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Rearing of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) and control with entomopathogenic nematodes

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Declaration

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Abstract

The banded fruit weevil, *Phlyctinus callosus* (Schönherr), is a key pest of apples, nectarines and grapevines in the southern areas of the Western Cape. The control of *P. callosus* is not satisfactory and the insecticides used to control this insect have not proved to be effective since the development of tolerance to pyrethroids and acephate. A control method that can be used, despite it being very labour-intensive, is that of tree trunk barriers. The use of such a method will prevent the weevils from reaching the fruit, as they are unable to fly. Alternative control options, such as the use of entomopathogenic nematodes, are urgently needed for the control of *P. callosus*.

Entomopathogenic nematodes belonging to the Steinernematidae and Heterorhabditidae are ideal biocontrol agents for incorporation into an integrated pest management programme. In order to develop control strategies for *P. callosus,* large numbers and a predictable quantity of different weevil stages are needed. Especially large numbers of larvae are needed, as this is the stage that will be targeted with nematodes. One of the aims of the current study was to assess various artificial diets for rearing larvae of *P. callosus*. Though adult weevils were easily collected from orchards, it was very difficult to obtain large numbers of larvae. Modified versions of an agar diet, as well as different carrot based diets, were tested at 21˚C. The highest percentage survival obtained for the agar diet was 50% and 60% for one type of carrot diet. A better rearing method proved to be that of planting full-grown carrots in pots, kept at 25˚C, resulting in the attainment of the highest percentage survival rate of 90%. A study was undertaken to assess how long, and at what temperature, *P. callosus* eggs could be stored. A mean percentage hatch of 45.7% was obtained when eggs were stored at 4˚C for 70 days. Eggs started hatching after 47 days and 10 days, when stored at temperatures of 11˚C and 14˚C, respectively. If the aim of the employment of such a method is only to delay egg hatching, the two temperatures (11˚C and 14˚C) will be suitable.

For the following part of the study, several entomopathogenic nematode isolates were evaluated for their potential use as biological control agents against *P. callosus*. The susceptibility of *P. callosus* larvae and adults to nematode infection was assessed in the laboratory by screening for their mortality, using different nematode isolates. Larvae were found to be more susceptible to nematode infection than adults. *Heterorhabditis* isolates were found to cause higher levels of mortality than the *Steinernema* isolates during screening, when a concentration of 400 infective juveniles (IJ) per insect

was used. Biological characteristics, such as the effect of different temperatures on nematode activity and the minimum concentration of nematodes needed to obtain acceptable levels of control for *P. callosus*, were also investigated. The percentage mortality ranged from no infection to 75% after four days for the larvae, and the SF41 isolate of *Heterohabditis zealandica* was selected as the most promising isolate for further laboratory experiments. The vertical movement of nematodes in sand, compared with such movement in sandy loam soil, and the biology of *H. zealandica* in *P. callosus* larvae was also investigated in laboratory bioassays. After four days, the LD_{50} and LD_{90} values were 96 IJ/50 µl and 278 IJ/50 µl, respectively. Nematodes were found to be inactive at 11˚C, with the highest mortality rate of *P. callosus* resulting from nematode infection being recorded at 25˚C. A higher percentage mortality rate was obtained with the sandy loam soil (95.2%) than with the sand (77.5%). *Heterorhabditis zealandica* could successfully complete its life cycle in 6th instar *P. callosus* larvae. The study showed that *P. callosus* larvae are suitable hosts for *H. zealandica*, and that the control of *P. callosus* in the field by the selected isolate holds promise.

The persistence of the SF41 isolate of *H. zealandica* at different concentrations was investigated in the last part of the study. The experiment took place in a blueberry orchard, subject to a high rate of infestation by *P. callosus*. Concentrations of 0, 20, 30 and 45 IJ/cm^2 were topically applied, with persistence being evaluated for days 1, 35 and 84. Percentage persistence for 30 IJ/cm^2 was calculated as 87.5% for days 35 and 84. The persistence of soil samples taken on day one, and kept in plastic containers at room temperature, was again evaluated on day 128, with the finding that both 30 IJ/cm^2 and 45 IJ/cm^2 caused 100% mortality of *Tenebrio molitor* (L.). Results indicated good persistence of *H. zealandica* after 84 days in field conditions, with a high maintenance of *P. callosus* populations. The study indicated the potential use of *H. zealandica* for the control of *P. callosus*, with the possibility of persistence for at least three months.

Future research into the control of *P. callosus* with nematodes should aim to investigate the technical aspects of field application. The current study shows that entomopathogenic nematodes have potential for controlling the soil stages of *P. callosus.* The capacity to rear large numbers of *P. callosus* larvae in the laboratory, for later use in laboratory and field trials, is of key importance.

Opsomming

Die gebande vrugtekalander, *Phlyctinus callosus* (Schönherr), is 'n groot plaag in appel- en nektarienboorde sowel as wingerde in die suidelike gebiede van die Wes-Kaap. *Phlyctinus callosus* word nie voldoende beheer nie, en plaagdoders wat voorheen gebruik is om dié insek in toom te hou, het doeltreffendheid ingeboet weens weerstandontwikkeling teen piretroïede en asefaat. 'n Alternatiewe beheermetode is stamsperbande. Omdat die kalanders nie kan vlieg nie, moet hulle teen stamme uitklim om die vrugte te bereik. Stamsperbande versper dus die insekte se toegang tot die vrugte, maar is baie arbeidsintensief. Meer haalbare metodes vir die beheer van *P. callosus* is daarom dringend nodig, en die gebruik van entomopatogeniese nematodes blyk 'n besliste moontlikheid te wees.

Entomopatogeniese nematodes, wat tot die Steinernematidae en Heterorhabditidae behoort, is uitstekende biobeheermiddels vir insluiting by geïntegreerde plaagbeheerprogramme. Om doeltreffende beheerstrategieë vir *P. callosus* te bedink, is groot en voorspelbare hoeveelhede kalanders nodig veral groot hoeveelhede larwes, aangesien nematodes op hierdie ontwikkelingstadium gemik sal wees. Die eerste doel met die studie was dus om 'n kunsmatige dieet vir die teling van *P. callosus* larwes te ontwikkel. Volwasse kalanders kon maklik in vrugteboorde ingesamel word, maar groot hoeveelhede larwes was moeiliker bekombaar. Aangepaste weergawes van 'n agardieet sowel as verskillende worteldiëte is by 21˚C beproef. Die hoogste persentasie larwale groei en -oorlewing op die agardieet was 50%, en 60% op een bepaalde soort worteldieet. Die beste teelmetode blyk egter volgroeide wortels te wees wat in potte geplant is en by 25˚C gehou word. Dié metode het 'n oorlewingspersentasie van 90% opgelewer. 'n Studie is onderneem om te bepaal hoe lank en by watter temperature *P. callosus* eiers vir toekomstige gebruik geberg kan word. 'n Gemiddelde uitbroeipersentasie van 45.7% is verkry toe eiers vir 70 dae by 4˚C geberg is. Eiers wat onderskeidelik by 11˚C en 14˚C geberg is, het ná 47 en 10 dae onderskeidelik begin uitbroei. Indien die doel is om die eiers slegs stadiger te laat uitbroei, sal hierdie twee temperature dus geskik wees.

Hierna is verskeie entomopatogeniese nematode-isolate vir moontlike gebruik as biologiese beheermiddels vir *P. callosus* beoordeel. *Phlyctinus callosus* larwes en volwassenes se vatbaarheid vir nematode infeksie is in die laboratorium bepaal deur dit met behulp van verskillende nematode-isolate vir mortaliteit te toets. Dié toetse het getoon dat larwes meer vatbaar is vir nematode infeksie as volwassenes. In die proefnemings het die *Heterorhabditis*-isolate hoër mortaliteit as die *Steinernema*-isolate veroorsaak teen 'n konsentrasie van 400 infektiewe larwes (IJ) per insek. Biologiese eienskappe, soos die uitwerking van verskillende temperature op nematode aktiwiteit, sowel as die minimum konsentrasie nematodes om aanvaarbare vlakke van beheer uit te oefen, is ondersoek. Die persentasie mortaliteit vir die larwes het ná vier dae tussen 0% en 75% gewissel, en die SF41-isolaat van *Heterohabditis zealandica* is as die belowendste isolaat vir die res van die proefnemings gekies. Die vertikale beweging van nematodes in sand teenoor leemgrond, sowel as die biologie van *H. zealandica* in *P. callosus* larwes, is ook bestudeer. Ná vier dae was die LD_{50}- en LD_{90}-waardes onderskeidelik 96 en 278 IJ/50 µl. Wat temperatuur betref, is daar bevind dat nematodes onaktief is by 15˚C, terwyl die hoogste mortaliteit van *P. callosus* larwes as gevolg van nematode infeksie by 25˚C aangeteken is. Die mortaliteit was hoër in die leemgrond (95.2%) as in die sandgrond (77.5%). *Heterorhabditis zealandica* kon sy lewensiklus suksesvol in 6de instar *P. callosus* larwes voltooi. Die studie het derhalwe getoon dat *P. callosus* larwes geskikte gashere is vir *H. zealandica*, en dat hierdie isolaat dus in die praktyk 'n doeltreffende beheermiddel vir *P. callosus* kan wees.

Die oorlewing van verskillende konsentrasies *H. zealandica* is ten slotte bestudeer. Die proefneming is in 'n bloubessieboord met 'n groot populasie *P. callosus* uitgevoer. Konsentrasies van 0, 20, 30 en 45 IJ/cm^2 is op die grond (uitwendig) toegedien, en oorlewing is op dag 1, 35 en 84 gemeet. Die persentasie oorlewing vir die 30 IJ/ cm^2 konsentrasie was 87.5% op sowel dag 35 as 84. Oorlewing in grondmonsters wat op dag een ingesamel en by kamertemperatuur in plastiekhouers geberg is, is weer op dag 128 beoordeel. Daar is bevind dat sowel die 30 IJ/cm^2 as die 45 IJ/cm^2 konsentrasie 100% mortaliteit by *T. molitor* veroorsaak het. *Heterorhabditis zealandica* blyk 'n goeie oorlewing te hê ná 84 dae in veld kondisies wat erg met *P. callosus* besmet is, en is dus 'n moontlike beheermiddel vir *P. callosus*, met potensiële oorlewing vir minstens drie maande.

Toekomstige navorsing oor *P. callosus* beheer met behulp van nematodes kan op die tegniese aspekte van nematode-toediening konsentreer. Hierdie studie bewys dat entomopatogeniese nematodes groot potensiaal toon om, hetsy op sigself of saam met ander beheermetodes, die grondstadia van *P. callosus* te beheer. Vir enige verdere laboratorium- en praktiese toetse sal groot hoeveelhede *P. callosus*-larwes egter eers kunsmatig geteel moet word.

Contents

Chapter 1

Literature Review

Phlyctinus callosus (Schönherr), the banded fruit weevil

(Coleoptera: Curculionidae)

Origin

The banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), is indigenous to South Africa and a key pest of apples and nectarines in the South Western Cape. The weevil originated from Africa and, over time, spread to other continents. In South Africa *P. callosus* has been a recognized pest since 1896 (Lounsbury, 1896). *Phlyctinus callosus* (Fig. 1.) was first reported in New Zealand in 1899 (Kuschel, 1972), with a sporadic occurrence in gardens and paddocks around Auckland (Kuschel, 1990). From there it spread to the warmer areas of North Island and to Nelson, located in the South Island (Butcher, 1984). *Phlyctinus callosus* first established in Tasmania, from where it spread to Australia, and now resides in all the southern Australian states.

Fig. 1. Newly emerged *Phlyctinus callosus* adult (left) without clear band and yellow colouring and adult *P. callosus* (right) showing characteristic white band on abdomen.

Host damage

Phlyctinus callosus has the ability to cause significant damage to numerous crops. The larval stages of the weevil cause damage by feeding on the roots or tubers of plants. Adult weevils damage leaves, young shoots and fruits, resulting in the formation of shallow scars. This can severely impact the quality of the final marketable produce. Fruit types that are particularly susceptible to weevil damage include apples and nectarines, while pears, plums and peaches are damaged to a lesser extent. In South Africa *P. callosus* is also regarded as a pest of grapevines (De Klerk, 1981; Barnes, 1989).

In South Africa most damage occurs during November and December when grape bunches are developing. Damage caused after berry set is detrimental to the development of the vine itself, as the insects chew on the stems of young bunches or individual berries, causing fruit to drop prematurely (Myburgh et al., 1973; Annecke & Moran, 1982). In New Zealand *P. callosus* is also regarded as a pest on grapevines grown in glasshouses (Lo et al., 1990). In apple and plum orchards most of the damage is inflicted on the lower parts of trees. When adult population densities are high enough young fruit trees can be defoliated entirely (Barnes, 1989). Damage caused by *P. callosus* generally ranges from less than 1% up to 66% in orchards where control measures are not adequate. The average crop loss varies from 5 to 29% (Barnes & Giliomee, 1992). In the Western Cape province it is estimated that *P. callosus* has the ability to cause up to 40% damage on apples (Witt et al., 1995).

Phlyctinus callosus is also present in Australian states with a Mediterranean climate, where it is a polyphagous pest of all economically important crops. In Australian nurseries the weevil is particularly problematic: it causes mostly cosmetic damage to the nursery plants and kills new buds. The larvae are the most damaging to nursery plants because they feed on the roots (Horne, 1997). In South Africa *P. callosus* adults are the most significant weevil problem in deciduous fruit orchards. The larvae are not significant as they cause no known damage of economic importance. In Tasmania the situation is however different; the larvae are regarded as the most important life stage because they attack the roots of vegetable crops, causing significant economic injury (Miller, 1979).

Host range

Phlyctinus callosus feeds on many species of monocotyledons and dicotyledons, including grasses, herbs and woody plants, and is therefore regarded as a polyphagous pest. In various countries *P.*

callosus is associated with different host plants. In South Africa, for example, *P. callosus* is the main pest of grapevines, apples and nectarines (Annecke & Moran, 1982; Barnes & Pringle, 1989). In New Zealand *P. callosus* mainly prefers carrots and parsnips but is also regarded as an important pest of apples, grapevines and nectarines (Fisher, 2003). It is a minor pest of ornamental plants and attacks the bulbs and corms of these plants (Butcher, 1984). It is also a minor pest of potatoes in New Zealand as well as in Australia (Matthiessen & Learmonth, 1994). In Tasmania *P. callosus* is mainly a pest of root vegetables (Miller, 1979).

Description

The adult weevil can reach up to 7 mm in length. Its colouring can be described as a dull greyish-brown and it has a characteristic white V-shaped band prevalent near the rear of the abdomen. The tip of the rostrum is shiny and black, and its abdomen is bulbous. The elytra are noticeably lumpy and each lump contains numerous setae (Annecke & Moran, 1982).

The eggs are oblong and approximately 0.9 mm long. As the eggs mature they turn black at the ends (Butcher, 1984). The larvae are up to 6 mm long and have a creamy white colour (Fig. 2.). They are legless, have orange head capsules with black jaws, and their bodies are covered with long hairs. There are four to eleven larval instars, but most larvae only pass through six to nine instars (Walker, 1978). The pupae are 7 to 8 mm long and have hooked bristles (Butcher, 1984).

Fig. 2. Six-week-old larvae of *Phlyctinus callosus* (left), larva infected with nematodes (right)

Life cycle

Phlyctinus callosus will start egg-laying approximately three weeks after emerging from the soil, and this can continue for three to six months. The female adults lay their eggs just below the soil surface or in loose organic litter on top of the surface. Egg-laying takes place in late summer or early autumn when temperatures range between 15 and 20˚C. The adult weevil female produces fewer than five eggs per week during the first four weeks (Walker, 1981). Subsequently, the adults can lay batches of up to 70 eggs at 7-day intervals. A female can produce approximately 350 eggs over a period of 20 weeks at an ambient temperature of 20˚C (Butcher, 1984). The eggs will hatch within 10 to 14 days, depending on the temperature. Eggs remain viable for a period of 12 weeks if they are stored at 5 to 8˚C. Eggs stored at temperatures ranging between 10.5 and 25˚C have a high survival rate (76–86%). However, when temperatures are above 30˚C the percentage survival of the eggs drop to 1.7%. Intermediately, when eggs are stored at 8˚C for 65 days, 50% of the eggs will hatch (Walker, 1981). It was estimated that the theoretical minimum threshold temperature for egg development is 6˚C (Walker, 1981).

When first instar larvae hatch they burrow into the soil and start feeding on roots, tap roots or tubers. The majority occur in the top 10 cm of soil (Barnes, 1989). This is also where overwintering takes place. The larvae pass through a varying number of instars. Because the width of the head capsules overlap between different instars, it is difficult to determine the number of instars. Curculionids vary a great deal in the number of larval instars. Most of the *P. callosus* larvae pass through six to eight instars, but some of them can pass through up to eleven instars (Barnes, 1989). It has also been reported that *P. callosus* passes through six to nine larval instars (Walker, 1978).

The mortality rate is highest during the early and middle larval instars. It was documented that 42% of the first instar larvae do not reach the second instar stage and 70% of those larvae die by the third instar stage (Barnes, 1989). When larvae were reared at a constant temperature of 10.5, 15, 20, 25 or 30˚C, only those reared at 15, 20 and 25˚C survived to adulthood. The survival of the larvae reared at 20˚C was four times higher than larvae reared at 15˚C (Walker, 1981). Pupation of larvae in the top 10 cm of the soil can last from 7 to 22 days, but on average it lasts approximately 14 days (Barnes, 1989).

Emergence of the adults takes place in the late spring and early summer. They feed in the night and hide during the day underneath rough bark, clumps of earth and rough organic material on the soil surface (Myburgh et al., 1973). The adults do not have wings and cannot fly because the elytra are fused together, but they are nonetheless very mobile (Annecke & Moran, 1982). They climb up the trunks of trees to reach the canopy of the host. They can also reach the canopy using weeds tall enough to touch the canopy or they use trailing canes or posts.

When adults were reared at a constant temperature survival was better at 15˚C (15% mortality) than at 20˚C (70% mortality) (Annecke & Moran, 1982). The majority of literature states that *P. callosus* does reproduce sexually, except in Tasmania where there are only females present, which suggests that reproduction takes place parthenogenetically (Miller, 1979).

Phlyctinus callosus normally has one generation per year. In irrigated orchards and the Western Cape province of South Africa there are two generations per year; the second-generation adults emerge in autumn when conditions are optimal (Barnes, 1989; Nel & Addison, 1993).

Control

Unfortunately, the control of *P. callosus* is unsatisfactory because the weevil has developed a high tolerance to many pyrethroids, with indications of cross-tolerance to acephate (Barnes et al., 1994). It is the only curculionid that causes primary damage to deciduous fruit in South African orchards. It is also regarded as one of the most serious pests that occur on grapevines (Myburgh et al., 1973; Annecke & Moran, 1982). Because *P. callosus* cannot fly trunk barriers can be used to prevent weevils from reaching the fruit, but this method of control is very labour-intensive. There is therefore a need for alternative control options, particularly options applicable to the larval stages in the soil.

Entomopathogenic nematodes (*Heterorhabditidae* and *Steinernematidae)* are used for the biological control of insect pests. They have also been used successfully for other weevil species such as the black vine weevil (Simons, 1981). These nematodes are very effective against soil insect pests. Nematodes offer an environmentally safe and an integrated pest management (IPM) compatible option. The use of nematodes is a good alternative control option to the use of chemicals in an IPM strategy to reduce chemical inputs.

Monitoring

The control of *P. callosus* adults is hindered because of the lack of a practical monitoring system. Growers need to be able to detect the emergence of the adults in early spring (Barnes, 1991). Corrugated cardboard bands can be placed around the trunks of vines and apple trees (Myburg, 1951; Whitehead, 1961) to capture adults which hide during the day. Cone traps are not very sensitive for detecting the emergence of weevils, although these traps are practical for growers to use. Monitoring devices that are labour-intensive and time-consuming will not be accepted for use by deciduous fruit growers (Barnes, 1991).

Phlyctinus callosus has the potential to be a pest on blueberries (*Vaccinium corymbosum*). Bredenhand et al. (2010) developed a practical monitoring technique in blueberry orchards to estimate the presence and relative abundance of *P. callosus*. Four different methods of monitoring, namely night-time beating, pitfall trapping, use of cardboard bands and leaf litter sampling, were compared. The most effective monitoring system proved to be night-time beating. There are numerous advantages of using this method, including low cost, short evaluation time, low labour-intensity and a much higher success rate in establishing the presence *of P. callosus.*

Rearing of Coleoptera

Studies of weevil biology are critical to the management of this pest. A large number of weevil larvae are needed to study the biology and control options of an insect. In order to develop control strategies, a large, consistent and predictable quantity of all life stages of the insects must be available throughout the year (Fisher & Bruck, 2004). Currently, there are no effective artificial media available for the rearing of *P. callosus*. The collection of weevil larvae in the field is generally very labour-intensive and tedious (Fisher & Bruck, 2004).

***Otiorhynchus sulcatus* (F.) (Curculionidae)**

An artificial diet for *O. sulcatus*, the black vine weevil, was initially developed by Shorey and Hale (1965), and modified first by Shanks, Jr. and Finnigan (1973) and then again by Shanks, Jr. (1980). This diet sustains the larvae of *O. sulcatus* and it is commercially available. An improved diet was later developed by Fisher and Bruck (2004), on which larval survival and growth was considerably

increased. The main differences between the standard diet and the improved diet are, first, a decrease in the methyl paraben content and, second, the replacement of sorbic acid with potassium sorbate. The reduction in the methyl paraben content is to decrease the preservatives, while still inhibiting the growth of bacteria and mould on the diet. Phytophagous insects need more potassium than other animals, but only trace amounts of sodium (Reinecke, 1985; Nation, 2002). The brewer's yeast present in the improved diet of *O. sulcatus* contains 844 mg K and the potassium sorbate contains 325 mg K (35% increase in potassium in comparison with the standard diet). Thus, increased vigour and a higher percentage survival rate may result from a diet that is contamination-free and has a higher potassium content (Fisher & Bruck, 2004).

***Hyperodes bonariensis* (Kuschel) (Coleoptera: Curculionidae)**

The rearing of *H. bonariensis* has previously been done on host plants, Italian ryegrass (*Lolium multiflorum*) and its hybrids (Power & Singh, 1974). Unfortunately, this method of rearing requires a substantial amount of space, is expensive and labour-intensive, and the growth of the insect and plant has to be synchronised. There are several advantages of rearing insects on an artificial medium rather than using the natural host (Pritam, 1977). On an artificial medium insects are available throughout the year, laboratory handling is easier, and their nutrition is known and therefore easy to replicate. The chemical and physical aspects of the diet can also be regulated; hence nutrition and metabolism can then be studied. Environmental conditions can also be regulated and, because it is a controlled environment, the insects are of good quality. Eight generations of *H. bonariensis* were reared on the artificial medium. The rate of development and the mortality of the generations were the same throughout the rearing programme. When using this diet, six generations can be reared annually (Power & Singh, 1974).

***Anoplophora glabripennis* (Motschulsky) (Cerambycidae)**

This beetle has the potential to destroy numerous urban and forest trees. It is therefore important to use an artificial diet when studying this beetle. Three different diets were tested. One of the diets is for *A. glabripennis* from China and the other two diets were developed for other members of the Lamiini family. Dubois et al. (2002) found that males grew the fastest on diets containing sawdust or phloem-cambium. Pupation time of males was the shortest on the *A. glabripennis* diet. Diets that contained

cellulose rather than sawdust produced the fastest growing females, while the females lived the longest on the *A. glabripennis* diet. In order to develop an optimum diet, the published *A. glabripennis* diet was modified by increasing the water content from 50% to 64.6% and substituting the phloem-cambium component with cellulose.

