72-91)	29.5 (27.2-30.9)	7.3 (6.7-7.9)	11 (9.7-11.9)	49 (44-56)	73 (68-84)	25	Yoshida, 2004
S. silvaticum	860 (670-975)	30 (26-35)	62 (51-73)	96 (75-109)	121 (100-141)	75 (63-86)	28.6 (22.5-32.5)	7.1 (6.3-7.7)	11.4 (9.9-13.1)	50 (46-56)	- -	21	Sturhan *et al.*, 2005
S. kraussei	951 (797-1102)	33 30-36	63 50-66	105 (99-111)	134 (119-145)	79 (63-86)	29 -	7.1 -	12.1 -	47 -	80 -	?	Mrácek, 1994
S. feltiae	849 (736-950)	26 (22-29)	62 (53-67)	99 (88-112)	136 (115-150)	81 (70-92)	31 (29-33)	6 (5.3-6.4)	10.4 (9.2-12.6)	45 (42-51)	119 (69-86)	25	Poinar, 1992
S. akhursti	812 (770-835)	33 (33-35)	59 (55-60)	90 (83-95)	119 (115-123)	73 (68-75)	24 (23-26)	6.8 (6.6-7.2)	11 (10-12)	47 (45-50)	77 (73-86)	20	Qiu *et al.*, 2005a
Steinernema n. sp.	754 (623-849	26 (23-28)	56 (46-67)	98 (83-108)	125 (118-137)	71 (63-81)	30 (25-34)	6.0 (5.1-7.1)	15 (13-14)	44 (39-58)	110 (85-132)	25	-
S. texanum.	756 (732-796)	30 (29-34)	59 (52-62)	92 (84-102)	115 (111-120)	73 (60-79)	25 (22-27)	6.5 (6.2-7.0)	10.4 (9.6-12.5)	51 (46-53)	81 (76-88)	20	Nguyen *et al*., 2007
S. sangi	753 (704-784)	35 (30-40)	51 (46-54)	91 (78-97)	127 (120-138)	81 (76-89)	22 (19-25)	5.9 (5.6-6.3)	9.3 (8.7-10.2)	40 (36-44)	62 (56-70)	50	Phan *et al*., 2001
S. weiseri	740 (586-828)	25 (24-29)	57 (43-65)	84 (72-92)	113 (95-119)	60 (49-68)	29 (25-33)	6.6 (5.7-7.2)	12 (10-14)	51 (44-55)	95 -	20	Mrácek *et al.,* 2003
S. jollieti	711 (625-820)	23 (20-28)	60 (53-65)	- -	123 (115-135)	68 (60-73)	30.5 (25-34-1)	5.7 (4.9-6.4)	10.5 (9.0-11.7	48 (46-50)	88 -	25	Spiridonov *et al.,* 2004
S. monticolum	706 (612-821)	37 (32-46)	58 (54-62)	88 (81-93)	124 (120-131)	77 (71-95)	19 (14-22)	5.7 (5.0-6.4)	9.3 (7.6-11.1)	47 (44-50)	76 (63-86)	?	Stock *et al.,* 1997
S. hebeiense	658 (610-710)	26 (23-28)	48 (43-51)	78 (73-83)	107 (100-111)	66 (63-71)	26 (24-28)	6.2 (5.7-6.7)	10 (9.4-11)	45 (40-50)	72 (65-80)	20	Chen *et al.,* 2006
S. kushidai	589 (424-662)	26 (22-31)	46 (42-50)	76 (70-84)	111 (106-120)	50 (44-59)	22.5 (19.3-25.2)	5.3 (4.9-5.9)	11.7 (10-13)	41 (38-44)	92 -	50	Mamiya, 2008

[a]Abbreviations as in Table 1.
- Measurements not available

Table 3: Comparative morphometrics of first-generation males of *Steinernema* n. sp. and related *Steinernema* spp. (in descending order of spicule length). Measurements are in µm and in the form: mean (range).

	Morphometric characters[a] (Range)							
Species	Spicule	Gubern.	W	D%	SW%	GS%	MUC	n
S. akhursti	90 (85-100)	64 (58-68)	131 (115-150)	56 (52-61)	180 (140-200)	71 (65-77)	P	20
S. litorale	75 (67-89)	53 (44-64)	96 (82-111)	40 (34-56)	174 (154-200)	71 (62-81)	P	25
S. oregonense	71 (65-73)	56 (52-59)	138 (105-161)	73 (64-75)	151 -	79 -	A	20
S. feltiae	70 (65-77)	41 (34-47)	75 (60-90)	60 (51-64)	113 (99-130)	59 (52-61)	P	25
S. monticolum	70 (61-80)	45 (35-54)	160 (117-206)	55 (49-61)	140 (120-150)	60 (50-70)	P	20
S. weiseri	68 (62-72)	53 (46-57)	112 (84-138)	49 (39-60)	180 (150-140)	80 (70-85)	A	20
S. jollieti	64 (55-70)	54 (45-60)	115 (98-135)	64 (53-83)	145 -	84 -	A	12
Steinernema n. sp	65 (57-80)	44 (32-59)	103 (87-113)	58 (47-67)	198 (156-233)	68 ± 12 (48-89)	P	20
S. kushidai	63 (48-72)	44 (39-60)	97 (75-156)	51 (42-59)	150 -	70 -	A	20
S. sangi	63 (58-80)	40 (34-46)	159 (120-225)	49 (42-63)	150 (120-160)	60 (50-70)	P	20
S. hebeiense	57 (51-63)	46 (38-50)	86 (74-98)	51 (48-59)	140 (120-170)	80 (60-90)	A	20
S. texanum	60 (55-66)	45 (39-53)	99 (81-116)	67 (58-73)	157 (127-203)	75 (62-84)	A	20
S. silvaticum	51 (42-64)	37 (30-43)	65 (52-78)	60 (45-63)	-	-	P	26
S. kraussei	49 (42-53)	33 (29-37)	128 (110-144)	53 -	110 -	67 -	P	?

[a]Abbreviations as in Table 1
- Measurements not available

Type host and locality

Steinernema n. sp. (141-C) was collected from a soil sample in the Western Cape Province, South Africa, by means of laboratory trapping with *G. mellonella*. The natural host is unknown.

Type material

Holotype and paratype of several males, females and IJ deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa.

Diagnosis and relationships

Steinernema n. sp. is characterised by morphometrics of the IJ with body length 754 *μ*m, distance from anterior end to the excretory pore = 56 *μ*m, tail length = 71 *μ*m, ratio a = 30, H% = 41 and E% = 110 (Table 1). Lateral field pattern of the new species is 2, 6, 2. Male of the first generation can be recognised by a body width of 103 μm, the spicule and the gubernaculum length and shape, position of the excretory pore, D% = 58 and GS% = 68. Female can be recognised by the vulva with a very low, single-flapped epiptygmata.

Steinernema n. sp. is closely related to species of the *feltiae*-group (Table 4), which include *S. akhursti* Qiu, Hu, Zhou, Mei, Nguyen & Pang, 2005; *S. feltiae*, *S. hebeiense* Chen, Li, Yan, Spiridonov & Moens, 2006; *S. jollieti* Spiridonov, Krasomil-Osterfeld & Moens, 2004; *S. kraussei* (Steiner, 1923) Travassos, 1927; *S. kushidai* Mamiya, 1988; *S. litorale* Yoshida, 2004; *S. monticolum*, *S. oregonense* Liu & Berry, 1966; *S. sangi* Phan, Nguyen & Moens, 2001; *S. silvaticum* Sturhan, Spiridonov & Mráček, 2005; *S. texanum* Nguyen, Stuart, Andalo, Gozel & Rogers, 2007; and *S. weiseri* Mráček, Sturhan & Reid, 2003.

The third-stage IJ of *Steinernema* n. sp. is the closest in body length to *S. texanum*, *S. sangi* and *S. weiseri*. It differs from *S. sangi* in body width of 26 μm versus 35 μm; from *S. texanum* and *S. weiseri* in the length of the pharynx of 125 μm versus 115 and 113 μm,

respectively. *Steinernema* n. sp. differs from *S. texanum*, *S. sangi* and *S. weiseri* in the E% of 110 µm versus 81, 62 and 95 µm, respectively.

The spicule length of the male of *Steinernema* n. sp. is shorter than those of *S. akhursti*, *S. litorale* and *S. oregonense,* in being 65 µm versus 90, 75 and 71 µm, but longer than *S. hebeienense*, *S. silvaticum* and *S. kraussei*, of 57, 51 and 49 µm respectively. This new species differs from *S. weiseri* and *S. jolleiti* in the length of the gubernaculum of 44 µm versus 53 and 54 µm, respectively. The body width of the male of *Steinernema* n. sp. of 103 µm, differs from *S. feltiae* and *S. monticolum* and *S. sangi*, which measure 75 µm, 160 µm and 159 µm respectively. The SW% of 198 for *Steinernema* n. sp. differs mainly from *S. feltiae*, *S. monticolum*, *S. jollieti, S. kushidai* and *S. sangi,* which are 113, 140, 145, 150 and 150 respectively. It differs from all closely related species (Table 3) in the morphology of the vulva of the first-generation female. The vulva can be best described as with a low, single-flapped epiptygmata.

Cross-hybridisation tests

Crossbreeding between males and females of *Steinernema* n. sp. with *S. feltiae* produced no progeny. In the control, when all species were self-crossed, males and females produced offspring normally.

Fig. 6. Light microscopy pictures of *Steinernma feltiae* development in a drop of *Galleria mellonella* blood. A: Developing female, B: Developing male; C, E: Progeny of males and females.

Conclusion

The morphological and biological differences of *Steinernema* n. sp. have been described. To complete the full description of this species, molecular evidence needs to be added (as it did not fall under the scope of the current thesis). The alignment of the full sequence of the ITS region of rDNA of this species with those available in Genbank (http://www.ncbi.nml.nih.gov), showed that *Steinernema* n. sp. has at least a 95% similarity with all other described *Steinernema* species (Nguyen, 2007). The Genbank accession number for the *Steinernema* n. sp. isolate 141–C is EU740970.

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Chapter 4

Long-term storage of Steinernema khoisanae and Heterorhabditis zealandica

Abstract

Entomopathogenic nematodes are used for the biological control of insect pests and interest in these nematodes as biocontrol agents is growing. Factors such as contamination and loss of the original genetic characteristics of new isolates have made it necessary to develop a method for their long-term storage. Cryopreservation in liquid nitrogen has been shown as a method for long-term storage of nematodes. A modified method for the successful cryopreservation of *Steinernema khoisanae* and *Heterorhabditis zealandica* infective juveniles (IJ) was developed. As a cryoprotectant, nematodes were incubated in 15% glycerol, 8% dimethyl sulfoxide or 8% ethylene glycol for varying incubation times, followed by their suspension in 70% methanol, before their subsequent immersion in liquid nitrogen. Nematode survival was significantly influenced by increasing the length of the preincubation period in the different cryoprotectants before their immersion in liquid nitrogen. The highest mean survival rate was 69% for *S. khoisanae* and 78% for *H. zealandica* after incubation in 15% glycerol for four days. Nematodes were able to infect *Galleria mellonella* larvae successfully after thawing when tested on day 42 of freezing, and completed their life cycle.

Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are important biological control agents against a number of economically important pests (Klein, 1990; Shapiro-Ilan *et al.*, 2002). The pest control ability of these species and strains can vary greatly (Shapiro-Ilan *et al.*, 2002), with one method of improvement being the isolation of new species and isolates. Several biocontrol successes have been based on such

discoveries (Burnell, 2002). Routine subculturing of entomopathogenic nematodes is labour intensive and can lead to loss of important genetic characteristics; run the risk of contamination by other nematode species, bacteria, andfungi; and a decrease in pest control abilities (Stuart & Gaugler, 1996; Shapiro *et al.*, 1996; Wang & Grewal, 2002). Wang and Grewal (2002) observed that useful biological traits of *Heterorhabditis bacteriophora* Poinar, 1976 were affected by repeated subculturing in *Galleria mellonella* L. of this nematode under laboratory conditions. Cryopreservation by freezing and storage in liquid nitrogen, has been shown to be an appropriate method for the long-term storage of entomopathogenic nematodes (Popiel & Vasquez, 1991; Curran *et al.*, 1992; Nugent *et al.*, 1996). It offers several benefits, such as the possibility of preserving entomopathogenic nematode isolates free from contamination by other isolates and of maintaining their original genetic makeup intact (Nugent *et al.*, 1996).

