The use of entomopathogenic nematodes to control citrus mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae) on citrus in South Africa

by
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Abstract

Planococcus citri (Risso) (Hemiptera: Pseudococcidae), the citrus mealybug, is a highly destructive pest of citrus in South Africa. The ability of mealybugs to develop resistance to chemicals, as well as their cryptic nature and protective wax coverings, all individually and combined, impair the ability of insecticides to control them. Furthermore, chemicals deplete natural enemy populations that usually establish control over mealybug populations well before harvest in March to May. The inability of chemicals to control *P. citri* successfully and the growing public awareness of the detrimental environmental effects and health risks that are associated with pesticides are pressuring citrus growers to find alternative methods to chemical control. Entomopathogenic nematodes of the Rhabditida order, belonging either to the families Heterorhabditidae or Steinernematidae, have proven to be valuable biocontrol agents of a variety of insect pest species. These nematodes are, however, not yet commercially available in South Africa.

Various laboratory bioassays were conducted to determine the potential of entomopathogenic nematodes to control *P. citri.* Adult female *P. citri* were screened for susceptibility to six indigenous nematode species. *Planococcus citri* was found to be most susceptible to *Steinernema yirgalemense* and *Heterorhabditis zealandica*, causing 97% and 91% mortality, respectively. Both *H. zealandica* and *S. yirgalemense* completed their life cycles inside adult female *P. citri.* Under optimal conditions, *H. zealandica* and *S. yirgalemense*, at an application rate of 170 infective juveniles (IJs) / insect (LD_{90}), were able to control *P. citri* efficiently with a discriminating dosage of 11(LD_{50}). The water activity (a_w) bioassay indicated that *S. yirgalemense* was two times more tolerant to lower levels of free water than *H. zealandica*, with a_{w50} = 0.96 and a_{w90} = 0.99, compared to *H. zealandica* with a_{w50} = 0.98 and a_{w90} = 1.01. Furthermore, *S. yirgalemense* proved able to locate and infect *P. citri* at a faster rate than *H. zealandica.* Nematode activity was not significantly affected when exposed to 15°C, 20°C and 25°C. Results of the exposure trial also showed that the first 2−4 h post-application was the most decisive time for establishing successful infection of mealybugs.

Further experiments were conducted to determine the ability of two polymer products, Zeba® and Xanthan gum, and a surfactant, Nu-Film-P®, to improve nematode application suspensions. Despite not being as effective as Xanthan gum, Zeba® still retarded sedimentation significantly. The addition of 0.02% Xanthan gum did not improve the ability of nematodes to control *P. citri* at 60% and 80% relative humidity (RH), while the addition of 0.03% Zeba® caused a significant increase in mortality. An aqueous suspension

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containing *H. zealandica* and 0.03% Zeba® increased mortality by 14% at 60% RH and by 22% at 80% RH. The same polymer formulation was tested with *S. yirgalemense*, with mortality being found to have increased by 21% at 60% RH and by 27% at 80% RH. The combined addition of Nu-Film-P® and Zeba® was able to retard sedimentation significantly, increasing the average number of nematodes deposited on 2-cm^2 leaf discs.

To illustrate those factors that should be taken into consideration before applying nematodes in an integrated pest management (IPM) programme for citrus, the compatibility of *H. zealandica* and *S. yirgalemense* with biological control agents and agrochemicals to which they are most likely to be exposed was determined. Bioassays showed the coccinellid beetle, *Cryptolaemus montrouzieri*, to be susceptible to both nematode species. Beetle larvae proved to be highly susceptible, with *H. zealandica* obtaining 80%, and *S. yirgalemense* obtaining 92% control. Adult beetles were found to be twice as susceptible to *S. yirgalemense*, with 64% mortality recorded, as to *H. zealandica*, with 30% mortality recorded. Tolerance of *H. zealandica* and *S. yirgalemense* infective juveniles to the affect of aqueous solutions of an insecticide (Cyperphos 500 E.C.®), two biopesticides (Cryptogran™ and Helicovir™), and two adjuvants (Nu-Film-P® and Zeba®) on the infectivity and survival of *H. zealandica* and *S. yirgalemense* was evaluated. *Heterorhabditis zealandica* proved to be highly compatible with all products tested. Significant increase in mortality of *S. yirgalemense* was recorded after 12 h exposure to Cryptogran™, Helicovir™ and Cyperphos 500 E.C.®, and after 6 h exposure to Nu-Film-P®. However, there was no decrease in the ability of *S. yirgalemense* to infect the insect hosts.

Towards selecting the appropriate nematode species for field studies, the ability of *S. yirgalemense* and *H. zealandica* to control *P. citri* was first evaluated under less harsh simulated glasshouse conditions. The ability of both the above-mentioned nematode species to control *P. citri*, with and without the addition of 0.03% Zeba® and 0.06% Nu-Film-P®, was evaluated in a growth chamber at 75 ± 8% relative humidity and 22°C. All treatments resulted in significantly higher mortality of adult female *P. citri* than did the control. The addition of 0.03% Zeba® and 0.06% Nu-Film-P® to an *H. zealandica* and an *S. yirgalemense* suspension increased resultant mortality from 26% to 30%, and from 34% to 45%, respectively. This increase in mortality was, however, not significant. The ability of this formulation to prolong the ability of *S. yirgalemense* to infect *P. citri* and to prolong nematode survival was also evaluated under the same conditions and showed the formulation to improve both infectivity and survival for up to 2–3 h post-application. In the semi-field trial, *S. yirgalemense* was unable to obtain significant control of *P. citri* without the addition of 0.03% Zeba® to

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nematode application suspensions. However, with the addition of 0.03% Zeba® *S. yirgalemnse* was able to obtain up to 53% control. The study established that the polymer product Zeba® improves the ability of *S. yirgalemense* to infect *P. citri* by retarding desiccation and by buffering nematodes from suboptimal environmental conditions.

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Opsomming

Planococcus citri (Risso) (Hemiptera: Pseudococcidae), die sitrus witluis, is 'n baie skadelike pes van sitrus in Suid Afrika. Die vermoë van witluise om weerstand teen chemiese middels te ontwikkel, hul kriptiese lewenswyse en die beskermende waslaag wat hul liggame omhul inhibeer gesamentlik en individueel die vermoë van insektisiede om witluis bevolkings te beheer. Chemiese middels verminder ook die natuurlike vyande wat gewoonlik witluis bevolkings beheer voor die sitrus oestydperk in Maart tot Mei. Die onvermoë van chemiese middels om *P. citri* suksesvol te beheer en verhoogde bewustheid van die publiek rond om die vernietigende omgewings impak en gesondheidsrisiko's verbonde aan chemiese insek beheer, noodsaak sitrus produsente om alternatiewe beheermetodes te ontwikkel. Entomopatogeniese nematodes, van die orde Rhabditida wat aan die families Heterorhabditidae of Steinernematidae behoort, is bekend as effektiewe biologiese beheeragente van 'n verskeidenheid insek pes spesies. Hierdie nematodes is egter tans nie kommersieel beskikbaar in Suid- Afrika nie.

Om ten einde die vermoë van plaaslike nematode spesies te bepaal om *P. citri* te beheer, is verskeie biotoetse in die laboratorium uitgevoer. 'n Vinnige siftings proses is uitgevoer om vas te stel watter plaaslike nematode spesies die hoogste persentasie mortaliteit van *P. citri* wyfies veroorsaak. Daar is bevind dat *P. citri* die mees vatbaarste is vir *Steinernema yirgalemense* en *Heterorhabditis zealandica* wat 97% en 91% mortaliteit respektiewelik veroorsaak het. Die ontwikkeling van beide *H. zealandica* en *S. yirgalemense* na die infektering van volwasse *P. citri* wyfies is gevolg en daar is bevind dat beide nematode spesies hul lewensiklusse kon voltooi in insek kadawers. Onder optimale toestande was *H. zealandica* en *S. yirgalemense* in staat om *P. citri* effektief te beheer, as hul teen 'n konsentrasie van 170 infektiewe larwes (JIs) per insek (LD_{90}) toegedien word, met 'n diskriminerende toedienings dosis van 11 (LD_{50}). 'n Water aktiwiteit biotoets het gewys dat *S. yirgalemense* twee keer so verdraagsaam is teenoor laer vlakke van vrye water as wat *H. zealandica* is met a_{w50} = 0.96 en a_{w90} = 0.99, in vergelyking met *H. zealandica* met a_{w50} = 0.98 en a_{w90} = 1.01. Resultate van 'n blootstellings toets het ook gewys dat *S. yirgalemense* die vermoë het om *P. citri* vinniger op te spoor en te infekteer as *H. zealandica.* Die blootstellings toets was uitgevoer teen 15°C, 20°C en 25°C en daar is bevind dat die aktiwiteit van *H. zealandica* nie beduidend verhoog het met 'n verhoging in temperatuur nie. Resultate vir die blootstellings toets het ook gewys dat die eerste 2 tot 4 uur na toediening van nematodes die mees kritieke tyd is om suksesvolle infektering van witluise te bevestig.

Verdere eksperimente is uitgevoer om te bepaal of die byvoeging van twee polimeer produkte nl. Zeba® en Xanthan gum en 'n benatter Nu-Film-P®, nematode toedienings suspensies kan verbeter. Deur

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0.02% Xanthan gum by nematode toedienings suspensies te voeg is die vermoë van nematodes om *P. citri* by 60% en 80% relatiewe humiditeit (RH) te beheer nie beduidend verhoog nie, terwyl die byvoeging van 0.03% Zeba® wel 'n beduidende verhoging in mortaliteit veroorsaak het. 'n Suspensie van *H. zealandica* en 0.03% Zeba® het beheer met 14% by 60% RH en met 22% by 80% RH verhoog. Die invloed van dieselfde polimeer formulasie was ook getoets op *S. yirgalemense* en mortaliteit het verhoog met 21% by 60% RH en met 27% by 80% RH. Ten spyte daarvan dat "Xanthan gum" nie so effektief was om afsakking van nematodes te verhoed nie, het die byvoeginging van 0.03% Zeba® by toedienings suspensies steeds 'n beduidende invloed gehad. Die gekombineerde byvoeging Nu-Film-P® en Zeba® was in staat om die gemiddelde aantal nematodes gedeponeer op 2-cm^2 blaar skyfies te verhoog.

Om te wys watter faktore in ag geneem moet word voor EPNs as deel van 'n geïntegreerde pes beheer program toegedien word, is die verenigbaarheid van *H. zealandica* en *S. yirgalemense* vir biologiese beheer agente en landbouchemikalieë, waaraan hulle heel waarskynlik blootgestel gaan word, bepaal. Biotoetse het gewys dat die liewenheersbesie, *Cryptolaemus montrouzieri*, vatbaar is vir beide nematode spesies. Die larwale fase was hoogs vatbaar met mortaliteit van 80% en 92% verkry deur die toedeining van *H. zealandica* en *S. yirgalemense* respektiewelik. Daar is ook bepaal dat volwasse besies twee maal so vatbaar is vir *S. yirgalemense* wat 64% mortaliteit veroorsaak het, as vir *H. zealandica* met 30% mortaliteit aangeteken. Verdraagsaamheid van IJs van *H. zealandica* en *S. yirgalemense* met oplossings van 'n insektisied (Cyperphos 500 E.C.®), twee bio-plaagdoders (Cryptogran™ en Helicovir™), en twee byvoegmiddels (Nu-Film-P® en Zeba®), vir infektiwiteit en oorlewing is bepaal. Resultate het gewys dat *H. zealandica* hoogs verenigbaar is met alle produkte wat getoets is. 'n Beduidende verhoging in mortaliteit van *S. yirgalemense* is aangeteken na 12 ure se blootstelling aan Cryptogran™, Helicovir™ en Cyperphos 500 E.C.®, en na 6 ure se blootstelling aan Nu-Film-P®. Resultate het egter ook gewys dat die vermoë van *S. yirgalemense* om om gashere te infekteer nie deur beinvloed word nie.

Om ten einde te bepaal watter een van *H. zealandica* of *S. yirgalemense* die beter isolaat sou wees om te gebruik in veldproewe, is hul vermoë om *P. citri* te beheer eers evalueer onder minder ongunstige gesimuleerde glashuis toestande. Die vermoë van beide nematode spesies om *P. citri* te beheer is bepaal in 'n groeikamer by 75 ± 8% (RH) met en sonder die byvoeging van 0.03% Zeba® en 0.06% Nu-Film-P®. Mortaliteit verkry deur alle behandelings was beduidend hoër as die kontrole. Deur 0.03% Zeba® en 0.06% Nu-Film-P® by toedienings suspensies van *H. zealandica* en *S. yirgalemense* te voeg is die gevolglike mortaliteit van *P. citri* verhoog van 26% na 30% en van 34% na 45%, respektiewelik. Hierdie verhoging in

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mortaliteit was egter nie beduidend nie. Die vermoë van die bo-genoemde formulasie om oorlewing van *S. yirgalemense* en infeksie tydperk van *P. citri* te verleng was bepaal onder dieselfde toestande en daar is gevind dat die formulasie beide die infektiwiteit en oorlewing van nematodes met 2 tot 3 uur na toediening verleng het. Tydens die semi-veldproef was *S. yirgalemense* nie in staat om beduidend hoër mortaliteit van *P. citri* te verkry, in vergelyking met die kontrole, sonder die byvoeging van 0.03% Zeba® by toedienings suspensies nie. Met die byvoeging van Zeba® is daar tot 53% beheer verkry. Die studie het onweerlegbaar gewys dat die polimeer produk Zeba® die vermoë van *S. yirgalemense* om *P. citri* te infekteer verbeter deur die uitdroging van nematodes te vertraag en deur hul te beskerm teen ongunstige omgewings toestande.

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CHAPTER 1

Literature review

Mealybugs (Hemiptera: Pseudococcidae)

Introduction

Mealybugs (Hemiptera: Pseudococcidae) are tiny, soft-bodied insects with piercing, sucking mouthparts used to extract plant juice (Downie & Gullan, 2004). The name mealybug is used to describe a large family, Pseudococcidae. The members of this family are characteristically covered by a waxy, mealy secretion, from which the common name is derived (Scholtz & Holm, 1985; Hattingh, 1993). The family Pseudococcidae comprises about 2 200 species, in almost 274 genera (Ben-Dov *et al.*, 2010), of which 109 species in 50 genera occur in South Africa (Millar, 2002). A total of twenty species are considered economically important pests of cultivated plants in South Africa (Annecke & Moran, 1982).

Origin, host range and distribution

Planococcus citri (Risso), commonly referred to as the citrus mealybug, was originally described by Risso in 1813 from specimens found on *Citrus* spp. from southern France (Cox, 1981). The citrus mealybug is globally distributed (Tauber *et al.*, 1985; Smith *et al.*, 1997; Blumberg & Van Driesche, 2001; Mustu *et al.*, 2008), highly polyphagous and generally the most destructive species of its family (Cadee & Van Alphen, 1997; Blumberg & Van Driesche, 2001). Commercially produced hosts include citrus, coffee, vineyards and a variety of ornamental plants (Cadee & Van Alphen, 1997; Mustu *et al.*, 2008). *Planococcus citri* is mainly a pest of ornamental plants in glasshouses in the temperate zone of Europe (De Jong & Van Alphen, 1989). The origin of the citrus mealybug remains highly speculative, because, of its wide distribution as a result of international trade (Franco *et al.*, 2004).

Paracoccus burnerae (Brian), commonly referred to as the oleander mealybug, is an important citrus pest in South Africa and is occasionally also found on granadillas (Annecke & Moran, 1982). Brian originally

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described *P. burnerae* in 1915 and it was re-described by De Lotto in 1967. *Paracoccus burnerae* is indigenous to southern Africa and has also been recorded in India (Ben-Dov *et al.*, 2010).

Morphology and identification

Females are wingless, soft, oval, distinctly segmented, and covered by a waxy or mealy secretion with the head, thorax and abdomen completely fused (Daly *et al.*, 1998). Mature males are tiny, fragile, short-lived insects, with non-functional mouthparts, one pair of functional wings and two long antennae and anal filaments (Smith *et al.*, 1997).

Citrus mealybug

For many years the citrus mealybug, *P. citri* was confused with the vine mealybug, *Planococcus ficus* (Signoret) (Cox, 1981; Annecke & Moran, 1982). These two species are morphologically very similar and can occur simultaneously in various agro-ecosystems, therefore the original morphological identification of these two species have been questioned (Demontis *et al.*, 2007; Saccaggi *et al.*, 2008). Genetic markers have since been identified to distinguish between the two species (Demontis *et al.*, 2007).

Cox (1981) notes that the citrus mealybug shows considerable morphological variation when reared under different environmental conditions. Generally, adult females are yellowish in colour, about 3 mm long, with a faint stripe running along the dorsal length of the body. They have eighteen pairs of waxy filaments around the margin of the body, which progressively lengthens towards the rear end, terminating in an elongated pair of filaments that can be up to one-quarter of the body length (Ebeling, 1959; Annecke & Moran, 1982; Hattingh & Moore, 2003).

Oleander mealybug

Adult females of the oleander mealybug, *P. burnerae*, are greyish in colour, slightly smaller than the citrus mealybug (approximately 2.5 mm) with a dense, uniform waxy or mealy covering, with no faint stripe along the dorsal length of the body and 17 pairs of waxy filaments around the margin of the body, of which the anal pair is elongated (Annecke & Moran, 1982; Hattingh *et al.*, 1998).

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General life cycle

Mealybugs are sexually dimorphic and therefore males and females have completely different life cycles. The immature stages, known as crawlers, superficially resemble adult females and progressively enlarge, maturing with each moult (Smith *et al.*, 1997). Although a few mealybug species are parthenogenetic, the majority reproduce sexually (McKenzie, 1967; Downie & Gullan, 2004). During sexual reproduction, mature females produce and secrete pheromones that attract males to copulate (Tauber *et al.*, 1985). Adult female mealybugs secrete thin, waxy threads along the dorsal length of the body, which develop into a cottony mass, in which 300 to 600 eggs are laid (Annecke & Moran, 1982; Smith *et al.*, 1997). The period required for oviposition and hatching is temperature dependent. During summer, oviposition lasts about six to fourteen days and eggs hatch after approximately six to ten days (Ebeling, 1959). During development, females undergo three nymphal (immature) instars, while males have four (Annecke & Moran, 1982). After a couple of instars, males spin a cocoon, in which they moult a number of times, after which an adult male emerges (Gullan, 2000). Males are fragile, short-lived, winged insects that live solely to reproduce, and have no mouthparts with which to feed (McKenzie, 1967).

Seasonal cycle

On *Citrus* spp., mealybugs occur throughout the tree canopy. Although they are primarily found on leaves and twigs, some females migrate to the main branches or trunk of their host tree to lay eggs (Martinez-Ferrer *et al.*, 2006). Mealybugs disperse from one tree to the next when crawlers are blown by the wind, or when carried by moving agents, such as ants (Mukhopadhyay, 2004).

Mealybugs overwinter in cracks and crevices or leaf axils throughout the tree canopy (Hattingh, 1993; Smith *et al.*, 1997). Although all three life stages occur during winter, the egg stage is predominant (Ebeling, 1959). During spring and early summer, shortly after fruit set, first-generation nymphs move to the foliage and colonise young growth and small fruit (Martinez-Ferrer *et al.*, 2006). Mealybugs settle under protected sites, such as the calyx of small fruit, to feed and reproduce (Hattingh & Moore, 2003). The population density of *P. citri* on grapefruit and orange varieties parallels fruit growth intensity, with the highest population numbers occurring between mid and late summer (Franco *et al.*, 2004).

According to Wakgari and Giliomee (2003), information on the phenology, as well as on the reproductive and developmental biology of *P. citri* in citrus orchards of South Africa, is still lacking and, for

this reason, they conducted a study to determine the biology of three mealybug species occurring on citrus in the Western Cape province of South Africa. *Planococcus citri* was found to be most common on *Citrus limon.* No distinct phenological pattern could be distinguished for *P. citri,* because multiple, overlapping generations occurred. The researchers also observed that population densities of *P. citri* vary considerably from one citrus orchard to the next, depending on the effect of different micro climates and host trees on female fecundity. Citrus cultivars, such as Eureka lemon producing fruit throughout most of the year, provide mealybugs with food and protective overwintering sites. As a consequence, population densities of *P. citri* are much higher on such cultivars, compared to those on others, such as Clementines that bears, for only a short period of the year. On Clementine trees, mealybug numbers declined drastically to almost undetectable levels after harvesting during April or May. Thus, the survival of individual mealybugs seems to be greatly affected by crop phenology. Another important observation noted in the study was that mealybugs prefer to infest fruit and to settle under fruit calyxes or other protective sites, such as in between fruit clusters, or within bud-mite induced fruit deformations.

Economic importance of mealybugs on citrus

Globally, South Africa is the second largest exporter of fresh citrus, following Spain. South Africa is, however, only the fourteenth largest citrus producing country, which means that the majority of citrus produced in South Africa is exported. Approximately 60% of total citrus produced in South Africa is exported, primarily to Europe and the Middle East (CGA, 2010).

Mealybugs were not considered major pests of citrus in South Africa prior to 1990, but have, since then, reached economically damaging population densities (Hattingh & Tate, 1996). On a global scale, the economic importance of mealybugs is low, but, from a phytosanitary perspective, they remain important citrus pests in South Africa (Hattingh *et al.*, 1998), as they are one of the major constraining factors when exporting citrus fruit to foreign markets (Wakgari & Giliomee, 2005). Indigenous species, such as *Delottococcus elisabethae* (Brian) and *P. burnerae*, occurring on citrus in South Africa are of phytosanitary concern (Millar, 2002).

Various species of mealybug attack citrus, of which seven are regarded as pests of economic, or potential economic, importance in South Africa, with *P. citri* the most common and destructive (Hattingh *et al.*, 1998). Wakgari and Giliomee (2005) developed a key to distinguish between adult and immature stages

of the spherical mealybug *Nipaecoccus viridis* (Newstead); the oleander mealybug, *P. burnerae*; the striped mealybug, *Ferrisia virgata* (Crockerell); the long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti); the citrophilous mealybug, *Pseudococcus calceolariae* (Maskell); and the citrus mealybug, *P. citri*. Specimens of *D. elisabethae* could not be found during the above-mentioned study and were, therefore, not described. Eggs and crawlers are difficult to identify, causing high numbers of fruit consignments to be rejected for export. Pieterse *et al.* (2010) developed a user-friendly molecular laboratory technique to distinguish between five of the mealybug species found on citrus in South Africa.

Planococcus citri has been recorded as a serious pest of citrus in Africa (Hattingh & Tate, 1996; Hattingh *et al.*, 1998), Australia (Smith *et al*. 1997; Gullan, 2000), the Mediterranean Basin (Blumberg *et al.*, 1995; Franco *et al.*, 2004), as well as North, Central and South America (Bartlett & Lloyd, 1958; Wakgari & Giliomee, 2003). When environmental conditions are optimal, citrus and oleander mealybug population densities can increase rapidly, and have the potential to infest up to 100% of fruit, even if early spring infestation levels could hardly be detected (Hattingh & Moore, 2003). A study undertaken by Moore and Kirkman (2005) showed that, during certain years, *P. burnerae* has become the dominant mealybug species in the Eastern and Western Cape provinces of South Africa.

Damage

Mealybug infestations cause a variety of direct and indirect forms of damage to citrus (Hattingh & Tate, 1996), by feeding on all parts of the tree, except the roots (Canhilal *et al*., 2001). Damage caused by mealybugs is associated with wilting, fruit and flower drop, and impaired fruit appearance, caused by growth deformations and sooty mould (Blumberg *et al*., 1995). It appears as though some strains of *P. citri* have a toxic affect on plant hosts and heavy infestations can cause stunting or, in severe cases, death to host plants (McKenzie, 1967).

In Southern Africa, early ripening cultivars (March to May), such as mandarins, grapefruit and navel oranges are more prone to damage caused by mealybugs than are late maturing cultivars (Smith *et al*., 1997). The reason for the higher degree of damage on early ripening cultivars is because natural enemies have less opportunity to suppress mealybug populations before harvest (Hattingh *et al*., 1998). According to Hattingh and Moore (2003), fruit damage generally occurs between petal fall and the time at which the fruit are approximately the size of a golf ball.

Calyx feeding causes fruit deformations, which are visible as bumps and indentations on fruit (Hattingh, 1993). Grapefruit is sensitive to feeding damage and prone to develop growth deformations (Hattingh *et al*., 1998). Young growth flush infestations can also cause severe leaf deformations (Hattingh & Tate, 1996), with such leaves being characteristically bent (Hattingh & Moore, 2003). The oleander mealybug prefers to feed on leaves and has the potential to cause extensive leaf damage (Hattingh *et al*., 1998). Early-season feeding damage caused by mealybugs can also cause increased fruit drop. Mandarins, navels and grapefruit are particularly sensitive to high mealybug infestations, which causes fruit to turn yellow and to drop prematurely (Hattingh *et al*., 1998). Easy-peeler cultivars are especially sensitive to early feeding damage and fruits infested with as few as one to two mealybugs may drop (Hattingh & Moore, 2003). Late-season feeding causes hyperpigmentation and stippling (Hattingh & Tate, 1996), with grapefruit and navels being particularly prone to such damage (Hattingh, 1993).

Mealybugs also cause indirect damage to fruit (Hattingh & Moore, 2003). As sap-feeders, mealybugs profusely secrete honeydew, which acts as a growth medium for sooty mould (Hattingh, 1993). Sooty mould deposits form a dark, superficial coating on twigs, leaves and fruit (Smith *et al*., 1997), which decreases the photosynthetic potential of the plant, with fruit covered in fungal growth being considered undesirable for the export market (Hattingh *et al*., 1998).

Chemical control

Although such a measure is only corrective, mealybugs are most commonly controlled with chemicals (Franco *et al.*, 2004). The chemical control of mealybugs is difficult, because they display cryptic behaviour, hiding in protected areas where chemicals cannot reach, and are covered with protective waxes (McKenzie, 1967; Michelakis & Hamid, 1995; Franco *et al.*, 2004). Mealybugs also have the ability to rapidly develop resistance to insecticides (McKenzie, 1967; Blumberg & Van Driesche, 2001; Mahfoudhi & Dhouibi, 2009). The continuous applications of broad-spectrum pesticides, such as organophosphates, are also partially responsible for citrus mealybug outbreaks (Michelakis & Hamid, 1995). According to Hattingh and Moore (2003), the most efficient time at which to apply insecticides is during spring, when the first crawlers emerge, as the efficiency of chemical control greatly reduces once mealybugs settle under calyxes and other protected areas.

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Biological control

If not disrupted by the application of insecticides or other detrimental treatments, mealybug population levels are usually kept under control by natural enemies well before harvest (Hattingh, 1993; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003) These natural enemies, however, recover slowly after winter, and population levels during early spring are usually insufficient to prevent feeding damage to young fruitlets (Hattingh, 1993; Franco *et al.*, 2004). Beneficial insects, such as the predatory lady beetle, *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), and the parasitoid, *Leptomastix dactylopii* (Howard) (Hymenoptera: Encyrtidae), have been mass- reared and sold to farmers as biological control agents since 1972 (Blumberg & Van Driesche, 2001; Canhilal *et al.*, 2001), and have since shown to be a valuable control strategy (Hattingh & Moore, 2003).