***Diaprepes abbreviates* (L.) (Curculionidae)**

Diaprepes abbreviatus is currently a serious pest in the citrus orchards of Florida in the USA and was first found infesting citrus in the Apopka region of Florida in 1964. Research done by Beavers and Selhime (1975) investigated the development of *D. abbreviatus* on potted citrus seedlings. Two tests were conducted and the first test was done by placing five neonate larvae each on 150 orange seedling plants. Each week three seedlings were uprooted and larvae were recovered by sifting the soil. Only 15.7% of the originally infested larvae were recovered of which there were six adults, six pupae and 107 larvae. The test started on the 5th of May and ended on the 8th of November. A second test was done where 93 plants were each infested with 10 neonatal larvae but these plants were held outdoor and the plants were only uprooted when they had been killed by larval feeding. During this test four adults were recovered after approximately 144 days. Eight pupae were recovered after a total of 238 days. There were 64 larvae recovered which were transferred to fresh plants. Results indicate that natural mortality of the immature forms are high and the developmental period of *D. abbreviatus* is highly variable (Beavers & Selhime, 1975).

Research conducted by Beavers (1982) developed an artificial diet for the rearing of *D. abbreviatus.* The ingredients of the diet consist of alphacel 307 g, cotton seed meal 250 g, soybean protein 104 g, sucrose 70 g, casein 70 g, wheat germ 60 g, cornstarch 44 g, Vanderzant's vitamin mix 31 g, Wesson® salts 16 g, methyl paraben 15 g, ascorbic acid 6 g, sorbic acid 5 g, cholesterol 3 g, choline chloride 2 g, agar 75 g, formalin (38.5%) 6 ml and water 2550 ml. Ingredients were mixed and heated to 95˚C. This was then poured into 35-ml platic cups. Larvae were placed in the cups with diet and larvae were transferred to fresh diet after 60 diet. Fresh diet was supplied at 45-day intervals until the insects pupated. Cups were held at 25˚C and 60-70% RH in a dark room. Results obtained from this research showed that in the average 1-year life cycle a weevil consumed approximately 150 ml of diet. There is a period of rapid growth after 3-4 months for the larvae. During the first 60 days mortality was highest for the larvae and mortality increased when cups contained more than one one

larva. When 1, 5, 10 and 25 larvae were placed in cups the percentage larvae recovered after 60 days were 30%, 12.8%, 10.6% and 2.5% respectively. The most number of adults were recovered when 10 larvae were placed in each cup. Eggs and larvae were not sterilised before placed on the diet. This diet facilitates the production of large numbers of four to six month old larvae for research focused on chemical and biological control methods (Beavers, 1982).

***Phlyctinus callosus* (Curculionidae)**

Temperature is the most important environmental factor that affects the growth and survival of many insects (Bengston, 1969; McLaren, 1971). Walker (1981) studied temperature as the only variable. He used two methods of rearing *P. callosus,* namely using carrot discs and potted carrot seedlings. Results showed that temperatures above 30˚C are fatal for eggs and above 25˚C are fatal for larvae. At temperatures between 15 and 20˚C females begin to oviposit more or less 15 to 21 days after emergence. This implies that if any chemical control measures are considered for the adults they should be applied within this pre-oviposition period, resulting in the population being significantly reduced. The survival of *P. callosus* is four times higher at 20˚C than at 15˚C. The size of the population can thus be estimated by looking at the quantity of weevils present in autumn, in conjunction with the ambient and soil temperatures. This information can be used to forecast the severity and timing of outbreaks the following season and ensure that preventative measures are taken (Walker, 1981).

Horne and Stacpoole (1989) describe a rearing technique that involves separating the adult weevils and placing them in oviposition chambers. Newly laid eggs are removed and placed in an incubation chamber, from where the first instar larvae emerge. These larvae are then placed onto potted carrot plants. When using this technique, only 54% of the larvae survived to the late instar stage. This rearing method facilitates the uninterrupted mass rearing of *P. callosus*.

Entomopathogenic nematodes

There are numerous nematode species that are associated with insects. The different types of association range from phoresis to facultative and obligatory parasitism. There have been 23 nematode families described that have parasitic associations with insects. Of these 23 families, 7 have species with potential for the control of insect pests: Mermithidae and Tetradonematidae (Order:

Stichosomida); Allantonematidae, Phaenopsitylenchidae, and Sphaerulariidae (Order: Tylenchida); Heterorhabditidae and Steinernematidae (Order: Rhabditida) (Poinar, 1979; Nickle, 1984; Nickle, 1991; Kaya & Stock, 1997; Kaya & Koppenhöfer, 2004). Currently, Heterorhabditidae and Steinernematidae are being produced commercially as biological insecticides. There are numerous companies worldwide that rear these nematodes for commercial uses (Koppenhöfer, 2000).

Biology and life cycle

Steinernematidae and Heterorhabditidae are obligate insect pathogens. Worldwide, many species have been recovered from soil (Hominick et al., 1996). They have the ability to kill the insect host within four days, depending on the nematode and insect species. Nematodes are facilitated by bacteria that are mutualistically associated with them. These bacteria are in the genus *Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis*. These nematode species share the same biological lifestyle although they belong to different families. Only the specially adapted third-stage infective juveniles (IJ) survive outside the insect host. This stage is non-feeding, outside the host, and is also known as the dauer juvenile. The cells of the bacterial symbiont are carried in the intestines of the IJ. When the IJ find a suitable host they will enter through natural openings such as the mouth, spiracles or anus. Areas on the host's cuticle that are thin can also be penetrated and access gained into the hemocoel of the host. This is more common for *heterorhabditis* equipped with a dorsal tooth (Peters & Ehlers, 1994). Once inside the insect host the IJ release the symbiotic bacteria (Fig. 4), which start producing exponentially.

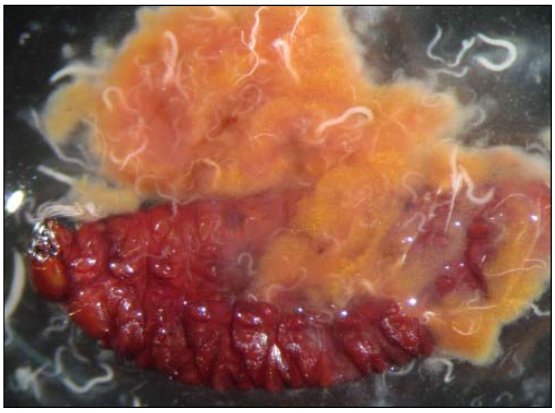

Fig. 4. *Phlyctinus callosus* larva infected with *H. bacteriophora* (left) and entomopathogenic nematodes emerging from a depleted host cadaver (right)

The host is killed by septicaemia and the bacteria metabolise the insect tissues. The nematodes feed and develop on the mixture of bacteria and metabolised host tissues. As these nematodes develop they go through one to three generations. A new generation of IJ will develop and emerge from the host and then search for a new host when the food in the insect cadaver is depleted (Fig. 5).

Fig. 5. Infective juveniles (indicated with an arrow) leaving a *Phlyctinus callosus* pupae under dry conditions.

Host range

Under laboratory conditions many insect species are killed by nematodes but this is not the case in the field. The natural host range of nematode species is narrower. The ecology of the nematodes are influenced by potential host insects and environmental factors (Peters, 1996). The effect of inundative applications of nematodes on non-target organisms has been intensively researched and nematodes are now regarded as safe biological control agents (Laird et al., 1990; Bathon, 1996). Some species are known to have a restricted host range, such as *S. scapterisci* Nguyen & Smart, 1990, which is

adapted to mole crickets (Parkman & Smart, 1996). *Steinernema kushidai* Mamiya, 1988, is more specific to scarab larvae (Mamiya, 1989). When new nematode strains or species are isolated from soil samples wax moth larvae are usually the bait insect. Therefore, the known host range of species is most likely to favour generalists or species that are adapted to Lepidoptera. Nematodes have been tested against numerous insect pest species and results have varied from no effect to excellent control (Begley, 1990; Klein, 1990; Bedding, 1993).

There are numerous factors that influence the success of a nematode application. Failures can be ascribed to not fully understanding the ecology of the pest or the nematodes. It is however important to carefully match the insect and nematode if one requires the nematode application to have a significant impact on the pest numbers (Gaugler et al., 1994). The foraging behaviour and the temperature requirements of the specific nematode species are important factors to be considered. Foraging strategies used by infective juveniles for the purpose of finding a host can vary along a continuum between ambushing and cruising (Campbell & Gaugler, 1993; Campbell & Gaugler, 1997). Nematode species are classified by certain behavioural aspects such as those that nictate are seen as ambushers and species that do not nictate are classified as cruisers. Species that only lift part of their body from the substrate for a few seconds are seen as intermediate (Campbell & Gaugler, 1997). The pest habitat should also be accessible to the nematodes and it should be suitable as a host. Nematodes have previously been applied in different habitats, for instance soil, cryptic habitats, manure, aquatic habitats and also foliage (Begley, 1990). The most general type of nematode applications is to the soil (Klein, 1990). In previous research periodic augmentation has been done to EPN communities using *S. riobrave* in citrus orchards. This method was effective in killing the *Diaprepes abbreviates* weevil larvae but following treatment, the occurrence of EPN and the mortality of the larvae dropped for short periods in treated plots, compared to untreated plots (Duncan et al., 2003; Duncan et al., 2007). It was speculated by Duncan et al (2003) that competition between the endemic EPN species and exotic *S. riobrave* resulted in eventually diminishing the number of EPN in the soil. Another possibility is that the density-dependant antagonists of the nematodes increase following an EPN augmentation which reduce the numbers of EPN below the previous equilibrium density (Ishibashi & Kondo, 1986; Koppenhöfer et al., 1996; Kaya, 2002). For these reasons, it is possible that a trophic cascade can be initiated by augmenting EPN and subsequently multiplication

of nematophagous fungi and other natural enemies, reducing the endemic as well as exotic EPN numbers. This will suppress the natural control of insect larvae (El-Borai et al., 2007).

Not all insects are susceptible to nematode infection because the portals of entry can be inaccessible (Eidt & Thurston, 1995). For example, the mouths of wireworms are blocked by oral filters (Gaugler et al., 1994). The mouth parts of sucking or piercing insects or young instars of insects that have chewing mouthparts are too narrow (Gaugler et al., 1994). Muscles or other structures can constrict the anus of insects and the spiracles can be covered in septa or sieve plates. The openings of the spiracles themselves can be too narrow for the nematodes to enter through (Gaugler et al., 1994). Nematodes themselves may also not have the ability to penetrate intersegmental membranes and the fore- and hind-gut cuticular linings of many insects as it can be a thick or dense peritrophic membrane, which hinders penetration at these specific sites. When insects groom themselves aggressively, have an evasive behaviour (Gaugler et al., 1994), or form impenetrable cocoons or soil cells (Eidt & Thurston, 1995), nematode infection is hindered. Social insects, such as formicidae, can withstand pathogens more easily as a result of social grooming, the removing of infected siblings or the translocation of the colony (Klein, 1990). The immune response is not the same for all insects penetrated by a nematode species; it depends on the type of insect host and the nematode species (Wang et al., 1995).

Movement in soil

The performance of IJ applied to the soil depends on two key factors: motility and persistence. The dispersal abilities of active IJ in the soil can be up to 90 cm in both a horizontal and a vertical direction, within 30 days. These nematodes therefore possess the ability to actively seek out the insect hosts (Kaya, 1990).

The most important factor that influences movement of nematodes in soil is moisture. A water film is needed for the IJ to effectively move in a forward motion. In soil the IJ move through the film of water that coats the interstitial spaces. Nematode movement is limited when this film is too thin or when the interspaces are completely filled with water (Koppenhöfer et al., 1995). When the water content is slowly reduced the IJ can survive desiccation to quite low moisture levels because they then have sufficient time to adapt to an inactive stage (Womersley, 1990). In natural soils the relative humidity

(RH) in the pores of the soil is generally close to 100%, except in soils that are sandy and low in organic matter in areas near the soil surface. IJ that are inactive can persist longer in dry soil (Kaya, 1990) but their ability to infect will be hindered. Moderate soil moisture is vital for good performance of nematodes (Georgis & Gaugler, 1991).

The survival of IJ is also dependent on the texture of the soil. Fine-textured soils result in nematode dispersal and survival being much lower (Kaya, 1990). The lowest survival of nematodes is in clay soils (Kaya, 1990). This is most probably linked to oxygen levels that are very low in these kinds of soils as a result of the smaller soil pores. Oxygen can also become a problem in soils that are water-saturated or soils that have a very high content of organic matter (Kaya, 1990).

Factors important for soil application

When nematodes are applied onto the soil surface persistence is affected as a result of ultra violet radiation and desiccation, and up to 50% of the IJ is lost within hours (Smits, 1996). The nematodes moving into the soil are reduced by 5% to 10% daily and after one to six weeks only 1% persist in the soil (Kaya, 1990; Smits, 1996). Nematodes should therefore only be applied when the susceptible stages of the target pest insect are present in the soil. There are various biotic and abiotic factors that influence the motility and persistence of applied IJ. Abiotic factors include extreme temperatures, soil moisture, soil texture, RH and UV radiation (Kaya, 1990; Smits, 1996), while biotic factors include competition and natural enemies (Kaya & Koppenhöfer, 1996).

Nematodes can be inactivated within minutes by exposure to UV light (Selvan et al., 1994). IJ should be applied early in the morning or in the evening to reduce the amount of exposure to direct sunlight. When soil applications are being done the timing is not that important if an adequate volume of water is used to wash the nematodes into the soil at the time of application or directly after application (Selvan et al., 1994).

Temperature plays a fundamental role in the performance of nematodes, and varies depending on the species or strain (Kaya, 1990). At lower temperatures (< 10–15˚C) IJ are normally more lethargic. At high temperatures (> 30–40˚C) IJ are completely inactivated. Nematodes perform very well at temperatures ranging from 20 to 30˚C. This is also true for most of the commercially available species (Georgis, 1990; De Waal, 2008). Exposure of nematode species to temperatures below 0˚C and

above 40˚C is lethal, but the effect of the temperature depends mainly on the period of the exposure. Georgis (1990) showed that temperature extremes do not affect IJ very much when they are in the soil environment, as the soil acts as a buffer. The IJ have sufficient time to disperse to deeper soil layers where the temperature is optimum. Survival temperatures for the majority of nematode species in the soil are between 5 and 15˚C. When the temperatures are higher the metabolic activity increases and results in energy reserves being depleted, which reduces the life span of the nematode.

Persistence of nematodes in soil

When nematodes are being applied for the control of a pest insect these nematodes can persist by recycling through a host. Numerous studies have yielded evidence of nematode recycling taking place in the soil after the inundative release of nematodes (Kaya, 1990; Klein, 1993).

The natural populations of nematodes have to recycle in a host because they are obligatory parasites. Only a few studies have focused on looking at how the dynamics of persisting nematode populations work and the factors that determine their persistence in one area but not in another. Studies have shown that the distribution of a nematode population within a site is very patchy (Stuart & Gaugler, 1994; Campbell et al., 1995; Strong et al., 1996). Environments that are favourable for the survival of IJ, can be very beneficial to manage pest insects when using nematodes. More than one generation of pests can be controlled when the pest has a short generation time (Kaya & Stock, 1997). Only one nematode application is needed when sufficient nematodes can survive in the environment (Kaya & Stock, 1997). If recycling can take place it can result in the nematodes establishing, and then control is subsequently improved (Kaya & Stock, 1997). It is very difficult to find nematode-killed insects in the soil. Therefore, a simpler and more practical method of determining whether they are still present is to trap the IJ from the soil. Soil samples need to be taken before the application of nematodes to establish whether they are present in the soil because they do occur naturally in the soil.

Control of Coleoptera with entomopathogenic nematodes

***Otiorhynchus sulcatus* (Fabricius) (Curculionidae)**

The black vine weevil, *O. sulcatus*, is a severe pest of cultivated plants. In field trials *Steinernema carpocapsae* Weiser, 1955, was tested against this weevil, but good control was not achieved (Weiser, 1955). Evenhuis (1978) found dead larvae of *O. sulcatus* in the soil near strawberry plants infected with *Heterorhabditis* sp. The nematodes were cultured and applied against this weevil (Simons, 1981). It was found that an increased number of weevil larvae were killed with higher nematode dosages. Better results were obtained when the nematodes were applied one or two weeks after deposition of insect eggs. The nematode dosage plays a lesser role when the nematodes are applied two weeks after the insect eggs are introduced. However, results were very poor when nematodes were applied five weeks after the introduction of insect eggs. Results of these studies have given rise to the proposal that newly hatched weevil larvae are too small for nematode infection and they do not provide enough food for the nematodes to complete their life cycle (Schmidt & All, 1978). It has been concluded that *Heterorhabditis* has the ability to provide good control against *O. sulcatus* larvae, but the time of introduction is very important (Simons, 1981).

Experiments were carried out in which the nematodes were applied against black vine weevil larvae in grow bags outdoors (Lola-Luz et al., 2005). The adult vine weevils feed on the plant foliage, but larvae cause the most damage (Schread, 1972; Bedding & Miller, 1981; Georgis & Poinar, Jr., 1984). The main objective of this study was to determine the potential of a commercial isolate of *Heterorhabditis megidis* Poinar Jackson & Klein, 1987, and an Irish isolate of *H. downesi* Stock et al., 2002. The nematode-treated grow bags had much less live *O. sulcatus* larvae compared to the control treatment. The insect stages targeted with nematodes were the last instar larvae and pupae because they were the most susceptible. The best results were obtained with two nematode applications in the same growing season. Results showed a drop in the number of larvae that were recovered from autumn to spring in the grow bags. This second application also reduced the number of adults emerging at the beginning of June (Lola-Luz et al., 2005).

A study was carried out in Ireland to detemine whether nematodes can be applied outdoors to control black vine weevil. This was done by using commercially available *H. megidis*. The highest level of

control was obtained using a triple application. The numbers of adult weevils that emerged were also significantly reduced when multiple nematode applications were applied, with infestation significantly less in the following season. This study indicates a window period for nematode application outdoors in Ireland in autumn and then again in spring when soil temperature is above 9˚C (Lola-Luz & Downes, 2007).

***Sitona lepidus* (Gyllenhal) (Curculionidae)**

The clover root weevil, *S. lepidus*, has the potential to be a serious pest of white clover in New Zealand (Willoughby & Addison, 1997; Murray & Willoughby, 1998). Larvae are a more serious problem as they feed on the roots of the plant, while adults feed on the foliage (Bell et al., 2000). As a result of the successful use of nematodes against *P. callosus* in New Zealand for asparagus (Jackson et al., 1985; Prestidge & Willoughby, 1990; Ferguson et al., 1990) these nematodes were also evaluated for their potential to control *S. lepidus* (Bell et al., 2000). A petri dish and pot experiment were conducted. The nematodes kill all the *S. lepidus* within approximately seven to 10 days after application. In laboratory and pot experiments *H. bacteriophora* proved to be more effective than the *Steinernema* spp. Research carried out in Poland also showed that *S. feltiae* Filipjev, 1934, and *H. bacteriophora* Poinar, 1975, are effective against *S. lepidus* in the laboratory (Wiech & Jaworska, 1990). Between August and September there is a peak in *S. lepidus* larvae numbers (Willoughby & Addison, 1997). During these months the temperatures are between 8.5 and 10.4˚C. A nematode active under cool temperatures could be effective against these larvae (Bell et al., 2000). *Heterorhabditis zealandica* Poinar, 1990, seems to be a good option as it is a cold-tolerant strain (Wharton & Surrey, 1994). Carrying out a field application of nematodes (De Waal, 2008) during August and September in New Zealand would advantageous because then the RH is high (MacVean et al., 1982) and UV radiation low (Gaugler & Boush, 1978).

***Anomala orientalis* (Waterhouse) (Scarabaeidae)**

The larvae of the oriental beetle, *A. orientalis,* feed on the roots of blueberry bushes. Research has shown that *S. scarabaei* Stock & Koppenhöfer, 2003, can effectively control the third instar larvae of this insect pest (Polavarapu et al., 2007). Larvae of *A. orientalis* are mainly found in the top 30 cm of soil in blueberry orchards. In New Jersey, USA, nematodes can be applied in late July, when the eggs

hatch, but temperatures on the soil surface and in the uppermost soil layers are then very high, and harmful to the nematodes (Grewal et al., 1994). Another option is to apply the nematodes as a curative treatment against the late second instar larvae that are present in the soil in late August and early September. The soil temperatures are then more nematode-friendly and the larvae are bigger, which means that higher nematode reproduction could be supported. Polavarapu et al. (2007) also showed that *S. scarabaei* was very effective in controlling third and late instar larvae of *A. orientalis*.

In Korea *A. orientalis* is the main pest insect on turfgrass in Korean golf courses (Choo et al., 1999; Choo et al., 2000; Choo et al., 2002). Six Korean nematode isolates were evaluated against *A. orientalis,* namely *S. carpocapsae* (Pocheon); *S. glaseri* Steiner, 1929, (Mungyeong), *S. longicaudum* Shen & Wang, 1992, (Gongju); *S. longicaudum* (Nonsan) and *Heterorhabditis* sp. (Gyeongsan). Laboratory studies showed the second instar larvae to be more susceptible to the nematodes than the third instar larvae. The two species that were most effective were *Heterorhabditis* sp. Gyeongsan and *S. longicaudum* Gongju, and Nonsan. These isolates performed best against second instar larvae in the laboratory and overwintering third instars in the field (Lee et al., 2002) demonstrating the value of preliminary laboratory studies.

***Otiorhynchus ligustici* (L.) (Curculionidae)**

The snout beetle, *O. ligustici*, is a serious pest of alfalfa in New York state, USA. This weevil is flightless, and is a good candidate for a biological control approach using multiple nematode species. It has a two-year life cycle in which it will move through the soil profile and also through different nematode niches. The combination of nematode species tested was *S. carpocapsae* NY001, *H. bacteriophora* Osweg and *S. feliae* Valko. All the nematode species persisted throughout the year. Much better control of weevil larvae was obtained when using a combination of more than one nematode species, in comparison to using only *S. carpocapsae*. There were significantly fewer plants with severe root damage when a combination of *S. carpocapsae* and *H. bacteriophora* was applied. Good results were also obtained when *S. feliae* and *H. bacteriophora* were combined, especially for plant protection and control of the larvae (Gabor & Shields, 2008).

White grub species (*Popillia japonica, Anomala orientalis*, *Cyclocephala borealis, Rhizotrogus majalis* and *Maladera castanea)*

The two white grub species, *P. japonica* and *A. orientalis,* are a major problem for turfgrass in the USA. They feed on the roots of grass and cause widespread destruction (Potter, 1998; Vittum et al., 1999). The pathogenicity of *H. bacteriophora* and *S. scarabaei* was tested against these two pest insects, as well as their different developmental stages. Results showed that the developmental stage has a significant effect on the susceptibility of the white grub to nematodes. The efficacy of *H. bacteriophora* against *A. orientalis* decreased from the first instar through to the third instar, and small third instars were more susceptible than large third instars. The susceptibility of *P. japonica* to both nematode species declined steadily as the insect went through the different stages, from actively feeding third instars until it reached pupating stage. The susceptibility of the different white grub developmental stages varies, depending on the white grub and nematode species, and no generalisation can be made (Koppenhöfer & Fuzy, 2004).