Cryoprotectants help to minimise intracellular and intercellular crystal formation, and to increase the glass formation of the samples being frozen. Dimethyl sulfoxide (DMSO) and glycerol are the most commonly used cryoprotective agents (Panis & Lambardi, 2005). Vitrification (the formation of a glasslike, noncrystalline solid at temperatures at or below the freezing point of an aqueous solution) is considered a standardised protocol for cryopreservation because it is simple, less expensive than other methods and requires niether specialised nor expensive equipment for the control cooling rates (Chao & Liao, 2001). Although vitrification can be successfully applied to a wide range of tissues and plant species, it may also cause injuries. For instance, chemical toxicity may result from the cryoprotectants used, or mechanical disruption due to ice formation may occur to the specimens during cooling or warming.

Prior studies have demonstrated that the length of the preincubation period, the speed of thawing, and the type and concentration of cryoprotectant used have a major influence on the survival of different species and isolates of nematodes following cryopreservation (Curran *et al.*, 1992; Nugent *et al.*, 1996). It was demonstrated by Bai *et al.*

(2004) that the concentration of IJ during the cryopreservation process can have a critical impact on their subsequent survival, when they achieved 100% post-cryopreservation survival of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, and 100% retention of the original virulence to *G. mellonella* larvae with a concentration of 12,000 IJ/ml in glycerol and 7,500IJ/ml in Ringer's solution. These results showed that nematode survival increases with increased IJ concentration up to optimum level and then decreases at higher concentrations. Popiel and Vasquez (1991) observed that, while *S. carpocapsae* could be successfully cryopreserved in 0.5-ml volumes of 70% methanol, postcryopreservation survival was considerably higher for *H. bacteriophora* when the pre-treated IJ were frozen on filter paper strips, rather than in liquid suspension. Curran *et al.* (1992) noted that the speed of thawing was critical, and that even slight delays in thawing resulted in a significant drop in the survival rate. The optimum survival rate for IJ of *Heterorhabditis zealandica* Poinar, 1990 was recorded when they were preincubated in 15% glycerol from four to six days (Nugent *et al.*, 1996).

Many nematode isolates, mostly from the Western Cape Province of South Africa, have been collected as part of a research project aimed at isolating nematodes from South African soils for use in biological control, (Malan *et al.*, 2006; De Waal *et al.*, 2008). Growing interest in these nematodes as biological control agents in South Africa has made it necessary to develop a successful technique for their long-term storage. Laboratory bioassays have been used to select specific nematode species and isolates for the control of codling moth *Cydia pomonella* L. (De Waal, 2008) and false codling moth, *Thaumatotibia leucotreta* (Meyeric.) (Malan, pers. comm.). The present study is aimed at modifying and optimising a method for the cryopreservation of infective juveniles (IJ) of steinernematids to prolong their survival by using different cryoprotectants and varying times of pre-incubation. The protocol was also applied for the preservation of heterorhabditid selected for the control of codling moth in South Africa.

Materials and methods

Source of nematodes

Nematodes used in the current study were obtained from previous surveys (Malan *et al.*, 2006) and are endemic isolates of *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006 (SF 87; DQ314287) and *H. zealandica* (J34; EU722436). They are maintained in the Department of Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch. Nematodes were cultured *in vivo* in last instar larvae of the wax moth larvae *G. mellonella* (L.). IJ were harvested daily for one week after their emergence from modified White traps (White, 1927; Poinar *et al.*, 1992; Cappaert & Koppenhöfer, 2003). They were stored horizontally in tissue culture flasks at 14ºC according to procedures described by Kaya and Stock (1997) and used within one to three weeks of harvesting. These isolates have been recycled many times through *G. mellonella* during maintenance, for the past five years.

Cryopreservation protocol

Freshly harvested IJ were preincubated in a cryoprotectant at room temperature. A 24-well plate (Flat bottom, Nunc™, Cat No. 144530) was used for the preincubation of the nematodes. The nematode concentration was adjusted to 4000 IJ/ml of water and 1 ml of the concentration was added to a well. After preincubation, the nematodes were pumped with a pipette to re-suspend them and were transferred to 2-ml Eppendorf tubes. The centrifuge was pre-cooled to 1ºC before use. The Eppendorf tubes with nematodes were centrifuged using Eppendorf Centrifuge 5415 at 12000 rpm for two minutes. Excess cryoprotectant was removed by pouring off the supernatant, with only the nematode pellet remaining at the bottom of the Eppendorf tube. The nematodes were resuspended in 70% methanol (directly from a freezer at -20ºC) and centrifuged at 12000 rpm for two minutes. In this step, the supernatant was removed by pipetting till only the nematode pellet with a small volume of methanol remained in the Eppendorf tube. The nematodes, with the remaining

methanol, were pipetted onto 13-mm filter paper discs, which were placed on ice blocks. The filter paper discs were folded and placed in precooled round-bottomed cryotubes, transferred to storage canes and immediately plunged into liquid nitrogen.

After 24 hours the cryotubes were removed from the liquid nitrogen and thawed by pouring 1 ml of 25ºC Ringer's solution (6g NaCl, 0.076g KCl, 0.1g $CaCl_3$, and 0.1g $NaHCO_3$) (Kaya & Stock, 1997) onto the cryotubes, closed, and left in a hot water bath at 25ºC for 5 minutes. Removal from liquid nitrogen and thawing of the nematodes was completed as rapidly as possible, to maximise survival. The nematodes were returned to the 24-well plates and the Ringer's solution was periodically replaced until nematode surfaces no longer appeared glossy. Survival was assessed 24 hours after thawing by microscopic observation of motility and response to prodding.

Different cryoprotectants and preincubation periods

Freshly harvested IJ of *S. khoisanae* were preincubated in 15% glycerol, 8 DMSO or 8% ethylene glycol at room temperature for two, three, four and five days. Using the cryopreservation protocol described above, IJ of *S. khoisanae* were cryopreserved with the survival rate being determined. For each treatment, six wells were used, with a control with IJ in water; one for the nematodes in the cryoprotectant; one to evaluate the survival of the nematodes after the methanol wash; and three replicates to determine survival of the IJ after freezing. A single ml of each cryoprotectant at double the concentration of the different cryoprotectants was added to each well, and only water for the control.

Cryopreservation of H. zealandica *IJ*

Infective juveniles of *H. zealandica* were preincubated in 15% glycerol for two, three, four and five days, using the cryopreservation protocol described above.

Infectivity after cryopreservation

An additional assay was conducted to determine whether the level of IJ virulence was retained in the cryopreservation process. The virulence of those *S. khoisane* IJ from the cryoprotectant and incubation time that exhibited the highest rate of survival during cryopreservation was compared with that of IJ (originating from the same culture flasks) that had been kept at 14ºC. Nematodes were stored for one, 21, and 42 days in liquid nitrogen. After each storage period, nematodes were thawed with Ringer's solution at 25ºC. After incubation for 24 hours at room temperature, the percentage survival was determined. The same number of IJ was extracted from fresh cultures and used as controls in infectivity experiments. The pathogenicity of thawed IJ was determined in a Petri dish assay with *G. mellonella* larvae. IJ in 50 µl tap water were dispensed onto each of 10 filter paper discs in 9-cm Petri dishes. Ten *G. mellonella* larvae were placed onto each filter paper disc, and the plates were maintained at 25ºC. Mortality was assessed 24 hours later and the results were compared to control bioassays in which non-frozen nematodes were used.

Data analysis

All statistical analyses were performed using Statistica 8.0 (StatSoft Inc., 2008). The data were analysed by using a one-way ANOVA, with post-hoc comparisons of means, using the Bonferroni's method, or the bootstrap multiple comparison method if residuals were not normally distributed (Efron & Tibshirani, 1993).

Results

Preincubation survival of IJ of S. khoisanae

The percentage survival of rate *S. khoisanae* IJ before freezing treatment for the control in water, cryoprotectant (15% glycerol, 8% DMSO and 8% ethylene glycol) and a methanol wash after four days was assessed. The results for the percentage survival

(before freezing) were analysed, using a one-way ANOVA, and bootstrap multiple comparisons were performed. Significant differences between the treatments were obtained ($F_{(4,36)}$ = 2.839; P = 0.0382). No significant differences were detected between the controls and the three different cryoprotectants used after an incubation period of four days (Fig.1). The only significant difference found was between the 8% ethylene glycol and 15% glycerol after the methanol wash (P < 0.001), although the 15% glycerol treatment before freezing did not differ significantly from the control (P = 0.3703). The lowest survival rate (of 85.6%) was recorded with 15% glycerol, followed by 8% DSMO (89.4%) and the highest survival occurred with 8% ethylene glycol (91.0%). The survival of IJ incubated in glycerol for four days declined from 85.6% to 76.2%; this was followed by a further decline to 70.1% when the samples were subjected to the methanol wash. After the methanol wash, there was a slight decrease in survival rate, from 85.6% to 70.1% with 15% glycerol; 89.4% to 84.9% with DMSO and 91% to 90% with ethylene glycol.

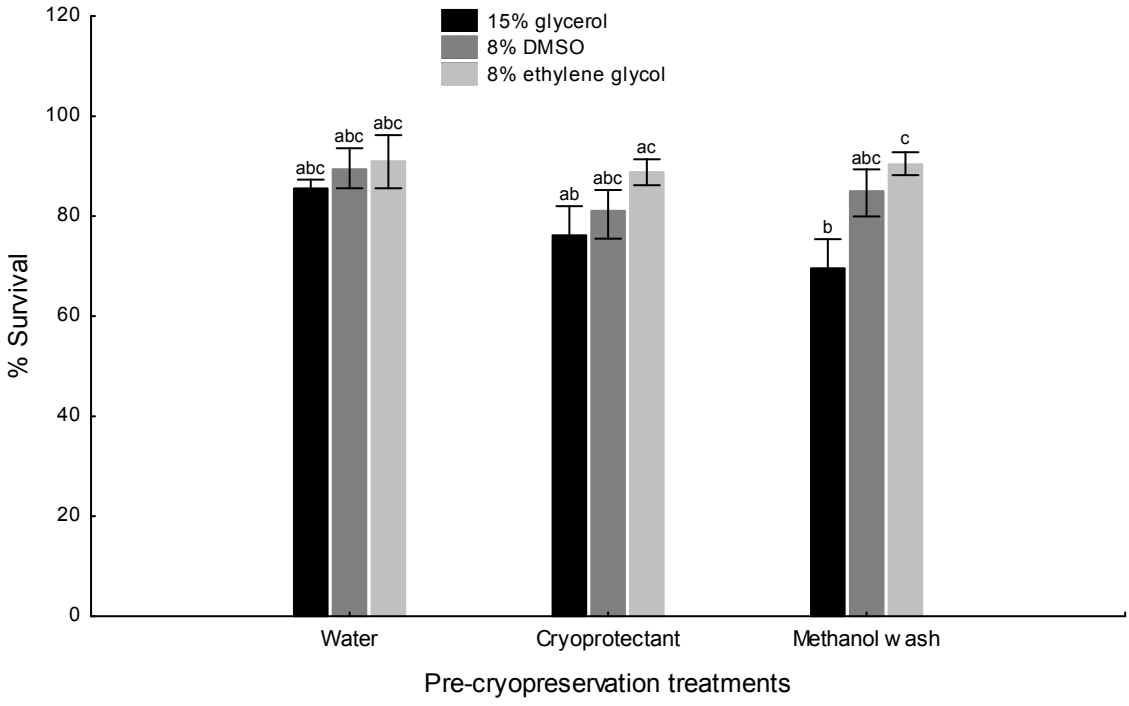

Fig. 1: Mean percentage survival (95% Confidence Interval) of *Steinernema khoisanae* (SF87) IJ after pre-incubation in water, three cryoprotectants (15% glycerol, 8% DMSO and 8% ethylene glycol) for four days and a methanol wash (without freezing) (one-way ANOVA; $F_{(4,36)}$ =2.839; P = 0.0382).