Parasitic wasps

Throughout Southern Africa, hymenopteran parasitoids are the most abundant biological control agents of mealybugs (Hattingh & Tate, 1996) and are therefore used to control mealybugs by augmentative releases (Demontis *et al.*, 2007). The parasitoids *Coccidoxenoides peregrinus* (Timberlake) (Hymenoptera: Encyrtidae) and *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) are widely distributed throughout southern Africa (Hattingh & Moore, 2003). In the Western Cape of South Africa, *Leptomastidea abnormis* (Girault) occur bountifully, while, in the Eastern Cape of South Africa, infestations of *P. burnerae* have frequently been associated with *Allotropa kamburovi* (Annecke & Prinsloo) (Hymenoptera: Platygasteridae) (Hattingh & Tate, 1996). *Leptomastix dactylopii, L. abnormis* and *A. pseudococci* are considered important natural enemies of the citrus mealybug (Bartlett, 1978).

The two parasitoids, *L. abnormis* and *L. dactylopii*, are often used as biological control agents against *P. citri* (De Jong & Van Alphen, 1989; Cadee & Van Alphen, 1997). *Coccidoxenoides peregrinus* is mass-produced and commercially available in South Africa; this parasitoid is, however, not as effective in controlling the oleander mealybug than the citrus mealybug (Hattingh & Moore, 2003).

Predators

Cryptolaemus montrouzieri was originally collected by Koebele in Australia and brought to California in 1982 (Canhilal *et al.*, 2001). This coccinellid predator has proven to have the capacity to control heavy mealybug infestation levels effectively, with releases of 1 000 to 2 000 beetles/ha (Hattingh & Moore, 2003).

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When releasing *C. montrouzieri* to control mealybugs, it is important to remember that the former might be sensitive to chemical control agents. Hattingh and Tate (1995) found that *C. montrouzieri* is negatively affected by the insect growth regulator, pyriproxyfen (Nemesis®), which is used to control red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), in South African citrus orchards.

Ants

Ants and mealybugs have a mutualistic relationship, as ants feed on droplets of honeydew secreted by mealybugs and, in exchange, ants protect mealybugs from natural enemies (Way, 1963; Annecke & Moran, 1982). When present in the tree canopy, ants can disrupt biological control agents significantly (Smith *et al.*, 1997), even at low activity levels (Hattingh & Tate, 1996). To suppress mealybug population levels, it is also important to decrease ant activity in the tree canopy (Annecke & Moran, 1982).

Cultural control

No cultural control methods can prevent mealybug infestations (Hattingh & Moore, 2003). Pruning, however, can aid in increasing the effectivity of control with insecticides. Removing dead or excess twigs and branches from the tree canopy increases the penetration of spray treatments (Hattingh & Moore, 2003), and mealybugs are also more exposed to natural enemies (Franco *et al.*, 2004).

Entomopathogenic nematodes

Introduction

Insect pests are traditionally controlled by applying chemical pesticides. These pesticides have detrimental effects on the environment, specifically through contaminated groundwater, causing food chain contamination, which, in turn, threatens human and animal health (Hussaini, 2002). Insects also develop resistance to insecticides, which is proving to be a major driving force for seeking alternative control methods (Ehlers, 1996). Many consider the application of natural enemies the most feasible alternative to chemical insect control (Hussaini, 2002). Entomopathogenic nematodes (EPNs) in the order Rhabditida, of the families Steinernematidae and Heterorhabditidae, are fatal pathogens of insects (Griffin *et al.*, 2005), and are valuable biological control agents for a wide range of economically important insect pests (Grewal *et al.*,

2005). Nematodes are used as inundatively applied biological control agents (Hazir *et al.*, 2003). The aim of inundative biological control is to control pest populations by repeatedly applying lage quantities of natural enemies (Mailleret & Grongnard, 2009). EPNs are considered excellent biological control agents, as they are able to actively seek a broad range of hosts and have no known harmful effects on mammals and the environment (Gaugler & Boush, 1979).

Life cycle

Both steinernematids and heterorhabditids advance through four immature stages before maturing (Glazer & Lewis, 2000). The third stage, in which they are known as dauer or infective juveniles (IJs), is the only free-living, non-feeding stage within the life cycle, and occurs naturally in the soil. Once a host is located, nematodes enter the host through natural openings (mouth, anus and spiracles), or by directly penetrating through thin layers in the cuticle (Shapiro-Ilan, 2009). Directly penetrating the host's cuticle commonly occurs in heterorhabditids that are equipped with a dorsal tooth (Peters, 1996). Once inside the haemocoel of the host, nematodes release a symbiotic bacterium that multiplies rapidly, killing hosts by septicaemia within 24 – 48 h (Adams & Nguyen, 2002; Dowds & Peters, 2002). Symbiotic bacteria associated with steinernematids and heterorhabditids fall in the genera, *Xenorhabdus* and *Photorhabdus*, respectively (Jagdale *et al.*, 2009). Nematodes feed on bacteria cells, as well as on host tissue, broken down by developing bacteria, and then advance to the fourth and adult stage. Depending on host size, nematodes moult and complete one to three generations within the host (Kaya & Gaugler, 1993). The bacteria also release antibiotic substances that aid in preserving the insect cadaver and that protect it against opportunistic organisms (Kondo & Ishibashi, 1986). Once the food source is depleted, IJs are produced that leave the host in search of a new host. IJs can survive in soil for several months by entering a near-anhydrobiotic state (Adams & Nguyen, 2002). Steinernematids and heterorhabditids have different modes of reproduction. First-generation heterorhabditids are self-fertile hermaphrodites while subsequent generations consist of cross-fertilizing males and females (Dix *et al.*, 1992). In steinernematids, cross- fertilizing males and females occur in each generation (Griffin *et al.*, 2005).

Safety and effect on non-target organisms

On examining the possible negative influence of EPNs and their symbiotic bacteria, Akhurst and Smith (2002) found no convincing evidence of any acute or chronic toxicity to humans or other vertebrates,

or any significant long-term influence on non-target invertebrate populations. No safety concerns that should prevent EPNs from being used as biological control agents have been identified (Ehlers, 2005).

Although EPNs are primarily aimed at controlling soil-dwelling insects, these biological control agents have proven to control some foliar pests (Arthurs *et al.*, 2004), but their broad host range is a concern, as they could possibly have a negative impact on beneficial organisms (Hazir *et al.*, 2003). Shapiro-Ilan and Cottrell (2005) compared the susceptibility of four ladybug beetle species to *Heterorhabditis bacteriophora* Poinar, 1976 and *Steinernema carpocapsae* (Weiser, 1955) Wouts Mráček, Gerdin & Bedding, 1982, in relation to that of the black cutworm, *Agrotis ipsilon* (Hüfnagel) (Lepidoptera: Noctuidae), a known susceptible host, and found that the former were significantly less susceptible than were the latter. Rojht *et al.* (2009) studied the possible non-target effect of *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1998; *S. carpocapsae* and *H. bacteriophora* on the larvae of the twospotted lady beetle, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae), and on the larvae of the lacewing species, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) using bioassays. The researchers concluded that the above-mentioned nematodes exhibit a pronounced non-target effect on the larvae of both predators, and suggested that a field study should be completed to confirm their findings. In the field, EPNs infect a significantly narrower host range than under optimal laboratory conditions (Kaya & Gaugler, 1993).

Compatibility of EPNs with agrochemicals

To apply EPNs successfully in an integrated pest management (IPM) programme, it is important to test their compatibility with agrochemicals. To save time and labour costs, it is often desirable to tank mix one or more agrochemicals or biopesticides. Nematodes may also be applied shortly after other chemicals, or simultaneously with other control agents to achieve better control of a single pest (Koppenhöfer & Grew, 2005).

Rovesti and Deseö (1990) tested the compatibility of 75 commercial pesticides with two nematode species. Both nematode species were tolerant to short periods of exposure (2– 24 h) to the majority of pesticides tested. Some pesticides, however, tend to decrease nematode longevity and infectivity (Zimmerman & Cranshaw, 1990; Patel *et al.*, 1997). It is also important to remember that different nematode species are likely to react differently to the same chemical, and differ in tolerance. EPNs are also exposed to surfactants and other ingredients that are used in pesticide formulations, which could also be toxic to

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nematodes, and they might react differently to different formulations of the same pesticide (Grewal, 2002). Incompatibility between nematodes and agrochemicals can be overcome by applying nematodes at intervals between chemical applications, depending on the persistence of the chemical applied (Koppenhöfer & Grew, 2005).

Foliar application

Generally, nematodes are applied to foliage as an aqueous suspension, using ordinary agrochemical spray equipment (Grewal, 2002; Hussaini, 2002). As nematodes are living organisms, the water used for application should not be heavily chlorinated, and the temperature should be within the range of 4 – 30°C (Wright *et al.*, 2005). Controlling foliar pests successfully with the aid of nematodes is extremely challenging, as above-ground conditions are not optimal for nematode survival (Mráček, 2002; Tomalak *et al.*, 2005).

To improve post-application nematode survival rates, high relative humidity is essential to retard desiccation, as nematodes require a water film to ensure their survival and to maintain mobility (Wright *et al.*, 2005). Rainy periods and tropical conditions improve nematode survival rates on foliage (Mráček, 2002). As wind also accelerates evaporation, it is advisable not to apply nematodes on windy days (Unruh & Lacey, 2001). Outstanding control (>80%) of the northern masked chaffer, *Cyclocephala borealis* (Arrow) (Coleoptera: Scarabaeidae) and the Japanese beetle *Popillia japonica* (Newman) was accomplished with 1.25–5 billion IJs/ha when plots of Kentucky bluegrass, *Poa pratensis* (L.) (Poales: Poaceae) (Coleoptera: Scarabaeidae) were irrigated 24 h prior to treatment application and again 24 h thereafter (Downing, 1994).

Application formulations can be improved to retard desiccation (Glazer *et al.*, 1992). Water-retention agents have been used successfully to obstruct the evaporation of application suspensions on foliage, thus increasing the duration of nematode survival (Webster & Bronskill, 1968; Shapiro *et al.*, 1985; Glazer & Navon, 1990). Schroer and Ehlers (2005) tested the influence of a formulation containing 0.3% Rimulgan® (surfactant) and 0.3% of the polymer xanthan (antidesiccant) on post- application nematode survival and infectivity in leaf bioassays. The results indicated that nematodes penetrate hosts within the first hour after application. The researchers concluded that foliar treatment formulations should aim to provide optimal environmental conditions for nematode host infection, rather than to enhance nematode survival. It is, therefore, beneficial to select a nematode strain or species that has the ability to actively locate and infect hosts. In Malaysia, some strains of indigenous *Steinernema* spp. have been identified by Manson and Wright (1997) as being better adapted to extreme environmental conditions than others.

When applying nematodes, temperature should be considered. The infectivity of most EPN species is considerably reduced at temperatures exceeding 32°C or below 15°C (Lacey *et al.*, 2005). Exposing nematodes to sunlight is also problematic, as they are sensitive to the ultraviolet (UV) radiation of certain wavelengths (Gaugler & Boush, 1978; Gaugler *et al.*, 1992). Such exposure can be prevented by applying nematodes at dusk (Lello *et al.*, 1996).

Previous work with entomopathogenic nematodes to control mealybugs

Stuart *et al.* (1997) examined the susceptibility of *Dysmicoccus vaccinii* (Miller and Polavarapu) to a variety of different EPN strains and species in sand-dish and sand-column assays. In the sand-dish assays, *H. bacteriophora, S. carpocapsae, Steinernema glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding 1882 and *S. feltiae* were tested at 10-, 50-, 100- and 500 IJs/mealybug. After 48 h exposure none of the nematode species tested caused significantly higher levels of mealybug mortality compared to the control. However, after 120 h exposure, *H. bacteriophora* (HP88 strain) caused significantly higher levels of mealybug mortality, reaching up to 63.6% mortality at a dose of 500 IJs/mealybug. In the study concerned, they also investigated whether mealybugs would be more susceptible to *H. bacteriophora* when their protective wax coatings are removed, and concluded that doing so made no difference. In the sand-column assay, the ability of *H. bacteriophora* and *Heterorhabditis indicus* Poinar, 1992 to move through sand to locate and infect the target host was tested. Both species proved to be effective against *D. vaccinii*, with *H. bacteriophora* causing 98% mortality at a dose of 500 IJs/mealybug and *H. indicus* causing 56% mortality at a dose of 100 IJs/mealybug and 100% mortality at 500 IJs/mealybug. Both nematode species tested were also able to reproduce and to emerge from infected *D. vaccinii* cadavers.

Alves *et al.* (2009) tested the potential of various *Heterorhabditis* strains to control the coffee root mealybug, *Dysmicoccus texensis* (Tinsley). Laboratory tests showed that most of the strains tested were highly virulent against the target host. In the greenhouse assay, the infectivity of two strains was tested in mealybug-infested pots using two application methods; infected insect cadavers and aqueous suspension. The results showed that the aqueous suspension application method was more efficient, with *Heterorhabditis* spp. (JPM3) causing up to 70% control. In the field study, nematode efficacy was compared to that of the insecticide Actra 250 WG® (thiamethoxam), which achieved 80% control. The nematode strain JPM3 caused 65% mortality, and was the only treatment that caused significantly higher mealybug mortality than did the control treatment.

Stokwe (2009) conducted various bioassays to determine the potential of nematodes to control the obscure mealybug, *Pseudococcus viburni* (Signoret). The study concerned showed that *P. viburni* differs greatly in susceptibility from one nematode species and strain to the next. Generally, *P. viburni* showed greater susceptibility to *Heterorhabditis* compared to *Steinernema* strains and species. In a biological study, mealybugs infected with *Heterorhabditis zealandica* Poinar, 1990 and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005 were dissected and showed that *H. zealandica* has greater penetration ability than did *S. yirgalemense.* Both nematode species tested could complete their life cycle inside *P. viburni.* When the susceptibility of various sizes of *P. viburni* to *H. zealandica* was tested, the results showed that all host sizes, from crawlers (22% mortality) to adults (78% mortality), were susceptible. The study concerned also showed that *H. zealandica* has the ability to locate and to kill hosts in cryptic habitats, such as inside the calyx and ovary of apples. A lethal dose (LD) and lethal time (LT) study was also undertaken on *P. viburni*, using *H. zealandica* (J34). Results showed that mortality is influenced by increasing dose and amount of time after inoculation with the LD_{50} and LD_{90} at 54 and 330 IJs/mealybug respectively, and the LT_{50} and LT_{90}, at 1.25 and 2.6 days respectively.

To date, only a few studies have been conducted on the susceptibility of Pseudococcidae to EPNs. No previous studies have been conducted on the susceptibility of *P. citri* to EPNs. Resistance, strict regulations and the detrimental effect of pesticides on natural enemies are just a few reasons why it is essential to find alternative control methods against mealybugs on citrus. The field of study concerned with the application of nematodes to control foliar pests in orchards is still relatively new. The best application method and formulation requires optimisation and novel methods of orchard application have still to be developed. According to Wright *et al.* (2005), EPNs are best used against foliar pests when they are applied synergistically together with other biological control agents and with compatible chemicals in IPM programmes. Although mealybugs are foliar pests, they are also cryptic in nature and tend to settle under protective sites, such as the calyx of young fruit and misshaped or bumpy leaves and will increase the ability of nematodes to control mealybugs, as the microclimates concerned are protected from UV light and desiccation. The cryptic nature of mealybugs is also one of the reasons why they are so difficult to control chemically, as they hide in protected sites where chemicals cannot reach. EPNs are therefore, a very attractive potential biological control method for mealybugs, as they have the ability actively to seek out their hosts in such protected areas.

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Aim and objectives of the study

In view of the above-mentioned literature review, the overall aim of the study was to contribute towards the development of knowledge regarding the control *Planococcus citri* in South African citrus orchards by means of entomopathogenic nematodes.

The specific objectives of the study were:

1) to identify a nematode strain that causes a high mortality rate on *P. citri*;
2) to determine the compatibility of nematodes with other agrochemicals and biological control agents; and
3) to determine the potential of nematodes to control foliar pests under greenhouse and field conditions.

The chapters of this study were written as separate papers and for that reason repetition of literature can be expected.

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CHAPTER 2

Potential of South African isolates of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) for control of *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae)

Abstract

Planococcus citri, the citrus mealybug, is the most important species of mealybug known to infest citrus in South Africa. Various laboratory bioassays were conducted to determine the potential of entomopathogenic nematodes to control *P. citri.* Adult female *P. citri* were screened for susceptibility to six indigenous nematode species. *Planococcus citri* was found to be most susceptible to *Steinernema yirgalemense* and *Heterorhabditis zealandica*, causing 97% and 91% mortality, respectively. The development of nematodes after infecting adult female *P. citri* showed both *H. zealandica* and *S. yirgalemense* were able to complete their life cycles inside the host. Further bioassays illustrated a linear relationship between mealybug mortality and the concentration of nematodes applied, with the highest level of control using a concentration of 80 infective juveniles (IJs)/insect. As nematodes would be used as an above-ground application to control *P. citri* in citrus orchards, available water is a major limiting factor. Insecticidal activity proved to be dependent on the available surface moisture after nematode application. The water activity (a_w) bioassay indicated that *S. yirgalemense* to be two times more tolerant to lower levels of free water, with a_{w50} = 0.96 and a_{w90} = 0.99, compared to *H. zealandica* with a_{w50} = 0.98 and a_{w90} = 1.0. After application, nematodes have a limited time frame in which to locate and infect hosts, as the level of available free water gradually decreases, as trees dry out. *Steinernema yirgalemense* proved able to locate and infect *P. citri* quicker than *H. zealandica.* Nematode activity was not significantly affected when exposed to 15°C, 20°C and 25°C. IJs were able to infect *P. citri* at an exposure time as short as half an hour. Results also showed that the first 2 to 4 h post application is the most decisive time for establishing successful infection of mealybugs. This is the first report on the potential use of nematodes for the control of *P. citri.*

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Introduction

Mealybugs (Hemiptera: Pseudococcidae) are regarded as pests of extreme economic importance on a wide range of field crops, fruit crops and ornamentals worldwide (Bartlett & Lloyed, 1958; Franco *et al.*, 2004). Although mealybugs occur globally, they are most abundant in the tropics and subtropics (Ben-Dov *et al.*, 2010). Throughout the world, more than 60 mealybug species have been noted to develop on *Citrus* spp., of which the majority are of minor economic importance (Franco *et al.*, 2004; Ben-Dov *et al.*, 2010). Out of the twenty mealybug species considered as economically important pests of cultivated plants in South Africa (Annecke & Moran, 1982), seven are regarded as pests of economic or potential economic importance on *Citrus* spp., of which *Planococcus citri* (Risso) is the most common and destructive (Hattingh *et al.*, 1998).

Mealybugs infest all parts of citrus trees, except the roots (Canhilal *et al.*, 2001). Damage caused by mealybugs on citrus includes: wilting, fruit and flower drop, fruit and leaf deformations, hyperpigmentation, fruit stippling and sooty mould growth (Hattingh, 1993; Blumberg *et al.*, 1995; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003).

In citrus orchards, mealybug population levels are usually suppressed by a complex of natural enemies and do not reach economically damaging levels (Hattingh, 1993; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003). However, if the natural enemy complex is disrupted by the application of chemical pesticides intended for the control of other important citrus pests, such as false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), citrus thrips, *Scriptothrips aurantii* (Faure) (Thysanoptera: Thripidae) or red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), mealybug populations can rapidly increase to damaging levels (Hattingh & Tate, 1995; Hattingh *et al.*, 1998). Mealybugs are difficult to control with insecticides, as they display cryptic behaviour by hiding in spaces where chemicals cannot reach them (McKenzie, 1967; Michelakis & Hamid, 1995; Franco *et al.*, 2004). Their waxy coverings also act as a barrier against insecticides and they have the ability to develop resistance to insecticides at an alarming rate (McKenzie, 1967; Blumberg & Van Driesche, 2001; Mahfoudhi & Dhouibi, 2009). According to Ehlers (1996), the development of resistance to insecticides is one of the major driving forces for seeking alternative insect control methods.

Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* (Rhabditida: Heterorhabditidae) and *Steinernema* (Rhabditida: Steinernematidae) have proven to control a broad range of economically important insect pest species (Grewal *et al.*, 2005) and are used as inundatively applied biological control agents (Hazir *et al.*, 2003). Both genera have a free-living non-feeding stage that can actively detect and

infect hosts (Glazer & Lewis, 2000). Once inside the host's haemocoel, nematodes release symbiotic bacteria that kill hosts by means of septicaemia within 24 to 48 h (Adams & Nguyen, 2002; Dowds & Peters, 2002). These nematodes have no known negative effect on the environment, humans or other vertebrates (Akhurst & Smith, 2002). No special measures are required for application, as nematodes can be applied as an aqueous suspension, using ordinary agrochemical spray equipment (Grewal, 2002; Hussaini, 2002). Unlike most other inundatively applied biological control agents, nematodes have proven to be tolerant to short periods of exposure (2-24 h) to a wide variety of agrochemicals (Rovesti & Deseö, 1990).

To date, very few studies have been done on the susceptibility of Pseudococcidae to EPNs. With the use of sand-dish bioassays, Stuart *et al.* (1997) determined the susceptibility of *Dysmicoccus vaccinii* (Miller & Polavarapu) to four nematode strains. Mealybugs were most susceptible to *Heterorhabditis bacteriophora* Poinar, 1976, which caused 64% mortality. In sand-dish assays, both *H. bacteriophora* and *Heterorhabditis indicus* Poinar, 1992 could complete their life cycle in *D. vaccinii* and caused significant mealybug mortality. They also showed that the susceptibility of mealybugs to EPNs is not influenced by the presence or absence of their protective wax coatings. Laboratory tests aimed at determining the susceptibility of the coffee root mealybug, *Dysmicoccus texensis* (Tinsley), to various strains of *Heterorhabdidits* showed that most of the strains tested were highly virulent (Alves *et al.*, 2009). Bioassays performed by Stokwe (2009) showed that the obscure mealybug, *Pseudococcus viburni* (Signoret), varies greatly in susceptibility from one nematode species to the next and was found to be generally more susceptible to *Heterorhabditis* spp. Bioassays also showed that all life stages of female *P. viburni*, from crawler to adult, were susceptible to nematodes to some degree. EPNs have also shown to have the ability to locate and to kill hosts in cryptic habitats, such as inside the calyx and ovary of apples.

Before laboratory bioassays were performed, the quality of infective juveniles (IJs) during a four-week storage period was tested. The overall objective of this study was to determine the potential of nematodes to control adult females of *P. citri.* The specific aims of the study were: to identify nematode species which are highly pathogenic to *P. citri*; to observe the life cycle of a heterorhabditid and steinernematid in *P. citri;* to determine the optimal nematode concentration at different humidity levels; to assess the effect of the water activity (a_w) on mortality; and to establish the exposure time at various temperatures required to ensure satisfactory levels of *P. citri* mortality.

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Materials and methods

Source of nematodes

Nematode species used in the current study (Table 2.1) were originally obtained from previous local surveys (Malan *et al.*, 2006; Malan *et al.*, 2011) and maintained in the Stellenbosch University nematode collection. IJs of the six nematode species (Table 2.1) were cultured in last larvae instar of the mealworm, *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) or the wax moth larvae, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) at room temperature, according to the procedures described by Kaya and Stock (1997). The IJs were harvested during the first week of emergence and stored horizontally at 14ºC, in 500-ml vented culture flasks containing 150 ml of distilled water. Nematodes were used within three weeks after harvesting. Flasks were shaken weekly to improve aeration and nematode survival. Nematode concentrations used in experiments were calculated using the method developed by Navon and Ascher (2000).

Table 2.1: *Heterorhabditis* and *Steinernema* species, strain, habitat, locality and GenBank accession number.

Species	Strain	Habitat	Locality	GenBank accession number
H. zealandica	SF 41	Natural	Patensie, Eastern Cape	EU699436
H. bacteriophora	SF 351	Disturbed	Wellington, Western Cape	FJ455843
H. safricana	SF 281	Disturbed	Piketberg, Western Cape	EF488006
S. khoisanae	SF 87	Disturbed	Villiersdorp, Western Cape	DQ314289
S. citrae	141-C	Disturbed	Piketberg, Western Cape	EU740970
S. yirgalemense	157-C	Disturbed	Friedenheim, Mpumalanga	EU625295

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

Source of insects

The identity of *P. citri* used in the current study was verified using morphological (Wakgari & Giliomee, 2005) and molecular techniques (Pieterse *et al.*, 2010). Mealybugs were reared in the laboratory on butternuts and sprouting potatoes. Cultures were kept in cages (650 mm × 350 mm × 590 mm) with substantial ventilation, allowing efficient airflow. Only adult female mealybugs were selected for use in experiments. Mealworm larvae were reared at room temperature in plastic containers on fine wheat bran. To

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improve humidity, potato peels or apple slices were laid over the surface of the colony. Last-instar mealworms were harvested regularly and kept at 4°C until needed. *Galleria mellonella* larvae were kept in a growth chamber at 25°C and reared on a diet consisting of baby cereal (Cerelac Nestlé™), brown bread flour, yeast, wheat germ, beeswax, glycerine and honey.

Bioassay protocol

Planococcus citri were individually exposed to IJs in multiwell bioassay plates (24 wells, flat bottom, Nunc™ Cat. No. 144530). Five bioassay plates were used per treatment and five control plates for each treatment, each containing evenly distributed adult female mealybugs were prepared. Each well was lined with a circular paper disc (13-mm-diameter) before mealybugs were added. Mealybugs were then inoculated individually with the required concentration of nematodes (Navon & Ascher, 2000). Control plates received 50 µl of distilled water only. To retain insects in their individual wells, each plate was covered with a fitted piece of glass. After inoculation, plates were placed inside plastic containers lined with moistened paper towels and closed with the lid to maintain high humidity levels (RH ± 95%). Plastic containers were then incubated in a dark growth chamber at 25 ± 2ºC for 48 h, after which the mortality of the mealybugs was determined by means of gentle prodding.

Influence of time on nematode quality

To ensure IJ quality, nematodes are usually used as inoculum within the first four weeks after being harvested. To determine the influence of time after harvest on nematode quality, the multiwell bioassay protocol was followed. *Heterorhabditis zealandica* Poinar, 1990 was chosen as the indicator species. After harvest, inoculum was stored in culture flasks at 14°C and tested for infectivity one, two, three and four weeks after harvesting. Five treatment plates and five control plates, each containing 12 evenly distributed adult female mealybugs were prepared for each predetermined test date (5 replicates; 60 insects per week). Mealybugs were then inoculated individually with a concentration 100 IJs/50 µl. Mealybug mortality was determined after 48 h. This experiment was repeated on a different test. The data of both trials experiments were pooled for analysis.

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Screening

The multiwell bioassay protocol was used to test the susceptibility of adult female *P. citri* to of six indigenous EPN species under optimal conditions. Three heterorhabditids, namely *H. zealandica, H. bacteriophora* and *Heterorhabditis safricana* Malan, Nguyen, De Waal & Tiedt, 2008, and three steinernematids, namely *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006, *Steinernema citrae* Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011 and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler & Adams, 2005 were used. Five treatment plates and five control plates, each with twelve evenly distributed adult female mealybugs, were prepared for each species and strain tested (5 replicates; 60 per nematode species). Mealybugs were inoculated individually with 200 IJs/insect and, after 48 h, mortality was determined. The experiment was repeated on a separate test date. The data of both experiments were pooled for analysis. The data of both experiments were pooled for analysis

Biological study

In thisstudy**,** the development of *H. zealandica* and *S. yirgalemense* in *P. citri* was determined. The multiwell bioassay protocol was followed. Adult female mealybugs were individually exposed to 50 IJs in multiwell plates. Thirty bioassay plates, each containing five evenly distributed mealybugs, were prepared for both *H. zealandica* and *S. yirgalemense*. Two days after inoculation, mealybugs were rinsed in distilled water to remove any remaining nematodes from their body surface and moved to clean Petri dishes (13-cm-diameter). Each new Petri dish contained a total of 50 mealybugs. To determine the developmental stage of each nematode species, 25 mealybugs were randomly selected and dissected every one to two days for ten days. To determine whether *H. zealandica* and *S. yirgalemense* could complete their life cycle in *P. citri*, five Petri dishes with ten infected mealybugs each were left undisturbed and placed in white traps. Mealybugs were also assessed for colour change, number of nematodes penetrated and quality of mealybug eggs, and the sex ratio of *S. yirgalemense* was determined.