Koppenhöfer et al. (2006) tested the virulence of *S. scarabaei, H. zealandica* and *H. bacteriophora* against five white grub species *Popillia japonica, A. orientalis, Cyclocephala borealis, Rhizotrogus majalis* and *Maladera castanea.* These species are all economically important pests of turfgrass in the USA. This research showed that both *H. bacteriophora* and *H. zealandica* have low virulence against *P. japonica, A. orientalis, C. borealis* and *M. castanea*. *Heterorhabditis zealandica* has a very low virulence against *R. majalis,* while *H. bacteriophora* was very inconsistent and caused low mortality. *Steinernema scarabaei is* moderately virulent against *C. borealis* and extremely virulent against *R. majalis, P. japonica, A. orientalis* and *M. castanea.*

Heterorhabditis bacteriophora and *S. carpocapsae* were applied in field trials to determine the control of this pest (Klein & Georgis, 1992). The population was reduced by 60% when *H. bacteriophora* was applied, and by 96% the following spring. When *S. carpocapsae* was applied control of up to 51% was achieved and 90% in the following spring. The data obtained showed that nematodes reproduce in larvae of *P. japonica* and they survive in turfgrass and in the field (Klein & Georgis, 1992).

***Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)**

On the North Island, New Zealand, *P. callosus* has become a major pest of asparagus in localised areas (Prestidge & Willoughby, 1990). Larvae in the soil feed on the crown, roots and root hairs of asparagus. Chemicals can be used successfully, but as this crop is harvested daily there is a need for low chemical residues (Prestidge & Willoughby, 1989), and thus the use of nematodes holds great potential (Clearwater & Wouts, 1980; Bedding & Miller, 1981; Barratt et al., 1989). *Heterorhabditis bacteriophora* was evaluated against *P. callosus* larvae and pupae. Nematodes were applied topically and inundatively to the soil. The weevil larvae were found to be very susceptible to nematodes (Prestidge & Willoughby, 1990). One hundred per cent mortality was achieved within 48 hours when weevil pupae were inoculated with *H. bacteriophora* at levels of 5 x 10^4 per pot. The mean temperature of 14.5˚C during the trial was at the lower end of the range for this nematode species. The identification of a strain that is more active at cool temperatures would be very useful. It is evident that the larvae and pupae of *P. callosus* are very susceptible to nematodes. The same level of mortality was achieved for studies with black vine weevil larvae (*O. sulcatus*) and *H. bacteriophora* with 3 x 10^5 nematodes per pot (Barratt et al., 1989).

Aims of the study

- Rearing of *P. callosus* for the purpose of evaluating the susceptibility of its different life stages against entomopathogenic nematodes.
- Selection of the most effective entomopathogenic nematode isolate for the control of the soil stages of *P. callosus,* using various laboratory bioassays.
- Evaluation of the selected entomopathogenic nematode strain in a field trial for persistence in an infested blueberry orchard.

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Chapter 2

Rearing of *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)

Abstract

The suitability of different artificial and carrot based diets for the laboratory rearing of *Phlyctinus callosus* larvae was tested. Modified versions of an agar diet were used, with the highest percentage survival rate being found to be 50% at 21˚C after five weeks. Modified agar diets were treated with formalin to minimise contamination and the effect of two differently sized containers was investigated. No survival was obtained on diets with formalin added, nor was there a significant difference noted between the production of the differently sized containers. Different carrot diets were tested, with the highest percentage survival rate of 60% being obtained for two carrot discs stacked on top of each other at 21˚C after five weeks. Full-grown carrots planted in pots produced the highest percentage survival rate of 90% after being kept for five weeks at 25˚C. Cold storage of *P. callosus* eggs used for rearing was investigated. Eggs can be stored at 4˚C for 70 days, with a mean percentage hatch rate of 45.7%. Storing eggs at temperatures of 11˚C and 14˚C is not recommended, as hatching was found to occur after 47 and 10 days, respectively. Egg hatch can, however, be delayed at such temperatures. A conclusion of this study is that the most effective method of rearing *P. callosus* tested is that of planting full-grown carrots in pots containing sterilised sand, kept at 25˚C.

Introduction

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae) or banded fruit weevil is a pest that causes economic damage to pome fruit, stone fruit, grapevine, apple and nectarine growers in the Western Cape province, South Africa (Myburgh et al., 1973; Annecke & Moran, 1982; Barnes & Pringle, 1989). The main factors influencing the number of generations of *P. callosus* occurring in any year are soil moisture and relative humidity in the cover crop. When there is sufficient moisture and humidity two generations instead of one, can occur in one year. First-generation adults start to emerge in October from those eggs that were laid by second-generation adults in the previous

autumn. In mid-November, the emergence of adults reaches a peak, with the second-generation adults starting to emerge from February to March. The latter adults emerge over a period of three months (Barnes, 1989). They feed on the above-ground parts of the plant, including the fruit, damaging it and making it unmarketable. *P. callosus* has been found to cause about 40% of the damage that is inflicted on apple crops (Witt et al., 1995). The control of *P. callosus* is currently inadequate, as the pest has developed tolerance to pyrethroids and, possibly, to acephate (Barnes et al., 1994), so that alternative control options require investigation.

For the development of biological control methods, the life table of the target pest needs to be understood. In addition, a constant supply of weevil larvae is required to study and develop effective control strategies (Fisher & Bruck, 2004). The rearing of insects is an important factor in the research and development of control strategies. Insects reared on an artificial diet in the laboratory are mainly used for the testing of the efficacy of biological control agents (Knipling, 1979). Though much research into the artificial diet of insects has already been undertaken, little attention has been paid to the development of such diets for weevils. The research that has been carried out thus far on banded fruit weevils is problematic, as the insects are sporadic and the larvae present in the soil are not easily obtainable through conventional methods, such as sieving (Barnes, 1989). Insects of a high quality are, nevertheless, important for guaranteeing that those studies undertaken on them are meaningful.

The preservation of healthy laboratory insects is essential to the formulation of a good insect diet (Cohen, 2004). The reliability of insect-rearing programmes is largely influenced by the health of the insects concerned, which, in turn, depends on the quality of the diets provided (Cohen, 2001).

Previous research has been undertaken into the biology and rearing of the pest insect. *Phlyctinus callosus* has been successfully reared by Walker (1981) in experiments that were designed with temperature as the only known variable. Two different cultivars of potted carrot seedlings were used for population growth studies. In laboratory rearing performed using cylinders containing carrot roots, 76% of the larvae passed through seven instars. An improved rearing technique was described by Horne and Stacpoole (1989), in which *P. callosus* first-instar larvae were placed on food plants for eight weeks. The mean survival rate of the larvae from first to late instar was 54%, with the employment of such a method allowing for the rearing of all developmental stages. Barnes (1987) reared *P. callosus* on carrot root discs, each containing a hole into which a newly hatched larva was

placed. The percentage survival rate during the first three instars was low. By the time of the first moult, 42% had died, with 70% having died by the time of the third moult. Such results agree with those of Walker (1978), who also reported very low survival rates for similar larval instars.

Walker (1981) also investigated the storage of *P. callosus* eggs. The results showed that *P. callosus* eggs can be stored for up to 12 weeks at 5˚C. Fisher and Bruck (2004) investigated the stockpiling of black vine weevil eggs for future use, as so doing would enable large experimental trials to be undertaken with large numbers of eggs. After the eggs were collected, they were stored at 4˚C for periods of 1, 2, 3 or 4 weeks, with the hatched weevils then being placed on the standard diet. Their testing concluded that such eggs can be stored for a period of up to four weeks at 4˚C, without a significant reduction in the later larval survival rate.

Research has been undertaken into rearing other weevil species, including the black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Fisher & Bruck, 2004); the stem weevil, *Hyperodes bonariensis* (Kuschel) (Power & Singh, 1974); and the Asian long-horned weevil, *Anoplophora glabripennis* (Motschulsky) (Dubois et al., 2002). Fisher and Bruck (2004) developed a technique for the mass rearing of the black vine weevil. A meridic diet was used, with additional modifications being made to the standard diet (Shorey & Hale, 1965; Shanks, Jr. & Finnigan, 1973; Shanks, Jr., 1980). The resultant diet was then referred to as the ‘improved’ diet. Fungal and bacterial inhibition was achieved, leading to improved larval survival and growth. A laboratory-rearing method was developed for the stem weevil (Power & Singh, 1974). An artificial medium was used by Power and Singh (1974) to rear eight generations of such a weevil from egg to adult. The weevil was reared at a temperature of 26˚C, with the development from first instar larva to adult taking approximately 28 days. The Asian long-horned beetle was reared on an artificial diet, which was developed by Dubois et al. (2002), who compared and modified three previously developed artificial diets. Of such diets, one was developed in China for *A. glabripennis*, with the other two diets having been developed for other members of the Lamiini group of beetles.

Research conducted by Beavers (1982) developed an artificial diet for the rearing of *D. abbreviatus.* The ingredients consist of alphacel 307 g, cotton seed meal 250 g, soybean protein 104 g, sucrose 70 g, casein 70 g, wheat germ 60 g, cornstarch 44 g, Vanderzant’s vitamin mix 31 g, Wesson® salts 16 g, methyl paraben 15 g, ascorbic acid 6 g, sorbic acid 5 g, cholesterol 3 g, choline chloride 2 g, agar 75

g, formalin (38.5%) 6 ml and water 2550 ml. Ingredients were mixed and heated to 95˚C. This was then poured into 35-ml platic cups. Larvae were placed in the cups with diet and larvae were transferred to fresh diet after 60 diet. Larvae were not sterilised before they were placed on the diet. Fresh diet was supplied at 45-day intervals until the insects pupated. Cups were held at 25˚C and 60-70% RH in a dark room. During the first 60 days mortality was highest for the larvae. When 1, 5, 10 and 25 larvae were placed in cups the percentage larvae recovered after 60 days were 30%, 12.8%, 10.6% and 2.5% respectively. The most number of adults were recovered when 10 larvae were placed in each cup. This diet facilitates the production of large numbers of four to six month old larvae (Beavers, 1982).

In the present study, a variety of methods and diets was tested for the rearing of *P. callosus* larvae. The codling moth diet, which is a modified version of the artificial diet developed for the black vine weevil, as well as variously shaped carrots, were tested. Differently sized containers, in which the diets were placed, were investigated. Full-grown carrots, carrot seedlings and potted chrysanthemums were tested as food media. The period that *P. callosus* eggs could be stored was also investigated.

Materials and Methods

Source of insects

Phlyctinus callosus adults were collected weekly from an organic fruit farm, Lorraine, in the Elgin district of the Western Cape province. They were kept in a ventilated Perspex cage (40 cm × 30 cm × 30 cm) (Fig. 1) in a laboratory maintained at room temperature (25˚C). Their diet consisted of *Coprosma repens* branches, which extended through the bottom of the cage into a container holding water. Layered cotton-wool pads were moistened and placed in the weevil cages as oviposition sites. The females oviposited overnight in between the layers of cotton wool. On the following day, the eggs were removed from the cotton wool and placed on wetted filter paper in Petri dishes, which were kept in the dark at room temperature. The filter paper was remoistened as necessary, in order to maintain high relative humidity (Giliomee, 1961; Shanks, Jr. & Finnigan, 1973). The eggs hatched within seven

to ten days at room temperature (Giliomee, 1961; Barnes, 1987). The newly hatched larvae were used to evaluate different diets and rearing methods.

Fig. 1. Perspex cage with *Phlyctinus callosus* adults inside feeding on *Coprosma repens* branches.

Basic diet

The improved diet of Fisher and Bruck (2004), which was used for the rearing of the black vine weevil, *O. sulcatus*, was used as a basis for the diet used for the rearing of *P. callosus*. Modifications were made to the diet by replacing lima beans as an ingredient with butter beans, and by the addition of carrots and wheat germ to it. The basic ingredients and quantities used are indicated in Table 1.

Agar in 65 ml water, beans in 65 ml water and dried carrots in 250 ml water were sterilised for 30 min in a kitchen pressure-cooker, and allowed to cool to 45˚C. Cooled beans with water were homogenised using a blender. The water was strained from the carrots, added to the beans and the rest of the dry ingredients, and then blended together again. Finally, the agar was added and the diet dispensed into sterile plastic containers, in which it was allowed to set. The diet was either used immediately, or stored at 4˚C.

Table 1: Ingredients and amounts used for the basic diet.

Ingredients	Amount
Butter beans	15 g
Sterile Millipore water	780 ml

Agar	3.75 g
Brewers yeast	5 g
Wheatgerm	6 g
L-asorbic acid	0.75 g
Methyl paraben	0.25 g
Sorbic acid potassium salt	0.32 g
Streptomycin sulfate	0.25 g
Dried carrots	22 g

Rearing conditions

Each of the experiments was replicated eight times, using five neonate larvae per container (*n* = 40). Before use, the containers were wiped out by hand, using 75% ethanol. Diets with larvae were incubated in the dark in a growth chamber at 21˚C. The percentage survival rate of the larvae was recorded after a period of five weeks.

Eggs on basic diet

A preliminary trial was undertaken with eggs of *P. callosus* placed on the basic diet, hereafter referred to as diet A. Diet A was poured into 40 sterilised plastic containers, which were 6.5 × 3.5 cm (diameter × depth) in size, to a depth of 2 cm. One *P. callosus* egg was placed on the diet in each container. The containers were incubated at 25˚C.

Different diets and containers used for the rearing of *P. callosus*

The different diets tested consisted of the following: A: basic diet; B: basic diet, with sawdust; and C: the codling moth diet (Guennelon et al., 1981). All three diets were prepared using two differently sized containers one with dimensions of 14 × 1.8 cm and the other with dimensions of 11.5 × 7 cm.

Addition of formalin

Formalin, at 0.175% concentration, already formed part of diet C. The same concentration of formalin was added to diets A and B. The container that was used for the trial was 11.5 × 7 cm in size.

Wax paper versus plastic lid on container

To test whether the condensation in the containers affected the survival of larvae, diets A and B were prepared in the 11.5 × 7-cm-sized containers, and covered with either a plastic lid or with perforated wax paper secured by means of an elastic band.

Comparison of carrots in different forms

Carrots in various shapes and sizes were tested. The carrots, with their leaves still attached, were purchased at a retail outlet. Three different forms of carrots were compared, namely single carrot disc with a hole in the centre, two carrot discs placed on top of each other, and grated carrots. Neonate larvae of *P. callosus* were placed individually in 2 mm diameter holes cut into the carrot root discs, which measured approximately 1 cm to 2 cm in diameter, with the discs being 1 cm to 2 cm thick. The 14 × 1.8-cm-sized Petri dishes were used.

Trials using two carrot discs were compared with those using grated carrots and long, flat pieces of carrot. Various 2-mm-diameter holes were made in the long, flat pieces of carrot. The same protocol as that which was used for the previous trial was used for this trial. Smaller and deeper containers sized 11.5 × 7-cm were used for the latter trial.

Full-grown carrots in two different soil types

A trial was undertaken using full-grown carrots with leaves, planted in pots with sterilised sand. Two types of soil, very coarse sandy soil and sandy loam soil, were compared. The sandy loam soil was obtained from the Welgevallen experimental farm in the Stellenbosch area. The sand and soil was frozen overnight before use. The pots were incubated in a chamber with 12 l: 12 d hours at 25˚C. The soil was regularly watered. The five replicates were allocated 40 larvae per pot for both types of soil. The larvae were washed, using water, into the sand. The percentage survival rate was assessed after a period of five weeks. The larvae were retrieved by washing the sand from the soil through a sieve.

Different types of plants

Neonate larvae were placed in pots alternately containing carrot seedlings, chrysanthemums and full-grown carrots with leaves on. The carrot seedlings and chrysanthemums were purchased from a

nursery. The full-grown carrots were purchased from a retail outlet and planted in sterilised sand. The pots were kept in the laboratory at room temperature. There were three replicates of each plant, with 40 larvae per pot. A similar protocol to that used for the full-grown carrots was used for the trial.

Storage of eggs

Eggs (*n* = 960), which were collected from cotton-wool pads in a weevil cage over a period of one week, were placed on wet filter paper in a Petri dish. The eggs were stored at four different temperatures: 4˚C, 11˚C, 14˚C and 25˚C. For each temperature eight replicates, containing 30 eggs each, were used. The Petri dishes were placed in plastic containers lined with moistened paper, covered with a lid to guarantee high humidity. The Petri dishes in the plastic containers were incubated at the four different temperatures in the dark. Thirty eggs (the egg contents of one Petri dish) were removed every ten days from the 4˚C, 11˚C and 14˚C growth chambers and placed in a 25˚C growth chamber to determine their viability. The eggs were monitored daily to determine the time–percentage hatching ratio.

Data analysis

The statistical analysis was performed using Statistics 8.0 (Statsoft Inc., 2007). The data were analysed using a one-way ANOVA with post-hoc comparisons of means, using Bonferroni's method, or a bootstrap multiple comparison, whenever the residuals were found not to be normally distributed (Efron & Tibshirani, 1993). The results of the full-grown carrots in two different soil types were analysed, using a one-way ANOVA with post-hoc comparisons of means, using the Mann–Whitney method (Maritz, 1995). The results, which were obtained for the two differently sized containers, were compared by using the test for equality of proportions for the independent group. The null hypothesis, H_0: $\pi_1 - \pi_2$, was tested under the assumption that the difference in sampling proportions, $P_1 - P_2$, was approximately normally distributed (Steyn, 1994). The results obtained for the cold storage of eggs were in addition to those obtained by means of the one-way ANOVA, which was also analysed using a generalised linear model (McCulloch et al., 2008).

Results

Eggs on basic diet

After a period of 21 days, no eggs were found to have hatched and, therefore, neonate larvae were used from that time onwards.

Different diets and containers for the rearing of *P. callosus*

The percentage survival rate of *P. callosus* neonate larvae in the 14 × 1.8-cm-sized Petri dishes used for the three different diets was found to be the highest (30%) for diet A, followed by diet B (17.5%), with no larvae being found to have survived on diet C (Fig. 2). Analysis with a one-way ANOVA indicated that the percentage survival rate ($F_{2, 9} = 4.881$; $p = 0.037$) differed significantly between the diets. No significant difference ($p = 0.483$) in percentage survival rate was found for larvae surviving on diet A or B.

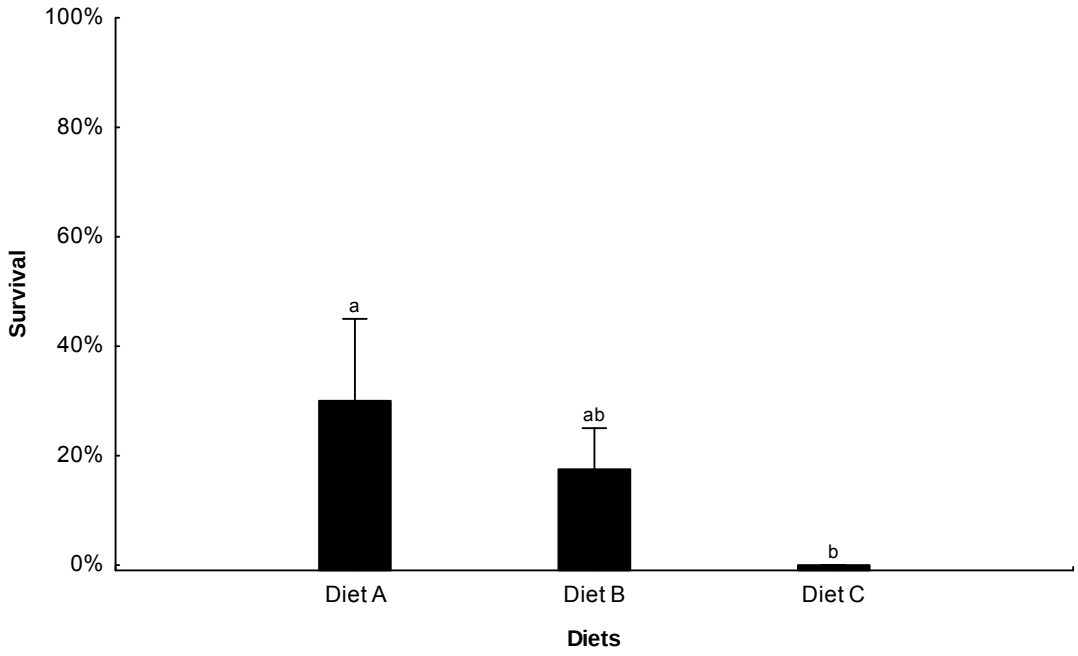

Fig. 2. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* larvae on three different diets: A: basic diet; B: basic diet, with sawdust; and C: codling moth diet, assessed after a period of five weeks (one-way ANOVA: $F_{2, 9} = 4.881$; $p = 0.037$). Different lettering above vertical bars indicate significant differences.

Using the smaller and deeper containers, which were 11.5 × 7-cm in size, the percentage survival rate of the newly hatched larvae for diets A and B was 45% and 47.5%, respectively (Fig. 3). The analysis was undertaken using a one-way ANOVA ($F_{2,\ 9}$ = 11.828; p = 0.003). No significant difference was found between the percentage survival rate of those larvae surviving on diet A and those surviving on diet B (p = 1). No larva was found to have survived on diet C.

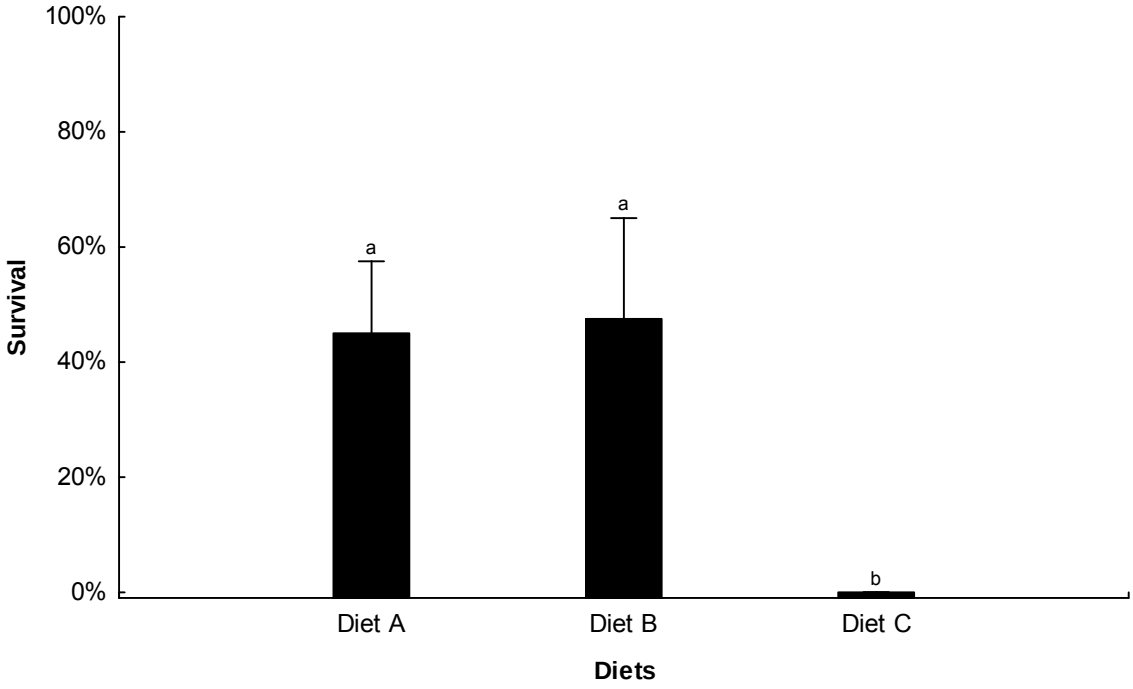

Fig. 3. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* larvae on three different diets in 11.5 × 7-cm-sized plastic containers, assessed after a period of five weeks (one-way ANOVA: $F_{2,\ 9}$ = 11.828; p = 0.003). Different lettering above vertical bars indicate significant differences.