Different cryoprotectants and preincubation periods

Results were analysed using a one-way ANOVA with significant differences between the type of cryoprotectant and length of preincubation $F_{(6,168)} = 9.853$; $P < 0.0001$). As residuals were not normally distributed, a bootstrap multiple analysis was performed which is presented in Fig. 2. Nematode survival was reduced in all three treatments. When glycerol was used as a cryoprotectant, the survival rate was low after two days (24.2%) but increased with increasing time, until an optimum survival rate of 69.2% was obtained after four days. Although there was no significant difference ($P = 1$) in the survival rate of *S. khoisanae* IJ between three and four days, the highest percentage survival rate was achieved after four days. When IJ were preincubated for five days, survival rate decreased to 53.3% but was still not significantly different ($P = 0.355$) from survival obtained after three days preincubation. When using 8% DMSO as a cryoprotectant, the rate of survival increased from 31.1% after two days to 49.8% after three days, after which it declined to 33.3% and 28.3% after four and five days, respectively. When 8% ethylene glycol was used as a cryoprotectant, the rate of survival of the IJ increased over time, from 7.9% after two days up to 41.6% after three days before it dropped to 9.3% and 11.3% after four and five days, respectively.

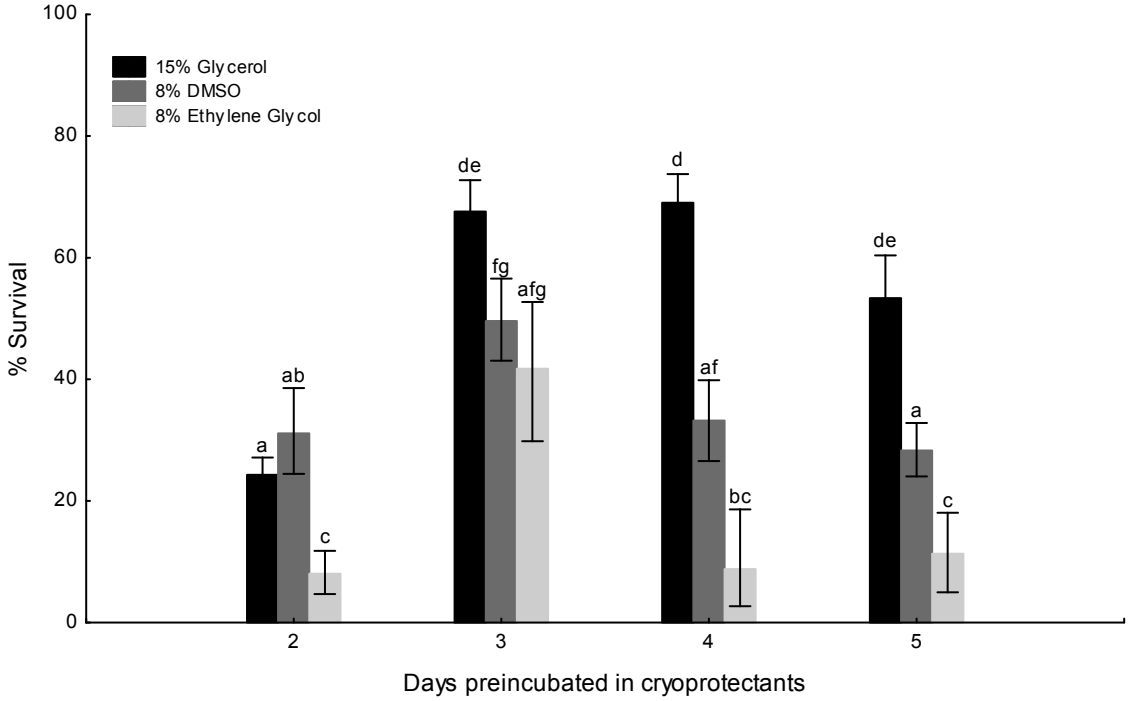

Fig. 2: Mean percentage *Steinernema khoisanae* survival (95% Confidence Interval) after incubation for two, three, four and five days in three cryoprotectants, glycerol (15%), DMSO (8%) and ethylene glycol (8%); 70% methanol (–20°C) wash and freezing in liquid nitrogen for 24 hours (one-way ANOVA; $F_{(6,168)} = 9.853$; $P < 0.0001$).

Cryopreservation of IJ of H. zealandica

The results of the survival of IJ of *H. zealandica* which were preincubated in 15% glycerol with and without freezing were analysed using a one-way ANOVA and significant differences ($F_{(3,72)} = 11.093$; $P < 0.0001$) in the survival rate were found between different days of incubation. Since residuals were not normally distributed, a bootstrap analysis was performed. This is graphically presented in Figure 3. There were no significant differences in the preincubation periods before freezing. After preincubation in 15% glycerol for three days, a significant difference ($P < 0.001$) in the survival rate was obtained. There was a significant difference in the percentage survival on each day before and after cryopreservation, however (Fig. 3). The survival of IJ of *H. zealandica* after preincubation in 15% glycerol for two days and for four days differed significantly ($P < 0.001$), but were no

significant differences between day three, day four and day five there (P = 1). The lowest numbers of active nematodes (34.3%) occurred in the treatment with the shortest incubation time being two days, and was maximal (77.8%) in samples that were incubated in 15% glycerol for four days before their immersion in methanol. A decline in the survival rate was observed when samples were preincubated in 15% glycerol for five days.

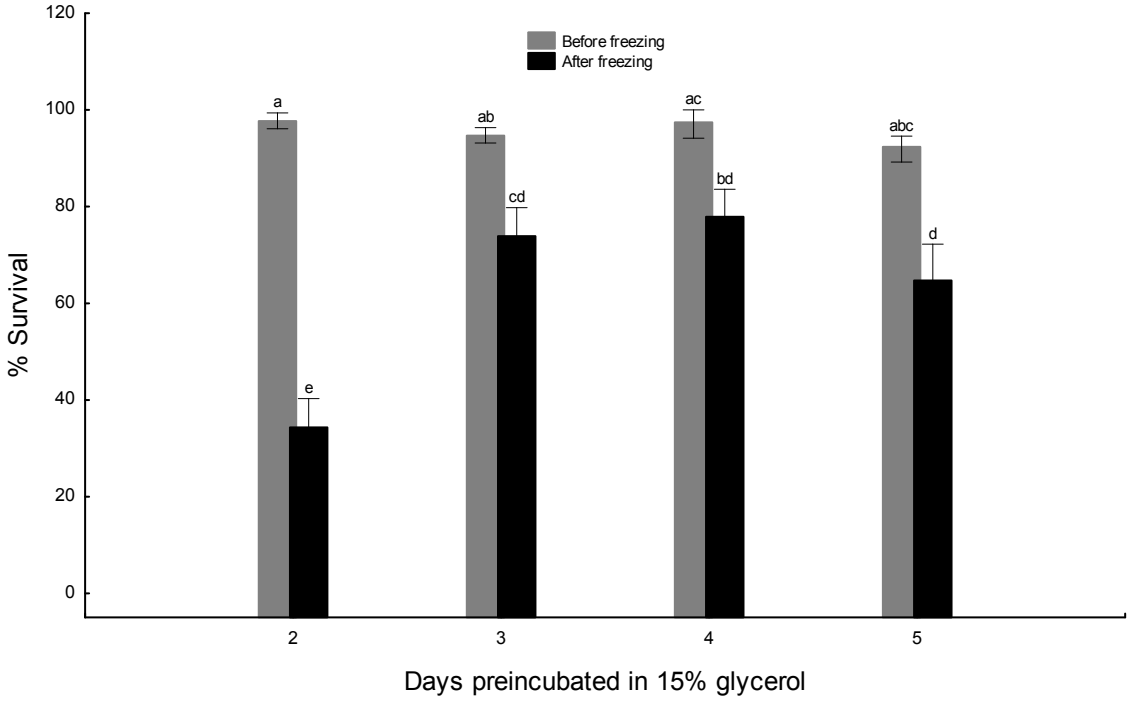

Fig. 3: Mean percentage survival (95% Confidence Interval) of *Heterorhabditis zealandica* (J34) as influenced by preincubation in 15% glycerol for two, three, four, and five days before and after freezing in liquid nitrogen for 24 hours (one-way ANOVA; $F_{(3,72)} = 11.093$; $P < 0.0001$)

The mean overall survival rate for *H. zealandica* (62.7%) was more than for *S. khoisanae* (53.6%). When the results were analysed using a one-way ANOVA, a significant difference was observed ($F_{(1,112)} = 16.551$; $P < 0.0001$) between the percentages for the survival rate of these two species.

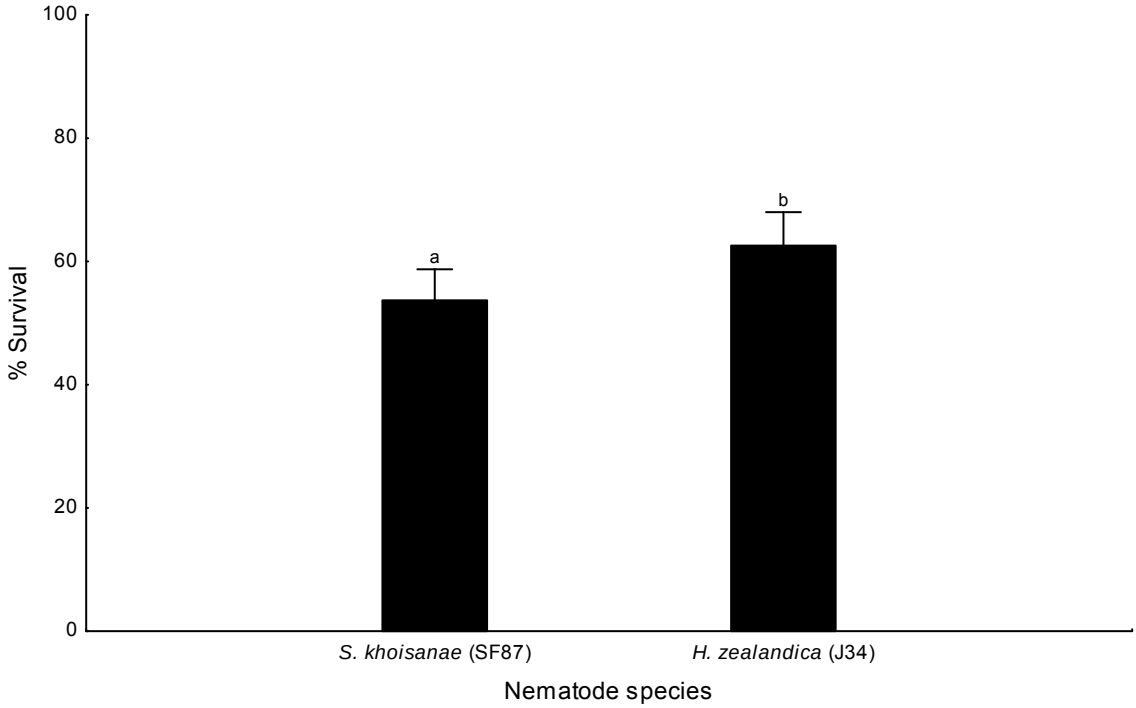

Fig. 4: Mean percentage survival (95% Confidence Interval) of *Steinernema khoisanae* and *Heterorhabditis zealandica* after preincubation in 15% glycerol and freezing in liquid nitrogen for 24 hours before thawing in Ringer's solution in a hot-water bath at 25ºC (one-way ANOVA; $F_{(1,112)} = 16.551$; $P < 0.0001$)

Survival after cryopreservation

In bioassay tests that were conducted on *G. mellonella* larvae, all the nematodes kept in liquid nitrogen for 24 hours were able to infect *G. mellonella* larvae, as were nematodes that were cryopreserved for 21 and 42 days. They were also able to complete their life cycle successfully until the production of IJ.