Influence of nematode concentrations and humidity on *P. citri* mortality

The influence of increasing concentrations of *H. zealandica* was determined at three humidity levels (80%, 60% and 100% RH). The lethal concentration of *S. yirgalemense* was determined at 100% RH only. To achieve the required humidity levels, airtight containers with solutions of glycerol (60% RH), KNO_3 (80%

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RH) and moistened tissue paper (100% RH) were prepared (Winston & Bates, 1960). Five multiwell plates, with each containing eight evenly distributed adult female *P. citri*, were prepared (5 replicates; 40 insects) for each of the different nematode concentrations (0-, 5-, 10-, 20-, 40- and 80 IJs/mealybug) at each of the different levels of humidity (60%, 80% and 100% RH) tested. Multiwell plates were lined with filter paper discs and covered with fine netting to allow airflow while preventing mealybugs from escaping. After inoculation, mealybugs were incubated in a growth chamber with a day cycle starting at 22°C for 14 h and at 11°C for 11 h. After 72 h mealybug mortality was assessed. The experiment was repeated on a separate date. The data of both experiments were pooled for analysis.

Effect of water activity levels on mortality

To assess the performance of *H. zealandica* and *S. yirgalemense* against adult *P. citri* females at reduced moisture levels, the two species were tested at different water activity (a_w) levels. The required a_w values were achieved by adding different volumes of water containing 50 IJs/insect to small Petri dishes (3-cm-diameter) lined with filter paper. Control treatments contained water only. The a_w values were measured using a Decagon Pawkit water-activity-meter (Decagon Devices Inc., Pullman, WA, USA) at a constant temperature of 25°C. Four adult mealybugs were added to each dish, covered with cling wrap and sealed with a lid to ensure an airtight seal. Dishes were incubated 22°C for 72 h, after which mealybug mortality was assessed. Five replicates were prepared for both treatments and control dishes for each nematode species and a_w-level tested (5 replicates; 20 insects per a_w-level and nematode combination). The experiment was repeated on a separate date. The data of both experiments were pooled for analysis.

Influence of exposure time and temperature on mortality

The activity of *H. zealandica* IJs at three different temperatures (15°C, 20°C and 25°C) was assessed, with the activity of *S. yirgalemense* being assessed at 25°C only. Five Petri dishes (13-cm- diameter) lined with filter paper, each containing six adult female mealybugs (5 replicates; 30 insects), were prepared for each of the time intervals and temperatures tested. Nematodes were applied to Petri dishes at a concentration of 80 IJs/insect in 1 ml of water and incubated at 100% RH in sealed plastic containers lined with moistened filter paper. An equal number of Petri dishes were prepared for control treatments that received 1 ml distilled water only. After inoculation, mealybugs were removed from Petri dishes after 30-, 60-, 180-, 240- and 480- min intervals, rinsed with distilled water to remove excess surface nematodes and

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placed in clean Petri dishes. Mealybugs were then incubated at 25°C for 48 h, after which mortality was assessed. The experiment was repeated on a separate date. The data of both experiments were pooled for analysis.

Data analysis

Data of all trials, apart from the lethal concentration and humidity trial, were corrected using Abbott's formula (Abbott, 1925), to compensate for mealybugs that died of natural causes prior to analysis. All statistical analyses were performed by means of Statistica 9.0 software (StatSoft Inc. 2009). Data were analysed using ANOVA, with post-hoc comparison of means using Bonferroni's method, or a bootstrap multi comparison if residuals were not evenly distributed (Efron & Tibshirani, 1993). Significant differences were determined on a 95% probability level. To determine lethal concentration (LC), water activity (a_w) and exposure time (min), a probit analysis (Finney 1971) was conducted using Polo PC (LeOra Software 1987).

Results

Influence of time on nematode quality

The results of insect mortality, as influenced by time, were analysed using a one-way ANOVA, and showed significant differences ($F_{(3,36)} = 4.722$; $P < 0.01$) between weeks (Fig. 2.1). Nematode mortality was significantly lower at week four than at week three ($P= 0.006$), but not significantly lower than during the first two weeks (Fig. 2.1). Nematodes caused the lowest mortality (81%) four weeks after harvest, compared to mortality (89% to 95%) obtained during the first three weeks after harvest.

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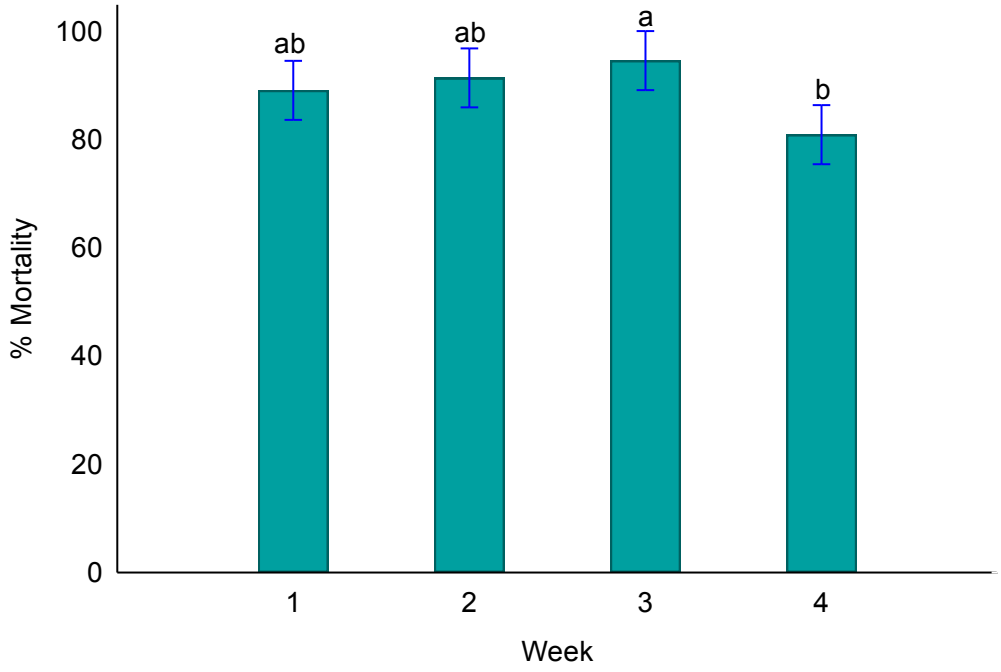

Fig.2.1: Mean percentage (95% confidence interval) mortality for adult female *Planococcus citri* determined 48 h after being inoculated with 100 *Heterorhabditis zealandica* infective juveniles/insect at weekly intervals after IJs had been harvested. Means with the same letter are not significantly different.

Screening

The percentage mortality of *P. citri* caused by the six nematode species was analysed using a one-way ANOVA. Significant differences were obtained ($F_{(5,\ 54)}$ = 18.91; P < 0.01) for percentage mortality between the different nematode species. Mortality (91% - 97%) caused by *H. zealandica* and *S. yirgalemense*, was significantly higher than mortality (63% - 74%) caused by the other four nematode species tested (Fig. 2.2). Although not significant, *S. yirgalemense* caused a higher average percentage mortality of 97%, compared to mortality of 91% obtained for *H. zealandica*.

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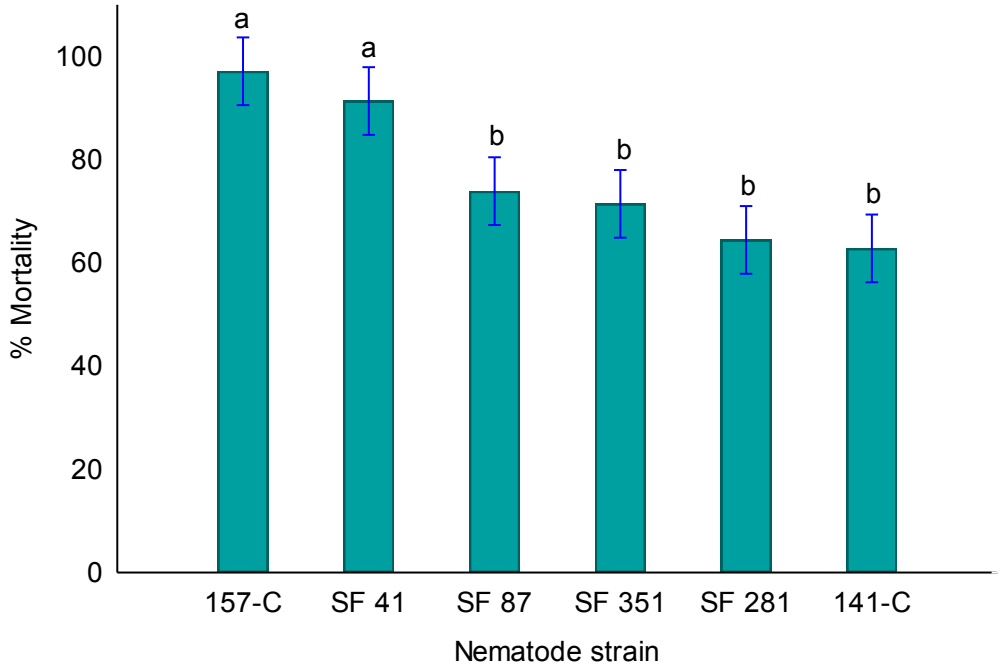

Fig. 2.2: Mean percentage (95% confidence interval) mortality for adult female *Planococcus citri*, using six species of entomopathogenic nematodes (157-C = *Steinernema yirgalemense;* SF 41 = *Heterorhabditis zealandica;* SF 87 = *S. khoisanae;* SF 351 = *H. bacteriophora;* SF 281 = *H. safricana;* 141-C = *S. citrae*) at a concentration of 200 infective juveniles/insect, after a period of 48 h in multiwell bioassay plates. Means with the same letter are not significantly different.

Biological studies

Changes in infected mealybug and nematode development were documented for ten days after exposure to IJs of *H. zealandica* and *S. yirgalemense* (Table 2.2). Mealybugs infested with *H. zealandica* became a rusty-brown colour compared to those infested with *S. yirgalemense*, which varied in colour change from none to various shades of brown (Fig. 2.3). No colour changes were observed for mealybug eggs that remained viable**.** Hermaphrodites with eggs were observed three days after infection of mealybugs with *H. zealandica* (Fig. 2.4A). Larvae were observed inside hermaphrodites after four days (Fig. 2.4B), and after eight days IJs emerged and the life cycle was completed. Mature males (Fig. 2.4C) and females with eggs (Fig. 2.4D) were observed three days after infecting mealybugs with *S. yirgalemense.* After four days, females with larvae inside were observed, and after six days IJs emerged and the life cycle was completed. Both nematode species completed one generation before IJs emerged. Although the majority of hosts

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infected with *S. yirgalemense* produced IJs, in some hosts nematodes only developed up to a certain point and then died. This was generally observed when the number of nematodes that penetrated the host was too high, with no colour change being observed in such hosts. The mean number of IJs that penetrated hosts (n = 25) was five for *H. zealandica* and nine for *S. yirgalemense*. The number of male and female *S. yirgalemense* was also documented and showed that there were only slightly more females than males, with a sex ratio of 1:1.

Table 2.2: Nematode development in adult female *Planococcus citri* inoculated with infective juveniles of *Heterorhabditis zealandica* and *Steinernema yirgalemense*.

Nematode species	**No. of days**	**Stage of nematode development**
H. zealandica	2	Immature
	3	Hermaphrodites with eggs
	4	Hermaphrodites with larvae
	6	Hermaphrodites with larvae
	8	Infective juveniles
	10	Infective juveniles
S. yirgalemense	2	Immature
	3	Males and females with eggs
	4	Males and females with larvae
	6	Infective juveniles
	8	Infective juveniles
	10	Infective juveniles

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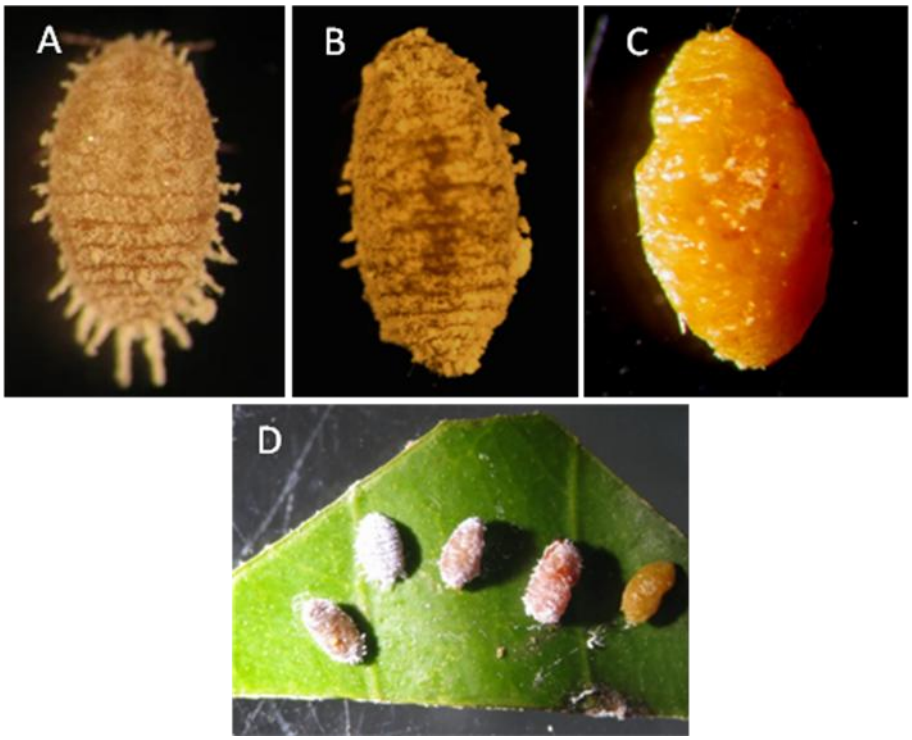

Fig. 2.3: Change in *Planococcus citri* appearance (A) before infection, and 48 h after infection with (B) *Heterorhabditis zealandica* and (C and D) *Steinernema yirgalemense.*

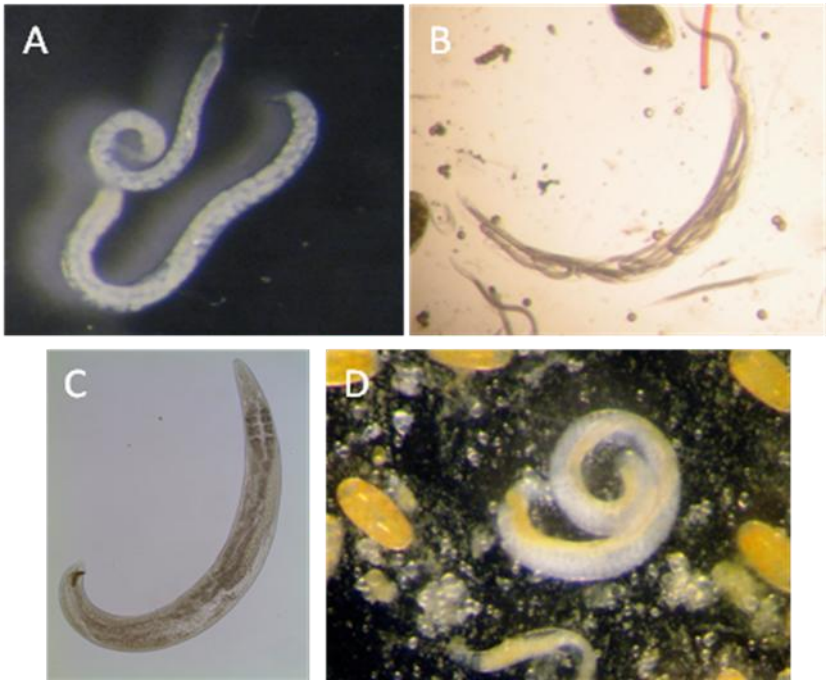

Fig. 2.4: Nematode development documented after infection showing (A) *Heterorhabditis zealandica hermaphrodite* with eggs present after three days, (B) *H. zealandica* hermaphrodite with larvae present after four days, and (C) *Steinernema yirgalemense* male and (D) female with eggs present after three days.

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Influence of nematode concentration and humidity on mortality

Results were analysed using a two-way ANOVA and showed no interaction between main effects species (2 levels: *S. yirgalemense* and *H. zealandica*) and nematode concentration (6 levels: 0, 5, 10, 20, 40, 80 IJs/insect) ($F_{(5, 108)}$ = 0.692; P = 0.630). Species behaved constantly at each nematode concentration level and no significant differences were observed between species at any of the nematode concentration levels. A one-way ANOVA for total mortality caused by each species further indicated that they did not differ significantly from each other ($F_{(1, 108)}$ = 2.35; P = 0.13). The data of both species were thus pooled together for further analysis, using a one-way ANOVA ($F_{(5, 108)}$ = 84, 734; P < 0.01) to determine the effect of increasing nematode concentrations on mealybug mortality at 100% relative humidity. The results illustrated a positive relationship between insecticidal activity and nematode concentration, with an accumulative increase in mortality as nematode concentrations increased (Fig. 2.5). At a concentration of 5 IJs/insect, 43% mortality was obtained, which was significantly higher than the control (P < 0.001) that caused 13% mortality. Although not significant, mortality increased gradually from a concentration of 10 to 40 IJs/insect, with mortality increasing from 59% to 71%. Mealybug mortality, however, increased significantly when a concentration of 80 IJs/insect was applied, obtaining 89% control.

Results of the probit analysis showed the probit regression lines for *H. zealandica* and *S. yirgalemense* to be the same (χ^2 = 0.12; d.f. = 2; P = 0.99) indicating the insecticidal activity of *H. zealandica* and *S. yirgalemense* to be identical (Fig. 2.6). The common probit regression line for the two species was Y = 3.85 + 1.09 (X) were Y is the probit mortality and X is log (nematode concentration). The data fitted this model well (χ^2 = 8.97; d.f. = 8; P = 0.35) indicating a positive relationship between nematode concentration and insecticidal activity of the two nematode species. The LC_{50} and LC_{90} values were 11.40 (90% fiducial limits: 8.53 – 14.34) and 107.00 (90% fiducial limits: 107.95 – 342.75) respectively.

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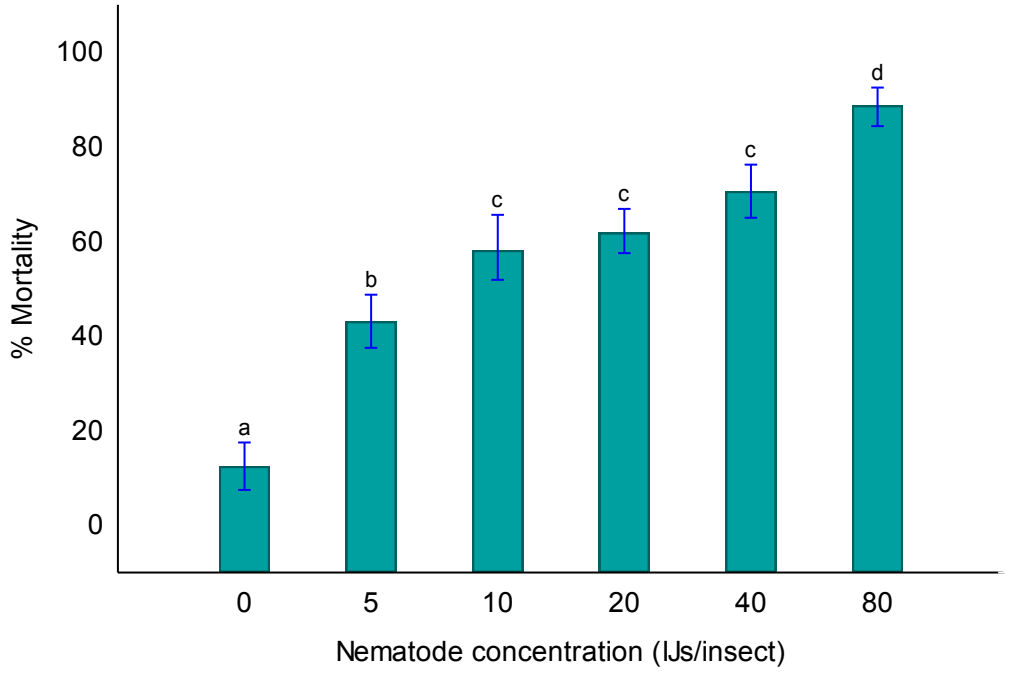

Fig. 2.5: Pooled mean percentage mortality (95% confidence interval) of adult female *Planococcus citri* after exposure to different concentrations of infective juveniles at 100% humidity. Different letters above vertical bars indicate significant differences.

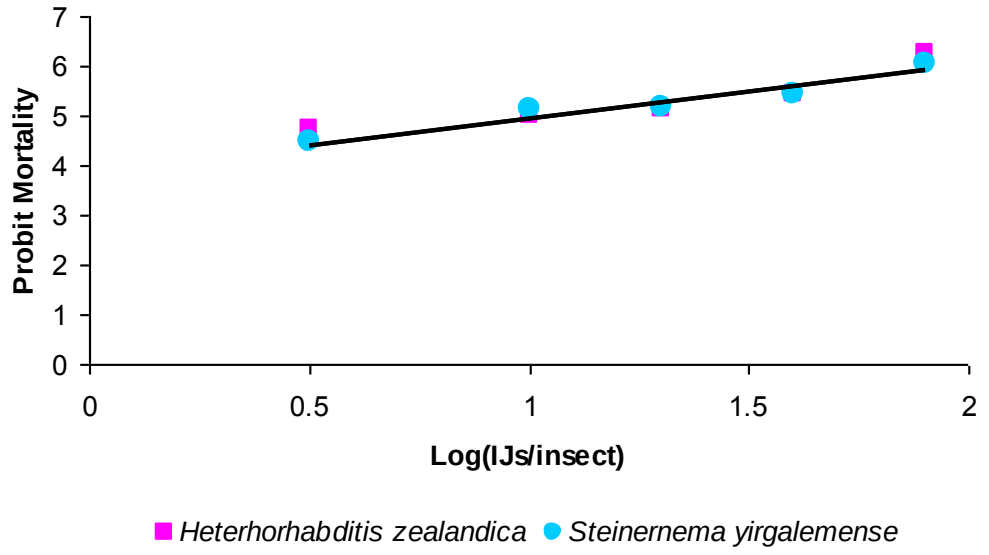

Fig. 2.6: Probit mortality of *Planococcus citri* of the logarithm of the number of infective juveniles per insect at 100% humidity and 25°C.

The results obtained from the humidity and nematode concentration experiment that were analysed using a probit analysis showed the probit regression lines for 60% -, 80% - and 100% RH to differ from each other (χ^2 = 204.10; d.f. = 4; P = 0.001), indicating the insecticidal activity of *H. zealandica* to differ when exposed to the various humidity levels (Fig. 2.7). The regression lines were however parallel to each other (χ^2 = 1.36; d.f. = 2; P = 0.506). The probit regression lines for 60% -, 80% - and 100% RH were Y = 2.17 + 1.12 (X), Y = 2.50 + 1.12 (X) and Y = 3.83 + 1.12 (X) respectively where Y is the probit mortality and X is log (nematode concentration). The data fitted this model well (χ^2 = 10.89; d.f. = 11; P = 0.45) indicating a positive relationship between nematode concentration and insecticidal activity of nematodes when exposed to the various humidity levels. The LC_{50} and LC_{90} values were 331.40 (90% fiducial limits: 198.05 – 671.61) and 4593.60 (90% fiducial limits: 1882.50 – 17175.0) at 60% RH, 169.33 (90% fiducial limits: 113.12 – 105.76) and 2345.60 (90% fiducial limits: 1076.80 – 7444.10) at 80% RH and 11.14 (90% fiducial limits: 8.12 – 14.51) and 154.33 (90% fiducial limits: 100.79 – 284.28) at 100% RH respectively. The relative potencies and their fiducial limits of nematodes when exposed to the different humidity levels are given in Table 2.3 which indicates nematodes to be 2 times more potent at 80% RH than at 60% RH, 15.19 times more potent at 100% humidity than at 80% humidity and 29.76 times more potent than at 60% humidity.

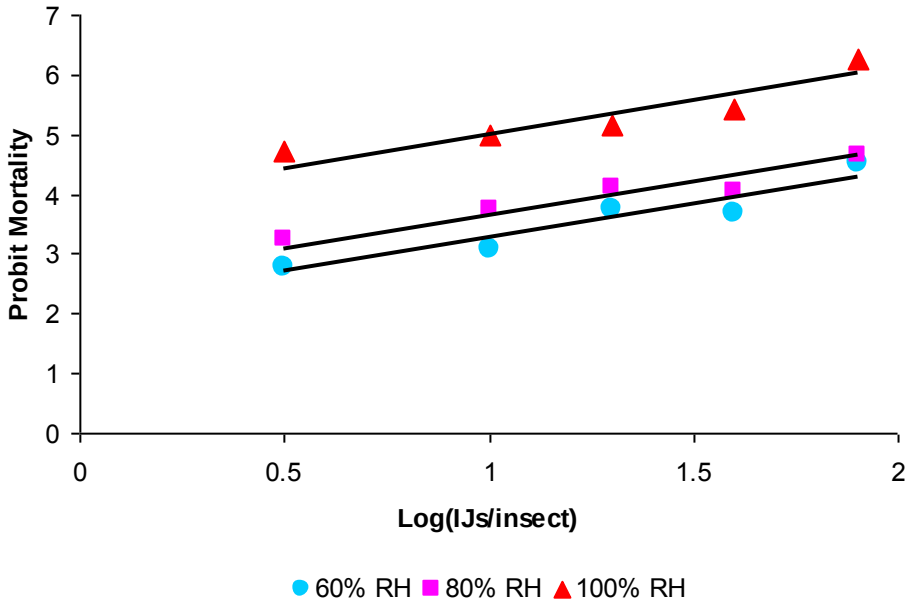

Fig. 2.7: Probit mortality of *Planococcus citri* of the logarithm of the number of infective juveniles per insect at 60%- 80%- and 100% relative humidity and 25°C

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Table 2.3: Relative potencies of nematodes when exposed to 60- 80 and 100% relative humidity at 25 °C

Potency at	Relative to	Potency	95% Fiducial Limits
100	80	1.96	1.02 – 3.85
100	60	29.76	14.23 – 88.33
80	60	15.19	13.26 – 23.00

Effect of water activity levels on mortality

The volumes of water used, with and without nematodes, to attain a_w values of 0.8 to 1.0 are indicated in Table 2.4. For the lowest a_w value of 0.8, 5.5 µl of water was used, with and without nematodes, and for the highest a_w value of 1.00, a volume of 27 µl was used.