A comparison was drawn between the two differently sized containers in which diets A and B were tested (Fig. 4). The data were analysed using the test for equality of proportions for independent groups. No significant difference (p = 0.166) was found between the groups in the two differently sized containers used for diet A, though a significant difference (p = 0.004) was found between the groups in the differently sized containers used for diet B.

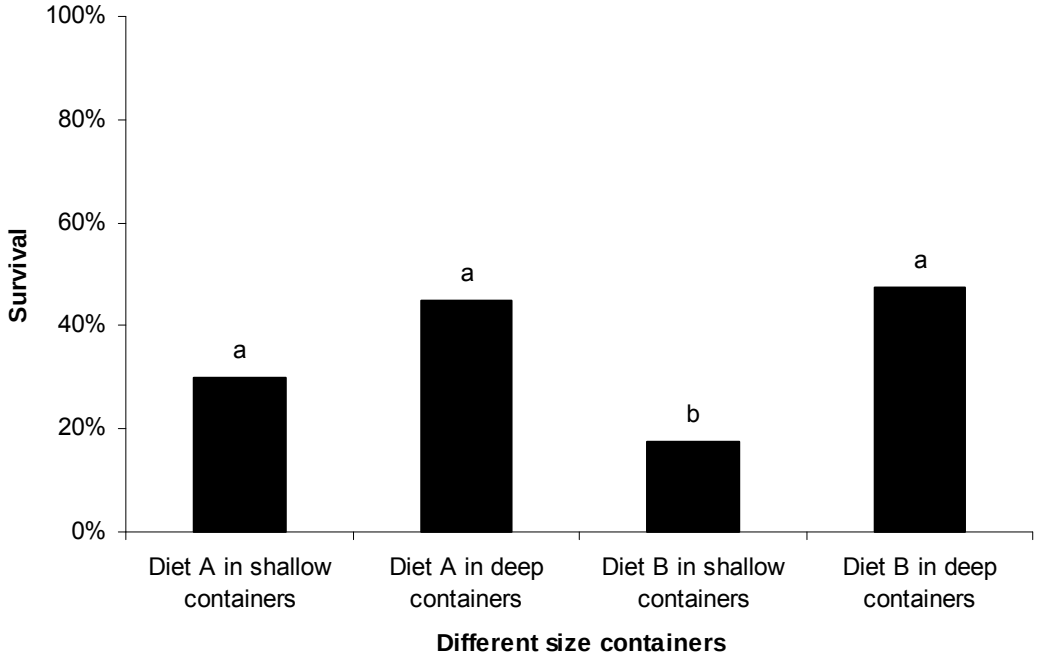

Fig. 4. The mean percentage survival rate (95% confidence interval) of newly hatched *Phlyctinus callosus* larvae, after a period of five weeks. Differently sized containers were compared using the test for equality of proportions for independent groups. Different lettering above vertical bars indicate significant differences.

Addition of formalin

Diet A, without formalin, had a percentage survival rate of 42.5%, compared with Diet A, with formalin added, on which diet no larvae survived. Diet B, without formalin, had a percentage survival rate of 47.5%, compared with Diet B, with formalin added, on which diet no larvae survived (Fig. 5).

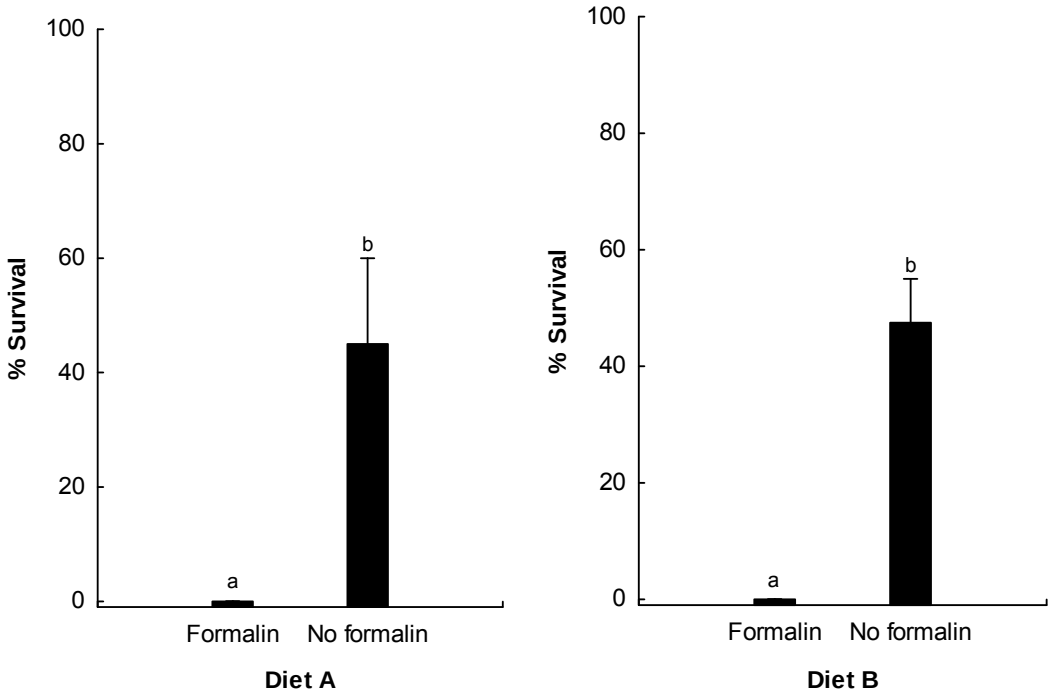

Fig. 5. Diets A and B, as tested with and without formalin, and the survival rate of *Phlyctinus callosus* larvae, as assessed after a period of five weeks.

Wax paper versus plastic lid covered container

The highest (50%) percentage survival rate was obtained using Diet B in a container covered with a plastic lid, whereas no survival was obtained using Diet B in a container covered with wax paper. A one-way ANOVA was used to analyse the data ($F_{3, 12} = 9.2667$; $p = 0.0019$). Though no significant difference ($p = 0.051$) was found between the percentage survival rate of larvae surviving on diet A in the lidded container and those surviving on diet A in the container covered with wax paper, more larvae were found to have survived in the lidded containers (Fig. 6).

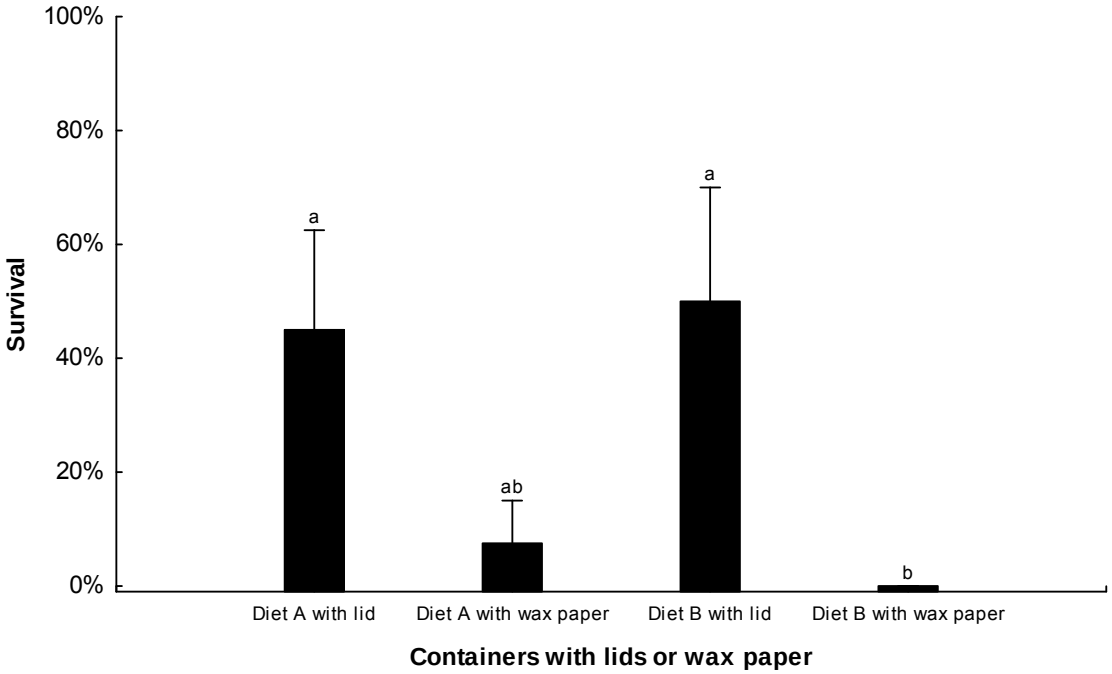

Fig. 6. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* surviving on diets A and B tested in containers covered either by a plastic lid or by a piece of wax paper, assessed after a period of five weeks (one-way ANOVA: $F_{3, 12} = 9.2667$; $p = 0.0019$). Different lettering above vertical bars indicate significant differences.

Comparison of carrots in different forms

The use of two carrot discs resulted in the highest survival rate (60%). The lowest (10%) percentage survival rate of larvae was obtained with the use of grated carrots (Fig. 7). A one-way ANOVA was used to analyse the data ($F_{2, 9} = 13.96$; $p = 0.0017$). No significant difference was found between the survival rate obtained for the single carrot discs with a hole and that obtained for the two carrot discs ($p = 0.825$), though the percentage survival rate obtained for both such samples differed significantly from that obtained for those surviving on a diet of grated carrots.

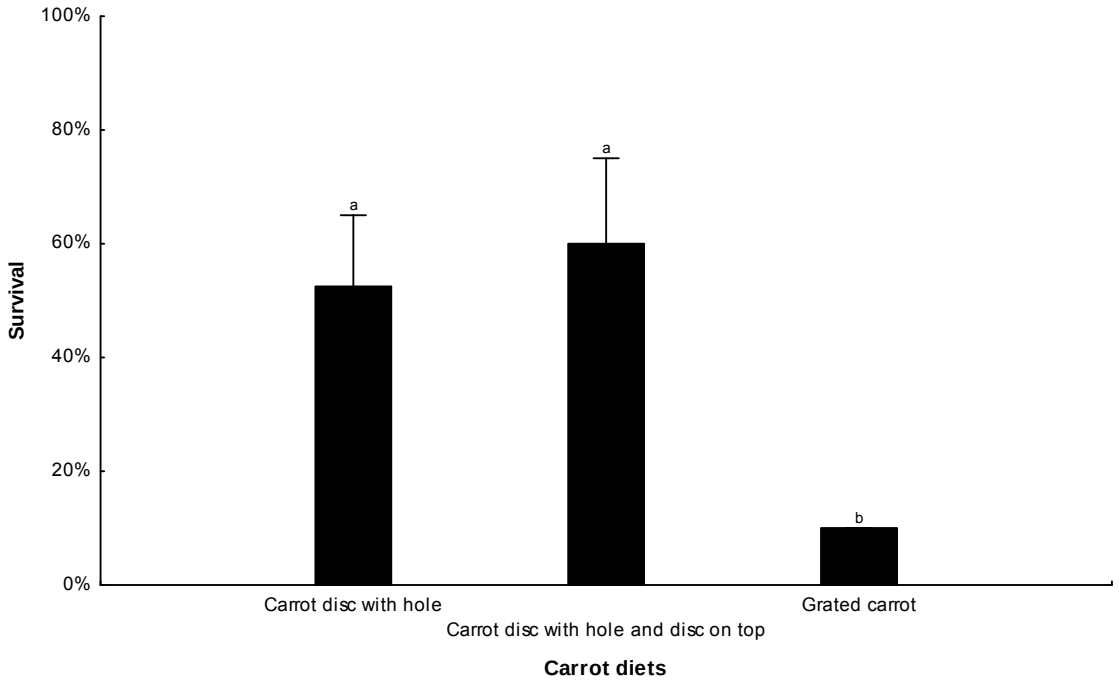

Fig. 7. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* on three different diets tested in 14 × 1.8-cm-sized Petri dishes, which were incubated at 21˚C, assessed after five weeks (one-way ANOVA: $F_{2, 9} = 13.96$; $p = 0.0017$). Different lettering above vertical bars indicate significant differences.

The percentage survival rates of larvae surviving on two carrot discs, on grated carrots and on long, flat pieces of carrot were compared. The highest (45%) percentage survival rate was obtained when using two carrot discs (Fig. 8). Grated carrots gave the lowest percentage survival rate of only 17.5%. The results obtained were analysed by means of a one-way ANOVA ($F_{2, 6} = 607.08$; $p = 0.00$). A significant difference ($p = 0.015$) was found between the percentage survival rate of larvae surviving on two carrot discs and those surviving on grated carrots, though no significant difference was found in the percentage survival rate between the grated and flat carrots.

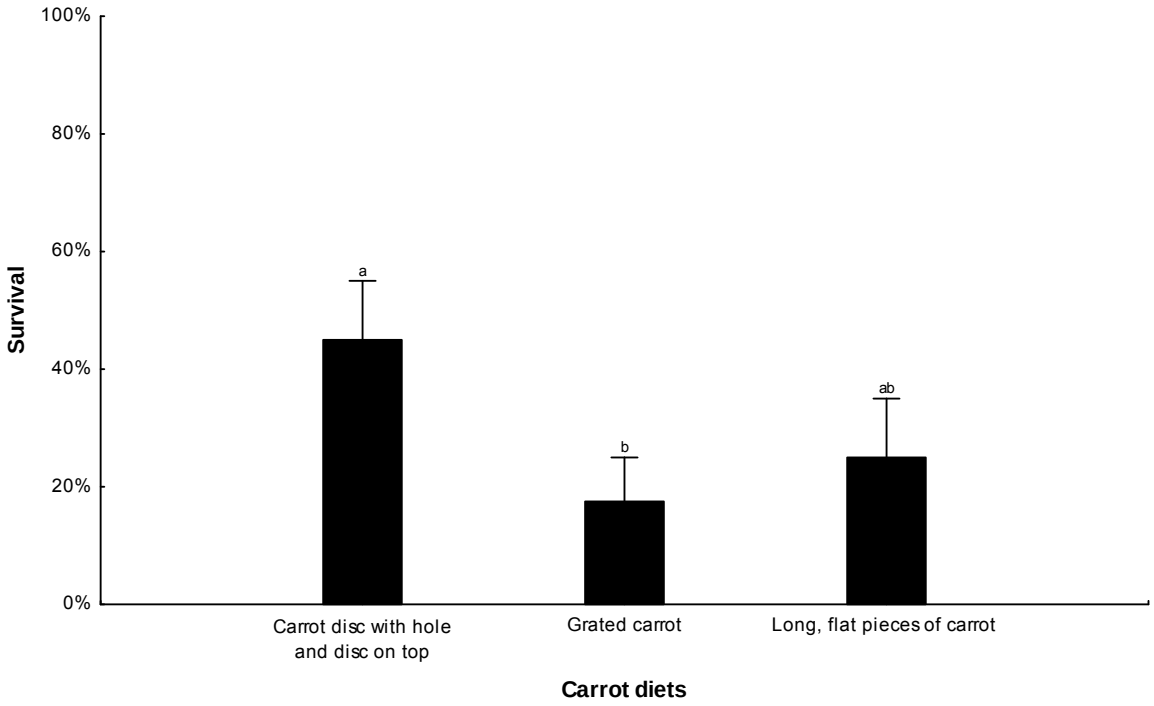

Fig. 8. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* surviving on three different diets was tested in 11.5 × 7-cm-sized plastic containers, which were incubated at 21˚C, assessed after a period of five weeks (one-way ANOVA: $F_{2,\ 6} = 607.08$; $p = 0.00$). Different lettering above vertical bars indicate significant differences.

Full-grown carrots in two different soil types

The highest percentage survival rate obtained for those larvae surviving in coarse sand was 90%, with the lowest being 82.5%. The mean percentage survival rate, given such conditions, was found to be 87.5%. The highest percentage survival rate for those larvae surviving in sandy loam soil was found to be 37%, with the lowest being 2.5% (Fig. 9). The data obtained were analysed using a one-way ANOVA ($F_{1,\ 8} = 119.13$; $p = < 0.01$), which showed that the mean percentage survival rates obtained for the two treatments differed significantly from each other ($p = 0.0069$).

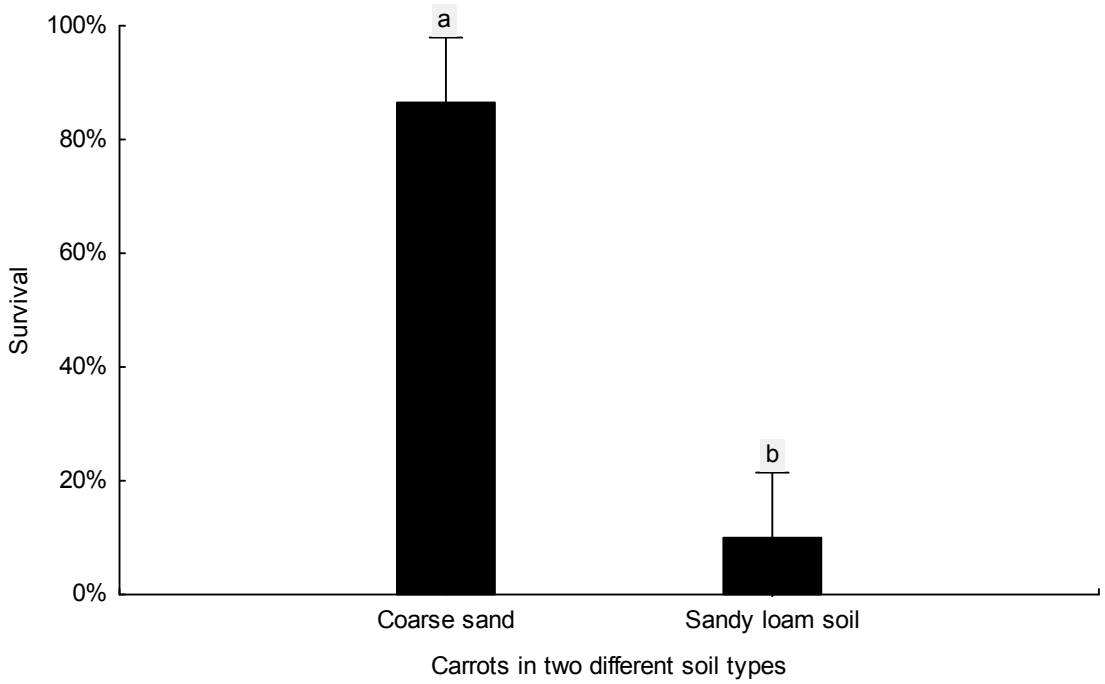

Fig. 9. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* larvae placed in pots containing two different soil types kept at 25˚C was assessed after a period of five weeks (one-way ANOVA: $F_{1, 8} = 119.13$; $p = < 0.01$). Different lettering above vertical bars indicate significant differences.

Different types of plants

The highest (79.3%) percentage survival rate of larvae was obtained using potted full-grown carrots. The lowest (5.8%) percentage survival rate was obtained using potted chrysanthemums. The data were analysed using a one-way ANOVA ($F_{2, 6} = 607.08$; $p = 0.00$). All the treatments differed significantly from one another. The percentage survival rate obtained for larvae living on chrysanthemums and carrot seedlings ($p = 0.0405$), chrysanthemums and full-grown carrots ($p = 0.0255$), and on carrot seedlings and full-grown carrots ($p = 0.0195$) differed significantly from one another (Fig. 10).

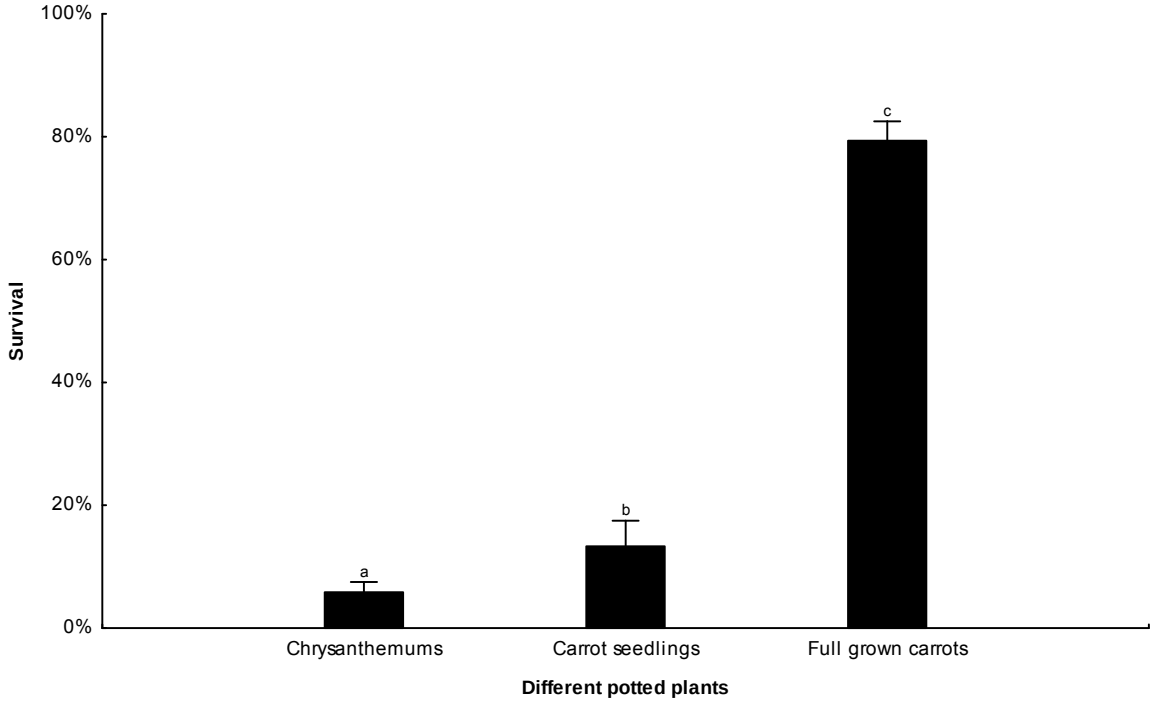

Fig. 10. The mean percentage survival rate (95% confidence interval) of *Phlyctinus callosus* on thee different potted plants kept at room temperature, after a period of five weeks (one-way ANOVA: $F_{2, 6} = 607.08$; $p = 0.00$). Different lettering above vertical bars indicate significant differences.

Storage of eggs

The mean percentage rate obtained for eggs that hatched within five days at 25˚C was 65.3%. This temperature served as the control for the trial. The percentage hatch rate of eggs stored for 30 days at 4˚C, 11˚C and 14˚C was 13.2%, 90% and 70%, respectively. The percentage hatch rate of eggs stored for 60 days at 4˚C, 11˚C and 14˚C was 40%, 86.6% and 86.6%, respectively (Fig. 11).

The percentage hatch rate of eggs stored at 4˚C was found to decrease from 10 to 30 days. The highest (90%) percentage hatch rate was found after 10 days. From 40 to 70 days, the percentage hatch rate was found to stay more or less the same, at a mean percentage hatch of 39.9%. At 80 days, no more eggs were found to hatch (Fig. 12).

At the maintenance of a temperature of 11˚C, the mean percentage hatch rate was 86.6%. A percentage of the eggs started hatching before they could be removed and placed at 25˚C. With the eggs being kept at 11˚C, 10% of the eggs were found to have hatched after 47 days; 20% of the eggs

were found to have hatched after 57 days; and 30% of the eggs were found to have hatched after 67 days.