Discussion

In the current study, a simplified cryopreservation protocol for *S. khoisanae* was developed and optimised. Different cryoprotectants (15% glycerol, 8% DMSO and 8% ethylene glycol) were tested, followed by a 70% methanol wash. The protocol described is a modification of that developed by Nugent *et al.* (1996), who used an ice-cold methanol wash,

2-ml round-bottomed cryovials, filter paper strips and a centrifuge. In the first step of their experiment, they used filter paper and a Sartorius funnel (a 25mm glass vacuum filter holder with a 30-ml funnel, Cat. No. SM 16315) and a vacuum flask attached to a vacuum pump, to remove excess cryoprotectant. In an attempt to simplify the method, centrifugation was used in the current study to condense the nematodes into a pellet, with the supernatant simply being poured off. Round-bottomed cryotubes were used instead of conical-bottomed cryotubes as suggested by Curran *et al.* (1992). It was shown by Popiel and Vasquez (1991) that, although preincubation of nematodes in glycerol alone was sufficient, their survival rate was enhanced by a 10-minute methanol wash. In the current study, filter paper strips were used as they had been proven to enhance post-cryopreservation survival rates, compared with those achieved by freezing nematodes as a liquid suspension (Nugent *et al.*, 1996).

Various modifications of the same protocol were tested on IJ of *S. khoisanae* in order to develop a more efficient and practical method directed at the long-term cryopreservation of these nematodes. All three cryoprotectants used in the current study proved to be non-toxic to the nematodes. A subsequent methanol wash resulted in a slight decrease in the survival rate, but was deemed necessary because it was assumed that the methanol protected the nematodes from slower cooling by reducing the cooling rate required for vitrification (Popiel & Vasquez, 1991). The effects of the length of exposure time of IJ to three different types of cryoprotectants at room temperature on the viability and infectivity of IJ after freezing were studied. The results indicate that the survival rate of IJ after storage in liquid nitrogen not only relies on the type of cryoprotectant used, but is also highly dependent on the legth of the preincubation period. Nematode survival increases with increasing incubation time up to an optimum level before it decreases again. Preincubation periods in 15% glycerol after three days resulted in significantly lower survival rates than after four days, indicating that a preincubation period of less than four days was probably insufficient to provide optimum protection for *S. khoisanae*.

We have successfully applied this method for the cryopreservation of *H. zealandica,* one of the important isolates in our collection, as it was selected for the control of codling moth, *Cydia pomonella* (L.). Sayre and Hwang (Sayre & Hwang, 1975) suggested that certain conditions, such as the age and vigour of nematodes before freezing; the length of time nematodes are in contact with the cryoprotectant; and concentration of the cryoprotectant before and after freezing affect the survival of nematodes. It therefore is important to use freshly harvested IJ.

According to Curran *et al.* (1992), larger nematode species such as *Steinernema glaseri* (Steiner, 1929) Wouts, Mrácek, Gerdin & Bedding, 1982, show lower survival rates after thawing and results from this study have confirmed their observations, with an overall lower survival rate of (53.5%) for *S. khoisanae,* in comparison with *H. zealandica*. The cause of the difference in survival post-cryopreservation is due to the difference in size between the two nematode species; *S. khoisanae* is a larger nematode with a body length of 1076 µm (Nguyen *et al.*, 2006), compared to *H. zealandica,* which has a body length of 685 µm (Poinar, 1990).

Prior studies on cryopreservation demonstrated that acceptable survival of all species and isolates of entomopathogenic nematodes can be obtained with the use of 15% glycerol, and Curran *et al.* (1992) obtained a mean survival rate of 69% for isolates of *Steinernema* and 68% for isolates of *Heterorhabditis*. The data obtained in the present study confirm their observations, for example, maximum survival of 69% was obtained for *S. khoisanae* after preincubation in 15% glycerol for four days, while optimum survival rate was 78% for *H. zealandica* when IJ were preincubated for four days in 15% glycerol. Even though Riga and Webster (1991) discovered that isolates of the pinewood nematode, *Bursaphelenchus* spp. can be best cryopreserved in 15% glycerol, Smith *et al.* (1990) observed that it was impossible to cryopreserve the IJ of *Steinernema feltiae* by a technique using 15% glycerol.

When Song *et al.* (2001) used DMSO for the cryopreservation of *Romanomermis culicivorax* Ross & Smith, 1976, a high percentage of motile nematodes was recovered but

the nematodes lost their infectivity during the cryopreservation procedures. Results from Brown and Gaugler (1998) showed that the infectivity and pathogenicity of IJ are reduced by prolonged exposure to cryopreservation. Jagdale and Grewal (2003) observed that trehalose helped to preserve the original virulence of nematodes; a result, when entomopathogenic nematodes were either heat- or freeze-stressed, their original virulence against *G. mellonella* larvae was largely preserved. The decrease in survival and infectivity of cryo-juveniles is entirely due to the freezing, storage and thawing procedure. The current study supports findings by Van der Beek *et al.* (1996) that there is no significant difference in infectivity after long periods of storage in liquid nitrogen.

Just as cryopreservation achieves success with entomopathogenic nematodes, it also offers the same potential with plant parasitic nematodes. Bridge and Ham (1985) described the cryopreservation of J2 of the root-knot nematode, *Meloidogyne graminicola* Golden & Birchfield, 1965 and their results clearly demonstrated that the exposure time of nematodes to the cryoprotectant is crucial. Earlier work with *Ditylenchus dipsaci* (Kühn) Filipjev, 1857 has established that most freezing injuries occur during the initial freezing and no further significant decrease in survival occurs during storage, even after 540 days (Sayre & Hwang, 1975). Triantaphyllou and McCabe (1989) observed that J2 of several *Meloidogyne* and *Heterodera* species start moving within one hour of thawing and can be used immediately as inoculum. They also predicted that freezing for at least 10 and even 100 years may not significantly decrease survival and infectivity of J2 juveniles of a *Meloidogyne* spp. Results of a Galway and Curran (1995) study showed that better survival of *Pratylenchus spp.* was obtained when fresh nematodes were used and that juveniles survived cryopreservation better than adults. Carneiro *et al.* (2001) discovered that, although the number of *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 larvae is reduced post cryopreservation, those collected after thawing infected tomato roots and produced a large number of eggs.

Long-term cryopreservation of entomopathogenic nematodes offers many benefits over the normal laboratory maintenance of nematode cultures. It obviates labour,

requirements for space and the maintenance of controlled environmental conditions, all of which are costly. The cryopreservation protocol described here can be applied to preserve genetic variation of nematode species, and to minimise the risk of contamination. Wang and Grewal (2002) showed the maintenance of stress tolerance and other fitness traits by the cryopreserved nematodes. They also suggested that keeping the strains in the laboratory for only few generations could lead to laboratory selection. It would be good practice to cryopreserve each new species or isolate of entomopathogenic nematodes found during surveys in South Africa. These cryopreserved nematodes may have many additional future advantages when tested against genetically manipulated nematodes.

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CHAPTER 5

Bioassays with South African isolates of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) against *Pseudococcus viburni* (Hemiptera: Pseudococcidae)

Abstract

Pseudococcus viburni (Signoret) (Pseudococcidae), commonly known as the obscure mealybug, is a common and serious pests of apples and pears in South Africa. The potential use of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) against *P. viburni* in laboratory bioassays was assessed. Screening was done to identify virulent strains of entomopathogenic nematodes against *P. viburni* by exposing the mealybugs to a concentration of 200 infective juveniles (IJ)/insect for 48 hours. Five nematode isolates were selected from the 16 isolates initially tested and from these only one isolate was selected as the best candidate for *P. viburni*. The development of nematodes after infection of adult females of *P. viburni* was followed and the number of nematodes inside the host was counted every two days until a new consort of IJ emerged. The effect of mealybug size on infectivity was assessed by infecting three stages of mealybugs (adults, intermediates and crawlers). To indicate whether nematodes can locate and infect mealybugs in the ovary and calyx of apples, mealybug-infested apples from an orchard were sprayed with IJ of *H. zealandica* (J34). To determine the lethal time and dose, *P. viburni* adult females were exposed to concentrations of 52, 73, 102, 143, and 200 IJ/insect for 12, 24, 36 and 48 hours. The isolate J34 of *Heterorhabditis zealandica* was the most effective and caused 80% mortality of *P. viburni*, 48 hours post inoculation at 25ºC. *Heterorhabditis zealandica* (J34) and *S. yirgalemense* (157-C) successfully reproduced in *P. viburni* and *H. zealandica* (J34) had greater penetration ability than *S. yirgalemense* (157-C). Mealybug size affected infectivity and crawlers were less susceptible (22%) than adults and intermediates with mortalities of 78% and 76%, respectively. *Heterorhabditis zealandica* (J34) applied to *P. viburni* field-infested apples were able to enter the apple core, where they infected *P. viburni*.

After 48 hours at a concentration of 200 IJ/insect, the LD_{50} and LD_{90} values were 54 and 330 nematodes per insect, respectively, and LT_{50} was 30 hours, while the LT_{90} was 62.5 hours. This work represents the first report on using entomopathogenic nematodes to control *P. viburni*.

Introduction

Mealybugs (Pseudococcidae) are among the most common and important pests of woody or perennial plants in commercial greenhouses and conservatories (Blumberg & Van Driesche, 2001). About 20 species of Pseudococcidae are of economic importance on cultivated plants in South Africa (Annecke & Moran, 1982). Three species of mealybug from the genus *Pseudococcus* common on pome fruit are *P. viburni* (Signoret), *P. longispinus* (Targioni-Tozzetti), and *P. calceolariae* (Maskell) (Myburgh *et al.*, 1975; Swart, 1977; Van der Merwe, 2000; Wakgari & Giliomee, 2003). These insects are commonly known as mealybugs due to the typically secreted white, powdery or mealy wax that covers the body. All mealybugs are sap feeders (Kriegler & Basson, 1962) and feed on plants using their piercing, sucking mouthparts. Many species are important agricultural pests. Their feeding may cause deformation or the death of plant shoots, with some species being capable of transmitting plant virus diseases (Millar, 2002). Large populations contaminate foliage with sticky honeydew excretions, which leads to the growth of sooty mould and, as a result, fruit infested with mealybugs can lose its market value (Kriegler & Basson, 1962).

Female *P. viburni* have three nymphal instars, while males have four (first, second, third or prepupal, and fourth or pupal) (Gullan, 2000). The identification of *P. viburni* in the field is extremely difficult, due to its close morphological resemblance to other mealybug species (Wakgari & Giliomee, 2004a). In the case of fruit consignments destined for foreign markets, misidentification of *P. viburni* results in the rejection of fruits when immatures and adults of this non-quarantine mealybug species are not identified or are confused with

quarantine species. As a result, growers have sustained heavy financial losses (during 2002, 30% of apples destined for export to the U.S.A. from South Africa were rejected because of the presence of eggs and immature mealybugs). Correct identification is also a prerequisite for effective control of mealybugs, enabling the use of specific management practices against the most susceptible developmental stage (Wakgari & Giliomee, 2004a).

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae and their symbiotic bacteria have generated significant interest as inundative biological control agents against insect pests (Klein, 1990; Kaya & Gaugler, 1993; Liu *et al.*, 2000). These nematodes have a mutualistic symbiosis with a bacterium, *Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively (Poinar, 1990). Increased understanding of nematode biology, host range and epizootiology as well as concurrent advances in commercial production, storage and formulation, have led to nematode-based products being marketed worldwide as safe alternatives to chemical insecticides (Friedman, 1990; Ehlers, 1996).