Table 2.4: The volume of water containing 50 infective juveniles/insect, or water only for control treatments, added to Petri dishes (3-cm-diameter) lined with filter paper to attain corresponding water activity (a_w) values.

a_w Value	Inoculum (µl)	a_w Value	Inoculum (µl)
0.8	5.5	0.95	16
0.9	7	0.96	17.5
0.91	8	0.97	20
0.92	9	0.98	23
0.93	10.5	0.99	25.5
0.94	13	1.00	27

The data obtained on the effect of water activity (a_w) on the two nematode species for mortality of mealybug females were analysed using a two-way ANOVA. No interaction was shown between the main effects nematode species (2 levels; *H. zealandica* and *S. yirgalemense*) and water activity level (12 levels; a_w = 0.8, 0.9, 0.91…..1.00) ($F_{(11, 216)}$ = 0.80; P = 0.64), indicating nematode species to have behaved constantly at each a_w value. Because no significant differences were observed between the two species, data were pooled for mortality and analysed using a one-way ANOVA ($F_{(11, 216)}$ = 39.05; P < 0.01). The results showed that *S. yirgalemense* caused significantly higher mortality (37%) in comparison to *H. zealandica* (24%). Data for the effect of water activity (a_w) on mortality of both species were also pooled for further analysis. A one-way ANOVA showed a significant positive effect of increasing a_w values on insecticidal activity ($F_{(11, 216)}$ = 39.05; P < 0.01). Very low levels of mortality (≤ 20%) were observed at a_w values 0.80 to 0.94. Mortality (8%-

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58%) increased gradually at a_w values 0.95 to 0.97, with the highest mortality being observed at a_w values 0.98 to 1.00, causing 59% to 96% mortality.

Results of the probit analysis showed the probit regression lines for *H. zealandica* and *S. yirgalemense* to differ from each other (χ^2 = 26.13; d.f. = 2; P = 0.001), indicating the insecticidal activity of *H. zealandica* and *S. yirgalemense* to differ when exposed to the various water activity levels (Fig. 2.8). The regression lines were however parallel to each other (χ^2 = 2.85; d.f. = 1; P = 0.091). The probit regression lines for *H. zealandica* and *S. yirgalemense* were Y = 6.53 + 95.98 (X) and Y = 5.94 + 95.98 (X) respectively where Y is the probit mortality and X is log (nematode concentration). The data fitted this model well (χ^2 = 23.13; d.f. = 21; P = 0.66) indicating a positive relationship between water activity and insecticidal activity of the two nematode species. The a_{w50} and a_{w90} values were 0.96 and 0.99 for *S. yirgalemense* and 0.98 and 1.01 for *H. zealandica* respectively. Furthermore results showed *S. yirgalemense* to be 2 times more potent than *H. zealandica.*

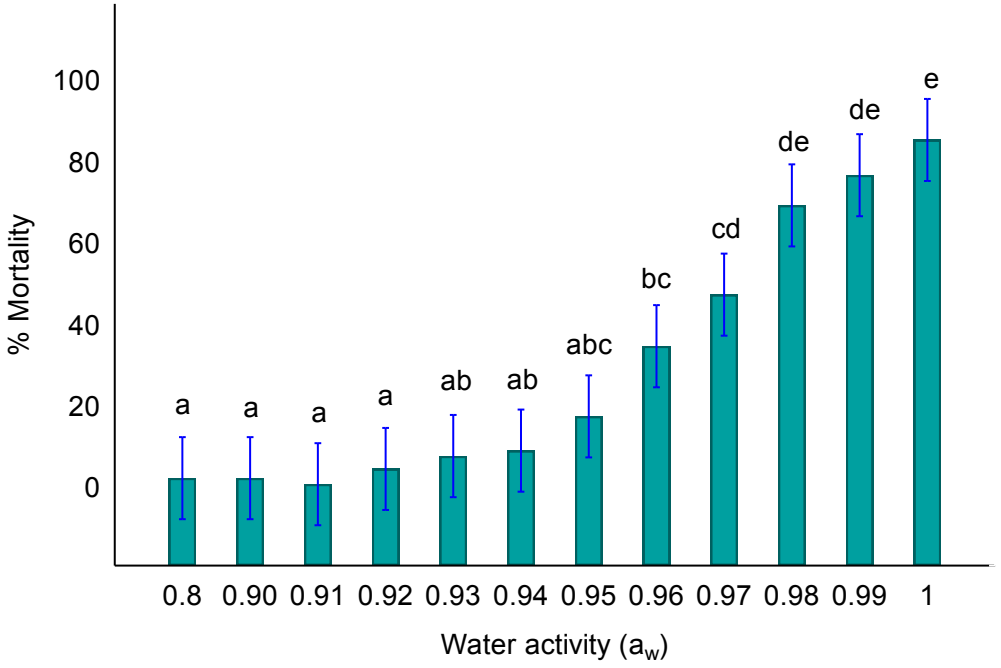

Fig. 2.8: Pooled mean percentage mortality (95% confidence interval) recorded for *Planococcus citri* after exposure to 50 infective juveniles/insect at different water activity (a_w) levels. Different letters above vertical bars indicate significant differences.

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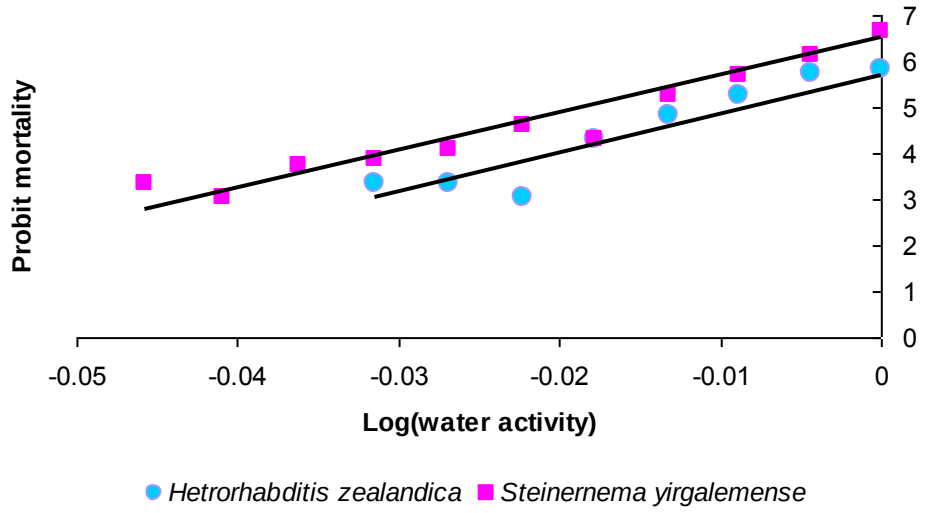

Fig. 2.9: Probit mortality of *Planococcus citri* of the logarithm of the water activity level to which *Heterorhabditis zealandica* and *Steinernema yirgalemense* were exposed.

Influence of exposure time and temperature on mortality

The two-way ANOVA showed no interaction between main effects exposure nematode species (6 levels; 0- 30- 60- 120- 240- 480 min) and nematode species (2 levels; *H. zealandica* and *S. yirgalemense*) ($F_{(4, 90)}$ = 1.44; P = 0.23). Although no significant differences were observed between species at any of the exposure intervals (Fig. 2.10), *S. yirgalemense* consistently caused higher mortality than did *H. zealandica* at each exposure time. A one-way ANOVA for pooled mortality caused by each species showed that *S. yirgalemense* caused significantly higher mortality than did *H. zealandica* ($F_{(1, 90)}$ = 1.44; P = 0.001). Low levels of mortality (10%-29%) were recorded when mealybugs were exposed to nematodes for 30 to 120 min (Fig. 2.10). The highest mortality levels were observed after 480 min exposure and ranged between 27% and 54%. Using a one-way ANOVA, data for both species were pooled for further analysis to illustrate that mortality (40%-43%) obtained after 240 and 480 min exposure to nematodes was significantly higher ($F_{(4, 135)}$ = 12.825; P > 0.01) than mortality (14%-20%) after 30, 60 and 120 min.

Results of the probit analysis showed the probit regression lines for *H. zealandica* and *S. yirgalemense* to differ from each other (χ^2 = 13.47; d.f. = 2; P = 0.001) indicating the insecticidal activity of *H. zealandica* and *S. yirgalemense* to differ when exposed to mealybugs for the various time intervals (Fig. 2.11). The regression lines were however parallel to each other (χ^2 = 0.54; d.f. = 1; P = 0.46). The probit

regression lines *H. zealandica* and *S. yirgalemense* where Y = 1.31 + 1.23 (X) and Y = 1.83 + 1.23 (X) respectively where Y is the probit mortality and X is log (minutes). The data fitted this model well (χ^2 = 7.28; d.f. = 7; P = 0.99) indicating a positive relationship between exposure time and insecticidal activity of nematodes. The LC_{50} and LC_{90} values were 971.58 (90% fiducial limits: 595.05 – 2125.05) and 10644 (90% fiducial limits: 4086.0 – 59691.00) for *H. zealandica* and 371.43 (90% fiducial limits: 261.019 – 631.15) and 4101 (90% fiducial limits: 1847.60 – 17227) for *S. yirgalemense* respectively. Furthermore results showed *S. yirgalemense* to be 2.59 times more potent than *H. zealandica.*

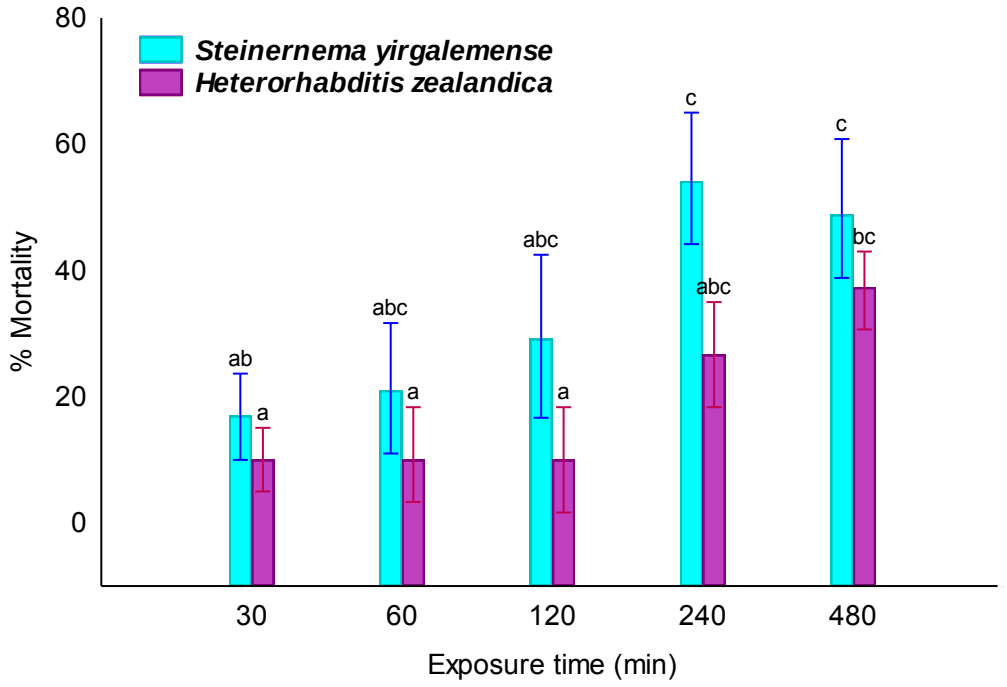

Fig. 2.10: Percentage mortality (95% confidence interval) recorded for *Planococcus citri* after exposure to 80 infective juveniles/insect of *Heterorhabditis zealandica* and *Steinernema yirgalemense* for different lengths of time at three different temperature levels. Different letters above vertical bars indicate significant differences.

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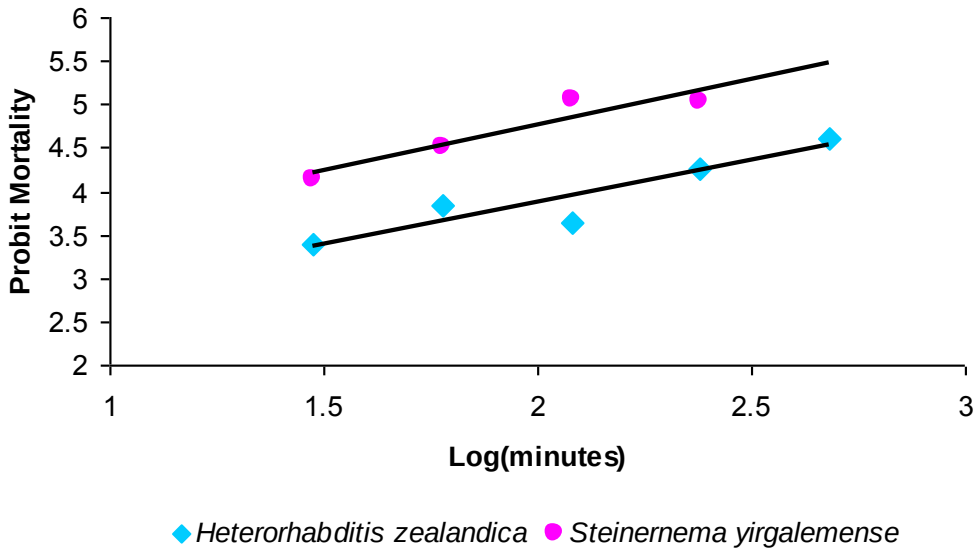

Fig. 2.11: Probit mortality of *Planococcus citri* of the logarithm of exposure time (minutes) interval in which mealybugs were exposed to *Heterorhabditis zealandica* and *Steinernema yirgalemense* at 25 °C.

The results obtained from the temperature and exposure time experiment that were analysed using a probit analysis which showed the probit regression lines for 15°C, 20°C and 25°C to be the same (χ^2 = 8.21; d.f. = 4; P = 0.84) indicating the insecticidal activity of *H. zealandica* to be unaffected (Fig. 2.12). The common probit regression line for the three temperatures species was Y = 1.30 + 1.04 (X) were Y is the probit mortality and X is log (minutes). The min_{50} and min_{90} values were 1544.09 (90% fiducial limits: 953.00 – 3549.11) and 23080.00 (90% fiducial limits: 8013.7 – 0.15245E + 06) respectively.

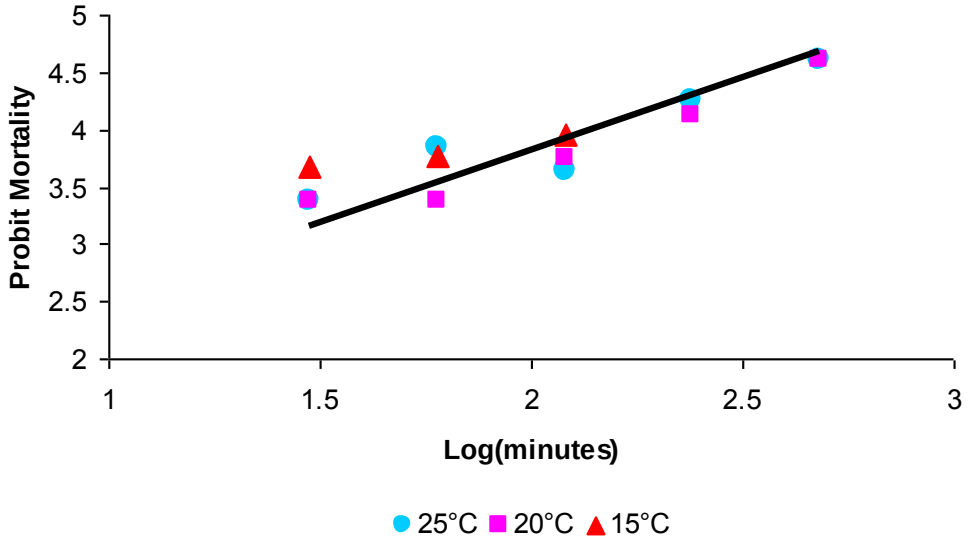

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Fig. 2.12: Probit mortality of *Planococcus citri* of the logarithm of exposure time (minutes) to which *Heterorhabditis zealandica* and *Steinernema yirgalemense* were exposed to mealybugs at 25 °C, 15 °C and 25 °C.

Discussion

No previous research has been conducted on the use of EPNs for the control of *P. citri*. The biology of both nematode and host contribute to the possibility of achieving successful control of mealybugs in general. On citrus trees, *P. citri* prefer to infest fruit and reside in protected areas, such as under fruit calyxes or within bud-mite-induced deformations (Wakgari & Giliomee, 2003). Mealybugs are difficult to control chemically, as they are covered by a wax coating; the cryptic lifestyle of mealybugs also prevents chemicals from reaching them. The use of chemicals also disrupts the natural enemy complex, which usually keeps mealybug populations under control (Hattingh, 1993; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003). Furthermore, mealybugs are known to develop resistance to insecticides (McKenzie, 1967; Blumberg & Van Driesche, 2001; Mahfoudhi & Dhouibi, 2009) which is one of the major reasons for seeking alternative control measures. In contrast, EPNs are able to crawl into hiding places after their hosts. The presence of wax coatings on mealybugs has also proven to have no influence on nematode pathogenicity (Stuart *et al.*, 1997). As biological control agents, EPNs also have the added advantage of being tolerant to short periods of exposure to a wide range of agrochemicals, so that nematodes can be applied together with or shortly before or after the application of such chemicals (Rovesti & Deseö, 1990).

Since nematodes are not currently commercially available in South Africa as a formulated product, IJs used in the study were stored horizontally in ventilated culture flasks and kept at 14°C. During storage, IJs do not feed and are dependent on reserve energy sources to survive until a host is located (Glazer, 2002). IJs are generally stored and used for bioassays within the first four weeks after harvesting (Kruitbos *et al.*, 2009; Ennis *et al.*, 2010; Malan *et al.*, 2011). Variation in nematode pathogenicity can occur during this period, so that a quality test was conducted with the same batch of IJs for infectivity of *P. citri* at weekly intervals for a period of four weeks. No significant difference in mealybug mortality caused by IJs one week after harvest in comparison to mealybug mortality caused after four weeks was obtained. However, significant difference was observed between mealybug mortality caused by IJs three weeks after harvest compared to their mortality after four weeks. The results indicate that IJs should rather be used within three

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weeks after harvesting them, as their quality seems to become unreliable after four weeks. The finding, however, requires verifying by testing quality for a further two to three weeks, as IJ quality seems to start decreasing after four weeks. It was concluded that IJs should be used within the first three weeks after harvest in order to ensure that the best results are obtained in laboratory experiments.

All six indigenous nematode species tested were able to cause high, but variable, levels of *P. citri* mortality (> 60%) under optimal conditions. *Heterorhabditis zealandica* and *S. yirgalemense* caused significantly higher mortality (> 91%) compared to the other four nematode species tested. Although not significant, *S. yirgalemense* caused a higher average percentage mortality of 97% in comparison to *H. zealandica*, which caused 91% mortality. The results are similar to those obtained by Stokwe (2009), which showed that both *H. zealandica* and *S. yirgalemense* were highly pathogenic to *P. viburni.* Using 24-multiwell bioassay plates, Malan *et al.* (2011) showed *S. yirgalemense* to be highly virulent to false codling moth larvae and, to a lesser degree, their pupa, with the pest being responsible for a great deal of destruction of citrus in South Africa, leading to significant financial losses. Of all the species tested, *S. citrae*, which was originally isolated from a citrus orchard near Piketberg in the Western Cape province of South Africa (Malan *et al.*, 2011), performed the worst percentage wise. To date, *S. citrae* has only been found in South Africa. Although not significantly so, *H. bacteriophora,* a commercially produced species in Europe and the USA, performed worse than did *S. khoisanae,* but better than *H. safricana* and *S. citrae.* Both *H. zealandica* and *S. yirgalemense* have shown great promise as potential biological control agents of *P. citri*, and were therefore selected for further use in laboratory studies.

In the study of the biology of the two nematode genera in *P. citri* as host, no sexually mature adults of *S. yirgalemense* were found to have developed after two days. After three days development, *S. yirgalemense* males and females were fully mature, and fertilised eggs were visible in females. The number of male and female *S. yirgalemense* was determined, with the sex ratio of males to females being found to be 1:1, with only slightly more females being present than males. Hermaphrodites of *H. zealandica* with eggs were also present after three days development. After four days, *H. zealandica* hermaphrodites with larvae and *S. yirgalemense* females with larvae inside were found to be present. The life cycle of *S. yirgalemense*, as shown by IJs emerging after six days, was found to be shorter than that of *H. zealandica,* whose IJs emerged after eight days. Both nematode species were able to complete their life cycle in *P. citri.*

Although IJs occur naturally in soil and *P. citri* is an above-ground pest of citrus, the ability of nematodes to complete their life cycle in *P. citri* could be advantageous in a citrus orchard. Infected hosts may fall onto the shaded, moist orchard floor and be recycled in the soil, potentially killing other citrus pests, such as false codling moth. IJs also have the ability to wait for favourable conditions before emerging (Brown & Gaugler, 1997). If IJs emerge after a rainy spell, or when trees are still moist from the morning dew, they could infect more mealybugs. Mealybugs tend to cluster together in protected areas on a tree, forming a microclimate that is suitable for nematodes. Crawlers that are less susceptible than adults (Stokwe, 2009) should increase in size after six to eight days, and could then possibly be infected.

The number of nematodes that managed to penetrate hosts was also determined, with the mean number of five and nine IJs being found to penetrate *H. zealandica* and *S. yirgalemense*, respectively. These results support those of Hominick and Reid (2009), who assume that nematodes with the highest invasion efficacy would also be more efficient in killing the target insect host. *Steinernema yirgalemense*, which caused higher mortality of *P. citri* than did *H. zealandica*, also had a higher mean penetration number of nine IJs, compared to *H. zealandica*, with a mean penetration number of five IJs. The general low numbers of IJs infecting mealybugs are due to the small size of adult females, which are approximately 3 mm long. Both the length of the life cycle and the penetration rate obtained indicate *S. yirgalemense* to be a better candidate than *H. zealandica* for the control of *P. citri.*

The results of this study clearly illustrate that *P. citri* has a nematode-concentration-dependant susceptibility to both *H. zealandica* and *S. yirgalemense.* Neither nematode species caused significantly higher mortality than the other. The pooled mortality of both species illustrated the highest percentage mortality (> 83%) when mealybugs were inoculated with the highest concentration of 80 IJs/insect. The LC_{50} and LC_{90} values for *H. zealandica* were 11 and 162 nematodes per insect, respectively. These values were considerably lower than those which were obtained by Stokwe (2009), who obtained an LC_{50} value of 54 and LC_{90} value of 330, which clearly indicated *P. citri* to be more susceptible to *H. zealandica* than to *P. viburni.* The LC values obtained for *S. yirgalemense* were similar to those for *H. zealandica* and their combined lethal dosage was determined. The combined LC_{50} and LC_{90} values were 11.40 and 170.00 respectively.

Planococcus citri only occur above ground on citrus trees, which is an important consideration for evaluating the potential of nematodes to control them. Lacey and Unruh (1998) found that the ambient humidity to which nematodes are exposed has a great influence on their ability to infect hosts, as nematodes proved to be active only at humidity levels of 95% or higher. Such findings were also prevalent in the

concentration and humidity experiment, which demonstrated nematodes to be 2 times more potent at 80% RH than at 60% RH, 15.19 times more potent at 100% humidity than at 80% humidity and 29.76 times more potent than at 60% humidity.

To enable nematodes to reach the target host, a water film is required for propulsion (Blackshaw, 1987). The a_w values indicate the available free water on the surface of leaves, fruit, barks and twigs that is required to enable nematodes to move forward to locate the sought-after hosts (Koppenhöfer, 2007). The first study on the influence of water activity on nematode efficacy was conducted by Navaneethan *et al.* (2010) using *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1998. Results of the above-mentioned study showed that *S. feltiae* could still infect codling moth larvae, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) at an a_w value as low as 0.90 with a_{w90} = 0.99. The similar results that were obtained by De Waal (2011) showed that *H. zealandica* could infect codling moth larvae, *C. pomonella,* at an a_w value as low as 0.92 with a_{w90} = 0.96. When the influence of water activity on the insecticidal ability of *H. zealandica* and *S. yirgalemense* was tested on *P. citri,* both nematode species were found capable of infecting hosts at an a_w value as low as 0.95. *Steinernema yirgalemense*, with an a_{w50} value of 0.96 and a_{w90} value of 0.99, proved to 2 times more tolerant to lower water activity levels than were *H. zealandica*, with an a_{w50} value of 0.98 and a_{w90} value of 1.0. Although nematodes have been proven to be active at lower water activity levels than expected, a positive relationship between insecticidal activity and increasing water activity levels was still found to prevail. These results indicate that the insecticidal activity of nematodes will be optimal immediately after applying nematodes to trees (a_w = 1.00), after which insecticidal activity will gradually decrease as trees dry out and the amount of available free water on the tree surfaces decreases. However, nematodes would also be inclined to move from the exposed macro-environment to the protected micro-environment of the host, with further infection of *P. citri* benefiting from added humidity and free water. A possible method for preventing such a decrease in insecticidal activity of nematodes is to apply overhead irrigation to trees both before and after applying nematodes. Doing so is, however, most probably not feasible, as *P. citri* primarily occur on fruit and leaves that are covered by waxy coatings. The waxes covering citrus fruit and leaves impair the ability of nematodes to stick to the surfaces of such fruit and leaves. Irrigating trees before application could increase application runoff, while nematodes will most probably be washed off leaves if trees are irrigated after application. Such loss of nematodes could be compensated for by applying them during the late afternoon, which would retard desiccation and the possible chance of dew forming on trees the following morning.

As mentioned above, optimal a_w levels should prevail for as long as possible in order to ensure optimal invasion of the existing insects by nematodes. The amount of free water available on tree surfaces gradually decreases after application as water evaporates, which suggests that nematodes only have a limited period during which to locate and infect hosts before becoming desiccated. It is, therefore, important to identify and to apply a nematode species with an active host-searching ability. Factors that increase desiccation include high temperatures, low levels of relative humidity, and wind. Although higher temperatures increase the rate of desiccation, nematode activity has proved to increase from 15°C to 32°C (Lacey *et al.*, 2005), and nematodes should be able to reach hosts within a shorter time, when they are exposed to higher temperatures. The exposure time and temperature experiment demonstrated that nematodes were able to infect mealybugs in an exposure time as short as 30 min long at all three temperature levels tested (15°C, 20°C and 25°C), with no significant difference regarding such ability being shown among them. The influence of exposure time on both *H. zealandica* and *S. yirgalemense* was tested at 25°C, in response to which the results showed that *S. yirgalemense* caused significantly higher mortality than did *H. zealandica*. Furthermore a Probit analysis showed *S. yirgalemense* to be 2.59 times more potent than *H. zealandica* with min_{90} and min_{50} values of 971.58 and 10644 for *H. zealandica* and 371.43 and 4101 for *S. yirgalemense* respectively. The results, therefore, suggest that *S. yirgalemense* may possibly perform better than *H. zealandica* in glasshouse and field trials when nematodes are exposed to suboptimal environmental conditions. The results illustrate that the first 120 to 240 min post application is the most decisive time for establishing successful infection of mealybugs by nematodes.