At the maintenance of a temperature of 14˚C, the mean percentage hatch rate was found to be 77.5%. A large percentage of the eggs hatched before they were placed at 25˚C. With the eggs being kept at 14˚C, after 10 days, 10% of the eggs were found to have hatched. After 20 days, 55% of the eggs were found to have hatched; and after 30 days 65% of all the eggs were found to have hatched already.

The use of a generalised linear model showed that the difference in temperature was found to have had an effect on the percentage egg survival rate (df = 4; $\chi^2 = 44.52$; $p < 0.0001$). The length of time transpired was found to have an effect on the egg survival rate, but only at lower temperatures, as was shown by the significant interaction that occurred with the time and temperature combination (df = 4; $\chi^2 = 30.17$; $p < 0.0001$).

The data were also analysed using a one-way ANOVA ($F_{2, 21} = 13.075$; $p = 0.002$). No significant difference ($p = 0.3015$) was found between the percentage hatch rate of *P. callosus* eggs at 11˚C and 14˚C, though the percentage hatch rate for both differed significantly from that obtained at the maintenance of a temperature of 4˚C (Fig. 11).

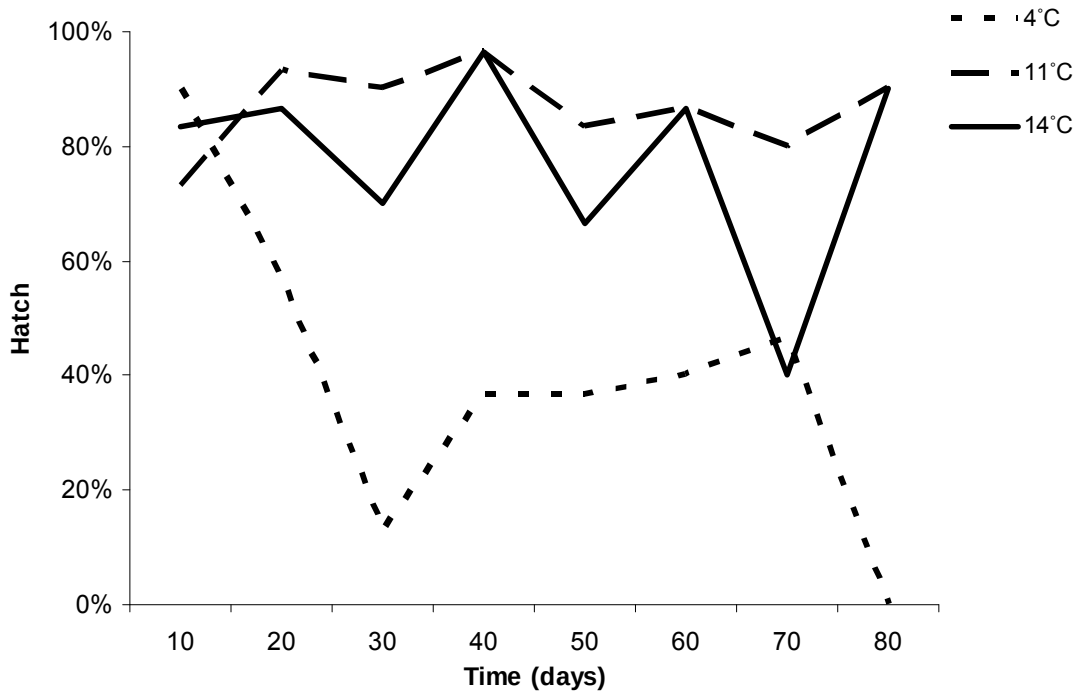

Fig. 11. The percentage of *Phlyctinus callosus* eggs that hatched after being stored at 4˚C, 11˚C and 14˚C for 10 to 80 days. The data were analysed using a generalised linear model, which showed that, only at lower temperatures, do both temperature (df = 4; χ^2 = 44.52; p < 0.0001) and time (df = 4; χ^2 = 30.17; p < 0.0001) have an effect on the egg survival rate.

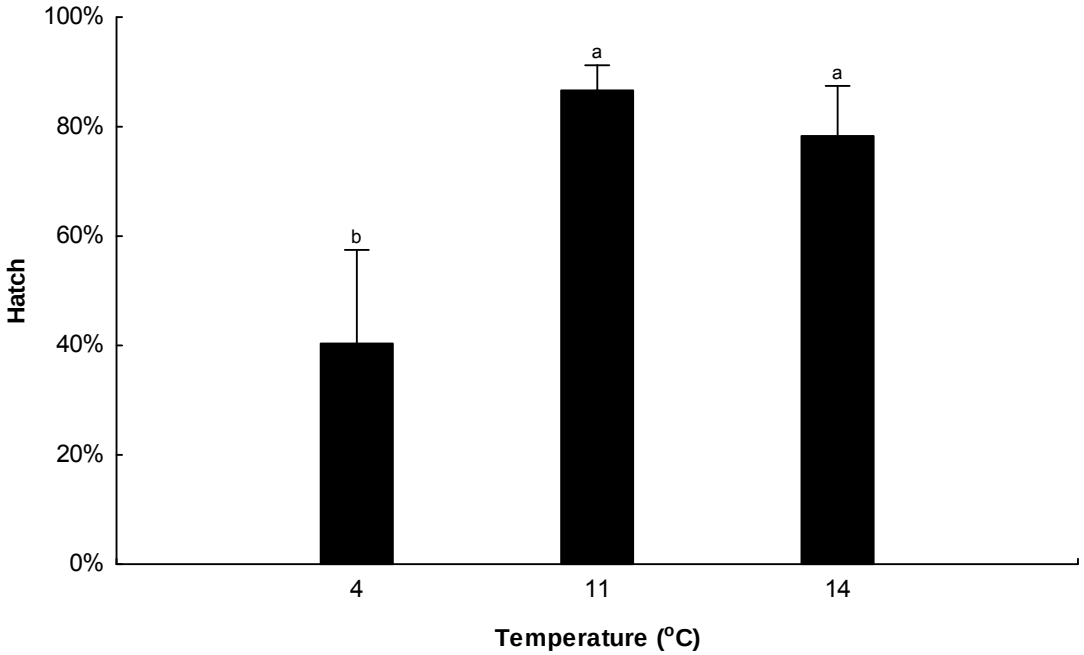

Fig. 12. The mean percentage hatch rate (95% confidence interval) obtained over a period of 80 days for *Phlyctinus callosus* eggs, kept at one of three different temperatures (one-way ANOVA: $F_{2, 21}$ = 13.075; p = 0.002). Different lettering above vertical bars indicate significant differences.

Discussion

The aim of the current study was to develop a diet for rearing larvae of *P. callosus*, as this would be the stage targeted with entomopathogenic nematodes. Adult weevils could easily be collected from orchards, but large numbers of larvae were very difficult to obtain from sieving the orchard soil. Throughout the study described in this thesis, a shortage of larvae was experienced as a result of the setbacks that were encountered with their artificial rearing.

The eggs, which were placed on wet filter paper at room temperature, were found to hatch within seven days (Giliomee, 1961; Walker, 1978; Barnes, 1984). The larvae were then placed on the different diets described earlier. Walker (1981) and Barnes (1984) used a temperature of 20˚C for the larval stages of *P. callosus*. Neonate larvae, rather than eggs, were used to test the different methods, in keeping with the protocol established by Horne and Stacpoole (1989), in accordance with which the neonate larvae were placed on the potted carrot seedlings.

Diet A had carrots added, as Walker (1981) obtained good results with carrot root discs and carrot seedlings. The diet, however, did not produce high numbers of larvae after a period of five weeks. Therefore, sawdust was added to make the diet more conducive to the burrowing of the larvae, for which soil is the natural habitat. More larvae were found to have been produced on diet B (with sawdust added), though no significant difference was detected when the number of larvae produced on the two diets was compared. Low larval survival might have resulted from the contamination experienced with both diets. No larva was found to have survived on diet C (the codling moth diet). Diet C was tested, due to its being readily available at the time, with a much lower contamination being experienced with such a diet.

Different size containers were tested, as it was assumed that a deeper container would allow larvae to burrow into the diet more easily than would soil. More larvae were, indeed, found to survive in the deeper containers, with a significant difference being found to exist between the larvae surviving in the two containers used for diet B.

The large amount of condensation present in the containers was suspected as adding to the fungal and bacterial growth encountered. However, when formalin was added to diets A and B, no larva

feeding on either of the said diets was found to have survived. Though the addition of fungal and bacterial inhibitors to artificial diets is necessary for the rearing of large numbers, weevils, in particular, are extremely susceptible to such inhibitors in general (Bass & Barnes, 1969; House et al., 1971).

In the current study, the large amount of condensation encountered also caused larval mortality due to drowning, as was experienced by Walker (1978). The research that was undertaken by Dubois et al. (2002) tested both perforated and non-perforated lids to solve the problem of too much moisture occurring. No larvae survived when the containers used for diet B were sealed with a piece of wax paper, rather than a plastic lid. With the use of the former, the diet was found to dry out, resulting in the occurrence of an insufficient supply of moisture. A significant difference was only found in the case of diet B. The fine balance that existed between too low and too high humidity was evident (Barnes, 1984).

Carrots tested because diet A and B, as well as the modification of these diets, did not yield high numbers of larvae. As a result of the carrots used in the study being purchased from a retail outlet, they varied in quality. The younger carrots most likely had higher nutritional value than did the older carrots, and were most likely to have been the cause of the larvae not developing at the same rate. Barnes (1984) encountered a similar obstacle with carrots purchased from a retail outlet.

When the larvae were enclosed in the hole in between two carrot discs, and so restricted in their movement, their survival percentage rate increased. Although no significant difference existed in the survival percentage rate when the larvae were enclosed within a carrot disc placed on top of another carrot disc, and when larvae were placed on a single carrot disc, a higher percentage survival rate was obtained with the former technique. The larvae had the tendency to move around, becoming trapped in water droplets when they were not enclosed. The research that was undertaken by Power and Singh (1974) also encountered such a problem; some of the larvae in their study died, due to moving around rather than feeding. As such a rearing method was found to be very labour-intensive, other rearing methods were investigated.

Grated carrots yielded a very low larval survival rate. High fungal and bacterial growth occurred with the grated carrots, despite the fact that the environment was sterile. *Phlyctinus callosus* eggs and 1st instar larvae were however not sterilised. Excessive moisture seemed to have been present with the

grated carrots. To reduce the amount of labour required, long flat pieces of carrots were tested. Excessive moisture was also found to be present with carrots cut up in this way, resulting in the larvae moving around too much, rather than feeding on the carrots. Consequently, a low larval survival rate was obtained.

Better results were obtained when neonate larvae were placed in pots containing sterilised sand, or soil together with full-grown carrots. A meaningful difference was found in the percentage survival rate experienced when the rate was compared between the two types of media, namely coarse sand and loamy orchard soil. The larvae performed noticeably better when placed in the coarse sand, with a difference of 76% in their mean survival rate. Although they were kept at 25˚C, with temperatures exceeding 25˚C being known to be lethal to larvae, the soil acted as a buffer, resulting in the larvae being unharmed (Walker, 1981). From this trial, it was evident that such a relatively low-intensity method of rearing, using coarse sand, is much superior to the use of carrot discs or artificial diets.

In addition, trials were undertaken with other potted plants. Walker (1981) and Horne and Stacpoole (1989) obtained good results with the use of carrot seedlings as host plants for *P. callosus*, leading to such seedlings also being included in the trial. Whereas Walker (1981) obtained 25% larval survival at 20˚C, Horne and Stacpoole (1989) obtained 54% larval survival rate at temperatures ranging from 18˚C to 28˚C. Both Walker (1981) and Horne and Stacpoole (1989) used potted carrot seedlings, with the use of such a rearing method producing an 87.5% larval survival rate at 25˚C, using full-grown carrots in pots. In the current study, chrysanthemums and carrot seedlings were found to produce a low larval survival rate. The use of full-grown carrots resulted in the attainment of a much higher percentage larval survival rate, which was, however, still not as high as was obtained when the larvae were kept at 25˚C. The mean percentage larval survival rate obtained for larvae surviving on a diet of full-grown carrots at 25˚C was 87.5%.

The ability to store eggs for an extended period of time benefits the researcher. The egg-laying ability of the adult female weevil in the laboratory decreases over time (Butcher, 1984; Fisher & Bruck, 2004). Therefore, the cold storage of eggs was tested at three different temperatures. The hatch percentage rate of eggs stored at 4˚C was found to remain constant from 40 to 70 days, though no hatching was found to take place after 80 days. The percentage hatch increasing from 30 to 40 days was possibly due to phenotypic plasticity (Stillwell & Fox, 2005). The phenotypic responses might

have resulted from adaptation to different thermal environments, in terms of where the eggs adapted to a temperature of 4˚C and the percentage hatch increasing from 30 to 40 days and then more or less staying the same from 40 to 70 days, until 80 days had passed, when no more adaptation was possible.

At a constant temperature of 11˚C and 14˚C, the eggs were found to start hatching, prior to their relocation at 25˚C. Maintaining lower temperatures can clearly be used to delay egg-hatching, though eventually the eggs do hatch when they are kept at such temperatures. When eggs are stored for no longer than 70 days, maintaining a temperature of 4˚C is recommended. In order to delay hatching, the eggs should, consequently, be kept at either 11˚C or 14˚C. To optimize storage of eggs temperatures between 4˚C and 11˚C should be tested in the future.

The aim of the current study was to develop an agar-based diet, based on the diet developed for the black vine weevil by Fisher and Bruck (2004), conducive to the rearing of mass numbers. The percentage larval survival rate on the two modified diets was low, with it not being high for the different carrot diets either. The findings of the study clearly show that the most effective rearing method currently for use with *P. callosus* is that of using full-grown carrots, which have been planted in pots with sterilised sand. Such a method is much less labour-intensive than that of using carrot discs or artificial diets. The percentage larval survival rate obtained with such use has been found to be much higher than that which has been obtained with the other methods described in this thesis.

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Chapter 3

Potential of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to control the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) in laboratory bioassays

Abstract

Several endemic entomopathogenic nematode isolates were evaluated for their potential use as biological control agents for *Phlyctinus callosus*. The susceptibility of *P. callosus* larvae and adults to entomopathogenic nematodes was screened in the laboratory at a concentration of 400 infective juveniles (IJ) per insect after four days. Larvae were found to be more susceptible to infection by nematodes than were adults. The percentage mortality for larvae for the different nematode isolates after four days ranged between no infection to 75%. Only two species, *Heterorhabditis zealandica* (SF41) and *H. bacteriophora* (SF134), caused a mortality rate higher than 60% for the larvae concerned. After four days the percentage mortality rate for *P. callosus* adults ranged between 13% and 45% for *H. zealandica* (SF41), *S. khoisanae* (106-C) and *H. bacteriophora* (SF134). The SF41 isolate of *H. zealandica* was selected as the most promising isolate, so that it was used for the rest of the study. The effect of concentration, temperature, vertical movement in sand and sandy loam soil, and the biology of *H. zealandica* in *P. callosus* larvae was investigated in laboratory bioassays. The LD_{50} and LD_{90} values after four days' incubation were 96 and 278 IJ/50 µl, respectively. Nematodes were inactive at 15˚C, with the highest mortality of *P. callosus* as a result of nematode infection being recorded at 25˚C. A higher (95.2%) percentage mortality rate was obtained with the sandy loam soil, than with the use of sand (77.5%). *Heterorhabditis zealandica* could successfully complete its life cycle in 6^{th} instar *P. callosus* larvae. The study showed that *P. callosus* larvae are suitable hosts for *H. zealandica*. The selected isolate also showed promise for the field control of *P. callosus*.

Introduction

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae), or banded fruit weevil, as it is more commonly known, causes damage to fruit by chewing away the skin, as well as the underlying flesh,

resulting in shallow lesions, with a scooped-out appearance. The weevil also feeds on the bark of fruit stalks. When damage is severe, the fruit prematurely wilts and drops. In the Western Cape, fruit damage occurs mainly from December to February. When fruit damage occurs earlier in the season, a corky tissue is produced, which forms a callus over the damaged area. As the fruit increases in size over time, the damaged area also enlarges and the “scar tissue” breaks away and falls off. The fruit is, consequently, left with russet-type lesions, which indicate damage caused by snout beetle (the group of beetles that includes *P. callosus*) infestation (Marais & Barnes, 2003; Fisher, 2004).

Current measures aimed at controlling *P. callosus* have proved to be ineffective. In many apple and pear orchards, snout beetles have been found to cause more damage than does any other pest insect. Previously, the control of snout beetles was excellent when arsenate spays were applied to control codling moth. The use of such sprays simultaneously kept the levels of *P. callosus* under control (Barnes & Swart, 1977). Arsenate sprays were later replaced with DDT, which controlled both *P. callosus* and codling moth well (Barnes & Swart, 1977). With the banning of DDT, other chemicals, such as organophosphate and carbamate, were incorporated into the control programme. Such noncontact insecticides lack a residual effect, which enables the snout beetle population to grow to harmful levels (Barnes & Swart, 1977). The use of spray applicators for such insecticides is also problematic in controlling *P. callosus*. Spray deposition tends to be ineffective on the trunk and scaffold branches of fruit trees, where it is most needed to control *P. callosus* effectively. The shy nature of the insect makes it difficult to target *P. callosus* during the day, when spraying takes place against the adults (Barnes & Swart, 1977).

Trunk exclusion barriers can be used as a cultural method for controlling *P. callosus* in deciduous fruit orchards. The creation of such barriers helps to reduce the number of insecticide sprays required (Barnes et al., 1996). However, constructing such barriers can be very labour intensive, as one such barrier must be placed around the trunk of each fruit tree. The barriers can also only be left on the trees for two seasons, and, even then, they have to be retreated with a suitable insecticide periodically. A general problem with trunk exclusion barriers is the dust and debris, which decreases the efficacy of the active ingredient. A commercially available trunk barrier called Bugband™ has been proved to work the best of all such barriers so far against *P. callosus.* The use of such a barrier

has, however, a major drawback, in that the active ingredient can be phytotoxic to the bark of trees. The barrier must, therefore, not be allowed to make contact with the bark (Barnes et al., 1996).

Entomopathogenic nematodes are lethal pathogens of insects that occur naturally in the soil. In combination with their symbiotic bacteria, they can be used in an integrated control programme against many insect pests (Griffin et al., 2005; Wright et al., 2005). Such biological control agents have a wide host range, and can actively search for their host, while remaining harmless to mammals (Gaugler & Boush, 1979). The most important entomopathogenic nematodes of commercial interest are those belonging to the families of Steinernematidae and Heterorhabditidae (Poinar, 1990). Prestidge and Willoughby (1990) tested the use of such nematodes against *P. callosus*, finding *Heterorhabditis bacteriophora* Poinar, 1975 to be the most effective. The results obtained were promising, leading to the deduction that entomopathogens can be effective against *P. callosus* (Prestidge & Willoughby, 1990).

Entomopathogenic nematodes have been used to control black vine weevil (*Otiorhynchus sulcatus*) (Fabricius) larvae, in the case of potted plants and crops grown in glasshouses (Fitters et al., 2001; Wilson et al., 1999; Lola-Luz et al., 2005). Lola-Luz and Downes (2007) infested potted and field strawberries with *O. sulcatus* larvae and then applied three soil-drenches using *H. megidis* Poinar, Jackson & Klein, 1987, after which no live weevils were recovered. The advantages to be gained from using entomopathogenic nematodes include the countering of a wide range of soil-inhabiting pests, their easy application and the safety of non-target arthropods (Kaya & Gaugler, 1993; Georgis & Gaugler, 1991).

Successful control of clover root weevils (*Sitona Lepidus*) (Gyllenhal) by entomopathogenic nematodes has been demonstrated in the New Zealand-based research that was undertaken by Bell et al. (2000). Petri dish and pot experiments were conducted using *H. zealandica* Poinar, 1990, *H. bacteriophora*, *Steinernema carpocapsae* Weiser, 1955 and *S. feltiae* Filipjev, 1934. Bell et al. (2000) showed that all nematode species were able to kill the larvae of the clover root weevil. The oriental beetle, *Anomala orientalis* (Waterhouse), has been controlled by the use of *S. scarabaei* (Stock & Koppenhöfer, 2003). In two greenhouse and two field trials, control ranged between 50% and 95% (Polavarapu et al., 2007).

In studies where nematode application was performed against the soil stages of *P. callosus*, the need for insecticide application against adults was reduced (Prestidge & Willoughby, 1990; Barnes & Giliomee, 1992). The soil stages of *P. callosus* have been found to occur from winter to spring (Brain, 1929; Barnes, 1989). Significant reduction of the *P. callosus* population in the soil at such a stage should provide widespread protection against damage to the leaves and fruit in the following growing season, as the larvae in the soil represent the following *P. callosus* population. Currently, the soil stage is not yet effectively controlled (Barnes, 1987). The soil environment is conducive to the use of entomopathogenic nematodes, as the soil acts as a buffer for such nematodes against environmental extremes (Koppenhöfer, 2000).

A laboratory bioassay was undertaken to select a nematode isolate capable of inflicting the highest mortality rate on *P. callosus* larvae. Other biological characteristics, such as the effect of temperature on nematode activity and the concentration of nematodes needed to obtain acceptable levels of control for *P. callosus*, were investigated. A sand bioassay trial was undertaken to determine the depth of movement into the soil required to infect and kill the insect host, and two different types of soil were compared with each other. A study was also undertaken to see whether the nematodes could complete their life cycle in *P. callosus* larvae.

Materials and Methods

Source of nematodes and insects

The entomopathogenic nematodes used in the current study were obtained from previous local surveys and maintained in Stellenbosch University's own collection (Malan et al., 2006; De Waal, 2008). Infective juveniles (IJ) of the different nematode isolates were cultured using *Galleria mellonella* (L.) or *Tenebrio molitor* (L.). The IJ were reared at room temperature, according to the procedures devised by Kaya and Stock (1997). The IJ were stored horizontally at 14˚C in 500 ml flat culture flasks. The flasks, which contained 150 ml filtered water, were shaken weekly for aeration. The nematodes were used within one month of harvesting. Details of the nematode species and isolates used in the study are given in Table 1. Adult *P. callosus* were collected from a farm in Elgin by means of corrugated cardboard bands tied to trunks of apple and prune trees.

Table 1. Entomopathogenic nematodes species, isolate numbers, Genbank accession number, locality and habitat.

Species	Isolate number	Genbank accession number	Locality	Habitat
H. zealandica	SF41	EU699436	Patensie	Natural vegetation
H. zealandica	J92	EU727166	Jonkershoek	Undisturbed soil
H. zealandica	J21	*Unavailable	Bonnievale	Undisturbed soil
H. zealandica	J34	EU722436	Brenton on Sea	Undisturbed soil
H. zealandica	J37	EU727165	Belvidere	Disturbed soil
H. zealandica	J182	EU727167	Kuilsrivier	Disturbed soil
H. bacteriophora	SF134	FJ423603	Piketberg	Apple
H. bacteriophora	159-C	EU715291	Nelspruit	Citrus
H. bacteriophora	J22	EU700310	Bonnievale	Disturbed soil
H. bacteriophora	J84	EU716331	Simondium	Undisturbed soil
H. bacteriophora	SF351	FJ455843	Wellington	Grapevine
H. safricana	J131	EU716336	Citrusdal	Undisturbed soil
S. khoisanae	106-C	EU683802	Porterville	Citrus
S. khoisanae	SF87	DQ314289	Villiersdorp	Apple

(Malan, 2006; De Waal, 2008)

Source of insects

Phlyctinus callosus larvae were reared on carrots discs, agar diets and potted plants (Chapter 2). The larvae of *T. molitor* were reared in the laboratory, using fine wheat bran in plastic containers. Potato peels were added for moisture. The larvae were regularly harvested, and then mixed with sawdust and stored at 4˚C. *Galleria melonella* larvae were reared on a diet that contained the following ingredients: five parts brown bread flour; five parts baby cereal; two parts wheat germ; two parts yeast; two parts glycerine; and one part honey. All the ingredients were mixed together with a beeswax comb (Bronskill, 1961; Woodring & Kaya, 1988).