A study which was conducted by García del Pino and Morton (2005) showed that entomopathogenic nematodes could be more successful control agents for neonate larvae of *Capnodis tenebrionis* L. than chemical pesticides. They are unique because they have evolved the ability to carry and introduce symbiotic bacteria into the body cavity of insects, and their host range includes the majority of insect orders and families (Gaugler & Kaya, 1990). Infective juveniles (IJ), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles) or, in some cases, through the cuticle. After entering the host's haemocoel, nematodes release their bacterial symbionts which are primarily responsible for killing the host within 24 to 48 hours, defending against secondary invaders, and providing the nematodes with nutrition (Dowds & Peters, 2002). The nematodes moult and complete up to three generations within the host, after which IJ exit the cadaver to find new hosts (Kaya & Gaugler, 1993).

Nematodes are used to control a variety of economically important insect pests such as the hairy fungus beetle *Typhaea stercorea* (L.) (Coleoptera: Mycetophagidae), the damp wood termite *Zootermopsis angusticollis* (Hagen), the black vine weevil, *Otiorhynchus sulcatus* (F.), diaprepres root weevil, *Diaprepres abbreviatus* (L.), fungus gnat (Jess & Kilpatrick, 2000), and various white grubs (Coleoptera: Scarabaeidae) (Klein, 1990; Svendsen & Steenberg, 2000; Shapiro-Ilan *et al.*, 2002; Wilson-Rich *et al.*, 2007).

Comparative studies have been conducted on the susceptibility of Homoptera to various strains and species of nematodes. Stuart *et al.* (1997) investigated the susceptibility of *Dysmicoccus vaccinii* (Miller & Polavarapu) to infection by various species and strains of entomopathogenic nematodes. They tested four nematode strains in a sand-dish assay in which 64% mortality was obtained when using *H. bacteriophora* Poinar, 1976. They also showed that removal of the waxy coating of the mealybugs had no effect on susceptibility. In a sand-column assay, both *H. bacteriophora* and *H. indicus* Poinar, 1992 were effective against *D. vaccinii*. When infected mealybugs were placed in White traps, nematodes successfully reproduced in mealybug cadavers.

De Waal *et al.* (2009) tested three South African species of nematodes (*H. zealandica* Poinar, 1990; *H. bacteriophora* and *S. khoisanae* Nguyen, Malan & Gozel, 2006) for their potential to infect the adult female stage of *Planococcus ficus* (Signoret). By measuring the possible entry points, they observed that the ostioles, vulva and anus of the mealybugs were wide enough, though the spiracles were too small for all three nematode species to enter the mealybugs. *Planococcus ficus* was more susceptible to *Heterorhabditids* species (with mortality ranging from 70-100%) compared to *S. khoisanae*, which caused 54% mortality. Their exploratory studies, together with those *D. vaccinii*, indicated that nematodes have good potential for use against mealybugs.

In this study, laboratory bioassays were performed to determine the potential of entomopathogenic nematodes for the control of *P. viburni*. In the first bioassays, endemic nematode species and isolates were screened to select an isolate tha produced high levels

of *P. viburni* mortality. The development of the life cycle of a steinernematid and a heterorhaditid in infected *P. viburni* was observed. In general, mealybugs are very small and the effect of size of *P. viburni* on nematode infection was therefore investigated. To use nematodes effectively for control, different concentrations were applied to indicate optimum nematode concentration. As humidity plays an important role in the mobility of nematodes, the lethal time for maintaining levels of high humidity promoting the infection of mealybugs by nematodes was determined. This study also attempted to see whether nematodes could infect *P*. *viburni* on the surface of , as well as inside field-infected apples.

Materials and methods

Nematode source:

Nematode species and isolates used in this study were obtained from previous surveys (Malan *et al.*, 2006; De Waal, 2008) maintained in the Stellenbosch University nematode collection (Table 1). Inoculum was reared using standard procedures (Woodring & Kaya, 1988) in last instar larvae of the wax moth, *Galleria mellonella* (L.) or the mealworm, *Tenebrio molitor* (L.) larvae. IJ were harvested within the first week of emergence and stored horizontally in tissue culture flasks at 14ºC till used within one to three weeks after harvest. Nematodes were normally used at a concentration of 200 IJ/50 µl calculated using the method developed by Navon and Ascher (Navon & Ascher, 2000).

Table 1: Nematode species, isolate, habitat, locality and Genbank accession number.

Species	Isolate	Soil type	Locality	Genbank accession number
H. zealandica	J34	Natural	Brenton on Sea, Western Cape	EU722436
H. zealandica	J182	Disturbed	Kuilsrivier, Western Cape	EU727167
H. zealandica	J21	Natural	Bonnievale, Western Cape	Problem
H. zealandica	J37	Disturbed	Belvidere, Western Cape	EU727165
H. zealandica	SF41	Natural	Patensie , Eastern Cape	EU699436
H. bacteriophora	SF351	Disturbed	Wellington, Western Cape	FJ455843
H. bacteriophora	SF19	Disturbed	George, Western Cape	FJ710787
H. bacteriophora	SF286	Disturbed	Porterville, Western Cape	Not available
H. bacteriophora	J172	Disturbed	Stellenbosch, Western Cape	EU716335
H. bacteriophora	SF285	Disturbed	Villiersdorp, Western Cape	Not available
H. safricana	SF281	Disturbed	Piketberg, Western Cape	EF488006
S. khoisane	SF87	Disturbed	Villiersdorp, Western Cape	DQ314289
S. khoisane	SF362	Disturbed	Rawsonville, Western Cape	Not available
S. khoisane	SF80	Natural	Tulbagh, Western Cape	DQ314287
Steinernema sp.	141-C	Disturbed	Piketberg, Western Cape	EU740970
S. yirgalemense	157-C	Disturbed	Friedenheim, Mpumalanga	EU625295

(Malan *et al.*, 2006; De Waal *et al.*, 2008; Malan *et al.*, 2009)

Twenty-four-well bioassay protocol

Adult females of *P. viburni* were individually exposed to IJ in 24-well culture plates (Flat bottom, Nunc™, Cat. No.144530). Each well was lined with a circular filter paper disc and nematodes were inoculated at a concentration of 200 IJ/50 µl in filtered tap water. Control plates received 50 µl tap water only. After inoculation, the plates were closed and placed in plastic containers which contained moistened paper towels to maintain a high humidity, closed and incubated in the dark in a growth chamber at 25±2ºC. After two days, mortality was determined by gentle prodding, dead mealybugs were removed, rinsed in tap water (to remove the external nematodes) and placed in small Petri dishes containing moistened filter paper for another two days at 25±2ºC in a growth chamber. Each cadaver was dissected with the aid of a dissecting microscope to confirm mortality due to nematode

infection. All infected mealybugs were recorded as infected while all those that died from other causes were recorded as naturally dead.

Screening

The 24-well bioassay protocol was used to test a total of 16 nematode species and isolates (Table 1) for their ability to infect adult female obscure mealybug under optimum conditions. Two 24-well plates, each with 12 mealybugs were used for each nematode isolate tested and one control plate inoculated with water only, with a total of 36 mealybugs for each nematode isolate. The same 24-well bioassay protocol was followed, but five bioassay plates, each with 12 mealybugs for *H. zealandica* (SF41, J34), *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005 (157-C), *H. bacteriophora* (J172), and an unknown *Steinernema* sp. were used, with a control plate for each nematode species.

Biological study

In this study the biology and development of *H. zealandica* (J34) and *S. yirgalemense* in *P. viburni* were studied. For each nematode species, 20 adult female mealybugs were placed in a 9-cm diam. Petri dish lined with filter paper and inoculated with 4000 IJ in 1 ml of filtered water. The Petri dishes were placed in a plastic box lined with damp paper towels to maintain a high humidity level, and incubated at 25±2ºC in a growth chamber. Dead mealybugs were rinsed in tap water to remove any nematodes from their body surface, moved to nematode-free Petri dishes and incubated at 25±2ºC in a growth chamber. Every two days, 10 mealybugs were randomly selected from each treatment, dissected under the microscope and the development of the nematodes noted. Individual mealybugs were assessed for colour change, infection, number of nematodes that penetrated, stage of nematode development, the quality of mealybug eggs and number of IJ that emerged. The first generation hermaphrodites for *H. zealandica* and males and females for *S. yirgalemense* were counted to determine the number of nematodes that penetrated. A total of 11 Petri dishes, each with 20 mealybugs, was prepared for each nematode species. Five Petri

dishes, each with 20 mealybugs, were left undisturbed and were placed on White traps to determine whether the life cycle would be completed, and the number of IJ that could be recovered.

Effect of mealybug size

To determine the effect of mealybug size on nematode infectivity, they were visually divided into three groups: adults, intermediates and crawlers. Ten mealybugs were placed in a 9 cm-diam. Petri dish lined with filter paper and inoculated with 2000 IJ of *H. zealandica* (J34) in 500 µl of filtered water. For each size of mealybug, there were six replicates and a control. Petri dishes were placed in a plastic container lined with damp paper towels to maintain high humidity, and incubated at 25±2ºC in a growth chamber. After two days, dead mealybugs were removed, rinsed in tap water (to remove the external nematodes) and placed in small Petri dishes containing moistened filter paper and left for another two days at 25±2ºC in a growth chamber. Each cadaver was measured using a motorised Olympus SZX12 stereomicroscope with a Colorview IIIu camera and AnalySIS 5 image analysis program. Afterwards they were dissected with the aid of a dissecting microscope to confirm mortality due to infection.

Ability of nematodes to infect mealybugs on and inside apples

To test the ability of *H. zealandica* (J34) to infect mealybugs on the surface, inside the calyx and ovary of apples, *P. viburni*-infested Starking apples from an orchard on the farm Molteno Brothers, Elgin were collected. Twenty apples were placed in five different plastic containers lined with damp paper towels to maintain high humidity, sprayed with IJ of *H. zealandica* (J34) and incubated at 25±2ºC. Controls were identical to the treatments, except that only water was added. After seven days insect mortality was checked by collecting mealybugs from the surface of the apple as well as by cutting each apple open to determine infection of mealybugs in the ovary and calyx.

Lethal time and concentration

Heterorhabditis zealandica (J34) was used to determine the optimal nematode concentration and lethal time. Petri dishes (9 cm diam.) with 20 adult mealybugs in each were inoculated with concentrations of 200; 143, 102; 73 and 52 IJ/insect. After application, the Petri dishes were placed in plastic containers with damp paper towels to maintain high humidity and incubated at 25ºC in a growth chamber. Five replicates were used for each treatment (concentration/exposure period) and for the control (only water) at each exposure period for a total of 30 replicates. Every 12 hours the mortality for each treatment was noted and after 48 hours the experiment was terminated and infection confirmed by dissection.

Data analysis

All statistical analyses were performed by using the data analysis software system Statistica 8 (StatSoft Inc., 2008). Data were analysed using a one-way ANOVA with post-hoc comparisons of means, using Bonferroni's method, or a bootstrap multiple comparison method if residuals were not normally distributed (Efron & Tibshirani, 1993). Probit analysis was conducted using Software NCSS 2007 ver. 07.1.1 (Hintze, 2007) to determine lethal concentration and time.

Results

Screening

Results obtained from screening the 16 nematode isolates were analysed using a one-way ANOVA with significant differences between the different isolates ($F_{(16.17)}$ = 14.127; P < 0.01) (Fig. 1). *Heterorhabditis zealandica* (J34 and SF41), *H. bacteriophora* (J172), *S. yirgalemense* (157-C) and *H. safricana* (SF281) induced the highest mortality rates – between 58.3% and 75.0%, while isolates of *S. khoisanae* (SF362 and SF80) and *Steinernema* n. sp. (141-C) resulted in 8.3% mortality and were not significantly different

from the control. *Heterorhabditis bacteriophora* (SF285), *S. khoisanae* (SF87) and *H. zealandica* (J21) did not infect any mealybugs.