The overall results obtained from the different bioassays conducted during this study indicate that both *H. zealandica* and *S. yirgalemense* hold great potential for the control of *P. citri.* They performed very similarly, with only the results of the exposure time experiment and water activity experiment suggesting *S. yirgalemense* to possibly be a more effective biocontrol agent under suboptimal field conditions than *H. zealandica.* To further determine the potential of *H. zealandica* and *S. yirgalemense* for the control of *P. citri*, glasshouse and field trials should be undertaken. As *P. citri* is an above-ground pest of citrus, this study also illustrates that desiccation is the most important hurdle to overcome before *P. citri* can be controlled successfully under suboptimal field conditions. Innovative ideas, such as the addition of anti-desiccants and surfactants to application formulations should be tested in order to determine whether desiccation could be retarded to enhance control of *P. citri.*

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CHAPTER 3

Evaluating the addition of adjuvants to improve control of *Planococcus citri* (Hemiptera: Pseudococcidae) using entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae)

Abstract

Planococcus citri is a globally distributed, highly destructive pest of citrus. Different adjuvants can be added to formulations of entomopathogenic nematodes to improve the control of *P. citri* in citrus orchards as a foliar application. In an aqueous suspension, nematodes settle rapidly to the bottom, resulting in an uneven distribution of nematodes. The ability of two polymer products, Xanthan gum, at a concentration of 0.01% and 0.02%, and Zeba®, at a concentration of 0.01%, 0.02% and 0.03%, to retard nematode sedimentation, was evaluated. Results showed Xanthan gum, at a concentration of 0.02%, to be highly effective, with 72% of the initial nematode number still in suspension after 1 h. Zeba®, at a concentration of 0.03%, despite not being as effective as Xanthan gum, nevertheless still retarded sedimentation significantly. The addition of Xanthan gum did not improve the ability of nematodes to control *P. citri* at 60% and 80% relative humidity (RH), while the addition of Zeba® caused a significant increase in mortality. An aqueous suspension containing *Heterorhabditis zealandica* and 0.03% Zeba® increased mortality by 14% at 60% RH and by 22% at 80% RH. The same polymer formulation was tested for *Steinernema yirgalemense* and mortality increased by 21% at 60% RH and by 27% at 80% RH. The addition of the surfactant, Nu-Film-P® and Zeba® to nematode suspensions did not significantly retard application run-off. The combination of Nu-Film-P® and Zeba®, however, was able to significantly retard sedimentation, increasing the average surface concentration of nematodes deposited on leaves. This is the first report of the potential of the use of adjuvants in nematode formulations, which is aimed at improving their performance as biological control agents against *P. citri* in citrus orchards, during aerial applications.

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Introduction

Planococcus citri (Risso) (Hemiptera: Pseudococcidae), the citrus mealybug, is globally distributed and has been reported as a serious pest of citrus in Africa (Hattingh & Tate, 1996; Hattingh *et al.*, 1998), Australia (Smith *et al*. 1997; Gullan, 2000), the Mediterranean Basin (Blumberg *et al.*, 1995; Franco *et al.*, 2004), as well as North, Central and South America (Bartlett & Lloyed, 1958; Wakgari & Giliomee, 2003). *Planococcus citri* are able to reproduce rapidly under optimal environmental conditions, potentially infesting up to 100% of fruit, even when spring populations could hardly be detected (Hattingh & Moore, 2003). Mealybugs are difficult to control chemically, as they display cryptic behaviour, are covered with protective waxes (McKenzie, 1967; Michelakis & Hamid, 1995; Franco *et al.*, 2004), and have been reported to develop resistance to insecticides (McKenzie, 1967; Blumberg & Van Driesche, 2001; Mahfoudhi & Dhouibi, 2009). The use of chemical control is also undesirable, because insecticides disrupt natural enemy populations (Hattingh, 1993; Hattingh & Tate, 1995, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003) and have detrimental effects on the environment (Hussaini, 2002).

Entomopathogenic nematodes (EPNs) are used as biological control agents for a wide range of economically important insect pests (Grewal *et al.*, 2005) and could possibly be another alternative to the chemical control of *P. citri.* The nematodes have an active host-seeking ability, which enables them to reach hosts in cryptic habitats (Gaugler & Boush, 1979). As above-ground conditions are not optimal for nematode survival (Mráček, 2002; Tomalak *et al.*, 2005), the successful control of mealybugs on citrus is extremely challenging. Such abiotic factors as extreme temperatures (Lacey *et al.*, 2005), ultraviolet radiation (Gaugler & Boush, 1978; Gaugler *et al.*, 1992), wind, and low ambient humidity (Unruh & Lacey, 2001), individually and combined, limit the efficacy of EPNs above ground as a biological control agent.

Desiccation, which is accelerated by low humidity levels and high wind speed, is the most limiting factor, as nematodes require a water film to maintain mobility and ensure survival (Wright *et al.*, 2005). Schroer *et al.* (2005) evaluated the ability of various surfactant-polymer formulations to improve the ability of *Steinernema carpocapsae* (Weiser, 1955) Wouts Mráček, Gerdin & Bedding, 1982 to control the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) on cabbage leaves under above-ground conditions. An application formulation containing 0.3% Rimulgan® (surfactant) and 0.3% of the polymer xanthan (antidesiccant) obtained the best control of diamondback moth, causing >90% at 80% relative humidity (RH) and >70% mortality at 60% RH. Schroer & Ehlers (2005) tested the same formulation

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for the control of diamondback moth on cabbage leaf bioassays under suboptimal conditions for nematode survival. Their results showed the survival time of *S. carpocapsae* applied with the formulation was found to be 22 h longer at 80% RH and 17 h longer at 60% RH than when the nematodes were applied with water only.

The objective of this study was to evaluate the effect of adding adjuvants to aqueous suspensions of two nematode species. The effect of the two polymers on the sedimentation of nematodes in aqueous suspensions and the mortality of *P. citri* in laboratory bioassays was investigated. Lastly, the effects of adjuvants on nematode deposition on citrus leaves were determined.

Materials and Methods

Source of nematodes and insects

Experiments were conducted using *H. zealandica,* Poinar, 1990 (SF 41) originally isolated from soil collected in Baviaanskloof near Patensie, Eastern Cape, South Africa (Malan *et al.*, 2006) and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler & Adams, 2005 (157-C), originally isolated from soil collected from a citrus orchard near Friedenheim, Mpumalanga (Malan *et al.*, 2011). Infective juveniles (IJs) were cultured according to the procedures described by Kaya & Stock (1997) in *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) and/or *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) larvae, at room temperature. Nematodes were harvested within the first week of emergence and stored in 150 ml of distilled water in 500-ml vented culture flasks. The flasks were stored horizontally at 14°C and shaken weekly to improve aeration. IJs were used for experiments within the first three weeks after harvest. Concentrations used in experiments were quantified by using the method developed by Navon & Ascher (2000). Mealybugs were cultured on butternuts and sprouting potatoes. The identity of *P. citri* used in this study was verified using morphological (Wakgari & Giliomee, 2005) and molecular techniques (Pieterse *et al.*, 2010).

Effect of polymers on nematode sedimentation

Two polymer products, Zeba® [starch-g-poly (2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch] and Xanthan gum [polysaccharide ($C_{35}H_{49}O_{29}$)] were evaluated in terms of their ability

to retard sedimentation of *H.* zealandica in a water suspension. Zeba®, at a concentration of 0.1, 0.2 or 0.3%, and Xanthan gum, at a concentration of 0.1 or 0.2%, were added to a nematode concentration of 1 000 IJs/ml. Both polymers were compared to a control that contained nematodes in water only. Treatments were added to 25-ml measuring cylinders (of 1.5 cm diameter) and stirred thoroughly to ensure that the nematodes were evenly distributed. To estimate sedimentation time, a 50 µl sample of the suspension was collected from a depth of 2 cm after 0-, 3-, 10-, 20-, 30- and 60-min intervals from each of three cylinders prepared per treatment (n = 3) and the number of nematodes determined. The experiment was repeated on a separate date. The data of both experiments were pooled for analysis.

Effect of two polymers on mortality

Bioassays were conducted by using multiwell bioassay plates (24 wells, flat bottom, Nunc™ Cat. No.144530). Polymer products, Zeba® or Xanthan gum, were added to nematode suspensions containing either *H. zealandica* or *S. yirgalemense*, and mealybug mortality was determined at 60% and 80% RH. Five treatment plates and five control plates, each containing ten evenly distributed adult female mealybugs (n = 50) were prepared for each treatment. Each well was lined with a circular paper disc (of 13 mm diameter) before mealybugs were added. Mealybugs were then inoculated individually with 50 µl containing either *H. zealandica* or *S. yirgalemense* at a concentration of 80 IJs/insect for each of three treatments, which included 1) Zeba® at a concentration of 3 g/L; 2) Xanthan gum at a concentration of 2 g/L; and 3) distilled water. Each treatment received its own control, in which mealybugs were treated with 50 µl of the treatment formulation containing no nematodes. The data of control plates were used to prepare data of treatment plates with Abbott's formula before analysis, in order to compensate for mealybugs that died of causes other than nematode infection (Abbott, 1925). Multiwell plates were covered with fine netting, which allowed airflow while preventing mealybugs from escaping. To achieve the required humidity levels, airtight containers with solutions of glycerol (60% RH) and KNO_3 (80% RH) were prepared (Winston & Bates, 1960). After treatment, mealybugs were placed in humidity chambers and incubated in a growth chamber with a day cycle starting at 22°C for 14 h and 11°C for 11 h. Mealybug mortality was determined after 72 h. The experiment was repeated on a separate date. The data of both experiments were pooled for analysis. The data of both experiments were pooled for analysis.

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Effect of adjuvants on nematode deposition

Nematode suspensions containing 1) nematodes in water only; 2) Nu-Film-P® (Poly-1-P-menthene, spreader, sticker; Hydrotech) at a concentration 0.6 ml/L + nematodes; 3) Zeba® at a concentration of 0.3 g/L + nematodes; and 4) Nu-Film-P® + Zeba® + nematodes were applied to citrus trees at the Welgevallen experimental farm, Stellenbosch, Western Cape. Each treatment was applied to randomly selected leaves on individual trees, with a spacing of two untreated trees between the treated trees. Nematodes were applied at a concentration of 1 000 IJs/ml with the aid of calibrated hand-held spray applicators. Leaves were left for 3 min to allow excess fluid to run off before randomly selected leaves were removed from the application area. Two 2-cm^2 discs were cut out from each of the five leaves for each treatment tested (n = 10). Each leaf disc was individually rinsed off in 5 ml tap water, and the number of nematodes present in each suspension was documented. The experiment was repeated on a separate date. The data of both experiments were pooled for analysis.

Data analysis

Nematode parentages for the effect of two polymers on nematode sedimentation were calculated as a percentage of the initial number of nematodes recorded directly after stirring had ceased. All statistical analyses were performed using Statistica 9.0 software (StatSoft Inc. 2009). Data were analysed using ANOVA, with post-hoc comparison of means using Bonferroni's method, or with a bootstrap multi-comparison if residuals were not normally distributed (Efron & Tibshirani, 1993). Significant differences were determined on a 95% probability level.

Results

Effect of two polymers on nematode sedimentation

Without the addition of a polymer, 91% of nematodes were recorded beyond a depth of 2 cm after 5 min. A repeated measures ANOVA for Zeba® showed interaction between main effects concentration (4 levels; 0%, 0.01%, 0.02%, and 0.03%) and time (5 levels; 5-, 10-, 15-, 30-, and 60 min) ($F_{(12, 80)}$ = 28.36; P = 0.001). Treatments did not behave consistently over time. Compared to the control, none of the polymer

concentration levels tested was able to retard sedimentation significantly 1 h after stirring had ceased. Only the 0.03% Zeba® formulation was able to retard sedimentation significantly (P = 0.001) after 30 min sedimentation with 71% of initial nematode number recorded (Fig. 3.1).

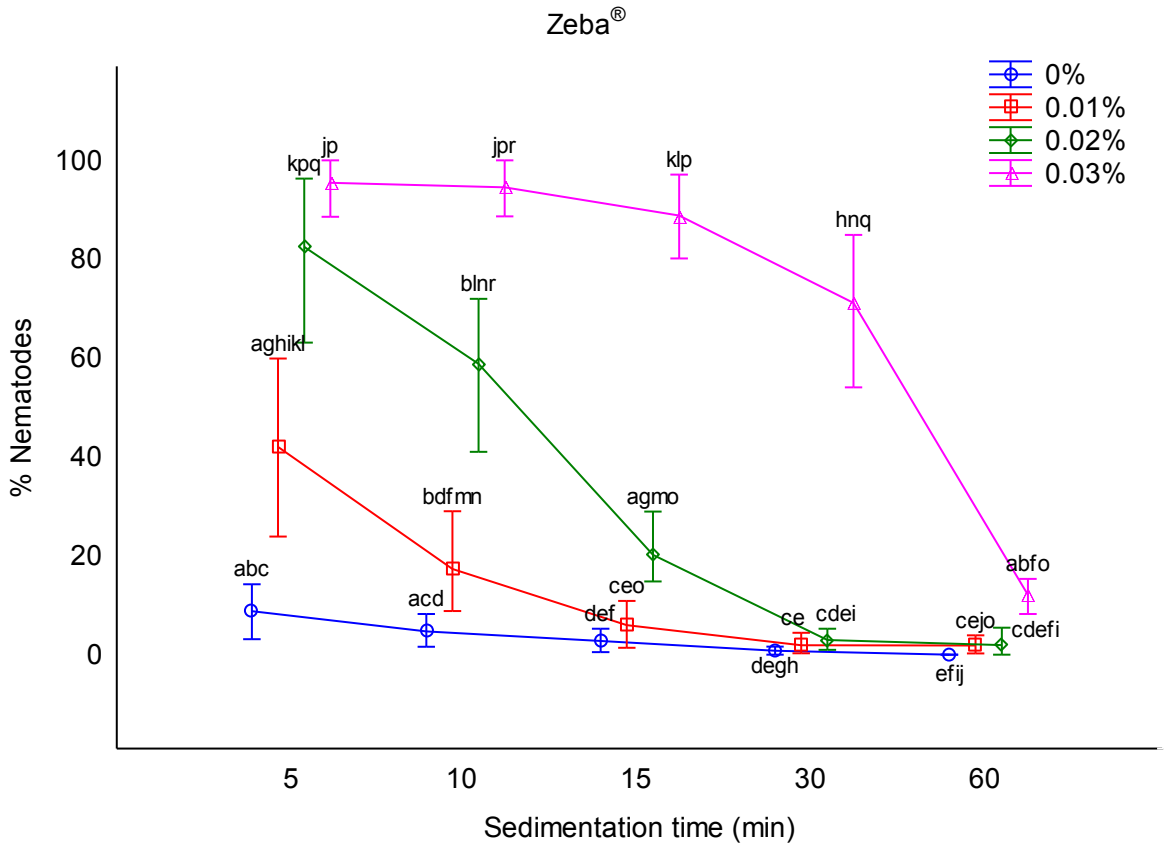

Fig. 3.1: Percentage nematodes recorded at a depth of 2 cm after stirring (95% confidence interval) at set time intervals for different concentrations of the polymer product Zeba®. Data points indicated with the same letters are not significantly different.

A repeated measures ANOVA for Xanthan gum showed interaction between main effects concentration (3 levels; 0%, 0.01% and 0.02%) and time (5 levels; 5-, 10-, 15-, 30-, and 60 min) ($F_{(12, 60)}$ = 5.45; P = 0.001). Treatments did not behave consistently over time. Both Xanthan gum concentrations levels tested were able to retard sedimentation significantly at all time intervals recorded, compared to the control (Fig. 3.2). No significant differences between the addition of 0.01% and 0.02% Xanthan gum were observed at any of the time intervals recorded. A one-way ANOVA ($F_{(1, 10)}$ = 2.95; P > 0.01) also showed the two concentration levels not to differ significantly. After 60 min sedimentation, 9%, 54% and 72% of the initial nematode number were recorded for 0%, 0.01% and 0.02% Xanthan gum, respectively.

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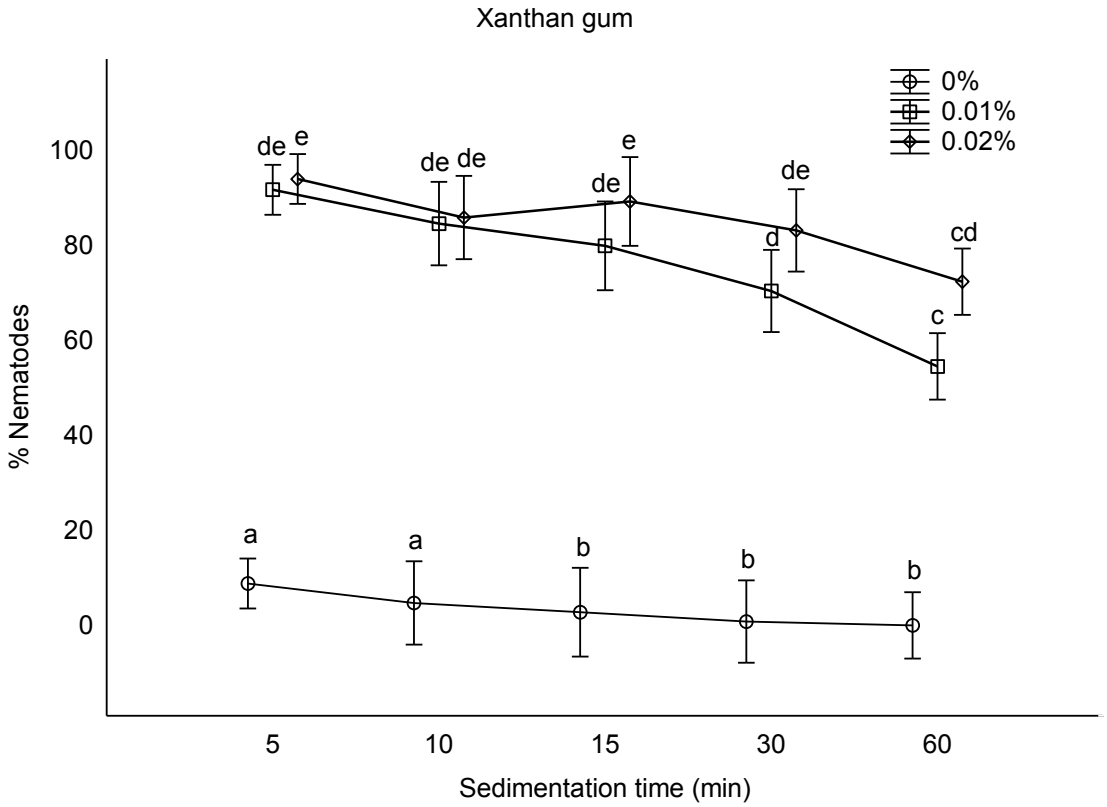

Fig. 3.2: Percentage nematodes recorded at a depth of 2 cm after stirring (95% confidence interval) at set time intervals for different concentrations of Xanthan gum. Data points indicated with the same lettering are not significantly different.

Effect of polymers on bioassays

Results of the effect of a suspension containing *H. zealandica* with 0.03% Zeba® or 0.02% Xanthan gum on mealybug mortality at 60% and 80% RH were analysed using a two-way ANOVA. No interaction was found between main effects humidity (2 levels; 60% and 80% RH) and adjuvants (2 levels; Zeba® and Xanthan gum) ($F_{(2,\ 54)}$ = 0.38; P = 0.67). Treatments reacted consistently at each humidity level tested. Although not significant, compared with the water containing nematodes suspension, the Zeba® formulation increased mortality by 14% at 60% RH (Fig. 3.3). The same formulation significantly increased mortality by 22% at 80% RH (P = 0.001). The Xanthan gum formulation performed worse than did the control, obtaining 14% control at 60% RH and 24% control at 80% RH, compared with the control that obtained 23% control at RH 60 and 30% control at RH 80. Data for both humidity levels were pooled together for further analysis using a one-way ANOVA ($F_{(2,\ 57)}$ = 15.27; P < 0.01), which confirmed that the Zeba® formulation obtained

significantly higher mortality (50%) than did either the Xanthan gum formulation (19% mortality) or the control (27% mortality).

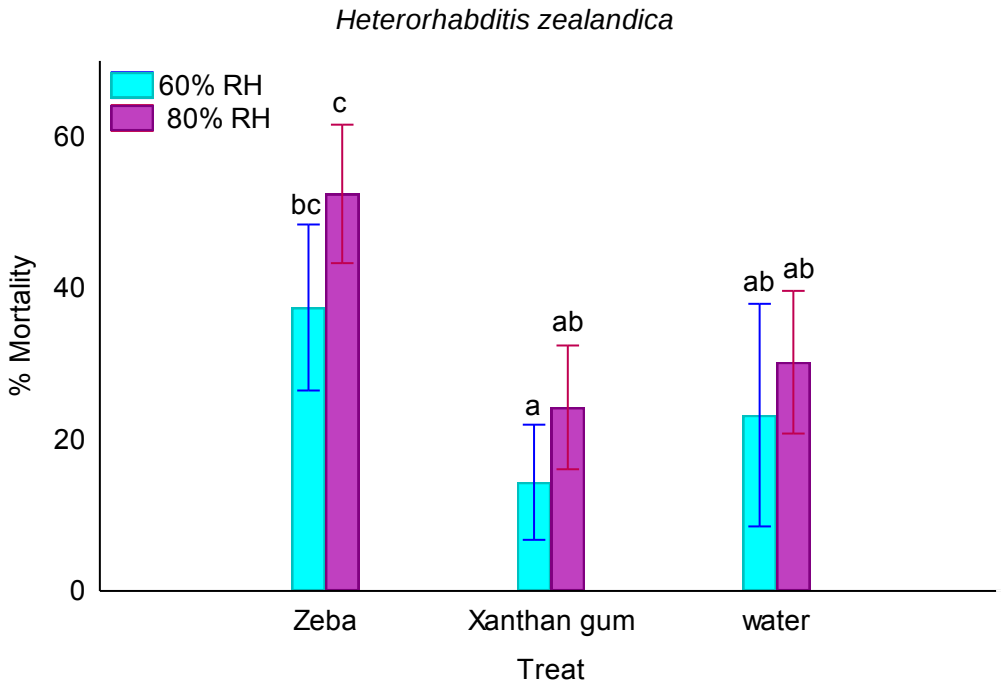

Fig. 3.3: Percentage mortality (95% confidence interval) of *Planococcus citri* after exposure to 80 IJinfective juveniles/insect of *Heterorhabditis zealandica* in a suspension of Zeba®, Xanthan gum or water only. Different letters above vertical bars indicate significant differences.

Results of the effect of a suspension containing *S. yirgalemense* and 0.03% Zeba® or 0.02% Xanthan gum on mealybug mortality at 60% and 80% RH were analysed using a two-way ANOVA that showed no interaction between main effects humidity (2 levels; 60% and 80% RH) and adjuvants (2 levels; Zeba® and Xanthan gum) ($F_{(2, 54)}$ = 0.22; P = 0.30). Treatments reacted consistently at each humidity level tested. The Zeba® formulation obtained significantly higher mortality of 36% at 60% RH and of 55% at 80% RH (55% mortality) compared with the Xanthan gum formulation (9% mortality at 60% RH and 34% mortality at 80% RH) and control formulation (9% mortality at 60% RH and 22% mortality at 80% RH) (Fig. 4.4). Data for both humidity levels were pooled together for further analysis using a one-way ANOVA ($F_{(2, 57)}$ = 20.68; P < 0.01), which confirmed that the Zeba® formulation obtained significantly higher mortality (46%) than did either the Xanthan gum formulation (22% mortality) or the control (16% mortality).

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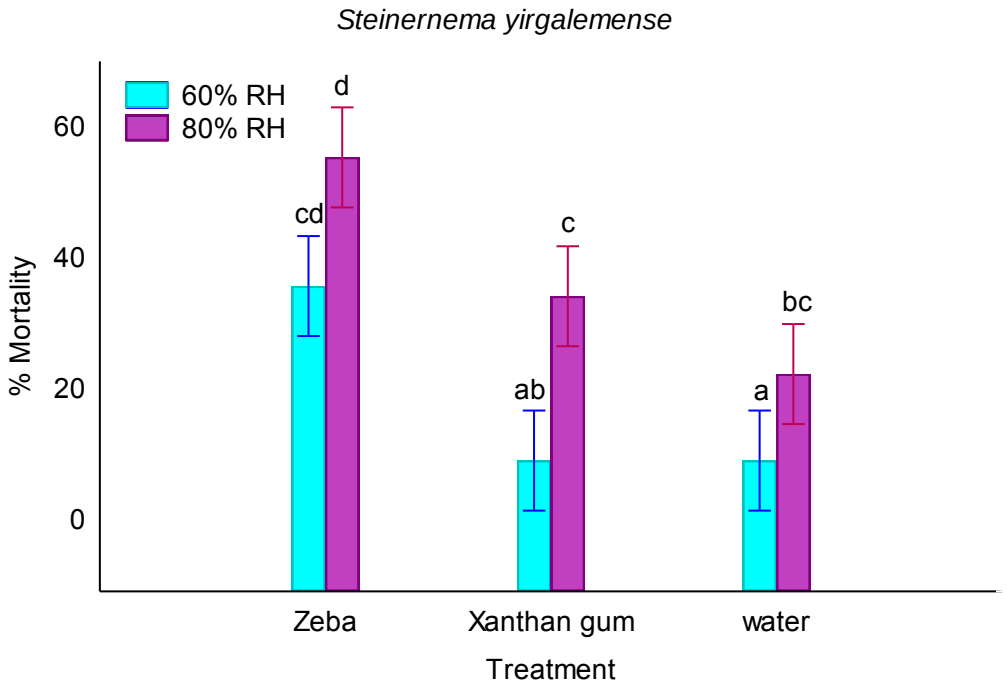

Fig. 3.4: Percentage mortality (95% confidence interval) of *Planococcus citri* after exposure to 80 infective juveniles/insect of *Steinernema yirgalemense* in a suspension of Zeba®, Xanthan gum or water only. Different letters above vertical bars indicate significant differences.

Data for all control plates used for Abbotts formula for corrected mortality were pooled to determine whether Zeba® or Xanthan gum had a toxic effect on mealybugs. A one-way ANOVA showed no significant differences ($F_{(2, 117)}$ = 0.32; P = 0.72) for mortality of mealybugs treated with Zeba®, Xanthan gum or water. All control plates caused less than 2.5% mortality of mealybugs.

Effect of adjuvants on nematode deposition

Results obtained for the effect of adjuvants on nematode deposition on citrus leaves were analysed using a one-way ANOVA. Significant differences were obtained for numbers of nematodes present on leaf surfaces ($F_{(3, 76)}$ = 3.03; P < 0.05). Although not significant, the addition of Nu-Film-P® and Zeba® to nematode application formulations increased the average number of nematodes deposited on 2-cm^2 leaf discs with four and seven nematodes respectively. Only the combined formulation of Nu-Film-P® and Zeba® significantly increased the average number of nematodes deposited on leaf discs with 10 nematodes (P =

0.009). However, the increase observed was not significantly higher than was that observed with the other two formulations tested.