Screening *P. callosus* larvae

For screening of the different nematode species and isolates (Table 1) against *P. callosus* larvae, 24-well plates (flat bottom, Nunc™, Cat. No. 144530) were used. Filter paper discs (of 13 mm diameter) were placed in 12 of the 24 wells of each plate, in such a way as to limit the movement of the

nematodes between the wells. A concentration of 400 IJ/50 µl was inoculated onto the filter paper discs (Navon & Ascher, 2000). An individual *P. callosus* larva was placed in each of the 12 wells. The plates were covered with a piece of glass, intended to retain each insect in its well, and placed in lidded plastic containers lined with moistened paper towels to guarantee maintenance of high humidity (RH 95%). Four plates were used per container. Due to the limited availability of *P. callosus* larvae, one plate was used for each isolate tested and one plate containing only water being added as controls and the experiment was done once. The plates in the plastic containers were incubated at 25˚C in darkness. Mortality was assessed after four days, on removal of the plates, with the infection rate being determined by dissection in Ringer's solution.

Screening of adults

Three nematode species, *H. zealandica* (SF41), *H. bacteriophora* (SF134) and *S. khoisanae* (106-C) (Nguyen, Malan & Gozel, 2006), were screened against infection of adult *P. callosus*. The same protocol as has been described for the larvae was used with 10 filter paper discs. Two plates were used for each nematode species tested, with two plates containing only water being added as controls. The experiment was repeated three times on different dates, using fresh nematode inoculum. The plates in the plastic containers were incubated at 25˚C in darkness. The mortality rate was assessed after four days, with the infection rate being determined by dissection in Ringer's solution. The number of first-generation nematodes in the adult weevils was counted to determine the mean number of IJ penetrated for each species.

Sand bioassay

The experiment was designed to simulate field conditions of the *P.callosus* larvae in the soil. *Heterorhabditis zealandica* (SF41) was evaluated using the same protocol described by Yu et al. (2006). Coarse river sand was frozen overnight before use. Centrifuge tubes (1.5 cm × 15 cm in diameter) were filled with sand, with 1.4 ml water being added to each tube. Larvae were buried 0 cm, 5 cm, 10 cm and 15 cm deep in the centrifuge tubes. To allow the nematodes to reach the larvae, and to simplify the retrieval of larvae, each larva was placed separately in an Eppendorf tube (0.2 ml thin-walled PCR tube) pierced with 10 holes, made using a heated needle, similar to the protocol described by Kehres et al. (2001). Nematode inoculum (400 IJ/100 µl) was added to each tube with

the final amount of liquid added being 1.5 ml. Control treatments received only water. After seven days at 25˚C, the rate of *P. callosus* mortality caused by nematode infection was confirmed by dissection.

Sand versus sandy loam soil

Phlyctinus callosus larvae were buried 10 cm deep in an Eppendorf tube. Soil was obtained from the Welgevallen experimental farm in the Stellenbosch area. A five fraction analysis was done by Bemlab, Somerset-West. The soil was frozen overnight before use. The same protocol as was described for the sand bioassay was followed in the sandy and sandy loam soil bioassay.

Nematode concentration

To determine the effect of different concentrations of *H. zealandica* (SF41) on the mortality rate of *P. callosus*, the 24-well bioassay protocol was followed. Concentrations of 0 IJ/insect, 200 IJ/insect, 400 IJ/insect and 800 IJ/insect of *H. zealandica* (SF41) were inoculated onto a circular piece of filter paper placed in each well, after which a larva of *P. callosus* was added. After closing the lid of the bioassay trays, the trays were then placed in a closed plastic container left in a growth chamber at 25 ± 2˚C. Due to the limited availability of *P. callosus* larvae this experiment was conducted only once. The mortality caused by the nematodes was confirmed by dissection after a period of two, four and six days.

Effect of temperature

The effect of three different temperatures (11˚C, 15˚C and 25˚C) on the larvicidal activity of *H. zealandica* (SF41) was studied. A concentration of 400 IJ per larva was inoculated onto filter paper discs in 24-well plates as was previously described for the screening procedure (Navon & Ascher, 2000), and 40 *P. callosus* larvae were used for each temperature tested. One *P. callosus* larva was placed in each of the 10 wells concerned. The plates were covered with a piece of glass, to retain each insect in its well, and then placed in lidded plastic containers lined with moistened paper towels to guarantee high humidity (RH 95%). The plates in the plastic containers were incubated, at the different temperatures specified, in darkness. Mortality was assessed after four days by removing the plates, with the infection rate being determined by dissection of the insects in Ringer's solution.

Biology of *H. zealandica* (SF41) in *P. callosus* larva

Ten multiwell plates, with six *P. callosus* larvae which were 5 weeks old in each plate (*n* = 60), were used in the trial. A concentration of 400 IJ/insect larva was used. The plates were placed in lidded plastic containers, lined with moistened paper towels to guarantee the maintenance of high humidity (RH 95%). On day four all infected larvae were rinsed in tap water to remove any surface nematodes and placed in 9-cm-diameter Petri dishes lined with moist filter paper and incubated at 25 ± 2˚C in a growth chamber. Six larvae (one Petri dish) were removed every three days and dissected using a stereo microscope and observations of *H. zealandica* life cycle were noted. The first generation hermaphrodites of *H. zealandica* were counted to determine the number of IJ that penetrated.

Eight Petri dishes (3.5 cm diameter), with one *P. callosus* larva each were left undisturbed and placed on White traps (White, 1927) for 22 days in a closed container, to determine the IJ production per larva.

Data analysis

The results were analysed using Statistics 8.0 (Statsoft Inc., 2007). Analysis of the data obtained by means of the screening of *P. callosus* adults, the sand bioassay, the comparisons of different soils, the concentration trial and the effect of temperature, was achieved by using a one-way ANOVA with post-hoc comparisons of means. Bonferroni's method or a bootstrap multiple comparison was used, where residuals were not normally distributed (Efron & Tibshirani, 1993) such as the screening of *P. callosus* adults, the sand versus sandy loam soil trial, the nematode concentration trial and the effect of temperature on mortality trial. Data obtained from the concentration trial and the effect of temperature were additionally analysed using a probit analysis (Hintze, 2007).

Results

Screening

Not all nematode isolates tested against the *P. callosus* larvae were effective in killing them (Fig. 1). The mean percentage mortality rate attained by larvae after four days ranged between no infection to 75%. Only two species, *H. zealandica* (SF41) and *H. bacteriophora* (SF134), resulted in mortality

rates higher than 60% after four days. For the four *H.* zealandica isolates (J21, J34, J37, J182) no mortality of *P. callosus* larvae was obtained. The two *S. khoisanae* isolates (SF87, 106-C) obtained mortality rates of 25% and 16% respectively. All three of the *H. bacteriophora* isolates (159-C, J22, J84) obtained mortality rates of 33% each. *Heterorhabditis safricana* (J131) and *H. zealandica* (J92) caused mortality rates of 58% and 41% respectively.

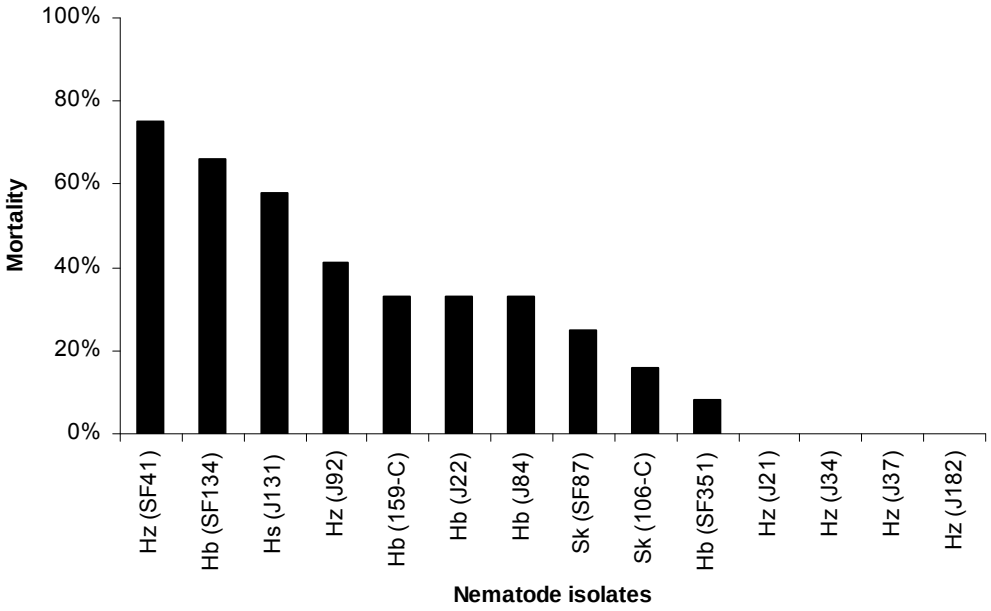

Fig. 1. The percentage mortality rate of *Phlyctinus callosus* larvae inoculated with *Heterorhabditis bacteriophora* (SF134, 159-C, J22, J84, SF351), *H. zealandica* (SF41, J92, J21, J34, J37, J182), and *Steinernema khoisanae* (SF87, 106-C), at a concentration of 400 IJ/50 µl, after a period of four days.

Adults

The percentage mortality rate for adult *P. callosus* (Fig. 2) ranged between 13% and 45% after a period of four days, at a concentration of 400 IJ/insect for *H. zealandica* (SF41), *S. khoisanae* (106-C) and *H. bacteriophora* (SF134). Whereas *H. bacteriophora* caused 45% mortality rate of adult weevils, the percentage mortality rate for *H. zealandica* mortality was 16%, with it being 13% for *S. khoisanae*. No mortality of adult weevils was found for the control after four days. Results from the three different test dates were analysed, using a two-way ANOVA. The interaction effects between the different dates and treatment proved to be insignificant ($F_{4, 9} = 2.36$; $p = 0.13$). The results, which were pooled

and analysed using a one-way ANOVA, indicated that *H. bacteriophora* differed significantly from *H. zealandica* and *S. khoisanae* ($F_{3, 20} = 26.581$; $p < 0.0001$), with no significant difference being found between *H. zealandica* and *S. khoisanae.* The mean number of IJ that was found to have penetrated *P. callosus* adults was 10 for *H. bacteriophora*, eight for *H. zealandica*, and six for *S. khoisanae.* An adult weevil infected with nematodes is shown in figure 3.

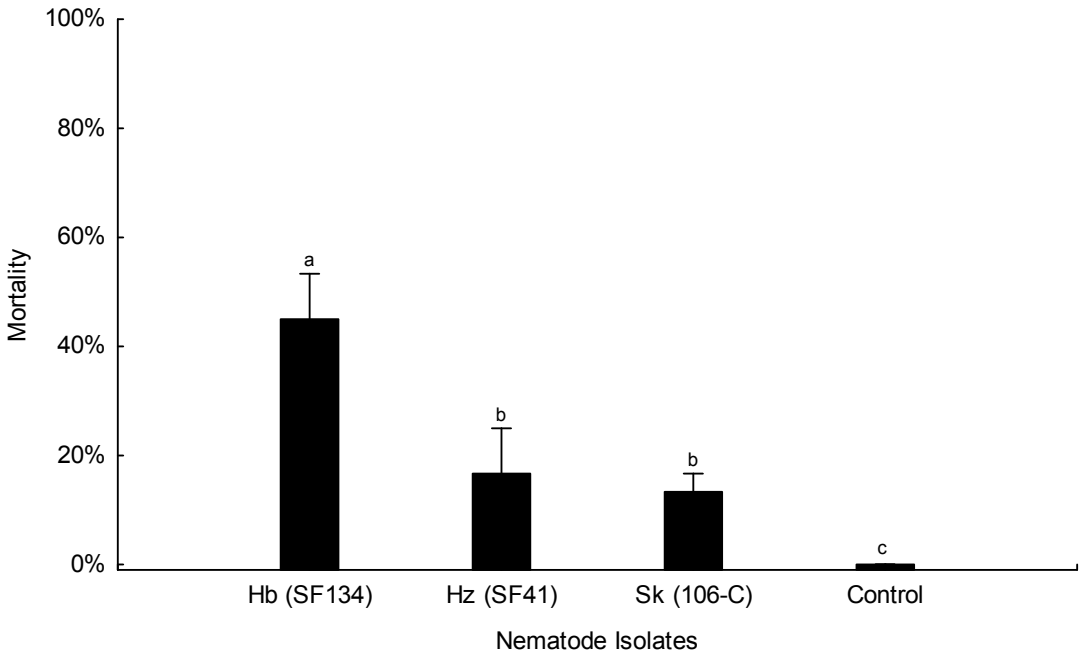

Fig. 2. The mean percentage mortality rate (95% confidence interval) of adult *Phlyctinus callosus* weevils inoculated with 400 IJ/insect of *Heterorhabditis bacteriophora* (SF134), *H. zealandica* (SF41) and *Steinernema khoisanae* (106-C) after four days (one-way ANOVA: $F_{3, 20} = 26.581$; $p < 0.0001$). Different lettering above vertical bars indicate significant differences.

Fig. 3. Dissected *Phlyctinus callosus* adult inoculated with nematodes.

Sand bioassay

The high percentage mortality rate for *P. callosus* larvae was obtained for the different depths of 0, 5, 10 and 15 cm, ranging between 77% and 85% (Fig. 4). No significant difference was found between the percentage mortality at different depths for *P. callosus* larvae, when a chi square test (df = 12; p = 0.59) was performed.

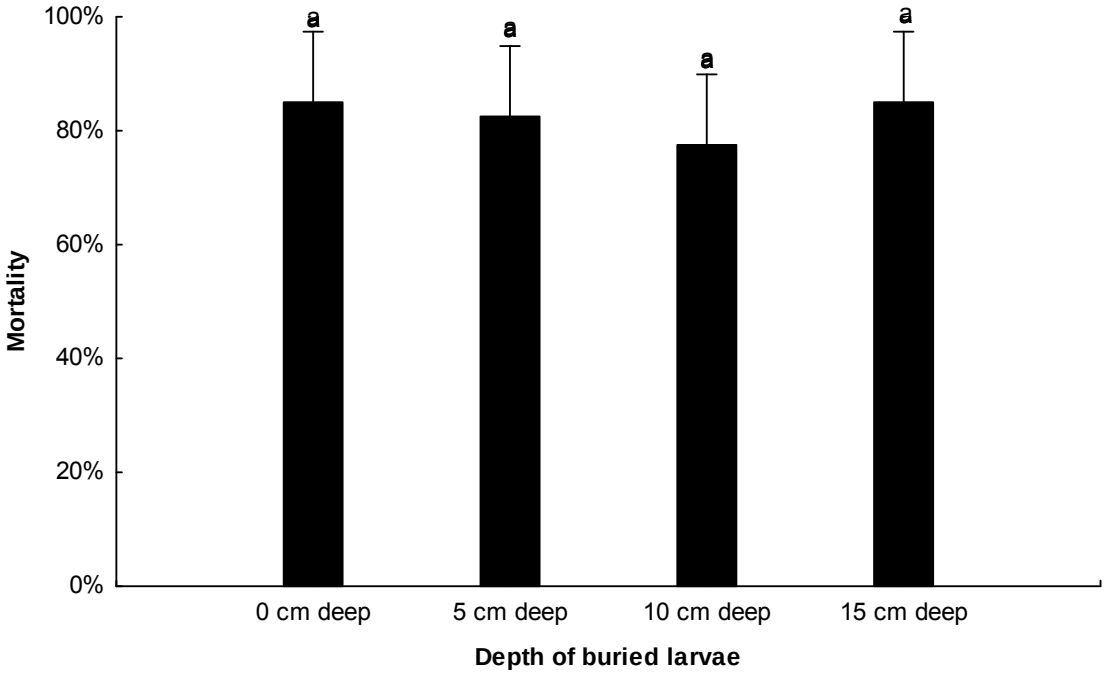

Fig. 4. The mean percentage mortality rate (95% confidence interval) obtained for *Phlyctinus callosus* larvae buried at four different depths of 15 cm, 10 cm, and 5 cm, as well as placed on the surface (0 cm) after inoculation of *Heterorhabditis zealandica* at a concentration of 400 IJ after seven days. Data were analysed using a chi-square test (df = 12; p = 0.59). Different lettering above vertical bars indicate significant differences.

Sand versus sandy loam soil

Soil obtained from the Welgevallen orchard, when analysed by means of a five-fraction analysis, was shown to contain a high percentage of fine sand (62.0% fine sand : 20.6% medium sand : 8.0 coarse sand : 6.0% silt : 3.4% clay), enabling its classification as a sandy loam soil.

The percentage mortality rate obtained for *P. callosus* larvae (Fig. 5) in coarse sand was 77.5%, compared to the markedly higher mortality rate of 95.2% obtained for sandy loam soil. The outcome for the sandy loam soil did not differ significantly from that obtained with the coarse sand (p = 0.105) when analysed by means of a one-way ANOVA. The rates of infected *P. callosus* larvae retrieved from the soil are indicated in figure 6.

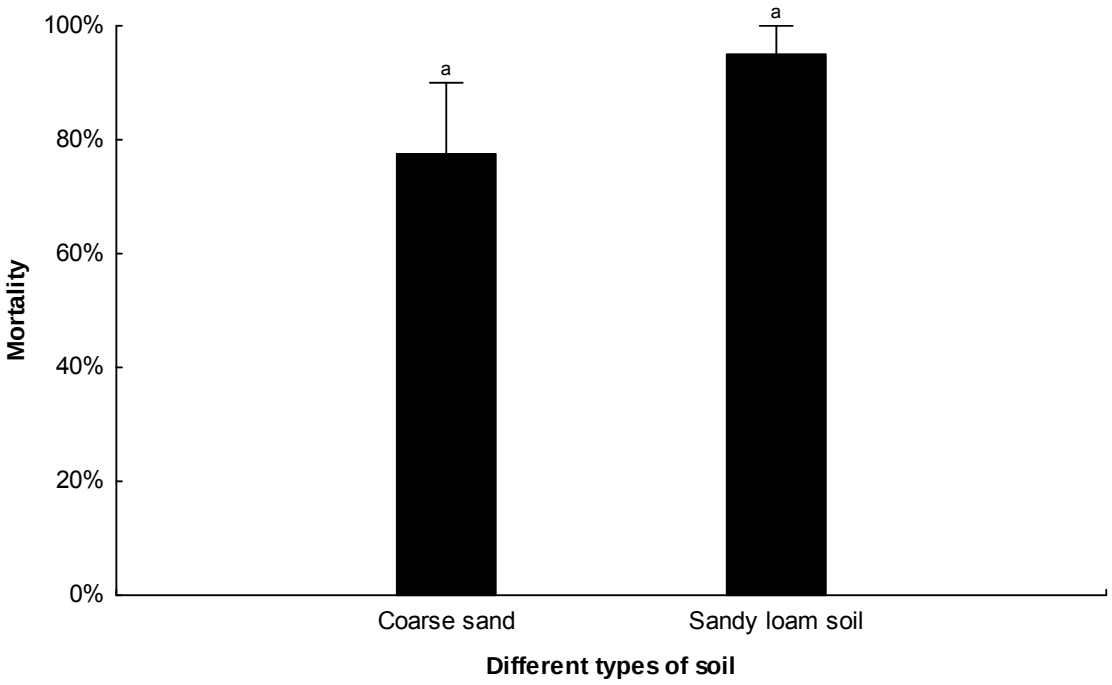

Fig. 5. The mean percentage mortality rate (95% confidence interval) recorded for *Phlyctinus callosus* larvae buried at 10 cm and inoculated with *Heterorhabditis zealandica* (SF41) at concentration of 400 IJ/100 µl and 1.4 ml water in sandy loam soil and coarse sand, after a period of seven days (one-way ANOVA; df = 1; p = 0.105). Different lettering above vertical bars indicate significant differences.

Fig. 6. Infected *Phlyctinus callosus* larvae inoculated with *H. zealandica* (SF41) and retrieved from the sandy loam soil after four days.

Nematode concentration

At a concentration of 200 IJ/50 µl of *H. zealandica*, 80% of the larvae were infected and killed by the nematodes after four days (Fig. 7). The percentage mortality rate recorded for the larvae ranged from 40% to 67% after two days, and from 80% to 100% after four days. After six days, the percentage mortality rate ranged between 82.5% and 100%. The data obtained were analysed using a one-way ANOVA ($df = 12$; $p < 0.01$). No significant difference was found after two days between the percentage mortality rate obtained for 200 IJ and that obtained for 400 IJ ($p = 0.298$), when the rates were compared between each respective dose. No significant difference was found between the percentage mortality rate obtained for 400 IJ and that obtained for 800 IJ either ($p = 1$). However, after a period of four days, a significant difference was found between the percentage mortality rate obtained for 200 IJ and that obtained for 400 IJ ($p = 0.038$), though no significant difference was found between the percentage mortality rate obtained for 400 IJ and that obtained for 800 IJ ($p = 1$) over the same period. However, after a period of six days, a significant difference was found between the percentage mortality rate obtained for 200 IJ and that obtained for 400 IJ ($p = 0.0119$), though no significant difference was found between the percentage mortality rate obtained for 400 IJ and that obtained for 800 IJ ($p = 1$) over the same period. A high percentage mortality rate, ranging from 17.5% to 30%, was found in the control treatments for the same periods.

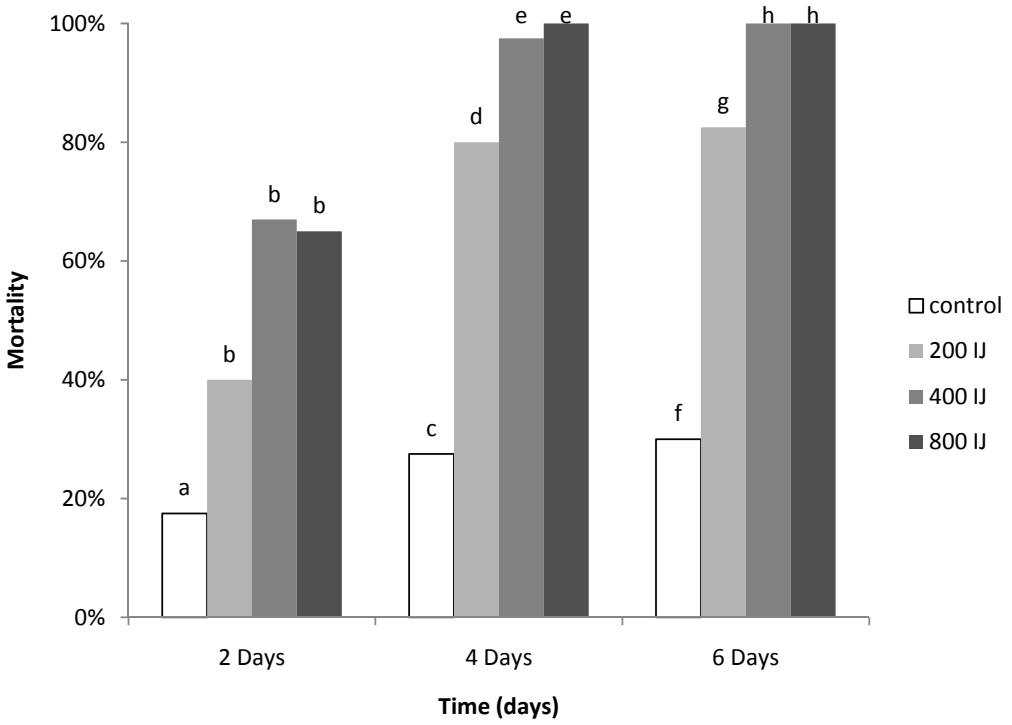

Fig. 7. The mean percentage mortality rate recorded for *Phlyctinus callosus* obtained after exposure to concentrations of 200, 400 and 800 IJ *of Heterorhabditis zealandica* (SF41) per larvae over a period of 2, 4 and 6 days (one-way ANOVA; df = 12; $p < 0.01$). Different lettering above vertical bars indicate significant differences.