The three isolates of *H. zealandica* (J34, SF41 and J182) did not significantly differ from one another ($P < 0.001$). They, however, differed significantly from the isolate J37, while the isolate J21 did not manage to kill any mealybugs. The *S. khoisanae* isolates (SF80, SF87, and SF362) with mortality rates of 8.3%, 0% and 8.3%, respectively, did not differ significantly from the control, while *S. yirgalemense* (157-C) gave significantly better results (66.6%) than did the *S. khoisanae* isolates.

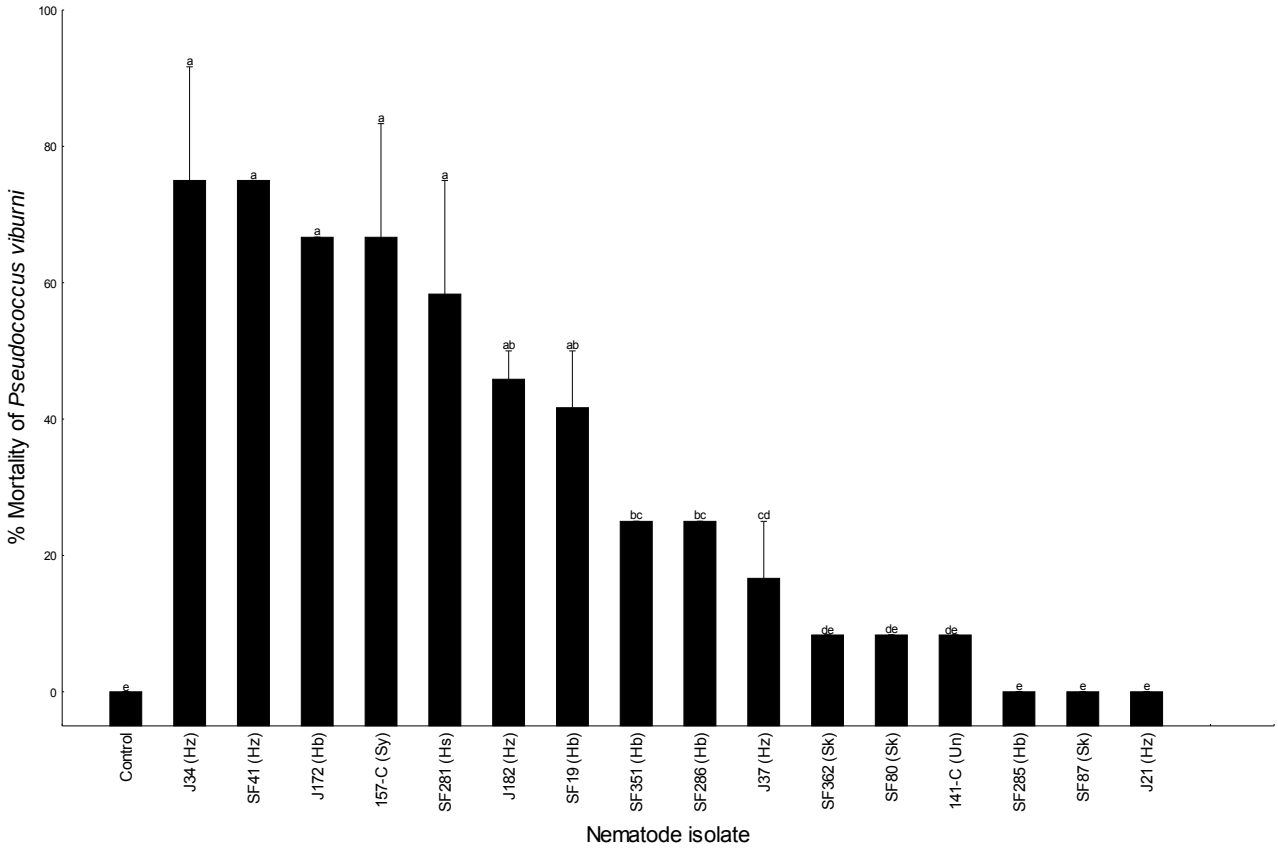

Fig. 1: Mean percentage (95% Confidence Interval) mortality for adult female *Pseudococcus viburni*, using different species and isolates of entomopathogenic nematodes at a concentration of 200 IJ/insect, after a period of 48 hours in 24-well bioassay plates, at 25ºC in a growth chamber. Means with the same lettering are not significantly different (one-way ANOVA; $F_{(16, 17)} = 14.127$; $P < 0.01$).

The results obtained with the five selected nematode isolates were analysed using a one-way ANOVA and significant differences were obtained ($F_{(5,24)}$ = 24.185; P = 0.01) for percentage mortality of *P. viburni*. Mortalities for all isolates ranged between 44% and 80% after 48 hours, with the highest percentage mortality for *H. zealandica* (J34) and the lowest for *H. bacteriophora* (J172). There were no significant differences between the isolates except for *H. bacteriophora* (J172), which differed significantly from *H. zealandica* (J34) (P = 0.004) (Fig. 2).

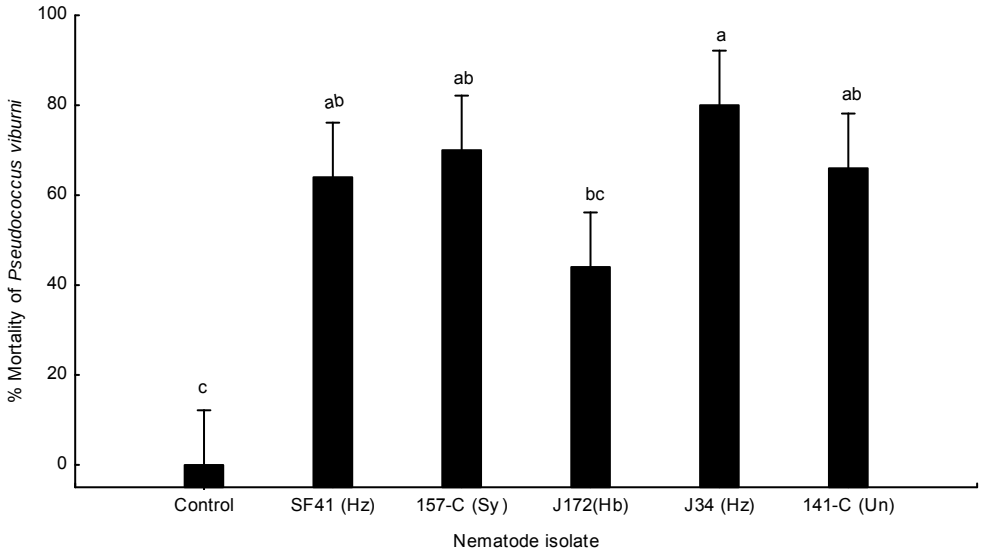

Fig. 2: Mean percentage mortality (95% Confidence Interval) for adult *Pseudococcus viburni* using *Heterorhabditis zealandica* (SF41, J34), *H. bacteriophora* (J172), *Steinernema yirgalemense* (157-C) and an unknown *Steinernema* sp. at a concentration of 200 IJ/insect after a period of 48 hours in 24-well bioassay plates at 25ºC in a growth chamber (one-way ANOVA; $F_{(5,24)}$ = 24.185; P < 0.01).

Biological studies

The observations of the development of the two species are presented in Table 2. The changes in mealybug biology were recorded for 12 days after exposure to IJ of *H. zealandica* (J34) and *S. yirgalemense* (157-C). The changes included change in colour; mealybugs infected with *H. zealandica* (J34) were red in colour (Fig. 3), compared to those

infected with *S. yirgalemense* (157-C) that became brown in colour. After infection, mealybug quality changed; they became soft, fragile and doughy.

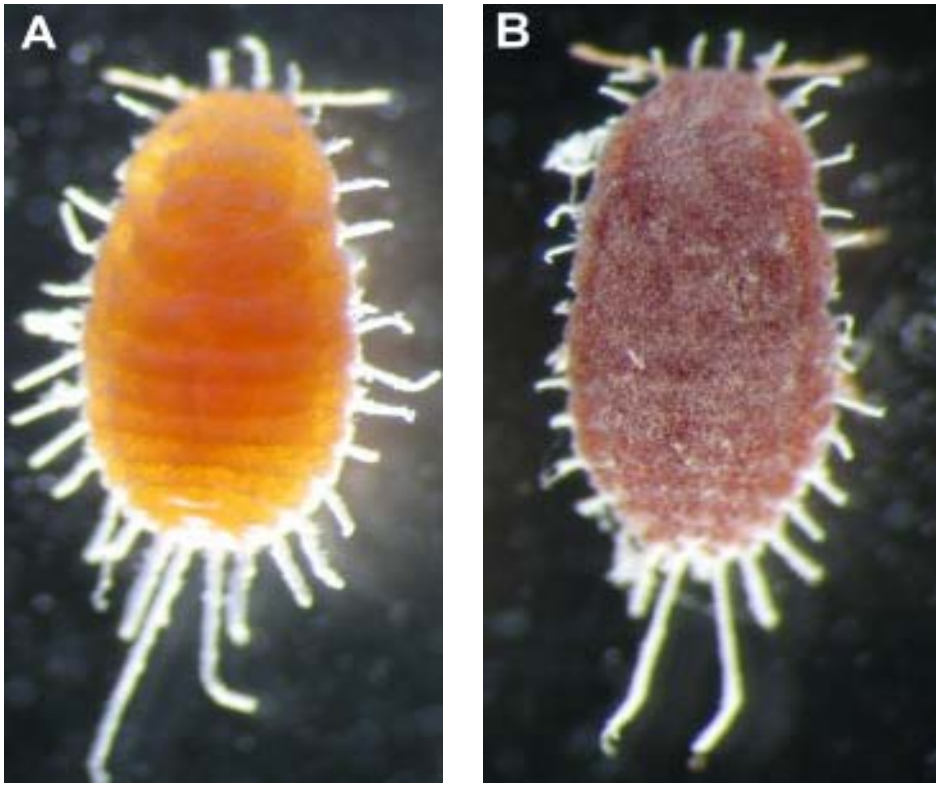

Fig. 3: Change in *Pseudococcus viburni* colour (A) before infection and (B) after nematode infection with *Heterorhabditis zealandica.*

Mealybug eggs did not change colour and remained intact and viable. On each observation date, the number of hermaphrodites for *H. zealandica* (J34) as well as males and females of *S. yirgalemense* (157-C) was counted (Fig. 4) (Table 1). Both *H. zealandica* (J34) and *S. yirgalemense* 157-C reproduced successfully and completed their life cycle in mealybug cadavers until IJ emerged. The mean number (n = 20) of nematodes that penetrated the hosts was 21.9 for *H. zealandica* and 15.2 for *S. yirgalemense*. The life cycle of *H. zealandica* was completed in 10 days while that of *S. yirgalemense* was completed in eight days.

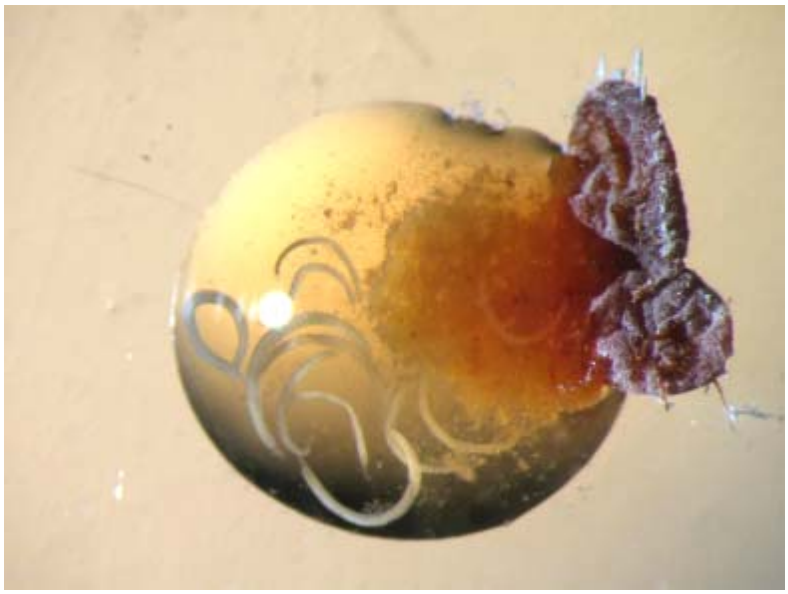

Fig. 4: Dissected *Pseudococcus viburni* showing the development of nematodes inside the cadaver.