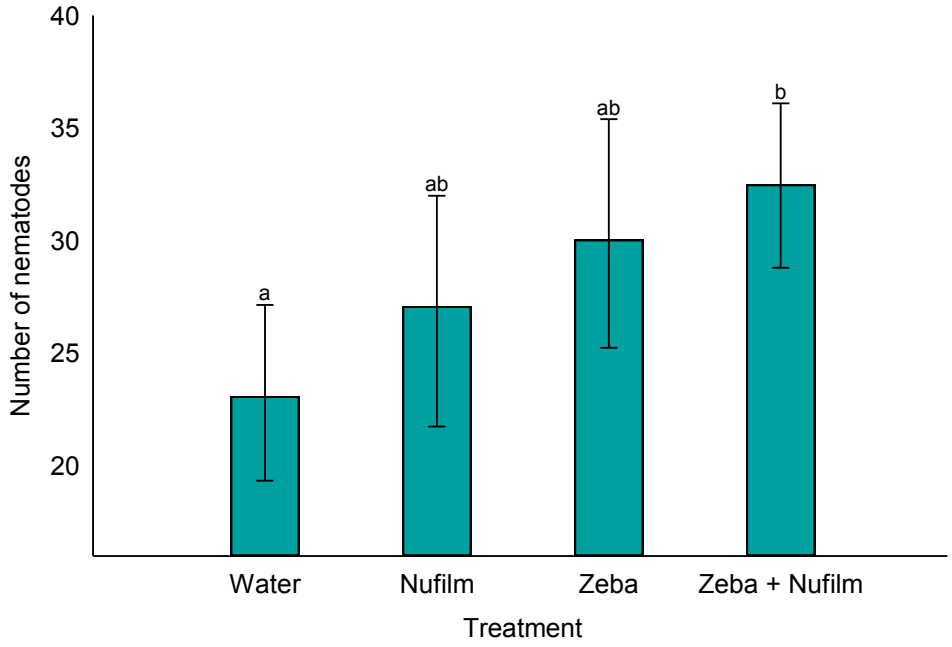

Fig. 3.5: Mean number of nematodes (95% confidence interval) on 2 cm^2 on citrus leaf discs sprayed with a suspension containing *Heterorhabditis zealandica* infective juveniles with Zeba®, Xanthan gum or water only. Different letters above vertical bars indicate significant differences.

Discussion

Planococcus citri is an important pest of citrus in South Africa, with the potential of infesting a high percentage of fruit under certain environmental conditions (Hattingh & Moore, 2003). Their cryptic life style and ability to develop resistance to pesticides necessitates research towards alternative control methods. EPNs, which are lethal pathogens with a wide host range, are considered as a valuable biological control method for a variety of insect pest species. The results discussed in Chapter 2 stressed the need to improve nematode application formulations in order to increase control of *P. citri* under variable environmental conditions. In the current study, sub-optimal conditions for nematode infection, with regard to nematode concentration and humidity, were maintained to simulate field conditions.

In an aqueous suspension, nematodes quickly settle to the bottom of spray tanks, causing uneven distribution. Sedimentation time is an important factor to consider, especially where larger nematode species, such as *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006 with an average length of 1 064 µm (Nguyen et al., 2006), are concerned. The polymer products Zeba® and Xanthan gum were evaluated at various concentrations for their ability to retard sedimentation of IJs. Xanthan gum was not evaluated at the highest concentration of 0.03%, as the suspension became too thick to pass through spray nodules, making its use impractical. *Heterorhabditis zealandica* was used in the sedimentation experiment, because IJs of this species have an average length of 685 µm and are larger than those of *S. yirgalemense*, with an average length of 635 µm (Nguyen, 2007). Results of the sedimentation trial showed *H. zealandica* to settle quickly on the bottom of 25-ml measuring cylinders with only 9% of the initial nematode number recorded at a depth of 2 cm, 5 min after stirring. None of the Zeba® concentrations tested was able to retard sedimentation significantly after 1 h. Only the 0.03% Zeba® formulation was able to retard sedimentation significantly after 30 min, with 71% of the initial nematode number recorded. Nematode suspensions containing Xanthan gum were able to retard sedimentation significantly at both concentration levels, tested after 1 h sedimentation. The above-mentioned results are similar to those that were obtained by Schroer *et al.* (2005) that showed Xanthan gum (0.01% and 0.02%) to retard sedimentation of *S. carpocapsae* effectively. Results also showed *S. carpocapsae* to settle quickly in water with 50% and 10% of the initial nematode number recorded at a depth of 2 cm after 5 min and 1 h sedimentation respectively. IJs of *S. carpocapsae*, being smaller in size (558 µm), settle much slower than do larger IJs (685 µm length) of *H. zealandica* (Nguyen, 2007), with 9% and 0% of the initial nematode number being recorded for *H. zealandica* after 5 min and 1 h respectively.

Although Xanthan gum performed much better than did Zeba® with regard to sedimentation, it was not the case for mortality of *P. citri* in bioassays. A nematode suspension containing 0.03% Xanthan gum performed poorly when the polymer's ability to increase the infectivity *P. citri* for both *H. zealandica* and *S. yirgalemense* was evaluated at 60% and 80% RH. The addition of Xanthan gum to nematode suspensions caused no significant increase in adult female *P. citri* mortality in any of the bioassays. Although not significant, the addition of Zeba® to *H*. zealandica application suspension increased mortality by 14% at 60% RH, and at 80% RH the same formulation significantly increased mortality with 22%. Similar results were obtained using *S. yirgalemense* and the addition of Zeba® to nematode suspensions increased mortality from 9% to 36% at 60% RH and from 34% to 55% at 80% RH. De Waal (2011) also tested Zeba® to improve control of diapausing codling moth, *Cydia pomonella* (Linnaeus) in tree trunk bioassays with *H. zealandica*, showing Zeba® to increase mortality significantly at both 60% and 80% RH, by 15% and 19% respectively.

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Zeba® and Xanthan gum proved not to be toxic to mealybugs, as less than 2.5% mortality was obtained after mealybugs were treated with Zeba® or Xanthan gum, and did not obtain significantly higher mortality than was obtained with mealybugs treated with water only. Due to the poor performance of Xanthan gum, it was not evaluated further for improvement of nematode deposition on citrus leaves.

Citrus leaves and fruit have a waxy cuticle and, therefore, the ability of nematode application suspensions to stick to their surfaces is greatly impaired. The possibility of using a surfactant, Nu-Film-P®, and the polymer product, Zeba®, to stick IJs to leaf surfaces was evaluated. Results showed the individual addition of Nu-Film-P® and Zeba® to nematode application suspensions increased the average number of 23 nematodes deposited on 2-cm^2 citrus leaf discs, by four and seven nematodes respectively. However, a significant increase was obtained when using the combination of Nu-Film-P® and Zeba®, increasing the average number of nematodes on leaf discs by 10 nematodes. Although nematodes will move to the same protected habitats as those occupied by *P. citri*, the additions of such adjuvants would not only increase their numbers on the leaves, but also protect them against desiccation on the exposed leaf area, which would be advantageous to movement and to the survival of the nematodes.

The investigation has shown that the addition of 0.03% Zeba® to nematode suspensions effectively hinders their sedimentation, resulting in a more even distribution. The same adjuvant also increased mealybug mortality in bioassays at 60% and 80% RH by retarding desiccation, extending nematode survival and improving mobility. Furthermore, the combined addition of both Nu-Film-P® and Zeba® increased application deposits on leaf surfaces, reducing the loss of nematodes by runoff. To further determine the ability of such adjuvants to improve the control of *P. citri* on citrus by nematodes, it should be tested under glasshouse and field conditions. Further improvement of nematode application formulations, aimed at the control of above-ground citrus pests, should be done by testing different surfactant and polymer combinations. The application of nematodes to control above-ground pests on a commercial scale is a relatively new field of study, so that there is still much room for improvement with regard to application techniques and technology.

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CHAPTER 4

Compatibility of *Heterorhabditis zealandica* and *Steinernema yirgalemense* with agrochemicals and biological control agents

Abstract

In this study, the compatibility of two indigenous South African entomopathogenic nematodes, *Heterorhabditis zealandica* and *Steinernema yirgalemense* with agrochemicals and biological control agents, likely to be used in an integrated pest management (IPM) programme for citrus in South Africa, was investigated. Bioassays were conducted to determine the susceptibility of the coccinellid beetle, *Cryptolaemus montrouzieri*, to both nematode species. Results showed the beetle larvae to be highly susceptible to *H. zealandica* and *S. yirgalemense*, with 80% and 92% infection respectively. Adult beetles were found to be twice as susceptible to *S. yirgalemense* with 64% mortality, as to *H. zealandica* with 30% mortality recorded. The ability of infective juveniles of *H. zealandica* and *S. yirgalemense* to tolerate exposure to aqueous solutions of an insecticide (Cyperphos 500 E.C.®), two biopesticides (Cryptogran™ and Helicovir™), and two adjuvants (Nu-Film-P® and Zeba®) was evaluated. *Heterorhabditis zealandica* proved to be highly compatible with all products tested, with no significant increase in nematode mortality. The formulations also did not affect the ability of *H. zealandica* to infect hosts, after exposure to products over a 24-h period. Significant increase in mortality of *S. yirgalemense* was recorded after 12 h exposure to Cryptogran™, Helicovir™ and Cyperphos 500 E.C.®, and after 6 h exposure to Nu-Film-P®. However, *S. yirgalemense* infectivity showed no decrease. This is the first report on the possible negative effect of an aerial application of EPNs against *C. montrouzieri,* a commercially produced biocontrol predatory insect, used against mealybugs. Results indicated that nematodes should be applied before the release of *C. montrouzieri* in citrus orchards. The results emanating from this study should be considered when incorporating nematodes in an IPM programme for citrus production in South Africa.

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Introduction

In South Africa, citrus are attacked by a wide range of economically damaging insect pest species, such as false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), citrus thrips, *Scriptothrips aurantii* (Faure) (Thysanoptera: Thripidae), American bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and a variety of mealybugs species, of which *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) is the most common and destructive (Bedford *et al.*, 1998). To apply entomopathogenic nematodes (EPNs) as a biological control agent efficiently and effectively in an integrated pest management (IPM) programme for citrus in South Africa, their compatibility with agrochemicals and other biological control agents needs to be known.

EPNs in the order Rhabditida, of the families Steinernematidae and Heterorhabditidae, are valuable biological control agents for a wide range of economically important insect pests (Grewal *et al.*, 2005). Although traditionally applied to control the soil stages of insects, nematodes have proven able to control some foliar pests (Arthurs *et al.*, 2004). The broad host range of EPNs is advantageous, as the possibility exists of controlling more than one pest species in a single IPM programme. However, when nematodes are applied to the aerial parts of trees, their broad host range could also be problematic if beneficial insects present during application are also susceptible (Hazir *et al.*, 2003).

Rojht *et al.* (2009) reported high mortality of larvae of the two-spotted lady beetle, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae), and those of the lacewing species, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) when exposed to *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin and Bedding, 1998; *S. carpocapsae* (Weiser, 1955) Wouts Mráček, Gerdin and Bedding, 1982, and *Heterorhabditis bacteriophora* Pionar, 1976. In contrast, Shapiro-Ilan and Cottrell (2005) found four ladybug beetle species to be significantly less susceptible to *H. bacteriophora* and *S. carpocapsae* than was the black cutworm, *Agrotis ipsilon* (Hüfnagel) (Lepidoptera: Noctuidae), which is a known susceptible host. The above-mentioned studies show that the susceptibility of beneficial insects to nematodes varies, depending on the nematode, insect stage and combination tested. Such beneficial insects as the predatory lady beetle, *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), and the parasitoid, *Leptomastix dactylopii* (Howard) (Hymenoptera: Encyrtidae), are mass reared and made commercially available to citrus farmers in South Africa. Such beneficial organisms have been shown to play a vital role in IPM programmes for citrus

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(Hattingh & Moore, 2003). The susceptibility of such beneficial insects to a specific nematode species should first be determined, before the nematode species are applied in an IPM programme.

In contrast, when applied as part of an IPM programme, nematodes are exposed to a variety of agrochemicals and biological control formulations that could be toxic and impair nematode performance. Although some pesticides retard nematode persistence and infectivity (Zimmerman & Cranshaw, 1990; Patel *et al.*, 1997), a study conducted by Rovesti and Deseö (1990) showed nematodes to be tolerant to short periods (2–4 h) of exposure to the majority of 75 commercial pesticides tested. When applying a specific nematode species, one should also consider the fact that nematodes tend to vary in reaction to pesticides, depending on the nematode species and pesticide formulation concerned (Grewal, 2002). Nematode tolerance to agrochemicals allows the possibility of tank-mixing nematodes with other agrochemical and biopesticide formulations that will save time and labour costs and/or help achieve better control of a target pest species (Koppenhöfer & Grew, 2005).

The objective of the current study was to evaluate the compatibility of two indigenous EPN species with a biological insect, biocontrol formulations and agrochemicals, to which they will most likely be exposed in an IPM programme for citrus in South Africa.

Materials and methods

Source of nematodes and biocontrol agents

Infective juveniles (IJs) of *H. zealandica* Poinar, 1990 (SF 41 strain) (Malan *et al.*, 2006) and *S. yirgalemense* Tesfamariam, Gozel, Gaugler and Adams, 2005 (157-C strain) (Malan *et al.*, 2011) were produced at room temperature in last-instar *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) by using the procedures described by Kaya and Stock (1997). After being harvested, IJs were stored horizontally in 500-ml culture flasks containing 150-ml distilled water at 14°C. Culture flasks were shaken weekly to improve aeration and nematode longevity. IJ concentrations used in experiments were quantified by using the method developed by Navon and Ascher (2000). Nematodes were used within the first three weeks after harvesting.

Mealworm larvae were reared at room temperature in plastic containers on fine wheat bran.

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Cryptolaemus montrouzieri (Mulsant) (Coleoptera: Coccinellidae) larvae and adult beetles were obtained from the commercial company Du Roy IPM, Letsitele, Limpopo Province, South Africa.

Susceptibility of *C. montrouzieri* to nematodes

To assess the susceptibility of the biological control agent *C. montrouzieri* to *H. zealandica* and *S. yirgalemense,* adult beetles and larvae were individually exposed to IJs in multiwell plates (24 wells, flat bottom, Nunc™ Cat. No. 144530). Five treatment plates and five control plates (5 replicates; 50 insects), each containing ten evenly distributed larvae or adult beetles, were prepared. Each well was lined with a circular paper disc (of 13-mm diameter) before adults and larvae were added. They were then inoculated individually with 80 IJs of either *H. zealandica* or *S. yirgalemense*. Control plates received 50 µl of distilled water only. To retain insects in their individual wells, each plate was covered with a piece of glass fitted as a lid. After inoculation, the plates were placed in plastic containers lined with moistened paper towels and closed to ensure high humidity levels (RH ± 95%). Plastic containers were then incubated in a dark growth chamber at 25 ± 2ºC for 48 h, after which the mortality of mealybugs was determined by means of gentle prodding. Insects were then rinsed to remove nematodes from their body surface, transferred to clean Petri dishes (9-mm-diameter) and incubated for another 48 h. To confirm mortality due to nematode infection, each cadaver was dissected with the aid of a dissection microscope. The experiment was repeated on a separate test date. The data of both experiments were pooled for analysis.

Influence of a pesticide, two biopesticides and two adjuvants on EPN survival and persistence

The compatibility of *H. zealandica* and *S. yirgalemense* with an insecticide, Cyperfos 500 E.C.® [450 g/L chlorpyrifos (organophosphate), 50 g/L cypermethrin (pyrethroid)], two biopesticides, Cryptogran™ [*Cryptophlebia leucotreta* granulovirus (CrleGC-SA)] and Helicovir™ (nucleopolyhedrovirus) and two adjuvants, Nu-Film-P® (Poly-1-P-menthene, spreader, sticker, Hydrotech) and Zeba® [starch-g-poly (2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch], were tested. Nematode suspensions containing 4000 IJs/ml were prepared for each nematode species tested. Product formulations were then prepared at twice the recommended dose (Table 4.1). One ml of nematode suspension and one ml of product formulation was then added to Petri dishes and kept in a growth chamber at 25 ± 2ºC. Five Petri dishes were prepared for each product and nematode species combination. Nematode survival was compared to five control dishes that contained nematodes in water only. To estimate nematode survival, 10

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µl samples were collected until the first 50 IJs were documented as being either alive or dead. Nematode mortality was assessed directly after preparing the treatments (0 h) and again after 6, 12 and 24 h.

Table 4.1: Recommended dosage of substance

Product	**Active ingredient**	**Use**	**Concentration/L**
Cyperphos 500 E.C. ®	Chlorpyrifos and Cypermethrin	Insecticide	1.00 ml
Cryptogran™	*Cryptophlebia leucotreta* granulovirus	FCM control	2.50 ml
Helicovir™	Nucleopolyhedrovirus	Bollworm control	0.12 ml
Nu-Film-P®	Poly-1-P-menthene	Spreader, sticker	0.60 ml
Zeba®	starch-g-poly (2-propenamide-co-2-propenoic acid) potassium salt	Antidesiccant	3.00 g

To determine whether the nematodes were still virulent after exposure to products, an additional 5 ml of each treatment was prepared and kept in the same growth chamber. After 24 h, each treatment was diluted in 1 L of distilled water. Nematodes were allowed to settle to the bottom of measuring cylinders, after which excess fluid was siphoned off to 10 ml. Five Petri dishes for each treatment and five control dishes, each containing ten mealworm larvae (5 replicates; 50), were prepared for each product tested. Treatment dishes were inoculated with a concentration of 100 IJs/insect. Control dishes received water only. After 48 h, insect mortality was assessed. This experiment in its entirety was repeated on a different test date. The data of both experiments were pooled for analysis.

Data analysis

Before analysis, data for mealworm mortality were corrected using Abbott's (1925) formula, in order to compensate for those mealworms that might have died of natural causes. All statistical analyses were performed by means of Statistica 9.0 software (StatSoft Inc. 2009). Data were analysed using ANOVA, with post-hoc comparison of means using Bonferroni's method, or with a bootstrap multi comparison if residuals were not evenly distributed (Efron & Tibshirani, 1993). Significant differences were determined on a 95% probability level.

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Results

Susceptibility of *C. montrouzieri* to nematodes

Adult beetles and larvae of *C. montrouzieri* were screened for susceptibility to *H. zealandica* and *S. yirgalemense.* As the mortality of both adults and larvae was high in control plates, Abbott's formula could not be used to compensate for those mealybugs that died of natural causes (Abbott, 1925), and results were given as a percentage of nematode infection. A two-way ANOVA ($F_{(1, 36)}$ = 5.31; P = 0.03) showed slight interaction between treatments and main effects could not be interpreted directly. Results showed *H. zealandica* to be significantly more virulent to beetle larvae, obtaining 80% infection (P = 0), than to adult beetles, with only 30% infection. Beetle larvae were also significantly more susceptible to *S. yirgalemense*, with 92% infection (P = 0.001), than to adults, with 64% infection. *Steinernema yirgalemense* obtained significantly higher control of adult beetles compared to that attained with *H. zealandica* (P = 0). Although not significant, *S. yirgalemense* obtained 12% higher infection of beetle larvae than did *H. zealandica*. Adult beetles in treatment plates were observed to secrete an obnoxious smelling yellowish substance compared to control plates were no such secretion was noted.

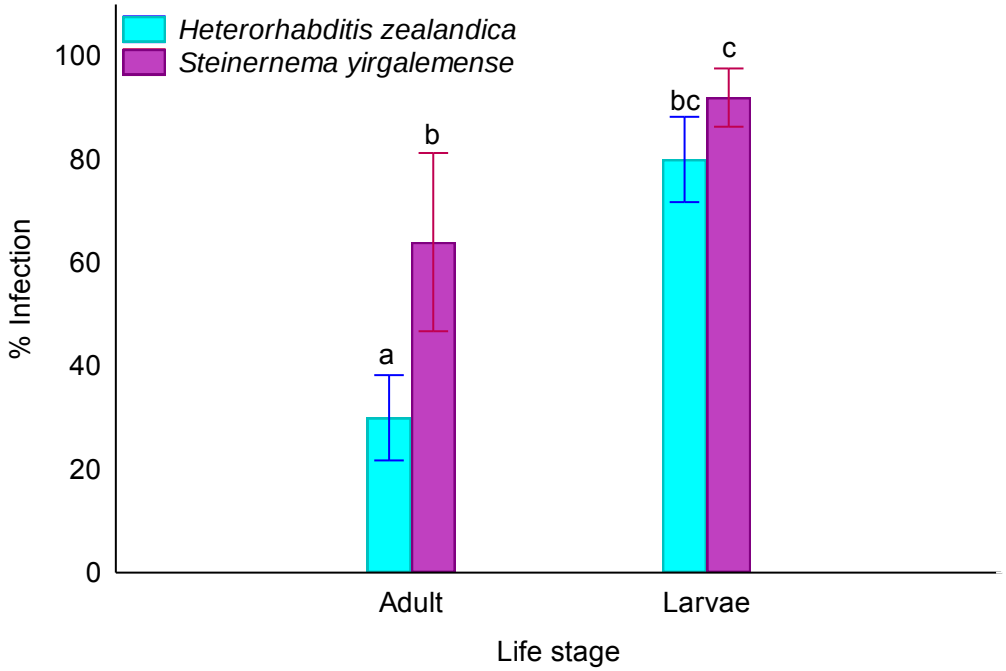

Fig. 4.1: Mean percentage (95% confidence interval) infection recorded for *Cryptolaemus montrouzieri* adults and larvae, 48 h after being inoculated with 80 IJs/insect in multiwell bioassay plates. Means with the same letter are not significantly different.

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Influence of a pesticide, two biopesticides and two adjuvants on EPN survival and persistence

Results of nematode survival over time were analysed by using a two-way ANOVA, with treatment (2 levels; agricultural product and water) and time (4 levels; 0, 6, 12, 24) as the main effects. Each treatment was compared to their corresponding control, containing either *H. zealandica* or *S. yirgalemense* only. No significant differences in *H. zealandica* mortality were observed over time, or compared to the control for any of the treatments tested.

Zeba® also proved to have no influence on *S. yirgalemense* over time, as no significant differences in nematode mortality were observed over time when the treatment was compared to the control. A two-way ANOVA that compared nematode response to Cryptogran™ over a specified length of time to their response in water only showed no interaction between main effects ($F_{(3,\ 72)}$ = 2.72; P = 0.07), which indicated nematodes to respond consistently to both the treatment and control over time (Fig. 4.2). Mortality of *S. yirgalemense* IJs exposed to Cryptogran™ increased significantly after 24 h, from 2% to 6% mortality. The increase in mortality noted was, however, not significantly higher than was the 3% mortality (P = 0.21) recorded for the control after 24 h.

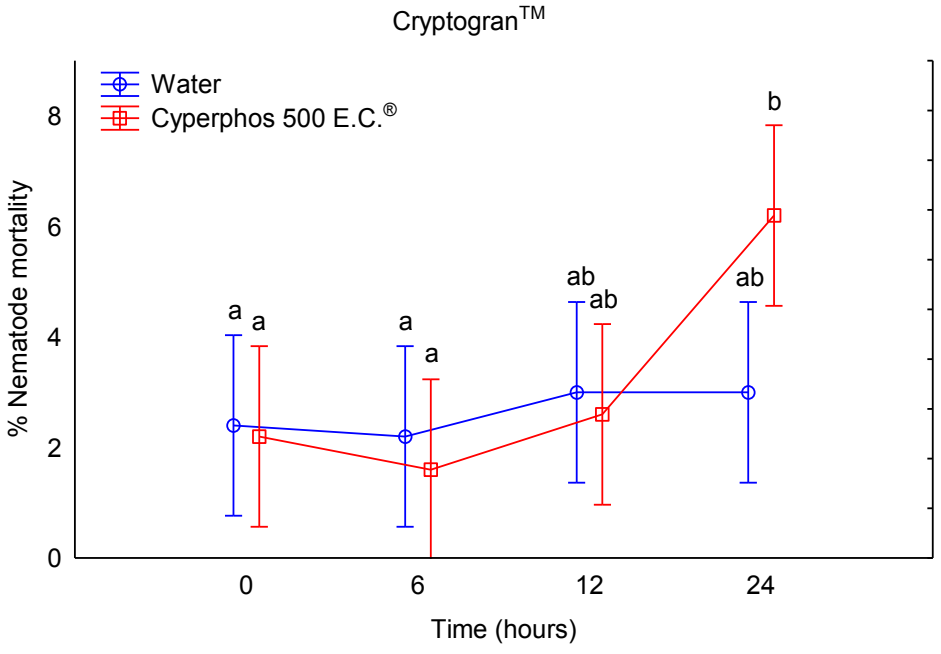

Fig. 4.2: Mean percentage (95% confidence interval) mortality of IJs of *Steinernema yirgalemense* after exposure to Cryptogran™ over time. Means with the same letter are not significantly different.

Results for the same control treatment for *S. yirgalemense*, as compared with Cryptogran™, were also individually compared to Helicovir™, Nu-Film-P® and Cyperphos 500 E.C.®. When results for nematode mortality over time were analysed using a two-way ANOVA, they showed interaction between main effects, indicating that nematodes did not respond consistently to treatments compared to the control. A significant increase in nematode mortality from < 6% to 16% was recorded after 24 h exposure to Helicovir™ (Fig. 4.3). This increase in mortality was also significantly higher than the 3% mortality recorded for the control after 24 h (P = 0.001).

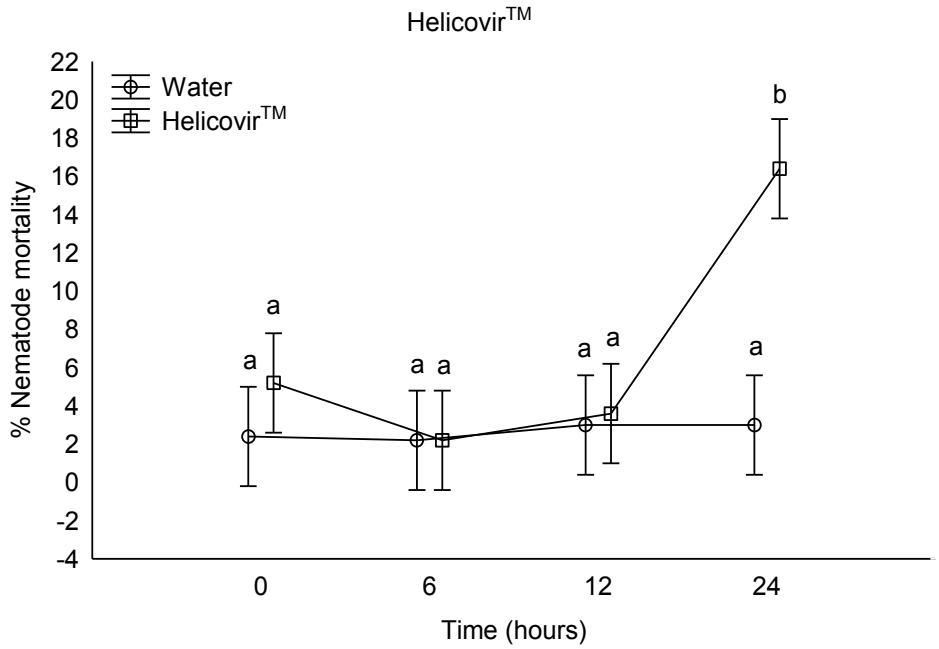

Fig. 4.3: Mean percentage (95% confidence interval) mortality of IJs of *Steinernema yirgalemense* after exposure to Helicovir™ over time. Means with the same letter are not significantly different.

After only 6 h exposure to Nu-Film-P®, nematode mortality increased significantly (P = 0.001) from 2% to 10% (Fig. 4.4). The increase was also significantly higher (P = 0.01) than the 2% mortality recorded for the control after 6 h. Nematode mortality increased after further exposure, with up to 26% mortality recorded after 24 h.