The LD_{50} and LD_{90} doses over a period of two days were 235 and 12 677 IJ/50 µl, respectively. After a period of four days, the LD_{50} and LD_{90} doses changed to 96 and 278 IJ/50 µl respectively. After a period of six days, the LD_{50} and LD_{90} doses were 88 and 254 IJ/50 µl, respectively (Fig. 8).

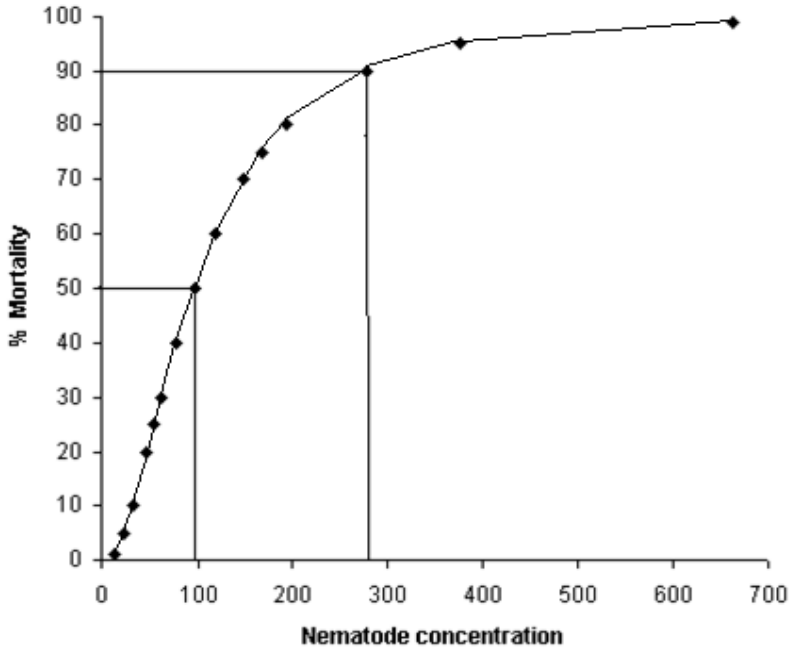

Fig. 8. LD_{50} and LD_{90} doses were 96 IJ and 278 IJ, after exposure of *Phlyctinus callosus* larvae to *Heterorhabditis zealandica* (SF41) for a trial period of four days, at 25˚C. Probit analysis software was used to analyse the data collected.

Effect of temperature on mortality

Nematodes were inactive below 15˚C, since no mortality for *P. callosus* larvae was observed. The percentage mortality for the larvae at the three different temperatures ranged between 0% and 70% (Fig. 9). The highest percentage mortality rate was observed at a temperature of 25˚C. Analysis was performed by means of a one-way ANOVA ($F_{2, 9}$ = 93.145, p = <0.01), with the percentage mortality rates all being found to differ significantly from one another when the nematodes were kept at a constant temperature of 11˚C, 15˚C and 25˚C. The control mortality rate using water as inoculum was 0% at all temperatures tested. Mortality of 50% was achieved at a temperature of 20˚C (Fig. 10) after four days, when using a concentration of 400 IJ per larva.

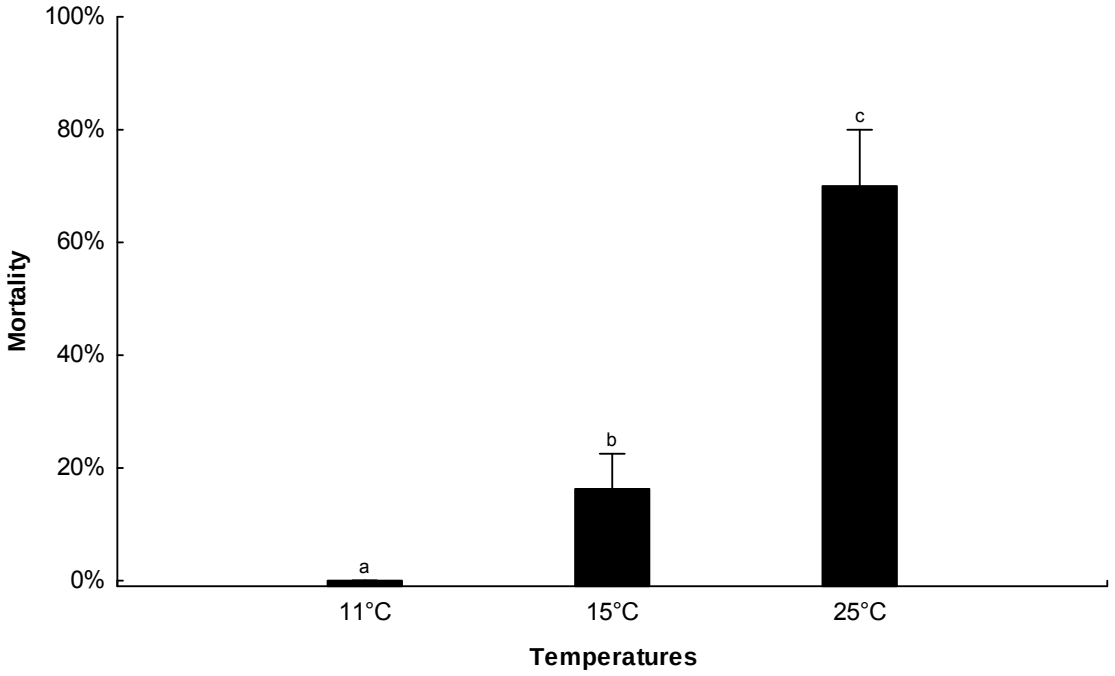

Fig. 9. The mean percentage mortality (95% confidence interval) for *Phlyctinus callosus* larvae after exposure to *Heterorhabditis zealandica* (SF41) at three different temperatures, and after inoculation with a concentration of 400 IJ over a period of four days (one-way ANOVA; $F_{2, 9} = 93.145$; $p = <0.01$). Different lettering above vertical bars indicate significant differences.

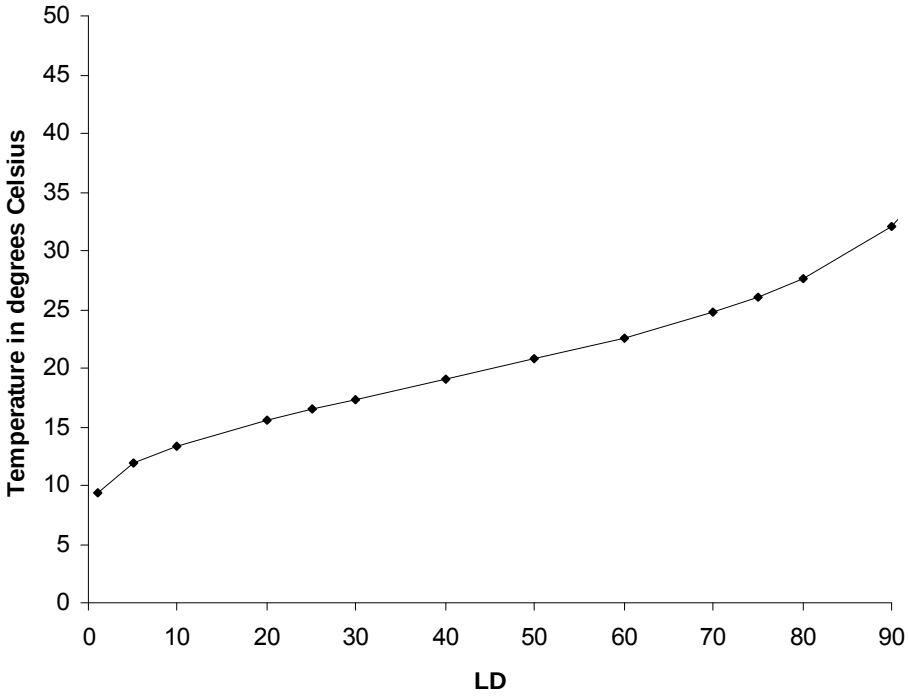

Fig. 10. The mortality rates were calculated for the different temperatures using a probit analysis, after exposing *Phlyctinus callosus* larvae for a period of four days to *Heterorhabditis zealandica* (SF41) at the specified temperatures. A concentration of 400 IJ/insect was used.

Biology of nematodes in *P. callosus* larvae

IJ of *H. zealandica* could not successfully infect and kill 1st instar larvae of *P. callosus*. Mortality of 74% was obtained after four days with 6th instar *P. callosus* larvae and the colouring of the host's body changed from a creamy white colour to brown. The number of hermaphrodites were counted until day 10, with a mean penetration rate of 48 IJ (Table 2). The time recorded from IJ entrance into the body until new IJ developed, was 16 days. During this period the nematodes passed through two generations. The first adult hermaphrodites were observed four days following the death of the insect. After seven days hermaphrodites were filled with larvae. By day 13 eggs were observed in females of the second generation. By the 16th day IJ started to develop. Females of the second generation and IJ were observed on day 19. On day 22 all IJ emerged and the cadavers were flattened and empty.

The mean IJ production of nematodes within *P. callosus* larvae were calculated and found to be 3300 per insect. The average size of the larvae used were 4.92 mm long and 1.45 mm wide. The mean width of the head capsule was found to be 84 µm indicating them to be the 6th larval stage.

Table 2. The biological development of *Heterorhabditis zealandica* in 6th instar larvae of *Phlyctinus callusus* exposed to a concentration of 400 IJ/insect for a period of four days at 25ºC in a growth chamber.

Number of days after inoculation	Stage of nematode development	Mean and range of IJ that penetrated (n=6)
4	Mostly 4th larval stage and hermaphrodites (some with eggs)	64 (49-102)
7	Hermaphrodites filled with larvae	28 (1-63)
10	Cuticle of hermaphrodites filled with larvae and developing second generation	53 (37-79)
13	Second generation males and females with eggs visible	-
16	Second generation females and infective juveniles	-
19	Infective juveniles and cuticle of the second generation females filled with larvae	-
22	Only infective juveniles	-

Discussion

Results obtained from the screenings showed *P. callosus* larvae to be susceptible to most nematode isolates tested. The larvae were found to be more susceptible than the adults to nematode infection. The adults were perhaps less susceptible, as a result of the insects exhibiting a grooming behaviour typical of Coleoptera (Gaugler et al., 1994; Koppenhöfer et al., 2000). *Heterorhabditis* isolates were found to be more effective in killing *P. callosus* larvae than were the *Steinernema* isolates. A possible explanation for such effectiveness might be the possession of a dorsal tooth by *Heterorhabditis*, which *Steinernema* lacks. The possession of such a tooth enables the nematode to enter directly into the host insect by abrasion of the thin parts of the cuticle. *Heterorhabditis* isolates were also successfully used in previous research, in which the susceptibility of the black vine weevil to entomopathogenic nematodes was examined (Simons, 1981; Lola-Luz et al., 2005; Lola-Luz & Downes, 2007). *Heterorhabditis* isolates are not always superior to *Steinernema* as seen in research done by Shapiro

and McCoy (2000). The objective of their research was to look at nine entomopathogenic species and 17 strains for the use against *Diaprepes abbreviatus*, which is a severe weevil pest of citrus in Florida. The species and strains tested were *H. bacteriophora* Poinar (Baine, NJ1, Hb, Hbl, HP88, and Lewiston strains), *H. indica* Poinar, Karunakar & David (original and Hom1 strains), *H. marelatus* Liu & Berry (IN and Point Reys strains), *H. megidis* Poinar, Jackson & Klein (UK211 strain), *H. zealandica* Poinar (NZH3 strain), *S. feltiae* (Flipjev) (SN and UK76 strains), and *S. glaseri* (Steiner) (NJ43 strain). The study was conducted at three temperatures namely 20˚C, 24˚C and 29˚C. At all three temperatures the greatest mortality was caused by *S. riobrave* and the results suggest that this specie has the greatest potential to control *D. abbreviatus* (Shapiro & McCoy, 2000).

The nematode isolate that was capable of causing the highest levels of mortality under controlled conditions for *P. callosus* was *H. zealandica* (SF41), which was further examined in additional bioassays in the laboratory. The same isolate of *H. zealandica* was also found to be the most effective against codling moth, as well as against false codling moth larvae (De Waal, 2008; Malan & Addison, 2008). Research done by De Waal (2008) proved *H. zealandica* (SF41) to be the most promising isolate for the control of codling moth larvae by initially screening 22 nematode isolates. The selected isolate was further evaluated by investigating optimum temperature, humidity and concentration. High mortality of codling moth larvae were obtained when temperatures were between 20˚C and 25˚C, relative humidity higher than 95% and a concentration of 160 IJ/ml (De Waal, 2008).

A different *Heterorhabditis* species was used in a previous study, in which *P. callosus* was controlled with entomopathogenic nematodes. Prestidge and Willoughby (1990) found that the weevil larvae and pupae were extremely susceptible to invasion by such nematodes. Research into the use of nematodes for the control of *P. callosus* has previously been undertaken in South Africa. Whereas *S. carpocapsae* (Wouts, Mracek, Gerdin & Bedding, 1982) were imported into South Africa from Biosys in California, *S. feltiae* (Wouts, Mracek, Gerdin & Bedding, 1982) were imported from Koppert in Holland in 1993 (Basson, 1993). Results showed that both of these species have the potential to control *P. callosus* larvae (Basson, 1993).

Heterorhabditis zealandica act as cruisers. Nematode isolates that are classified as intermediate or cruisers are expected to perform better than ambushers, when the insect hosts are sedentary below the soil surface (Bell, 1991; Huey & Pianka, 1981), as is the case with *P. callosus* larvae and pupae.

In the present sand bioassay trial, in which a number of different *P. callosus* larvae were buried at four different depths, the IJ were found capable of easily moving down to the lowest depth of soil within seven days of inoculation. Nguyen and Smart (1990) obtained comparable results in their earlier study of *S. scapterisci*. Basson (1993), when working on *H. megidis* and *S. carpocapsae*, obtained similar results, finding that both nematode species in their study penetrated the soil for at least 10 cm. The vertical downward (15 cm) movement of the nematodes tested in the current study was adequate to encounter *P. callosus* larvae in soil, considering the vertical distribution of the weevil reported by Barnes (1989).

Sandy loam soil has higher moisture retention than does coarser sandy soil, making the former a more suitable medium for mobility and infection of nematodes, possibly contributing to the good IJ performance found in the former type of soil (Kaya, 1990; Molyneux & Bedding, 1984). Moisture is a key factor in nematode survival (Wright et al., 2005), with heterorhabditids being known to be more susceptible to desiccation than are steinernematids (Surrey & Wharton, 1995). Although no significant differences were found between the mortality rates for larvae in the sandy soil and in the sandy loam soil, a markedly higher percentage mortality rate was found for *P. callosus* larvae in the latter type of soil.

In the concentration trial, a positive relationship was found between the concentrations of IJ and the percentage of larval mortality recorded after a period of four days. The LD_{90}-value was then found to be 278 IJ/50 µl after four days, giving a good indication of a field concentration with acceptable levels of control. The research undertaken by De Waal (2008) showed that 275 IJ/ml as an LD_{90} dose effectively controlled codling moth larvae after two days. Prestidge and Willoughby (1990) obtained satisfactory results in the laboratory by using 10 to 15 nematodes in 25 µl suspension for each *P. callosus* larva, which were far fewer than the number of IJ used in the current study. During the study they used *H. bacteriophora.* A pot experiment was conducted where twenty *P. callosus* larvae were placed in honey pots filled with 150 ml of Horotiu sand. Three concentrations of $5x10^3$, $50x10^3$ and $25x10^3$ nematodes were applied to the soil surface. The soil was then drenched with a further 12.5 ml of distilled water. After 28 days the percentage mortality were 55%, 70% and 80% for $5x10^3$, $50x10^3$ and $25x10^3$ respectively. The mean soil temperature during this experiment was 18.5˚C. It is clear from this study that the larvae are susceptible to nematode infection and a field trial would shed more

light on the possibility of using nematodes for the control of *P. callosus* larvae (Prestidge & Willoughby, 1990).

The results found in the current study suggest that the ideal time of year for applying the nematodes in order to achieve *P. callosus* control would be during winter and early spring. The larvae, which tend to be present in the soil during this time of year, are also at the stage during which they are most susceptible to targeting with entomopathogenic nematodes. In South Africa, during winter and early spring, temperatures are generally low, which might prove to be a challenge that must be overcome to ensure successful nematode application. Cool temperatures affect both host-finding ability and the rate of infection by entomopathogenic nematodes (Lacey & Unruh, 1998; Vega et al., 2000). In the current study, the performance of nematodes was found to be very poor at 11˚C. Nematodes start becoming inactive at temperatures below 15˚C. Koppenhöfer (2000) found that when the temperature rose above 35˚C, the IJ became inactive, whereas at temperatures between 30˚C and 40˚C, the nematodes were killed. Consequently, when the nematodes are applied in the field, the optimum time for such application would be when the ambient temperatures range between 20˚C and 25˚C. As *P. callosus* larvae are present in the soil during winter and early spring, nematodes should preferably be applied when the daytime temperature exceeds 15˚C.

The development and completion of the life cycle of *H. zealandica* in 6th instar larvae of *P. callosus* was found to be successfully concluded. The developmental rate of the nematodes in *P. callosus* was found to closely resemble that of *H. bacteriophora* in *G. mellonella*, though occurring at a slightly slower pace (Aguera De Doucet et al., 1996). As *H. zealandica* was found incapable of infecting the 1st instar *P. callosus* larvae, the size of the host is likely to determine the ability of IJ to infect and kill it (Kakouli-Duarte & Hague, 1999).

The current study showed that *P. callosus* larvae are suitable hosts for *H. zealandica*. The selected isolate might be used to control *P. callosus* in an integrated pest management programme. Further research into and development of such control methods is necessary, as there is much potential in using the specified type of nematode. However, *P. callosus* larvae were found to not be as susceptible to nematode infection in general, compared with the larvae of codling moth and false codling moth, requiring double concentrations and amount of time to give comparable results.

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Chapter 4

Persistence of *Heterorhabditis zealandica* (Poinar 1990) (Heterorhabditidae) in a blueberry orchard infested with *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)

Abstract

Persistence of a *Heterorhabditis zealandica* isolate SF41 at different concentrations was evaluated in a blueberry orchard with a high infestation of *Phlyctinus callosus*. Concentrations of 0, 20, 30 and 45 infective juveniles (IJ)/cm^2 were topically applied in a complete randomised block design, with persistence being evaluated on days 1, 35 and 84 after application. On both days 35 and 84, the percentage persistence rate was, calculated as being 87.5% for 30 IJ/cm^2. Persistence in soil samples taken on day 1 and kept in plastic containers at room temperature was again evaluated on day 128. Both 30 IJ/cm^2 and 45 IJ/cm^2 were found to cause 100% mortality of *Tenebrio molitor*. Nematode infection by endemics was found in the control samples, and identified by means of molecular techniques as being that of *H. bacteriophora*. The results of the current study indicate a good persistence of *H. zealandica* after 84 days under field conditions where a large *P. callosus* population was present. The study indicated the potential use of *H. zealandica* for the control of *P. callosus*, with the possibility of persistence for a period of at least three months.

Introduction

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae) originated in Africa, from where it spread to other continents. The pest is regarded as economically important in pome, stone, grapevine, apple and nectarine orchards in the south-western area of the Western Cape province, South Africa (Myburgh et al., 1973; Annecke & Moran, 1982; Barnes & Pringle, 1989). Though numerous crops have been found to be significantly damaged by *P. callosus*, the adults have been identified as causing most damage to the above-ground parts of the plants (De Klerk, 1981; Barnes, 1989). Those deciduous fruit orchards located in the south-western area of the Western Cape province are the only site in South Africa where *P. callosus* is the main curculionid, causing major damage to apples and nectarines (Barnes, 1989).

In the past, good control of snout beetles, including *P. callosus,* was maintained by means of the cautious use of DDT. Since the prohibition of the use of DDT, the pesticide has been replaced with acephate, as well as a variety of synthetic pyrethroids (Barnes et al., 1994). Such products have to be applied with caution in order to obtain good results, especially keeping in mind the tendency of weevils to retreat out of sight during the day (Barnes & Swart, 1977). Research which has been undertaken since the advent of currently used pesticides (Barnes & Knipe, 1994) has shown the development in *P. callosus* of a high degree of tolerance to pyrethroids. Cross-tolerance to acephate is also suspected.

Another method of controlling adult weevils is that of using trunk barriers (Barnes et al., 1996). However, such a cultural method is very labour intensive and time consuming.

The control of soil-inhabiting pests by entomopathogenic nematodes has previously been documented (Gaugler, 1981; Kaya, 1985; Klein, 1988; Capinera & Epsky, 1992). The use of nematodes to manage *P. callosus* is possible, because both the larval and pupal stages of the weevil have been found to be present in soil (Barnes, 1989) and in general, weevils (Coleoptera: Curculionidae) have proved to be extremely susceptible to entomopathogenic nematodes (Klein, 1990). The black vine weevil, *Otiorhynchus sulcatus* (Fabricius), has been successfully controlled with *Heterorhabditis* both indoors, in a greenhouse (Bedding & Miller, 1981; Simons, 1981; Georgis & Poinar, Jr., 1984; Stimmann et al., 1985), and outdoors, in a field (Shanks & Agudelo-Silva, 1990).

Wilson et al. (2003) have applied *H. bacteriophora* for the control of scarab beetle larvae (Scarabideae). The nematodes were applied in three different spatial distributions: uniform; one central circular patch; and nine individual patches. The results showed that, in all the treatments, the nematode levels declined to the same level. No significant differences were observed in the nematode numbers or spatial patterns concerned. Grub numbers were significantly reduced in cases where the nematodes were applied uniformly to nine individual patches, but no significant reduction was achieved when the nematodes were applied to only one patch. Therefore, spatial distribution do not have an effect on the persistence of nematodes in the soil but it does have an effect on the level of control that you can achieve (Wilson et al., 2003).

Susurluk and Ehlers (2008) investigated the persistence of *H. bacteriophora* Poinar ,1976, in different crops. The nematodes were applied to pasture, potato, wheat, pea, oil-seed and lupine crops. The results obtained showed that the more that the plant canopy was developed, the less the nematodes appeared able to reach the soil. In the pasture and potato fields, 77% and 78% of applied nematodes, respectively, were found to have reached the soil. In the wheat and pea fields, less than half of the nematodes applied reached the soil. Only 5%-6% of the nematodes were found to have reached the soil in the oil-seed field and the lupine fields. After the fields were tilled, the number of soil samples testing positive for EPN decreased substantially.