Table. 2**:** Results of the biological study in which mealybugs were exposed to IJ of *Heterorhabditis zealandica* (J34) and *Steinernema yirgalemense* (157-C) to assess development of the nematodes inside the mealybugs

Nematode species	No. of days	Stage of nematode Development
H. zealandica (J34)	2	Hermaphrodites
	4	hermaphrodites with eggs
	6	Hermaphrodites with larvae inside
	8	Hermaphrodites with larvae inside
	10	Infective juveniles
	12	Infective juveniles
	19	Infective juveniles
S. yirgalemense (157-C)	2	First generation males and females
	4	First generation. males and females
	6	First generation. males and females
	8	Infective juveniles
	10	Infective juveniles
	12	Infective juveniles
	19	Infective juveniles

Mealybug size

The measurements of the adults, intermediates and crawlers of *P. viburni* in comparison with *H. zealandica* are shown in Table 3. There was no overlap in the measurements and the three sizes could easily be visually distinguished. When comparing the susceptibility of the different sizes of *P. viburni,* adults and intermediates were the most susceptible to *H. zealandica* IJ infection, with mortalities of 78% and 76%, respectively, while crawlers showed 22% mortality. The size of the first-generation hermaphrodite after four days in comparison with an adult female *P. viburni* is indicated in Fig. 5.

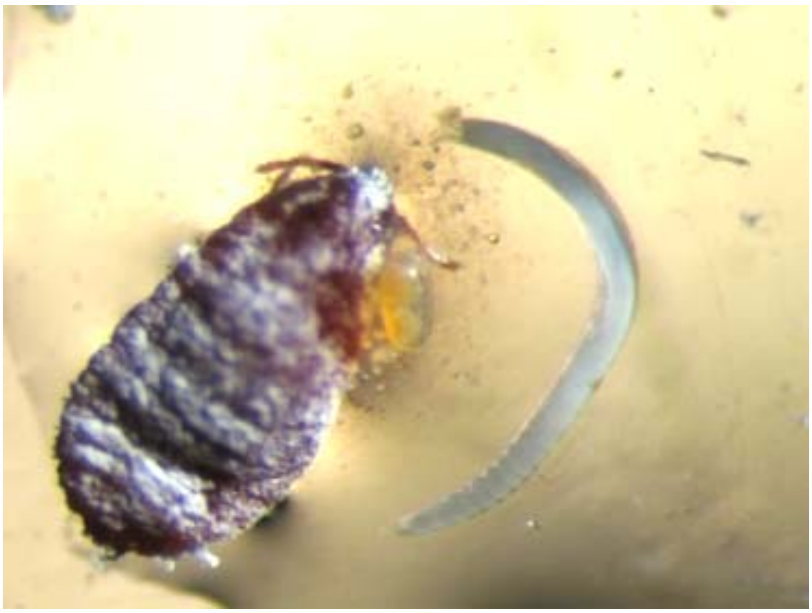

Fig. 5: First-generation *Heterorhabditis zealandica* from an infected adult female *Pseudococcus viburni*

Table 3: *Pseudococcus viburni* were visually divided into three groups: adults, intermediates and crawlers based on mean length and width.

Size	Mean length ±sd (range) (µm)	Mean width ± sd (range) (µm)	% infected
Adults (n = 60)	2992 ± 466 (1984 - 3626)	1437 ± 282 (952 - 1837)	78
Intermediate (n = 60)	1640 ± 141 (1528 - 1799)	733 ± 139 (620 - 889)	76
Crawlers (n = 60)	1241 ± 135 (1021 - 1417)	598 ± 98 (392 - 858)	22
H. zealandica	685 (570 - 740)	27 (22 - 30)	–

Ability of nematodes to infect mealybugs on and inside apples

A total of 35 mealybugs were collected from the surface of the apples of which 31 were infected. Of the 50 mealybugs recovered from the calyx, 19 were infected. From the ovary, 50 mealybugs were recovered and 19 of these were infected. Significantly higher mortality (87%) was obtained for mealybugs on the surface of the apple, compared to 33% and 18% mortality obtained for mealybugs in the calyx and ovary, respectively (Fig. 6). The data obtained from mealybug-infested apples were analysed using a one-way ANOVA, with significant differences found between the positions of the mealybugs on the apple ($F_{(2, 40)}$ = 16.568; $P < 0.001$).

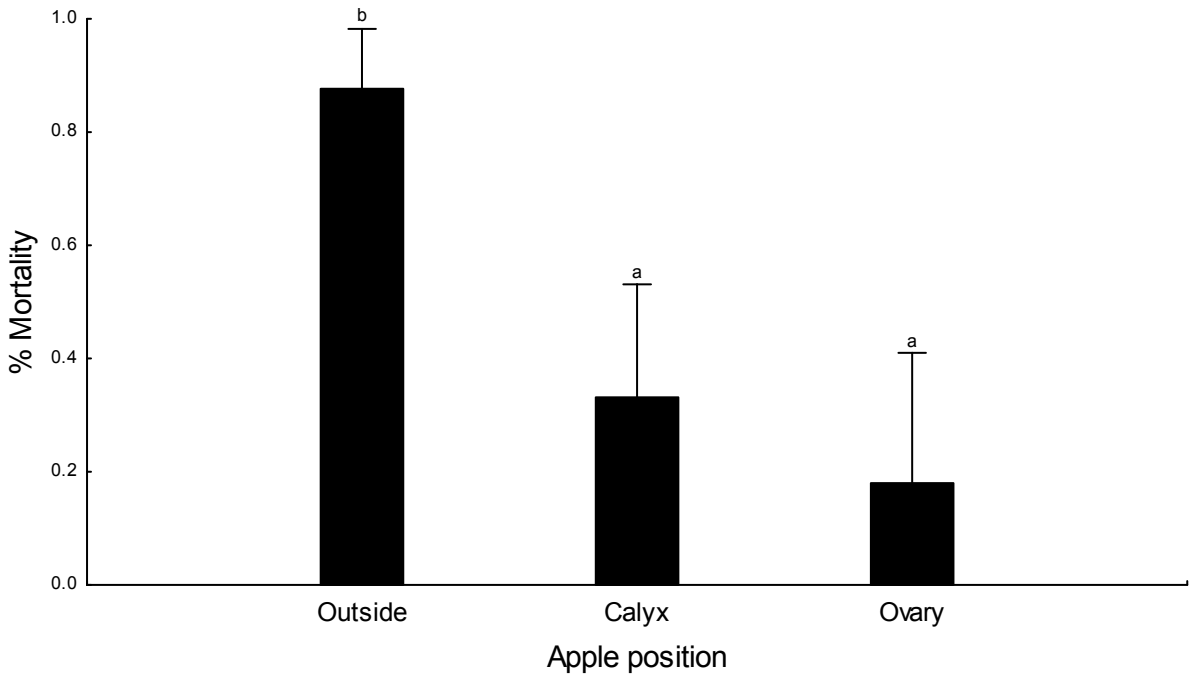

Fig. 6: Mean percentage mortality (95% Confidence Interval) for *Pseudococcus viburni* on the surface, and inside the calyx and ovary of field-infested Starking apples (one-way ANOVA, F(2, 40) = 16.568, P = 0.0001)

Lethal time and concentration

The percentage mortality following exposure to *H. zealandica* (J34) in concentrations of 52 to 200 IJ per host ranged from 0% to 8% after 12 hours, from 2% to 26% after 24 hours, from 30% to 60% after 36 hours, and from 46% to 84% after 48 hours (Fig. 7). The corresponding LD_{50} values were 1127, 322, 152 and 54 IJ per mealybug, respectively. The LD_{90} values at 12, 24, 36 and 48 hours were 5867, 1036, 2079 and 330 IJ/insect, respectively. At a dose of 200 IJ/insect, the time required to obtain 50% mortality (LT_{50}) was 30 hours and LT_{90} was 62.5 hours. The data obtained from the time and dose inoculation were analysed using a one-way ANOVA, with significant differences ($F_{(15,\ 96)}$ = 7.3879; P<0.001) as indicated in Fig. 7.

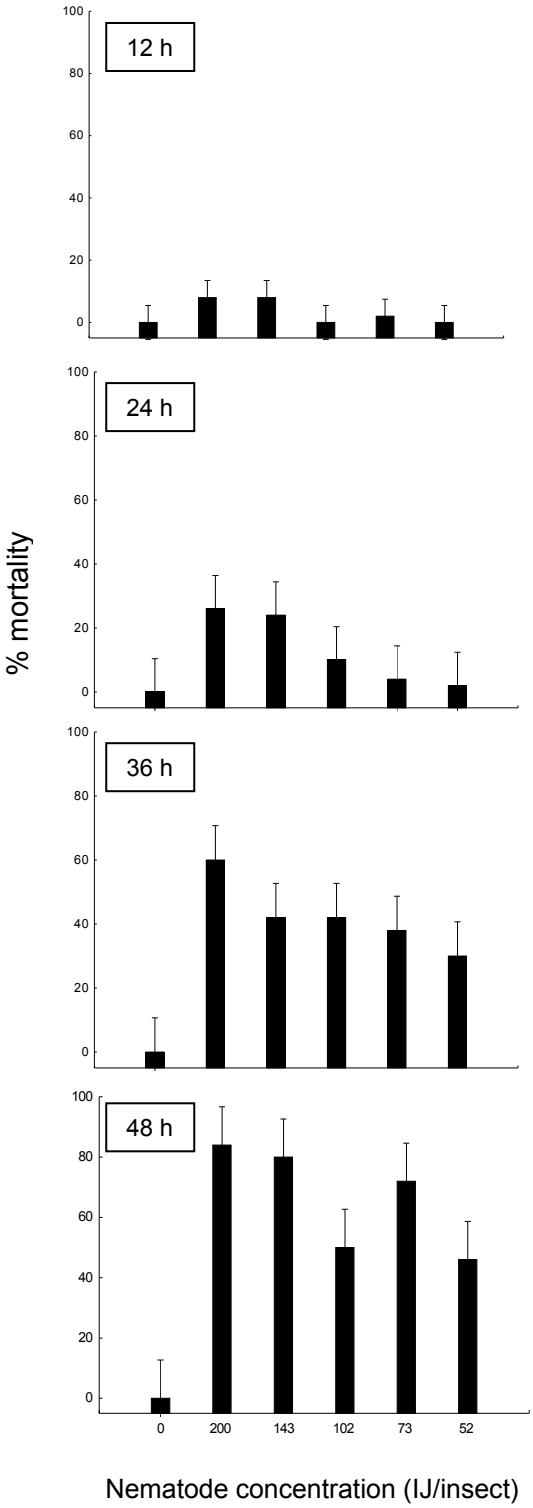

Fig. 7: Percentage mortalities of adult female *Pseudococcus viburni* at five different concentrations and four exposure periods. The mealybugs were individually exposed to nematodes in 24-well culture plates and incubated at 25ºC in a growth chamber (one-way ANOVA; $F_{(15, 96)}$ = 7.3879, P <0.001).