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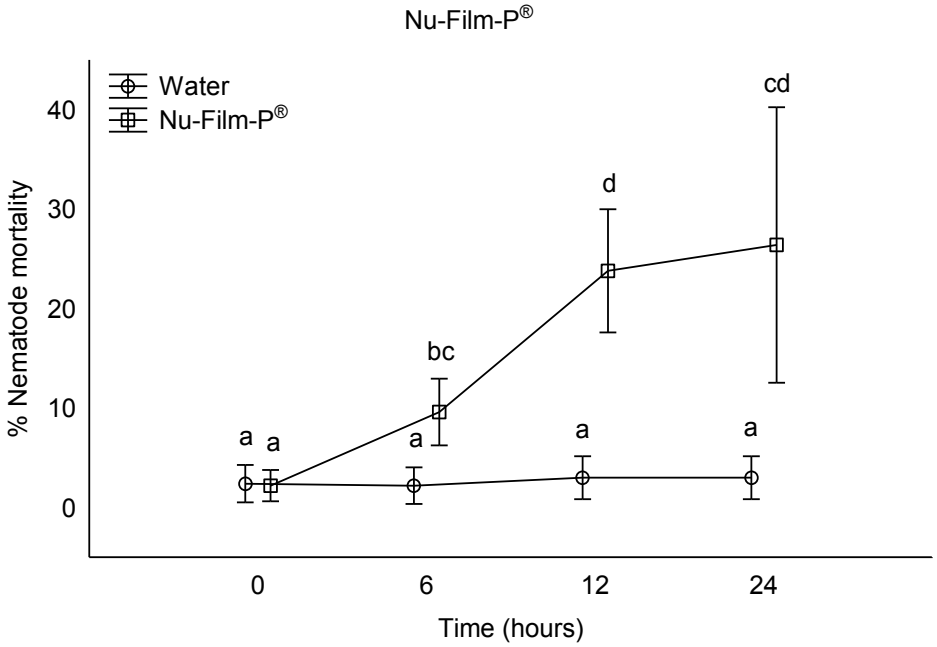

Fig. 4.4: Mean percentage (95% confidence interval) mortality of IJs of *Seinernema yirgalemense* after exposure to Nu-Film-P® over time. Means with the same letter are not significantly different.

A significant increase in nematode mortality from > 5% to 11% was recorded for nematodes after 12 h exposure to Cyperphos 500 E.C.® (Fig. 4.5). The increase in mortality noted was also significantly higher (P = 0.001) than the 3% mortality recorded for the control after 12 h. Nematode mortality significantly (P = 0.001) increased to 27% after an additional 12 h exposure to Cyperphos E.C.®.

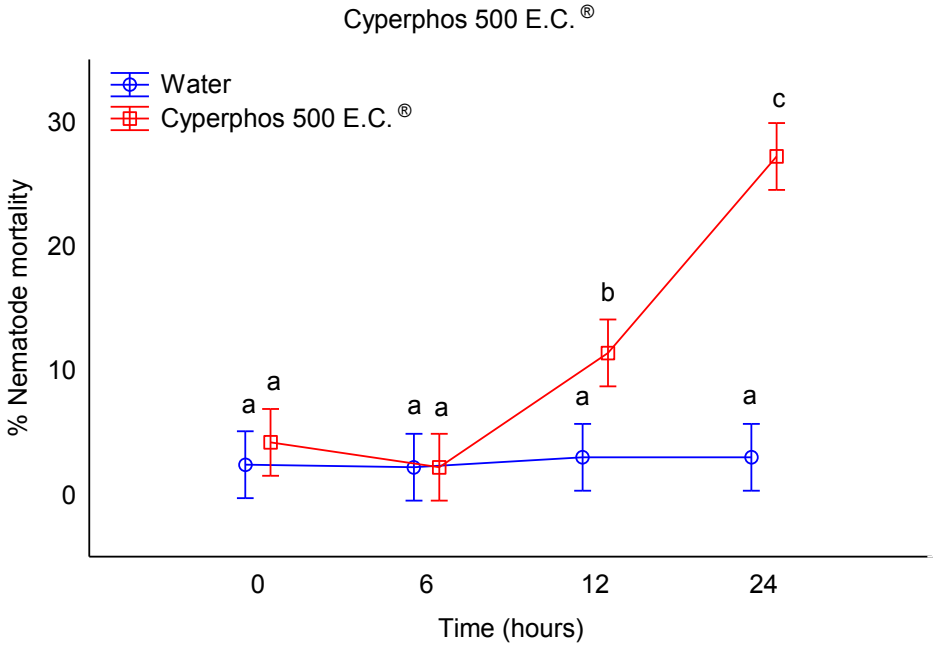

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Fig. 4.5: Mean percentage (95% confidence interval) mortality of IJs of *Steinernema yirgalemense* after exposure to Cyperphos 500 E.C.® over time. Means with the same letter are not significantly different.

Results for mealworm mortality obtained by *H. zealandica* and *S. yirgalemense* after exposure to the above-mentioned agrochemicals were individually analysed with the use of a one-way ANOVA, which showed treatments not to differ significantly ($P < 0.01$), and no significant differences were observed. Mealworm mortality of > 89% and > 98% were recorded, for *H. zealandica* and *S. yirgalemense* respectively, for all treatments of IJs after 24 h.

Discussion

In South Africa, citrus production is plagued by a complex of major and minor insect pest species (Bedford *et al.*, 1998). In order to save time and labour costs, to achieve better control of a single pest, or to target more than one pest, it would be desirable to tank mix and apply agrochemicals with a biocontrol agent, or with more than one biocontrol agent at the same time. The simultaneous application of pesticides and EPNs is possible, as some species have proven tolerant to short periods of exposure to certain pesticides (Rovesti & Deseö, 1990). Tolerance to agrochemicals is also advantageous, as nematodes can be applied within a short time interval after the application of chemicals. Above-ground insect pests in general are more susceptible to nematodes, as they did not develop resistance mechanisms over the aeons through cohabitation with nematodes (Kaya & Hara, 1981). When applying nematodes to control above-ground pests, it is important to remember that EPNs are lethal pathogens of a wide range of insect pest species (Griffin *et al.*, 2005), and could possibly also infect non-target organisms, such as the natural insect enemies of pests.

Planococcus citri is a serious pest of a variety of economically important crops and ornamental plants throughout the world (Cox, 1981), and is considered the most common and destructive species of mealybug to attack citrus in South Africa (Hattingh *et al.,* 1998). *Cryptolaemus montrouzieri* is a coccinellid beetle, also called the mealybug lady beetle or the mealybug destroyer, and is considered a valuable biological control agent aimed at *P. citri* (Hattingh & Moore, 2003). Both the adult and the larvae of the beetle

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are voracious feeders, preying dominantly on mealybugs. In South Africa, *C. montrouzieri* is produced by a commercial company, Du Roi IPM.

Results of the susceptibility of *C. montrouzieri* larvae to *H. zealandica* and *S. yirgalemense* showed beetle larvae to be highly susceptible to both nematode species, with an infection rate of 80% for *H. zealandica* and of 92% for *S. yirgalemense*. The adult beetle was also found to be susceptible to nematode infection, with an average mortality of 30% in the case of *H. zealandica* and of 64% for *S. yirgalemense*. Unlike adult beetles in control plates, beetles exposed to nematodes were observed to excrete an abnoxious smelling yellowish liquid indicating its secretion as a defence mechanism used to prevent nematode infection. Adult beetles are also very mobile which will further impair nematode infection. These results indicate that the susceptibility of natural enemies to nematodes depends on the nematode−insect species interaction, as adults were found to be twice as susceptible to *S. yirgalemense* as to *H. zealandica.* Shapiro-Ilan and Cottrell (1995) and Rojht *et al.*, (2009) found comparable results using different beetle species.

The high susceptibility of beetle larvae and even of adult beetles to both nematode species, which is especially high for *S. yirgalemense,* should be taken into consideration when both of these biocontrol agents are to be applied in a citrus orchard. Adults of *C. montrouzieri* live up to two months, with a female producing up to 500 eggs and having the potential of being persistent (Smith *et al.*, 1997). As nematodes have only a short window period for mealybug infection after application, they should be applied before *C. montrouzieri* release. In Chapter 2, bioassays on *P. citri,* in the lethal nematode concentration trial conducted under similar conditions to the bioassays in the current study, showed *H. zealandica* and *S. yirgalemense* to obtain up to 89% control of adult female *P. citri,* indicating *C. montrouzieri* larvae to be just as susceptible to nematodes as to the target pest species. Results also indicated that the nematode species should also not be applied when *C. montrouzieri* larvae are already present in high numbers on the trees concerned.

Nematode tolerance to three agrochemicals and two biocontrol formulations, to which they are likely to be exposed in an IPM programme for citrus in South Africa, showed *H. zealandica* mortality after exposure to Zeba®, Nu-Film-P®, Cryptogran™, Helicovir™ and Cyperphos 500 E.C.® over a 24-h period to be unaffected by any of the products tested. Significant increases in nematode mortality were, however, observed for *S. yirgalemense* after 12 h exposure to Cryptogran™, Helicovir™ and Cyperphos 500 E.C.®. Results indicate that *S. yirgalemense* should not be tank mixed with the above-mentioned products for prolonged periods before application. A significant increase in *S. yirgalemense* mortality was also observed

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after 6 h exposure to Nu-Film-P®. To determine exactly how long *S. yirgalemense* is tolerant to Nu-Film-P®, the same experiment should be conducted, but nematode mortality should be recorded every hour for six h. Although *S. yirgalemense* proved to be sensitive to some of the formulations tested, results for nematode infection showed that none of the products tested influenced the ability of either nematode species tested to infect hosts after 24 h.

The current study illustrates some of the factors that should be taken into consideration before nematodes are applied in commercial orchards in an IPM programme. Pest complexes that attack citrus vary from one orchard to the other and from one year to the next (Ebeling, 1959). Thus, the required agrochemicals and biological control agents also vary. When EPNs are available as commercial biological control agents in South Africa, a database of data relating to nematode species tolerance to agrochemicals, as well as susceptibility of pests and commercially available natural enemies used in IPM programmes for citrus, should be set up, in order to aid producers or consultants in decision making with regard to nematode application. Furthermore, studies should be conducted to determine whether the simultaneous application of chemicals and biopesticides could improve control of a target pest species.

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CHAPTER 5

Evaluating the efficacy of a polymer-surfactant formulation to improve control of *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) under simulated natural conditions

Abstract

Traditionally, entomopathogenic nematodes in the families Heterorhabditidae and Steinernematidae are targeted at the soil stages of insect pests. To improve control of *Planococcus citri,* which occur in the canopy of citrus trees, nematode application suspensions require the addition of adjuvants. In a growth chamber assay, simulating glasshouse conditions of 75 ± 8% relative humidity and 22°C, all treatments obtained significantly higher mortality of adult female *P. citri* than the control. The addition of 0.03% Zeba® and 0.06% Nu-Film-P® to a *Heterorhabditis zealandica* and a *Steinernema yirgalemense* suspension increased resultant mortality from 26% to 30% and from 34% to 45%, respectively. This increase in mortality was, however, not significant. The ability of the above-mentioned formulation to prolong the ability of *S. yirgalemense* to infect *P. citri* and to prolong nematode survival was also evaluated under the same conditions and showed the formulation to improve both infectivity and survival for up to 2–3 h post-application. In the semi-field trial, *S. yirgalemense* was not able to obtain significantly higher control of *P. citri* without the addition of 0.03% Zeba® to nematode application suspensions, resulting in up to 53% control. The study conclusively showed the polymer product Zeba® to improve the ability of *S. yirgalemense* to infect *P. citri* by retarding desiccation and by buffering nematodes from suboptimal environmental conditions.

Introduction

The family Pseudococcidae, commonly referred to as mealybugs, comprising about 2 200 species in almost 274 genera (Ben-Dov *et al.*, 2010), of which the citrus mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae), is considered the most destructive (Cadee & Van Alphen, 1997; Blumberg & Van

Driesche, 2001). *Planococcus citri* is highly polyphagous and is known to infest such commercially produced hosts as citrus, coffee, vineyards and a variety of ornamental plants (Cadee & Van Alphen, 1997; Mustu *et al.*, 2008).

In South Africa, seven mealybug species, of which *P. citri* is the most important, are regarded as economically damaging citrus pests (Hattingh *et al.*, 1998). Mealybugs feed on all parts of citrus trees, except the roots (Canhilal *et al*., 2001), causing both direct and indirect damage (Hattingh & Tate, 1996), such as wilting, premature fruit and flower drop, growth deformation and sooty mould growth (Blumberg *et al*., 1995). During winter, mealybugs occur throughout the tree canopy, generally residing in cracks and crevices or leaf axils (Hattingh, 1993; Smith *et al.*, 1997). First-generation nymphs emerge during spring or early summer, moving to the foliage to colonise fruitlets and young growth (Martinez-Ferrer *et al.*, 2006), settling in protected sites, such as under fruit calyxes or in between fruit clusters (Hattingh & Moore, 2003). Multiple overlapping generations of *P. citri* occur during a single growing season (Wakgari & Giliomee, 2003), with the highest population numbers occurring between mid- and late summer, parallel with fruit growth intensity (Franco *et al.*, 2004).

Although mealybugs are generally controlled with chemicals (Franco *et al.*, 2004), this method of control is not ideal. Mealybugs are known to develop resistance (McKenzie, 1967; Blumberg & Van Driesche, 2001; Mahfoudhi & Dhouibi, 2009) and the continuous applications of broad-spectrum pesticides has proven to be partially responsible for pest outbreaks (Michelakis & Hamid, 1995), as they disrupt natural enemies, usually keeping mealybug populations under control (Hattingh, 1993; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003). The success of chemical control is further impaired by mealybugs being covered with protective waxes and displaying cryptic behaviour, residing in protected sites where they cannot be reached by chemicals (McKenzie, 1967; Michelakis & Hamid, 1995; Franco *et al.*, 2004). Growing public awareness of detrimental environmental impact and of health risks associated with pesticides has further pressured citrus growers into trying to find alternative control methods (Hussaini, 2002).

The application of natural enemies is considered the most feasible alternative to chemical insect control (Hussaini, 2002). In citrus orchards, mealybug populations are usually controlled by means of natural enemies, if the behaviour of the latter is not disrupted by the application of pesticides (Hattingh, 1993; Hattingh & Tate, 1996; Hattingh *et al.*, 1998; Hattingh & Moore, 2003). After winter, however, natural enemy

population numbers tend to increase slowly and early spring population densities are usually insufficient to prevent early feeding damage (Hattingh, 1993; Franco *et al.*, 2004).

Entomopathogenic nematodes (EPNs) in the order Rhabditida belong to the families Steinernematidae and Heterorhabditidae and are fatal pathogens of insect. Such nematodes are used as inundatively applied biological control agents against a wide variety of economically important insect pests (Grewal *et al.*, 2005). EPNs are, however, primarily applied to control the soil stages of insects (Arthurs *et al.*, 2004). Controlling foliar pests with nematodes in orchards is still a relatively new field of study, and is extremely challenging, as nematodes require a water film to maintain mobility and to ensure survival (Wright *et al.*, 2005). Above-ground conditions are not optimal for nematode survival (Mráček, 2002; Tomalak *et al.*, 2005), as nematodes are exposed to such limiting abiotic factors as ultraviolet radiation (Gaugler & Boush, 1979; Gaugler *et al.*, 1992), extreme temperatures (Lacey *et al.*, 2005), low ambient humidity and wind (Unruh & Lacey, 2001).

Nematodes are usually applied to foliage as an aqueous suspension by means of ordinary chemical-spraying equipment (Grewal, 2002; Hussaini, 2002). Water retention agents can be added to application formulations to retard desiccation (Glazer *et al.*, 1992), thus increasing the duration of nematode survival on foliage (Webster & Bronskill, 1968; Shapiro *et al.*, 1985; Glazer & Navon, 1990). According to Tomalak *et al.* (2005), the successful control of western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) in glasshouses is a result of the sensible use of adjuvants, which improve nematode distribution on foliage. A nematode application formulation containing 0.3% Rimulgan® (surfactant) and 0.3% of the polymer xanthan (antidesiccant) obtained more than 90% control at 80% relative humidity (RH) and > 70% control at 60% RH of the diamondback moth (DBM), *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), on cabbage leaves (Schroer & Ehlers, 2005). The same formulation was also evaluated by Schroer and Ehlers (2005) for DBM control on cabbage-leaf disc assays, with results showing nematodes to survive 22 h longer at 80% RH and > 17 h longer at 60% RH than the control. Further field studies for DBM control on cabbage were conducted by Schroer and Ehlers (2005). The formulation significantly reduced the number of insects per plant, resulting in > 50% control after seven days. No significant effect was, however, recorded when compared to that achieved with a formulation containing nematodes only and with a surfactant, with the difference in effect being attributed to the high ambient humidity that prevailed in the experimental unit and the moist microclimate in the cabbage heads, favouring nematode survival.

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The objective of this study was to evaluate the potential of a surfactant-polymer formulation added to nematode application suspensions to improve the ability of nematodes to control *P. citri* on citrus under simulated glasshouse conditions and semi-field trials.

Materials and Methods

Source of nematodes and insects

Infective juveniles (IJs) of *Heterorhabditis zealandica,* Poinar, 1990 (SF 41) and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler and Adams, 2005 (157-C) were produced in last-instar mealworm larvae, *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae), at room temperature, according to the procedures described by Kaya and Stock (1997). After harvest, IJs were stored horizontally at 14°C in 500- ml vented culture flasks containing 150 ml distilled water. Flasks were shaken weekly to improve aeration and nematode survival. IJs were used within the first three weeks after emerging and harvested from white traps (White, 1927). Nematodes were kept at 22°C for 24 h prior to use in all experiments but the semi-field experiment, prior to which nematodes were kept at room temperature for 24 h. Before conducting experiments, IJ concentrations were quantified for all trials by using the method developed by Navon and Ascher (2000).

Mealybugs were laboratory-raised in ventilated cages (650 mm × 350 mm × 590 mm) on butternuts and sprouting potatoes. The identity of *P. citri* used in this study was verified using morphological (Wakgari & Giliomee, 2005) and molecular techniques (Pieterse *et al.*, 2010).

Growth chamber assay using leaves and fruit

To simulate glasshouse conditions, large plastic containers were filled with water and placed at the bottom of growth chambers to increase humidity. Leaves were obtained from a citrus orchard at Welgevallen experimental farm, Stellenbosch, Western Cape. Citrus fruits were obtained from a local supermarket. To eliminate other organisms, leaves and fruit were washed in a solution of water and 0.01% household bleach, rinsed thoroughly in tap water and left to dry before use. Leaves were cut to fit 13-cm-diameter Petri dishes lined with moist filter paper. Eight adult female mealybugs were transferred to each of eight leaves (8 replicates; 64 insects), for each treatment. After adding the mealybugs, the Petri dishes were covered with a lid to keep the mealybugs from escaping and the citrus leaves from drying out. Citrus fruit were cut in half, with each half being placed in a small, round plastic container (250-ml), with the open end facing to the

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bottom (Fig. 5.1A). Eight fruits with eight mealybugs each were prepared (8 replicates; 64 insects) and covered with a lid to prevent the mealybugs concerned from escaping. The mealybugs were then left for 24 h to settle on leaves and fruit before treatment.

The two adjuvants used were Zeba® [starch-g-poly (2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch] and Nu-Film-P® (poly-1-pmenthene, spreader/sticker, Hygrotech). Treatments were: 1) water, as control; 2) *H. zealandica*; 3) *H. zealandica* + Zeba® + Nu-Film-P®; 4) *S. yirgalemense*; and 5) *S. yirgalemense* + Zeba® + Nu-Film-P®. Nematodes were applied to leaves and fruit with the aid of calibrated handheld spray applicators at a concentration of 2000 IJs/ml. Zeba®, and Nu-film-P® were used in treatments at a concentration of 0.03% and 0.06% respectively. Treatment formulations were prepared 1 h before each trial. After treatment, plastic containers with fruit were covered with fine-mesh netting to allow airflow, while preventing mealybugs from escaping. Treatments were arranged according to a randomised design in a growth chamber at 22°C and 75 ± 8% RH.

Leaves were left for three minutes after treatment to eliminate excess runoff and placed in small pockets made out of the same fine-mesh netting that covered the plastic containers with fruit (Fig.5.1B). Pockets with leaves were then hung in a randomised block design in the same growth chamber as that which was used for the fruit. Leaves were hung directly above fruit containers receiving the same treatment. After 48 h, mealybugs were removed from the leaves and the fruit and mortality were assessed. The mealybugs were then washed to remove surface nematodes and placed in Petri dishes (13-cm-diameter), lined with moistened filter paper, and incubated for a further 48 h at 25°C. After further incubation, each cadaver was dissected with the aid of a dissection microscope to confirm mortality due to nematode infection. Temperature and humidity levels were monitored by Hobo® H8 Pro Series data loggers, which were placed inside the growth chambers. The experiment was repeated on a separate test date. The data of both experiments were pooled for analysis.

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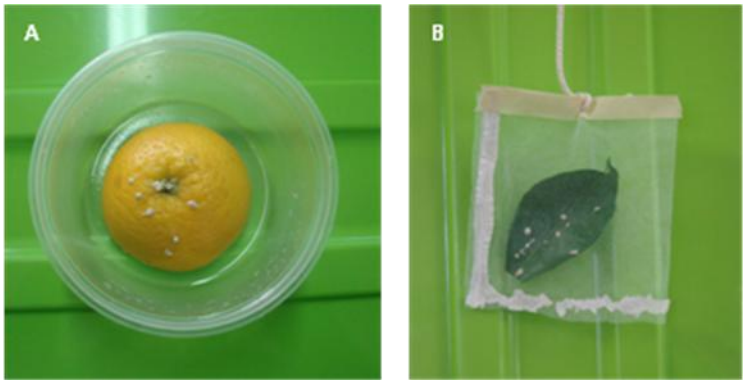

Fig. 5.1: Insect containment method used in growth chamber assay. (A) Plastic container with citrus fruit, and (B) pockets, made out of fine-mesh netting, containing a citrus leaf.

Effect of polymer-surfactant formulation on IJ infectivity

The ability of a polymer-surfactant formulation to increase the infectivity of *S. yirgalemense* under simulated gashouse conditions, as described in the growth chamber assay, was evaluated. The same procedure for preparing leaves before treatment and for determining mealybug mortality in the growth chamber assay was followed. Nematodes were applied to leaves with the aid of calibrated handheld spray applicators at a concentration of 2000 IJs/ml. A suspension of *S. yirgalemense* only was compared to a suspension containing *S. yirgalemense*, 0.03% Zeba®, 0.06% and Nu-film-P®. Five leaves were prepared for each treatment at each time interval. To estimate nematode infectivity potential after treatment application, leaves were left in a growth chamber for 0, 60, 120, 180 and 240 min, after which the leaves at each time interval, were removed and cut into smaller pieces to fit into Petri dishes (3-cm-diameter) (Fig. 5.2). Five mealybugs were added to each of five Petri dishes (5 replicates; 25 insects), closed with the lid and covered with cling wrap to ensure an airtight seal, and left in the growth chamber for 48 h, after which mortality was assessed, as was described in the growth chamber assay. The experiment was repeated on a separate test date. The data of both experiments were pooled for analysis.

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Fig. 5.2: Petri dish (3-cm-diameter) filled with cut citrus leaves to test the ability of *Steinernema yirgalemense* to infect *Planococcus citri* post-application.

Effect of a polymer-surfactant formulation on IJ survival

The ability of a polymer-surfactant formulation to retard nematode desiccation under simulated glasshouse conditions described in the growth chamber assay was evaluated. Mortality of nematodes on citrus leaves was recorded 0-, 30-, 60-, 120- and 240 min after applying nematodes to leaves. A suspension containing *S. yirgalemense* only was compared to a suspension containing *S. yirgalemense,* 0.03% Zeba®, 0.06% Nu-film-P® and *S. yirgalemense*. Nematodes were applied to leaves with the aid of calibrated handheld spray applicators at a concentration of 2000 IJs/ml. Three leaves were prepared for each treatment at each time interval. To determine percentage nematode mortality, two 2-cm² leaf discs were cut out of each leaf (3 replicates; 6 leaf discs), rinsed in 5 ml tap water and the number of live and dead nematodes recorded. Nematode mortality was determined as a percentage of the total number of nematodes recorded on each individual leaf disc. Nematodes that did not respond to light and prodding were recorded as dead. The experiment was repeated on a separate test date. The data of both experiments were pooled for analysis.

Field trial

The efficiency of adjuvants to increase the ability of *S. yirgalemense* to control adult female *P. citri* under semi-field conditions was evaluated in a citrus orchard on Welgevallen experimental farm, Stellenbosch, Western Cape, South Africa. The field experiment was conducted on 6 October 2011 in the early evening during spring. The experimental layout was in a completely randomised design, with seven rows, each containing six treatment trees, except for one row that contained four treatment trees (8

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replicates; 80 insects per treatment). Between the individual treatment trees stood two buffer trees, with two buffer rows separating the treatment rows from each other.

As was previously described in the growth chamber assay, pockets containing citrus leaves, each containing 10 adult *P. citi* females, were used for insect containment in the field experiment. Treatments were: 1) water only as control; 2) nematodes; 3) nematodes + Nu-Film-P®; 4) nematodes + Zeba®; and 5) nematodes + Zeba® + Nu-Film-P®. Nematodes were applied to leaves with the aid of calibrated handheld spray applicators at a concentration of 4000 IJs/ml, Zeba® at 3 g/L water and Nu-film-P® at 0.6 ml/L water. Treatment formulations were prepared 1 h before application. Pockets with leaves containing mealybugs were fastened onto the scaffold branches 1 m above ground of each of the 40 treatment trees on the day of the trial (Fig. 5.3) before applying treatment applications. After 26 h, the leaves were removed from the trees and taken back to the laboratory. The mealybugs were then removed from the leaves and rinsed to remove surface nematodes, and mealybugs from each leaf were placed in individual Petri dishes (9-cm-diameter) lined with moistened filter paper. The Petri dishes were then incubated for a further 48 h at 25°C, after which the mealybug mortality was assessed. Hobo® H8 Pro Series data loggers were placed in the middle of every second treatment row to document temperature and humidity in the orchard throughout the trial period.

Fig. 5.3: Pockets made out of fine-mesh netting containing a citrus leave and 10 *Planococcus citri* fastened to treatment trees prior to the experiment.

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Data analysis

All statistical analyses were performed by means of Statistica 9.0 software (StatSoft Inc. 2009). The data were analysed using ANOVA, with post-hoc comparison of means using Bonferroni's method, or a bootstrap multi-comparison test if residuals were found not to be evenly distributed (Efron & Tibshirani, 1993). Significant differences were determined on a 95% probability level.