According to Smits (1996), five phases can be distinguished from one another in the post-application persistence of entomopathogenic nematodes. Each phase is beset by a definite set of mortality factors. The survival rate of the nematodes before application is influenced by such factors as production, storage and transport conditions. Infective juveniles (IJ) are fairly tolerant to shear forces, so that application with a sprayer does not tend to harm them. Critical periods for survival are during the first few hours after spraying. Losses of 40% to 80% were recorded during this phase. Mortality factors, such as ultraviolet radiation and dehydration, are significant in nematode survival. The nematodes that remain in the soil have been found to decrease by 5% to 10% a day. Mortality factors that have been found to play a role during this stage are predation, infection by antagonists, depletion of energy, and desiccation. In many cases, after 2 to 6 weeks, less than 1% of the applied nematodes were found to remain. At such levels, the nematodes can persist for years in the soil by recycling themselves in host insects (Smits, 1996).

Despite the numerous successes that have been achieved with field trials using entomopathogenic nematodes for the control of the soil stages of insect pests, consistent control of such pests has not yet been achieved (Kaya, 1990). To achieve consistent control of an agricultural pest, an understanding of the ecological role played by nematodes is important (Fuxa, 1987; Kaya, 1990). In the current study, the persistence of different concentrations of *H. zealandica* was evaluated in a blueberry orchard, infested with a high *P. callosus* population.

Materials and Methods

Source of insects

Larvae of *Tenebrio molitor* (L) were raised on wheat bran in a closed, ventilated wooden box. Vegetable peels placed on the surface provided the necessary humidity (Woodring & Kaya, 1988).

Source of nematodes

An endemic isolate, SF41 of *H. zealandica* (EU699436) (Malan et al., 2006), was used, as it had been shown to be the most promising isolate for the control of *P. callosus* in previous laboratory bioassays (Chapter 3). Nematode inoculum was cultured in the laboratory on *T. molitor* (L.). The IJ were reared at room temperature, according to the procedures devised by Kaya and Stock (1997). The IJ were stored horizontally at 14˚C in 500 ml flat culture flasks. The flasks, which contained 150 ml filtered water, were shaken weekly for aeration. The nematodes were used within one month of harvesting. The IJ concentrations were quantified in the laboratory, using the procedures described by Kaya and Stock (1997).

Experimental orchard layout

The field trial was conducted in a blueberry orchard on the farm Zuurvlakte outside Porterville in the south-western area of the Western Cape province, South Africa. Straw was used as mulch in each blueberry bush row. A complete randomised block design was used for the experimental layout, consisting of four treatment rows, separated from one another by an untreated row. Each treatment row contained two blocks, consisting of four treated blueberry bushes, and allowing for the presence of two buffer bushes between all of the treated bushes. Thus, the trial consisted of four treatments with eight replicates of each treatment. The area that was treated underneath each blueberry bush was $1 \times 3\ m^2$.

Weather data

The temperature was recorded by a weather station that was located on the farm on which the field trial took place.

Application method

The different nematode treatments were concentrated in a small volume of water, poured onto nylon batting, and placed in plastic containers kept at a constant temperature of 11˚C the day before application. The nematodes were transported in the containers, which were kept in a cooler box, over a distance of 120 km to the specified field. Applications were made using shoulder-pump sprayers. In the field, the nematodes were washed from the batting into buckets containing water. The contents were poured into sprayers filled with the required volume of water. The contents were continuously agitated throughout the application period to ensure that the nematode suspension was aerated and that sedimentation did not take place. Three different IJ concentrations of 20 IJ/cm^2, 30 IJ/cm^2 and 45 IJ/cm^2 were applied, as well as the control water alone. The trial was conducted late in the afternoon to avoid the worst effects of ultraviolet (UV) radiation. There is a drip irrigation system in the orchard which kept the soil moisture fairly constant, thus water were not additionally sprayed after nematodes were applied. No pesticides or fertilizer were applied in the rows that were treated with nematodes. Pesticide were however applied in the rows adjacent to those that were sprayed with nematodes.

Method used in assessing persistence

Soil samples were individually taken from beneath the treated bushes on the following morning which was called the morning of day one, as well as on days 35 and 84. Soil samples were also taken on day 242. Only one soil sample per treatment were taken. In the orchard, a separate spade was used for the extraction of the control samples to prevent contamination. The soil-baiting technique, using *T. molitor* as the host, was used for trapping the nematodes and five *T. molitor* were used per soil sample. The original soil samples taken on day one were kept in plastic containers at room temperature, and again baited on day 128. *Tenebrio molitor* larvae were recovered from the soil after a period of 14 days, during which the samples were kept at a constant temperature of 25˚C. The larvae were then dissected to confirm that they had been infected with nematodes (Curran & Heng, 1992).

Data analysis

All statistical analyses were performed using Statistica 8.0 (Statsoft Inc., 2007). The persistence of nematodes over time and treatments was compared. As the responses were not normally distributed, three separate Kruskal-Wallis (Maritz, 1995) tests were undertaken, with one being undertaken on each separate test date. In order to allow for the degree of persistence over days to be reflected in one graph, a bootstrap multiple comparison was made.

Persistence after 128 days was analysed using a one-way ANOVA. As the residuals were not normally distributed, a Kruskal-Wallis test was done.

Results

Weather data

The maximum temperature recorded for the first 84 days after spraying was 34.8˚C, whereas the minimum temperature was 6.8˚C. The mean temperature recorded for the 84 days was 17.57˚C (Fig. 1).

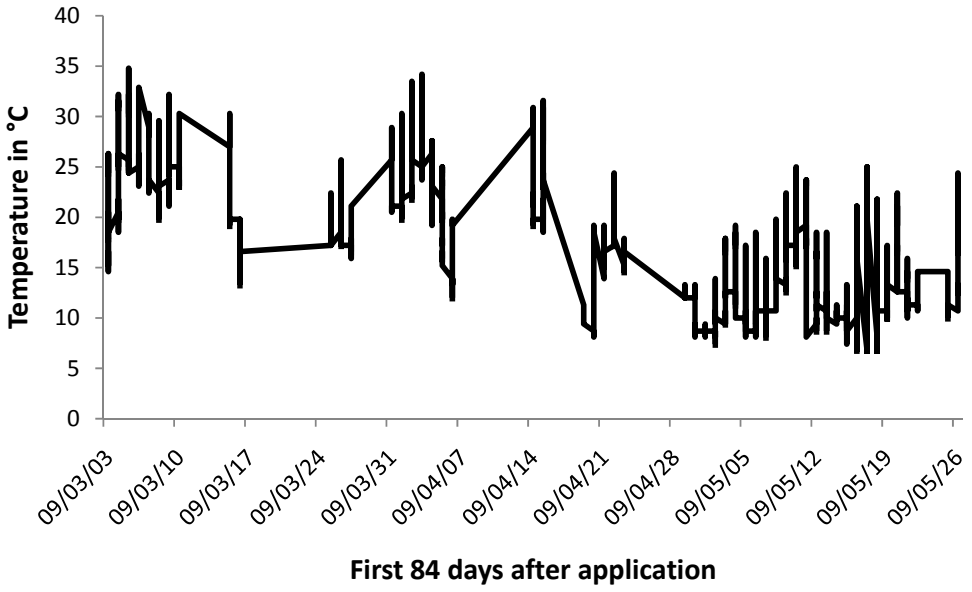

Fig. 1. Temperature recorded in an orchard for the first 84 days after treatment, as shown at weekly intervals, after application of nematodes.

Persistence

Persistence was estimated from the mortality of *T. molitor* in the days after treatment with various nematode concentrations (Fig. 2). On day one, the persistence of the nematode treatments was 100%. However, use of the control treatment led to 12.5% mortality rate, caused by nematode infection. After 35 days of being subjected to a concentration of 20 IJ/cm^2, 62.5% of the insects were found to have been infected. For both concentrations of 30 IJ/cm^2 and 45 IJ/cm^2, 87.5% infection rate was obtained. No significant difference in the levels of persistence was found between the samples subjected to 30 IJ/cm^2 and 45 IJ/cm^2, though both differed significantly from 20 IJ/cm^2. On day 84, the three nematode concentrations of 20 IJ/cm^2, 30 IJ/cm^2 and 45 IJ/cm^2 experienced 50%, 87.5% and 37.5% mortality rate respectively. The persistence levels all differed significantly from one another according to the nematode concentrations that had been applied. Significant differences were found between treatments for day one ($H_{3, 32}$ = 17.577; p = 0.0005), day 35 ($H_{3, 32}$ = 26.41; p = < 0.0001), and day 84 ($H_{3, 32}$ = 29.096; p = < 0.0001). The control treatment obtained a mortality rate of 25% for *T. molitor* on both days 35 and 84. Molecular analysis showed the control samples to have been infected with *H. bacteriophora*.

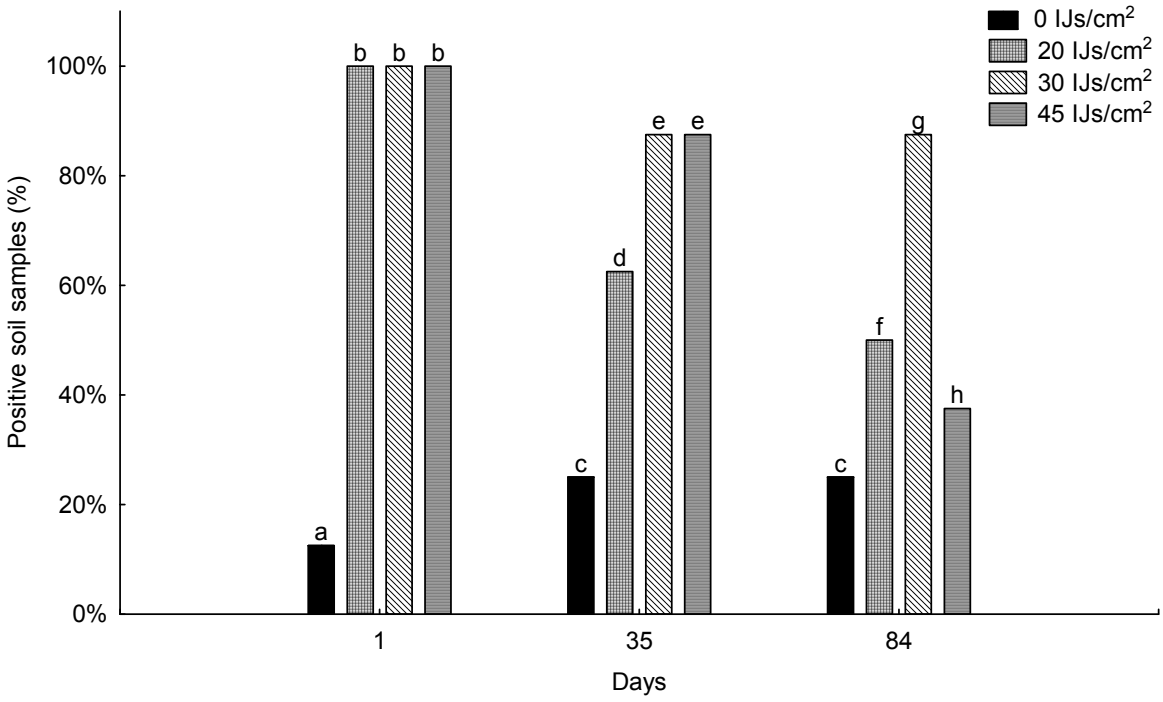

Fig. 2. Percentage persistence (95% confidence interval) after 1, 35 and 84 days for different concentrations of *Heterorhabditis zealandica* (SF41) in a blueberry orchard (in terms of a non-parametric one-way ANOVA for the days concerned: $H_{3,\ 32}$ = 17.577, p = 0.0005; $H_{3,\ 32}$ = 26.41, p = < 0.0001; $H_{3,\ 32}$ = 29.096, p = < 0.0001). Different lettering above vertical bars indicate significant differences.

Original samples, taken the morning after treatment (eight hours after application) and kept at constant room temperature in the laboratory, were again tested for persistence 128 days later (Fig. 3). The mortality rate found with a concentration of 20 IJ/cm^2 was 37%, and with concentrations of both 30 IJ/cm^2 and 45 IJ/cm^2, was found to be 100%. On day 128 the control samples showed 12.5% mortality rate for *T. molitor*. An analysis was undertaken using a one-way ANOVA ($H_{3,\ 32}$ = 26.79; p = < 0.0001). After 128 days had passed, no significant difference was found between the 30 IJ/cm^2 and 45 IJ/cm^2 (p = 1) samples, though both of the concentrations were found to differ significantly from the samples subjected to 20 IJ/cm^2 (p < 0.0001).

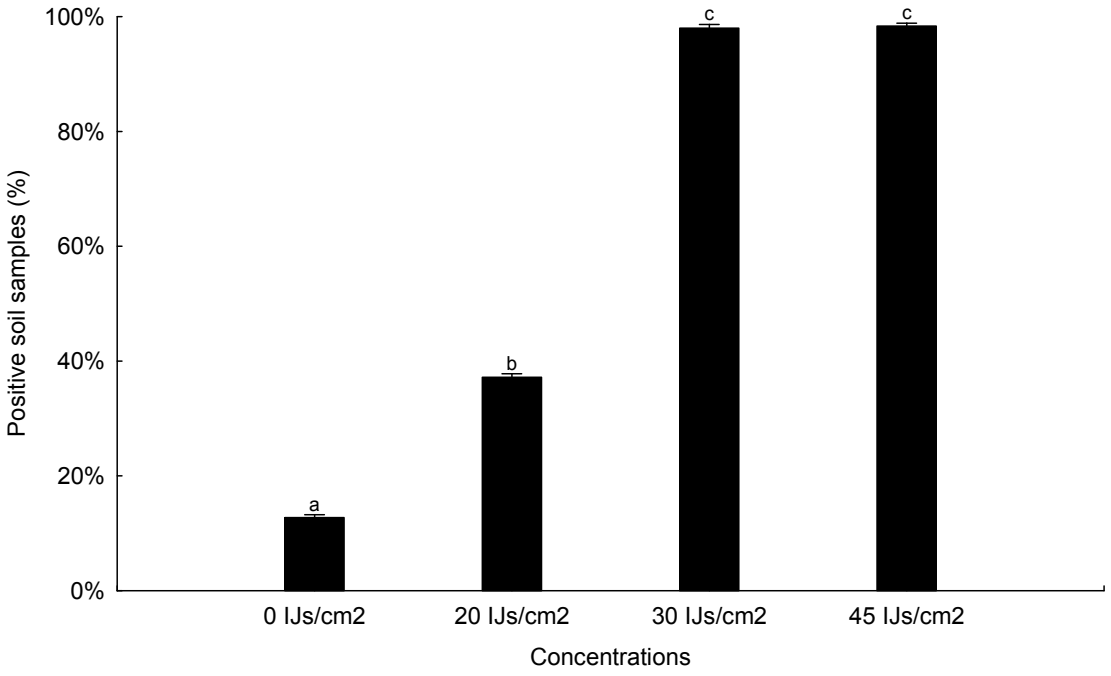

Fig. 3. The percentage persistence rate (95% confidence interval) found for different concentrations of *Heterorhabditis zealandica* (SF41) on day 128 (non-parametric one-way ANOVA: $H_{3, 32} = 26.79$; $p = < 0.0001$). Different lettering above vertical bars indicate significant differences.

Discussion

The current study focused on the persistence of *H. zealandica*, inundatively applied in a blueberry orchard subject to high levels of *P. callosus* infestation. Over time, there were significant differences in nematode survival between treatments containing concentrations of 0 IJ/cm^2, 20 IJ/cm^2, 30 IJ/cm^2 and 45 IJ/cm^2.

From days one to 35, a loss of ± 37 % was found in the mortality rate of *T. molitor* for the 20 IJ/cm^2 treatment applied. For both the 30 IJ/cm^2 and the 45 IJ/cm^2 treatments, a rate of only 12.5% mortality of *T. molitor* was found after 35 days had passed. The decrease in the levels of nematode persistence found may be attributable to the higher than normal concentration of nematodes in the soil, which can cause a strong food web response. Organisms such as nematophagous fungi, *Paenibacillus* spp., soil-borne nematodes, microarthropods and enchytraeid worms, prey on entomopathogenic nematodes, as well as competing with them (Duncan et al., 2007). Mortality factors could also include

depletion of energy, desiccation (Smits, 1996) and natural longevity (Shapiro…). An increase in the antagonists of entomopathogenic nematodes have the ability to reduce the number of endemic nematodes temporarily, as a result of the growth in the antagonistic population. Such an occurrence can sometimes reduce the natural mortality levels of weevil larvae temporarily until the natural equilibrium between EPNs and their antagonists is re-established (Duncan et al., 2003; El-Borai et al., 2007).

Soil samples taken on day one, which were kept in plastic containers at constant room temperature, were again baited on day 128. A 100% mortality rate for *T. molitor* was obtained for both 30 IJ/cm^2 and 45 IJ/cm^2, with 37% persistence rate being obtained for 20 IJ/cm^2. There were similarities between the results obtained from the current study and the research undertaken by Ferguson et al. (1995), though higher levels of persistence were obtained with the latter study. The researchers compared four nematode isolates, namely *H. bacteriophora* Poinar 1976 (Oswego), *H. bacteriophora* (NC), *S. carpocapsae* Weiser 1955 (NY001), and an undescribed *Steinernema* species (NY008-2E). The results showed that all the nematode isolates persisted, with no significant reduction in the percentage levels of infection of *G. mellonella* for six months after they had been applied to the soil. In the current study, high levels of persistence were obtained for three months after such application. In the study of Ferguson et al. (1995), seventeen months after application a significant reduction in infection rates was observed, except for that of *H. bacteriophora* (Oswego) which still showed high levels of infection. No persistence was observed after eight months of the current study, which was undertaken in the blueberry orchard.

Soil samples were taken on day 242, when spring temperatures started to rise. The reason for no persistence being obtained for all three nematode concentrations applied might have resulted from the low soil temperatures encountered during the winter, combined with the limited number of insect hosts, which would otherwise have enabled nematode recycling (Georgis & Gaugler, 1991). No effective recycling was found to have taken place during the winter.

The field trial was undertaken in March 2009 at the beginning of autumn, as temperatures were starting to decrease. During the first 84 days of the trial, the highest temperature recorded was 34.8˚C, whereas the lowest recorded was 6.8˚C. The average temperature recorded during the first 84 days was 17˚C. Zuurvlakte is located in the Groot Winterhoek Mountains outside Porterville, and,

as a result, reaches low temperatures during the evening. At low temperatures, nematodes become sluggish, whereas at higher temperatures, ranging from 30˚C to 40˚C, nematodes become inactive (Koppenhöfer, 2000). As the nematodes were in the soil, the degree of moisture to which they were subject remained fairly constant, due to the use of drip irrigation in the orchard. Drip irrigation, combined with the straw mulch layer placed on top of the soil, might have contributed to the good levels of persistence obtained (Koppenhöfer, 2000).

The current study showed good persistence of *H. zealandica* isolate SF41, when it was applied as a biological control agent in an orchard. Levels of persistence are influenced by such factors as the degree of temperature, relative humidity, and soil moisture, as well as the concentration of nematodes that is applied. Though the temperature in the orchard fluctuated after the nematodes had been applied, as the nematodes were present in the soil, which acted as a buffer, it is assumed that they were not negatively affected.

Similar research, undertaken by Susurluk and Ehlers (2008), showed that low temperatures did not eradicate *H. bacteriophora* from the soil. They found that *H. bacteriophora* persisted 23 months after release in a field of beans, with long-term persistence in the field being attributed to the recycling of nematodes in the insect hosts. Such reproduction is, however, restricted once the host population becomes unavailable, with the nematodes disappearing after being released.

In the current study, the soil moisture level was kept constant by irrigation. As no significant difference was detected between the persistence of 30 IJ/cm^2 and that of 45 IJ/cm^2 on day 35, combined with the fact that those samples subjected to 30 IJ/cm^2 showed a higher degree of persistence on day 84, the concentration should be sufficient for orchard application to be an appropriate means of control. When nematodes are applied to the soil, their numbers tend to increase temporarily, but then start to decline after a period of time (Smits, 1996). In addition, the necessity for nematodes to recycle frequently in insect hosts partly explains why they are not harmful to the soil ecosystem. Thus, the favourable traits of nematodes as a selective biological control agent for pest insects, such as *P. callosus*, are numerous, and the possibility of causing negative effects on non-target hosts has been found to be minimal (Smits, 1996).

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Chapter 5

General conclusion

The overall aim of this study was to evaluate the potential of entomopathogenic nematodes (Rhabditidae: Steinernematidae and Heterorhabditidae) to control *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae). Laboratory reared larvae of *P. callosus* were needed to do laboratory and field trials and as soon as enough adults were collected, the study embarked upon selecting the most optimal larval rearing technique. The most promising entomopathogenic nematode species and isolate for the control of *P. callosus* was selected by testing it under field-simulated conditions in the laboratory. Lastly, the selected isolate was applied in the field for the purpose of looking at persistence.

Rearing of *Phlyctinus callosus*

No artificial diet for the successful rearing of *P. callosus* is available. The diets that were tested did not perform as well as would be hoped. The highest percentage survival obtained for the agar diet was 50% and the highest for the carrot supplemented diet was 60%. Cold storage of *P. callosus* eggs was investigated as this can be of benefit to researchers. Egg hatching can also be delayed at certain temperatures, which is a added tool for researchers. The aim of this study was to assess an artificial diet for *P. callosus*. As a result of struggling with the rearing part of the research, the bioassays with the nematodes were influenced and placed under strain. There was however a rearing method developed that was successful in the end which was the full grown carrots planted in pots with sterilised sand, kept at 25˚C for five weeks. The mean percentage survival rate of larvae was found to be 87.5%.

Potential of entomopathogenic nematodes to control the banded fruit weevil, *Phlyctinus callosus* in laboratory bioassays

A total of 14 entomopathogenic nematode isolates were evaluated using a fast screening method for their potential as biological control agents for *P. callosus*. The susceptibility of *P. callosus* larvae to entomopathogenic nematodes were assessed and three nematode isolates were able to cause

infection levels of more than 50%. Only two species *H. zealandica* (SF41) and *H. bacteriophora* (SF134) caused mortality higher than 60%. When the susceptibility of *P. callosus* larvae were compared to codling moth larvae it was evident that *P. callosus* larvae were less susceptible to nematode infection but was still a suitable host for *H. zealandica.* Possible reason for this is that codling moth larvae is an above-ground pest and did not evolve with the nematodes in the soil, as *P. callosus* larvae did. An isolate that was able to cause sufficient mortality was the SF41 isolate *H. zealandica* and this isolate might be used to control *P. callosus* in an integrated pest management programme. Results obtained from this study indicate optimum conditions needed to ensure successful field application. *Phlyctinus callosus* larvae can potentially be managed in an orchard by using a biocontrol method such as entomopathogenic nematodes.

Persistence of *Heterorhabditis zealandica* in a blueberry orchard infested with *Phlyctinus callosus*

The last objective of this study was to evaluate *H. zealandica* persistence in a blueberry orchard infested with *P*. *callosus*. Percentage persistence was high when a concentration of 30 IJ/cm^2 was used. Results from this study highlighted the fact that persistence is influenced by factors such as temperature, soil moisture and the concentration of nematodes applied. This study also indicated the importance of firstly investigating factors that relate to *H. zealandica* being successfully applied in an orchard.

Future research on *P. callosus* and controlling it with nematodes, should be aimed at investigating the technical aspects regarding application in the field in particular the eficacy of SF41 against *P. callosus*, as a function of seasonal timing as well as rate and application method. Entomopathogenic nematodes have great potential to be effective on their own, as well as be used in combination with other control methods to control the soil stages of *P. callosus.*