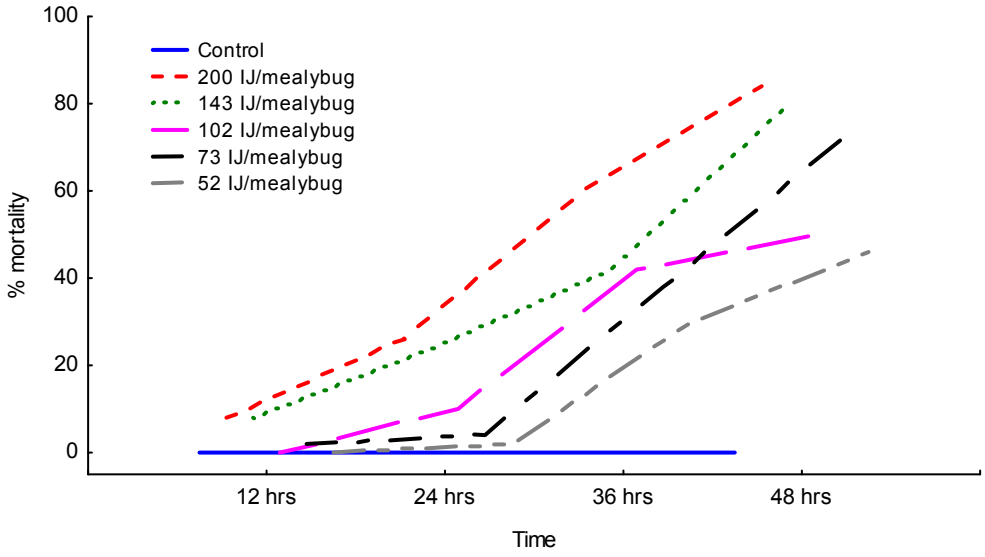

Fig. 8: Percentage mortality for *Pseudococcus viburni* in the lethal time and lethal dose assay when exposed to five different nematode concentrations at four exposure periods (Probit analysis)

Discussion

No research has yet been conducted on the control of *P. viburni* with entomopathogenic nematodes. On fruit trees, *P. viburni* usually occur on the trunk where the insects normally hide under the bark, in cracks in the tree and on the fruit itself. This cryptic lifestyle makes it difficult for chemicals to reach these insects. Options for the chemical control of *P. viburni* are becoming limited, especially for the export market, because of restrictions on chemical use. Furthermore, mealybugs have been reported to be capable of resistance to insecticides. Alternative biological control agents therefore need to be investigated. More recent research has focused on the use of natural enemies as biocontrol agents of *P. viburni*. As a result, some parasitoids, such as *Anagyrus* sp., *Acerophagus* sp., *Pseudaphycus maculipennis* (Mercet), *Pseudectroma* sp. and *Tetracnemoidea* sp. have been reared from *P. viburni* (Wakgari & Giliomee, 2004b) . Currently, little is known about the natural enemies of *P. viburni* and the potential use of entomopathogenic nematodes as biocontrol agents of *P. viburni* was investigated in this study. These nematodes can reach

colonies living underneath the bark, as the IJ stage is mobile. They hane no residue problems and are safe for both the environment and for humans.

This study has shown that the pathogenicity of entomopathogenic nematodes is strongly affected by the interactions between the nematode isolates and *P. viburni*; and as a result different nematode species and isolates were seen to differ greatly from each other in terms of pathogenicity. The *Heterorhabditis* isolates that were tested in the laboratory, on average, were more pathogenic to *P. viburni* than the *Steinernema* isolates and this is similar to the results obtained by De Waal *et al.* (2009). The effectiveness of heterorhabditids in this study may be attributed to their not being associated with soil, or the fact that they possess a dorsal tooth that might promote penetration through abrasion of the thin cuticle (Bedding & Molyneux, 1982), a mode of entry for the nematodes that could be especially important when the target insects are mealybugs. Great variation was observed among the *H. zealandica* isolates with percentage mortality ranging from highest to intermediate and even to no infection at all. In contrast, infection rates of Steinernematids were generally low and *S. yirgalemense* gave better results than the three *S. khoisanae* isolates. Two strains of *H. zealandica* (SF41 and J34), one strain of *H. bacteriophora* (J172), one of *S. yirgalemense*, and the unknown new species of *Steinernema* were found to be highly pathogenic to *P. viburni* and to show promise for development as biocontrol agents. From the second screening, *H. zealandica* (J34) was selected as the most promising isolate for the control of *P. viburni* and was therefore used in all further laboratory studies.

Inspection of cadavers exposed to nematodes for two days revealed that *H. zealandica* (J34) had greater penetration ability than *S. yirgalemense* (157-C). It was not easy to distinguish between males and females of *S. yirgalemense* after two days because the nematodes were not fully developed. After four days, *S. yirgalemense* were fully developed and 90% of the counted nematodes were females while 10% were males; *H. zealandica* (J34) had developed into hermaphrodites with eggs inside. After six days, the *H. zealandica* first-generation females had larvae inside and the second generation was also

present; the *S. yirgalemense* (157-C) second generation was present. The life cycle of *S. yirgalemense* (157-C) was shorter and IJ emerged after eight days, compared to that of *H. zealandica* (J34), with which IJ only started emerging after 10 days. *Heterorhabditis zealandica* (J34) and *S. yirgalemense* (157-C) were able to successfully reproduce and complete their life cycle in *P. viburni*. Our results support the assumption by Hominick and Reid (2009) that the nematode with the highest efficacy against a target insect would have the highest invasion efficiency. These results are in agreement with many other studies demonstrating that nematode invasion is in agreement with nematode insecticidal activity based on host mortality (Kondo & Ishibashi, 1986; Mannion & Jansson, 1993; Shannag *et al.*, 1994; García Del Pino & Morton, 2005). *Heterorhabditis zealandica* showed the highest mortality and penetration rate against *P. viburni*, compared to *S. yirgalemense*. In general, relatively few nematodes penetrated with a mean of 21.9 for *H. zealandica* and 15.2 for *S. yirgalemense.* This can be attributed to the relatively small size of the adult female mealybug, of 6 mm in length and 3 mm in width.

In comparing the efficacy of *H. zealandica* against different sizes of *P. viburni*, we have to consider that nematode efficacy can be affected by the *P. viburni* stage and that nematode applications may have to be timed accordingly for optimal efficacy. All host sizes from crawlers to adults were found to be susceptible to nematodes. Surprisingly enough, even though crawlers are 1.2 cm in length and 0.5 cm in width, in comparison with the IJ of *H. zealandica* with a length of 0.7 cm and width of 0.03 cm, they were also susceptible to nematodes, though low mortality was obtained. Therefore, if *P. viburni* colonies are targeted beneath the bark or on the roots of apple trees, all mealybug stages will be infected. This also applies to other mealybugs associated with roots, such as *P. ficus* in the case of grapevines (Walton, 2003).

Mealybugs cause indirect damage to fruit by forming colonies in the stem end, as well as in the calyx end of the apple. In many cases the mealybugs penetrate through the open calyx of the apple (Kriegler & Basson, 1962; Myburgh, 1962). Myburgh (1962) discovered

that chemical control of mealybugs on apple trees after the first week of January is inadequate because by this time, the insects are hiding in places where they cannot be reached by insecticides. Mealybugs spend the winter in cracks in the bark or on apple roots (Myburgh, 1962), making winter control impossible. Our results show the ability of *H. zealandica* to locate and kill mealybugs in cryptic habitats such as inside the calyx and ovary of the apple. Therefore the perfect time to spray nematodes in the orchards would be when mealybugs are hiding under the bark. Although it will not be practical to spray fruits in the orchard, it is interesting to see that infection inside the fruit does take place. This can have practical implications; for example, when nematodes are applied to soil there is a possibility that fallen citrus fruits infected with false codling moth can be infected and thus aid in orchard sanitation.

The results of our studies clearly demonstrate that *P. viburni* exhibits a dosage-dependent susceptibility to the nematode *H. zealandica* (J34). With increasing time and concentrations of *H. zealandica*, the mortality of the mealybugs increased. Mortality of *P. viburni* was highest after exposure to the normal concentration of 200 IJ for 48 hours used in this study, resulting in LD_{50} value of 54 and LD_{90} value of 330 nematodes per insect. These values are not very different from those of De Waal (2008) who obtained an LD_{50} value of 72 and LD_{90} value of 275. The time required to obtain 50% mealybug mortality (LT_{50}) was 1.25 days, while the LT_{90} was 2.6 days. At 12, 24, and 36 hours, the LD_{50} values were 1127, 323 and 152 IJ/insect, respectively. At 12 hours, mortality was still low and mortality obtained at all concentrations did not differ significantly from the control. After 48 hours the highest concentration caused significantly higher mortality than the middle concentration, which did not differ significantly from the lowest concentration (52 IJ/insect).

The use of the entomopathogenic nematode *H. zealandica* (J34) to control *P. viburni* has been demonstrated in the laboratory. There are many other biological factors that can affect the final choice of a nematode species or isolate for the control of *P. viburni*, some of which have been partially addressed in this study. Further studies with additional species

and isolates of nematodes in field-simulated conditions in the laboratory, in glasshouses and in the field are still needed. The susceptibility of entomopathogenic nematodes *to P. viburni* in the field might be influenced by some aspects of the *P. viburni* natural history. All host stages of *P. viburni* older than the crawlers seem to be more suitable for treatment with nematodes. Hence, control in the field should be made when the intermediates and adults are most abundant. We suggest that innovative methods to enhance the prevalence and activity of indigenous entomopathogenic nematodes should be developed and implemented for the control of *P. viburni*.

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Chapter 6

Conclusion

The first objective of this study was to use crossbreeding, light microscopy, SEM, morphometrics and morphology and drawings to describe a new species of *Steinernema*. This new species was recovered from a citrus orchard in South Africa during a previous survey for the presence of entomopathogenic nematodes. In crossbreeding tests, when this nematode was crossed with closely related *S. feltiae*, (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 (Russian strain), no progeny was produced. It is characterised by an infective third-stage juvenile with a body length of 754 (623-849) µm, distance from head to excretory pore of 56 (49-63) µm, tail length of 51 (42-57) µm, ratio E value of 110 (85-132). These measurements, together with the cross breeding results, provide ample evidence that this is indeed a new species. This objective was achieved. Molecular data should be added to complete the description.

The second aim was to develop a simplified protocol for the long-term storage of infective juveniles (IJ) of *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006. Glycerol (15%), dimethyl sulfoxide (8%) and ethylene glycol (8%) were used as cryoprotective agents. The effect of exposure of nematodes to different cryoprotectants for different time periods was assessed. Glycerol (15%) appeared to be the best of the three cryoprotectants resulting in optimum survival of 69% for *S. khoisanae* after nematodes were preincubated for four days. *This protocol was applied to H. zealandica*. Virulence of nematodes following cryopreservation was not lost and there was no difference in virulence between IJ kept in liquid nitrogen for two, 21, and 42 days. At 42 days they successfully completed their lifecycles in *Galleria mellonella* larvae.

The third and last objective of this study was to evaluate South African isolates of entomopathogenic nematodes for their potential in controlling *Pseudococcus viburni* (Signoret) and to select the most effective isolate. After a final screening, *H. zealandica*

(J34) was selected for use in further laboratory studies. Biological studies revealed that *H. zealandica* (J34) and *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005 (157-C) can reproduce in and complete their life cycle in cadavers of *P. viburni*. When the effect of mealybug size to infectivity was assessed, it was evident that crawlers present the least susceptible stage, compared to intermediates and adults. When *P. viburni*-infested apples from the field were sprayed with IJ of *H. zealandica*, nematodes were able to infect mealybugs both on the surface and in the core of the apple. Results obtained from a concentration and time study indicated that the highest mortality (84%) of *P. viburni* was obtained after exposure to 200 IJ for 48 hours. At this time and concentration, LD_{50} and LD_{90} values were 54 and 330 nematodes per insect, respectively. The time required to obtain 50% mealybug mortality (LT_{50}) was 30 hours, while the LT_{90} was 62.5 hours.

The new *Steinernema* species described here is the third report of a *Steinernema* species for South Africa. It is recommended that all newly-isolated nematodes should be cryopreserved before going through the laboratory cycles causing adaptation and loss of valuable biological traits Local isolates of nematodes have shown potential as biological control agents of *P. viburni*, but more research simulating field conditions in the laboratory and in glasshouse should be done before field trials commence.