Results

Growth chamber assay using leaves and fruit

Results obtained from the growth chamber assay were analysed using a two-way ANOVA. The analysis showed no interaction between main effects part of tree (2 levels; leaves and fruit) and treatments (5 levels) ($F_{(4, 150)}$ = 0.60; P = 0.66). The mortality of mealybugs on fruit and leaves was consistent with that encountered in the treatments and no significant differences were observed between the mortality of *P. citri* on leaves and fruit for any of the treatment suspensions tested. The one-way ANOVA for mortality observed separately on the fruit and leaves were pooled and showed the average percentage mortality (30%) on fruit not to be significantly higher ($F_{(1, 150)}$ = 0.84; P = 0.36) than the average percentage mortality (28%) observed on leaves. For further analysis, the results of mortality obtained from treatments of fruit and leaves were pooled and analysed using a one-way ANOVA. All treatments obtained significantly higher mortality than the control ($F_{(4, 150)}$ = 16.59; P = 0.001) with an average percentage mortality of 11% (Fig. 5.4). Although not significant, the combined addition of 0.06% Nu-Film-P® and 0.03% Zeba® to *H. zealandica* (T3) suspensions increased mortality from 26% to 30%. Even without the addition of Nu-Film-P® and Zeba®, a suspension of *S. yirgalemense* only (T4) obtained higher mortality (34%) than a suspension of Nu-Film-P®, Zeba® and *H. zealandica* (T3) (30% mortality). The highest average percentage mortality of 45% was obtained when mealybugs were treated with a suspension of *S. yirgalemense,* 0.06% Nu-Film-P® and 0.03% Zeba® (T5); the mortality obtained was significantly higher than when treated with suspensions of *H. zealandica* alone.

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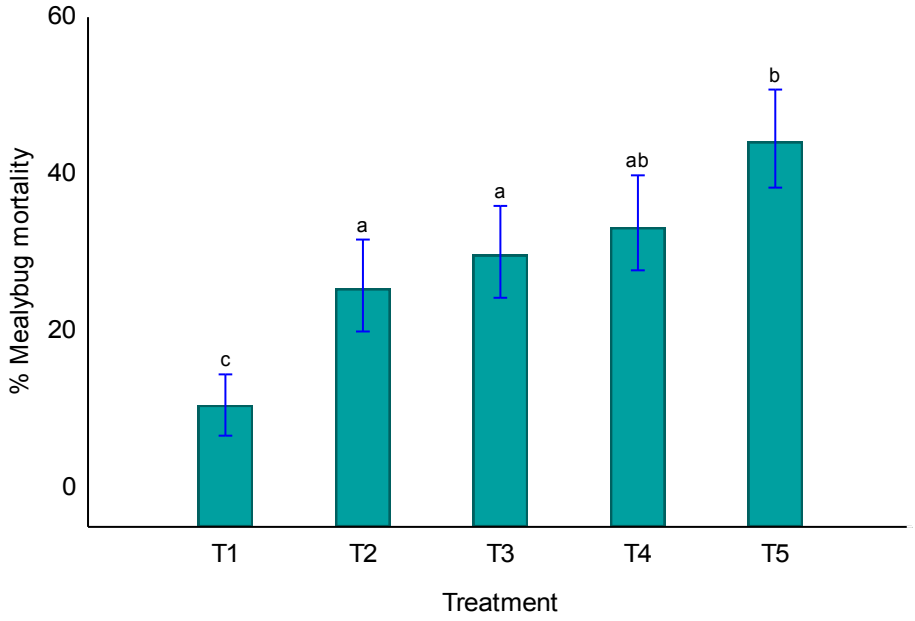

Fig. 5.4: Mean percentage mortality (95% confidence interval) recorded for adult female *Planococcus citri* on leaves after exposure to different formulations of *Heterorhabditis zealandica* and *Steinernema yirgalemense* during a growth chamber assay at 75 ± 8% RH, 22ºC and 2000 IJs/ml. Treatments were: T1, water as control; T2, *H. zealandica*; T3, *H. zealandica* + Zeba® + Nu-Film-P®; T4, *S. yirgalemense*; and T5, *S. yirgalemense* + Zeba® + Nu-Film-P®. Different letters above vertical bars indicate significant differences.

Effect of a polymer-surfactant formulation on IJ infectivity

The results for *S. yirgalemense* infectivity under simulated glasshouse conditions on citrus leaves were analysed using a two-way ANOVA. The analysis showed interaction between the main effects treatment (2 levels; treatment and formula) and time (6 levels; 0, 30, 60, 120, 180, 240) ($F_{(5, 108)}$ = 6.14; P = 0.001), indicating that the treatments did not behave consistently over time. No significant differences in nematode infectivity were observed during the first 60 min after applying nematodes to leaves, with mealybug mortality ranging between 72% and 84% (Fig. 5.5). Although not significant, 120 min after applying nematodes, the infectivity of nematodes applied in water only started to decrease to 54% control of mealybugs. Infectivity of nematodes applied with 0.03% Zeba® and 0.06% Nu-Film-P® was significantly higher (P = 0.007) after 120 min than with nematodes applied in water only, and obtained high mealybug mortality of 86%. Infectivity of nematodes applied with 0.03% Zeba® and 0.06% Nu-Film-P® only started to decrease after 180 min attaining 60% mortality; however, the percentage mortality was still not significantly lower than was the mortality of 84% (P = 0.21), obtained directly after applying nematodes. The infectivity

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potential of nematodes applied in water only, with 24% control after 180 min, was still significantly lower (P = 0.001) than when nematodes were applied with 0.03% Zeba® and 0.06% Nu-Film-P®. The lowest infectivity potential of nematodes was observed in both treatments after 240 min, with < 10% control.

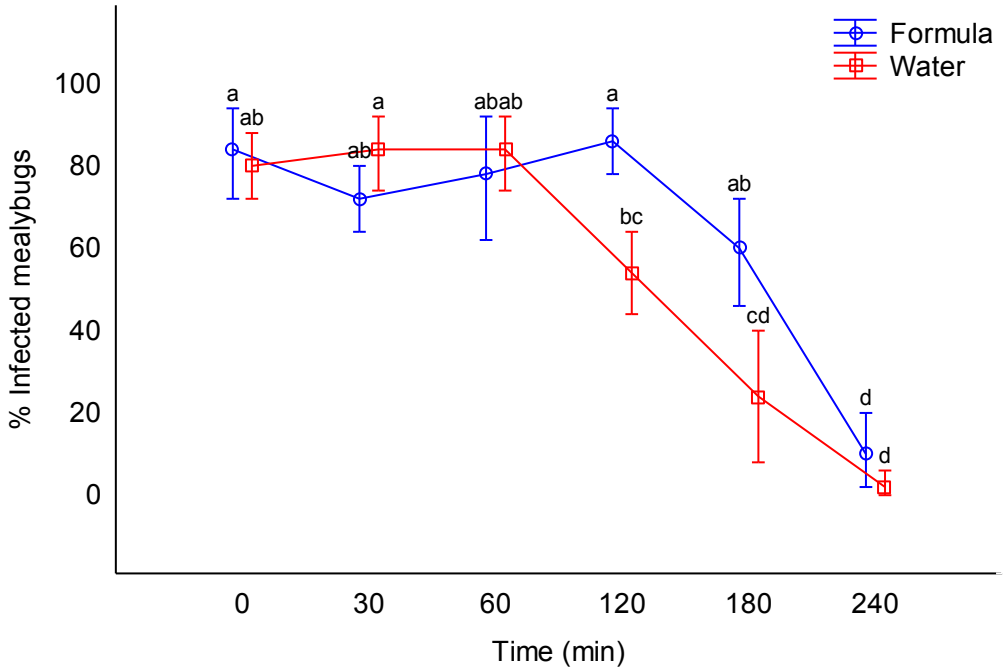

Fig. 5.5: Mean percentage (95% confidence interval) *Planococcus citri* mortality recorded after exposure to *Steinernema yirgalemense* for different time intervals during growth chamber assays at 75 ± 8% RH, 22ºC and 2000 IJs/ml. Data points with the same letter are not significantly different.

Effect of a polymer-surfactant formulation on IJ survival

The results for *S. yirgalemense* survival under simulated glasshouse conditions on citrus leaves were analysed using a two-way ANOVA. The analysis showed interaction between main effects treatment (2 level; formula and water) and time (4 levels; 30-, 60-, 120-, 240 min) ($F_{(3, 88)}$ = 4.77; P = 0.004) indicating that the treatments concerned did not behave consistently over time. Low nematode mortality (< 8%) for both treatments was observed 30 min after applying nematodes to the leaves (Fig. 5.6). The mortality of nematodes applied with water only increased significantly to 38% (P = 0.001) 60 min after application, while the mortality of nematodes applied with Zeba® and Nu-Film-P® increased only slightly, to 17%. Although not significant, after 120 min, the mortality (51%) of nematodes applied with Zeba® and Nu-Film-P® was lower than was the mortality (74%) of nematodes applied with water only. After 4 h, very few live nematodes were observed for either treatment tested.

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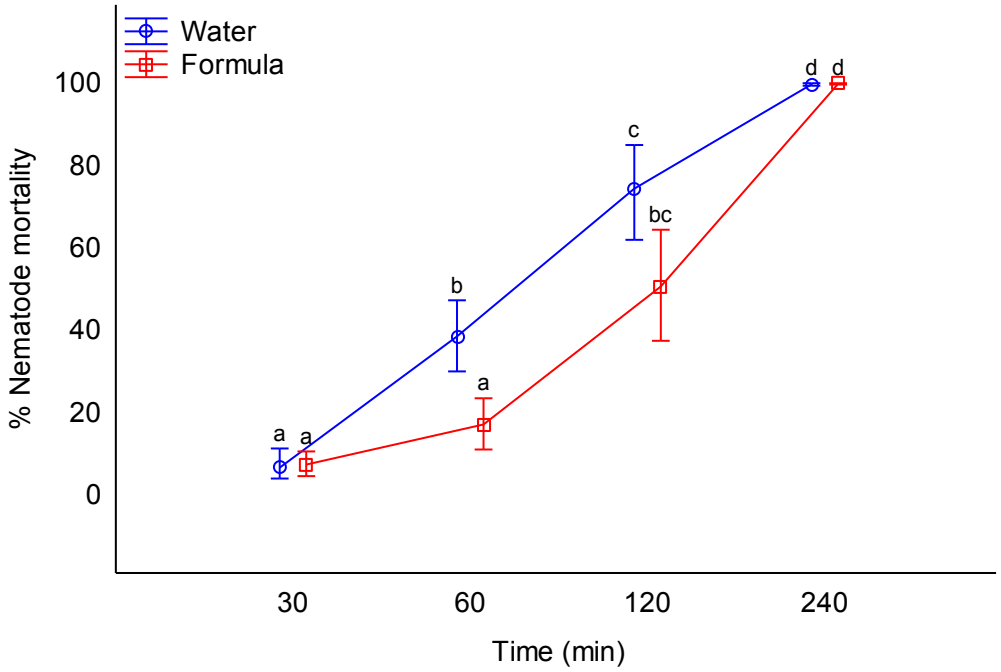

Fig. 5.6: Mean percentage (95% confidence interval) mortality of *Steinernema yirgalemense* recorded on 2-cm^2 leaf discs at different time intervals post-treatment at 75 ± 8% RH, 22ºC and 2000 IJs/ml. Data points with the same letter are not significantly different.

Field trial

Moderate temperatures, ranging between 9°C and 26°C, with an average of 11°C, were recorded throughout the trial period. The relative humidity in the orchard was average (≈ 52 % RH) at the time of application and started to increase after 1 h, as the temperature dropped after sunset (Fig. 5.7). The average temperature and humidity for the first four h post-application was 12ºC and 71% RH, respectively. From approximately 03:00 h dew formed on the trees, as the ambient humidity rose to 100% and the dew point equalled the temperature (±10°C). The ambient humidity remained 100% until 08:00 h. At 10.00 h, when pockets containing leaves were retrieved, the trees were still wet from the morning dew.

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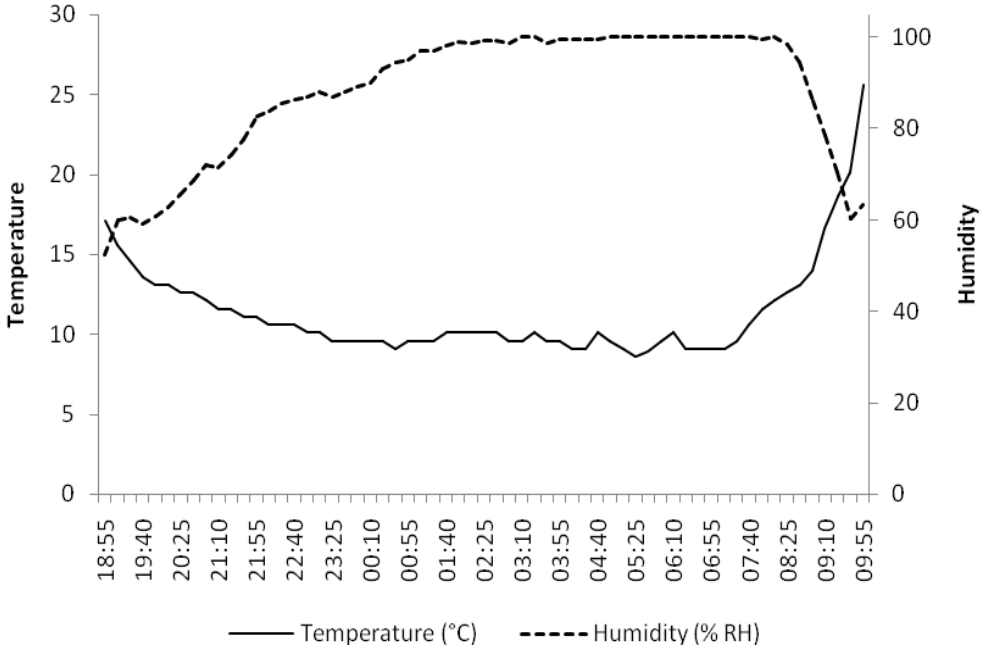

Fig. 5.7: Climatic data recorded over a 26-h period during a field experiment.

Mortality recorded for adult *P. citri* females during a field trail was analysed using a one-way ANOVA. Mortality (< 33%) obtained with a suspension of *S. yirgalemense* only (T2) and with a suspension of *S. yirgalemense* and Nu-Film-P® (T3) were not significantly higher than was that achieved with the control (11% mortality). The addition of 0.06% Nu-Film-P® to *S. yirgalemense* suspensions only increased mortality by 2%. Although not significant, the average percentage mortality increased considerably to 50% (T4), with the addition of 0.03% Zeba® and mortality obtained being significantly higher than with the control (P = 0.003). The highest average percentage mortality of 53% was observed for mealybugs treated with a suspension of nematodes, 0.06% Nu-Film-P® and 0.03% Zeba® (T5).

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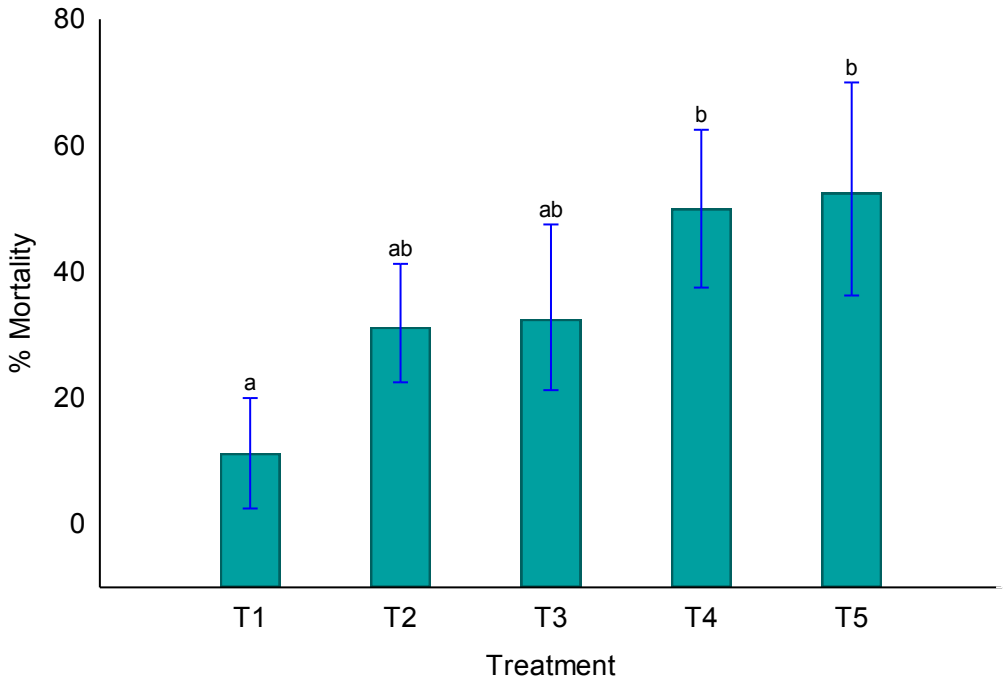

Fig. 5.8: Mean percentage mortality (95% confidence interval) recorded for adult *Planococcus citri* females after exposure to different formulations of *Steinernema yirgalemense* during a field trial conducted on 6 October 2011. Treatments were: T1, water only; T2, nematodes; T3, nematodes + Nu-Film-P®; T4, nematodes + Zeba®; and T5, nematodes + Zeba® + Nu-Film-P®. Different letters above vertical bars indicate significant differences.

Discussion

Desiccation, accelerated by such abiotic factors as high temperatures, wind and low humidity, limits the effectivity of nematodes to control above-ground insect pests (Wright *et al.*, 2005). To retard desiccation, water retention agents can be added to nematode application suspensions (Glazer *et al.*, 1992). The sensible use of adjuvants in combining water retention agents with surfactants has resulted in the successful control of western flower thrips in glasshouses (Tomalak *et al.*, 2005). In the current study, the ability of a polymer product, Zeba®, and a surfactant, Nu-Film-P®, to improve the ability of *H. zealandica* and *S. yirgalemense* to control *P. citri* on citrus was evaluated.

During a growth chamber assay, simulating glasshouse conditions of 75 ± 8% RH and 22°C, both *H. zealandica* and *S. yirgalemense* were able to increase *P. citri* mortality significantly compared to the mortality

attained with the control. Although not significant when using nematodes alone, the addition of 0.03% Zeba® and 0.06% Nu-Film-P® increased the control obtained with the use of *H. zealandica* from 26% to 30% and with *S. yirgalemense* from 34% to 45%. However, with the addition of adjuvants, the *S. yirgalemense* treatment was able to obtain significantly higher control than did the *H. zealandica* treatments. The general higher performance of *S. yirgalemense* correlated with the results obtained, as reflected in Chapter 2, which showed that the nematode species were able to locate and infect *P. citri* at a faster rate than they did *H. zealandica*, as well as being slightly more tolerant toward lower levels of free water.

As *S. yirgalemense* performed significantly better with the addition of adjuvants, only this species was further investigated. Under the same conditions as those mentioned above, adjuvants improved both the infectivity and the survival rate of *S. yirgalemense* 2–3 h post-application. When the ability of *S. yirgalemense* to infect mealybugs post-application was evaluated, the infection potential of nematodes in water significantly decreased 2 h post-application. The first decrease in infectivity potential of nematodes applied with the adjuvants was observed 3 h post-application, although the decrease concerned was not significant. The infectivity potential of nematodes applied with adjuvants decreased drastically 4 h post-application, obtaining only 10% control. When the mortality of nematodes was investigated, the average mortality of nematodes applied with water only increased significantly 1 h post-application, while a significant increase in the mortality of nematodes applied with adjuvants was observed 2 h post-application. Although not significant, the mortality of 51% observed for nematodes applied with adjuvants was considerably lower than was the nematode mortality of 74% observed for nematodes applied with water only, 2 h post-application. No live nematodes were recorded 4 h post-application. The death of nematodes, due to low humidity after 4 h, explained the loss of nematode infectivity observed.

The ability of the formulation to improve control of *P. citri* was further investigated during a semi-field trial. The adjuvants were added separately in order to compare their individual and combined influence on nematode performance. Results of the field experiment showed that, without the addition of Zeba®, suspensions containing *S. yirgalemense* were unable to obtain significantly higher control of *P. citri,* than that which was achieved with the control. The addition of 0.06% Nu-Film-P® to application suspensions had minimal effect on nematode performance, increasing *P. citri* control by only 3%. The highest mealybug mortality of 53% was obtained with *S. yirgalemense*, when applied together with both Zeba® and Nu-Film-P®. Similar results were obtained by Schroer and Ehlers (2005), who evaluated a formulation containing 0.3% Rimulgan® (surfactant) and 0.3% of the polymer xanthan (antidesiccant) aimed at improving the ability of *S.*

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carpocapsae to control DBM in a field study conducted on cabbage heads. The number of insects observed on cabbage heads declined significantly, resulting in ≥ 50% control after seven days. However, their formulation did not prove to have a significant effect, compared to that of the nematodes only, as the moist microclimate in cabbage heads, combined with high ambient humidity, favoured nematode survival.

Moderate temperatures ranging between 9°C and 26°C prevailed during the field trial, with a mean of 11ºC. Nematodes have proven to be most active at temperatures ranging between 15°C and 32°C (Lacey *et al.*, 2005), indicating that the low temperatures of between 10°C and 15°C, which occurred during the night, suppressed the performance of *S. yirgalemense.* In Chapter 2, the exposure time experiment showed the first 2–4 h post-application to be the most important. According to the findings of Lacey and Unruh (1998), the ability of nematodes to infect hosts was found to be greatly impaired when exposed to ambient humidity lower than 95%, thus suboptimal humidity levels ranging between 52% and 87% with an average of 72% prevailing during the first 4 h post-application would have suppressed nematode infectivity even further. In spite of the suboptimal environmental conditions which occurred during the specific semi-field trial undertaken in the current research, 53% control of *P. citri* was still obtained through a suspension of *S. yirgalemense*, Zeba® and Nu-Film-P®.

The feasibility of nematodes to control *P. citri* should be investigated by applying nematodes to citrus trees that have been naturally infected with mealybugs. The insect containment method used in the field trial was very limited. Although mealybugs occur on foliage, they are cryptic in nature and usually occur in more protected sites, such as between leaves, in bud-mite-induced growth deformations or in between fruit clusters (Wakgari & Giliomee, 2003), which sites also provide a much more favourable microclimate for nematode infection than does the exposed leaf surface. Arthurs *et al.* (2004) collected data of experiments conducted over the last two decades in order to develop a linear model for testing the efficacy of *S. carpocapsae* to control insect pests. His model showed nematode efficacy to be dependent on pest target habitat, with efficiency decreasing in the following order: firstly, bore holes; secondly, cryptic foliage; and thirdly, exposed foliage. Thus, control of *P. citri* obtained during natural conditions should theoretically be considerably higher than the control that was obtained in the current field study, in which mealybugs were much more exposed to the harsh macroenvironment.

The cost-effect efficiency of increasing nematode application concentrations should also be investigated, as the concentration of 4000 IJs/ml used in the field trial is relatively low. Also, mealybugs tend

to cluster together and often to infect only a few adjacent trees, which can be treated as hot spots, with the application of a high concentration of nematodes. In Chapter 2, the feasibility of irrigating citrus trees pre- and post-application to increase humidity and to improve nematode performance was discussed, with it being concluded that, in many cases, such irrigation would, most probably, not be practical, as water is a limited natural recourse in South Africa. Furthermore, the waxy coatings that cover citrus leaves and fruit impair the ability of nematode application suspensions to stick to their surface. Irrigating trees before application could increase application runoff, while nematodes would most probably be washed off from the exposed leaf surface if trees were to be irrigated after application. It was suggested that such loss of nematodes could be compensated for by applying them during the late afternoon, as was done in the current field trial. However, if, theoretically, nematodes had been applied at 05:00 h on 7 October 2011, the nematodes would have been subjected to approximately 7 h of moisture, allowing them to detect and infect their hosts. Dew formed at 03:00 h, when the relative humidity reached 100%. The ambient humidity remained 100% until 08:00 h. At 10.00 h, when treated leaf pockets were retrieved, the trees were still wet from the morning dew. If the nematodes had been applied at 05:00 h, they would have had at least five hours of moisture before the desiccation-retarding abilities of Zeba® would have been required. Consequently, at least an additional two hours would have been added to the lifetime of the nematodes, providing them with a total of seven hours in which to locate and infect *P. citri.* During this time, the temperature would also have increase, adding to the nematode infection potential.

Nematodes have been found to be best used to control above-ground pests in an IPM system (Wright *et al.,* 2005). As mealybugs tend to occur in all life stages on citrus trees throughout the year (Wakgari & Giliomee, 2003), nematodes can be applied at any time when environmental conditions are favourable. Adult mealybugs are difficult to control with chemicals, as, in addition to tending to hide in protected sites, where insecticides cannot reach them, they are also covered by protective waxes (McKenzie, 1967; Michelakis & Hamid, 1995; Franco *et al.*, 2004). Crawlers that emerge from protected hiding sites in search of food during early spring, despite being less susceptible to nematodes than are adults (Stokwe, 2009), tend to be more susceptible to chemicals (Hattingh & Moore, 2003). Although the use of chemicals is not desirable, nematodes can be applied in combination with an insecticide during early spring to target all life stages of mealybugs. Control of mealybug populations early in the growing season will tend to reduce the pesticide load that might otherwise be experienced later in the season. Furthermore, natural enemy populations recover slowly after winter, resulting in population levels that are inefficient in preventing feeding damage to young fruitlets (Hattingh, 1993; Franco *et al.*, 2004). Nematodes can, thus, be

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applied during this period to fill the gap left by natural enemies. If high mealybug numbers are recorded later in the growing season during late and midsummer, nematodes alone can be applied for control, without any problem of residues remaining on the fruit. Average temperatures will also be higher during the night at such times of the year, which will increase nematode efficiency.

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CHAPTER 6

Conclusion

Overall, the aim of this study was to contribute towards the control of citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) in South African citrus orchards with the use of entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae and Steinernematidae). The objectives of this study was firstly to identifying nematode species which show great potential for the control of adult female *P. citri* in the laboratory. Secondly, to evaluate whether the addition of adjuvants to nematode application suspensions could improve the ability of selected nematode species to control *P. citri* under suboptimal environmental conditions with regard to nematode mobility and survival. Thirdly, to evaluate the compatibility of selected nematode species with biocontrol agents and agrochemicals to which they most likely will to be exposed to in an IPM programme for citrus in South Africa. And fourthly, to evaluate whether the addition of adjuvants could improve the ability of selected nematode species to control *P. citri* under simulated glasshouse conditions and semi-field trials conducted under local conditions.

Prior to conducting laboratory bioassays, the quality of infective juveniles (IJs) during a four-week storage period was tested using *Heterorhabditis zealandica* Pionar, 1990 as indicator species. Results indicated that IJs should be used within the first three weeks after harvest, in order to ensure reliable results in laboratory experiments. The aim of the first part of the study was to select nematode species with the greatest potential to controlling *P. citri.* After screening six indigenous species, *H. zealandica* (SF 41) and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005 (157-C) were selected for further evaluation in laboratory bioassays. Biological control studies showed both *H. zealandica* and *S. yirgalemense* able to reproduce and complete their life cycles in *P. citri* cadavers, with *S. yirgalemense* having a higher penetration potential and shorter life cycle.

The ability of the above-mentioned nematode species to control *P. citri* was further evaluated under controlled laboratory conditions. Results obtained from laboratory bioassays conducted in Chapter 2, indicate both *H. zealandica* and *S. yirgalemense* to hold great potential for controlling *P. citri*. With different water activity levels *S. yirgalemense* proved to be 2 times more tolerant to dryer conditions than *H. zealandica.* A positive relationship between exposure time and effective control was observed with nematodes being able to infect mealybugs within 30 min. Results also showed that the first 2 to 4 h post application is the most decisive time for establishing successful infection of mealybugs. Only the results recorded in the exposure

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time experiment and water activity experiment conclusively suggested *S. yirgalemense* to possibly be a more efficient biocontrol agent than *H. zealandica* under suboptimal conditions.

The second objective of the study was to evaluate the ability of adjuvants to improve nematode application suspensions aimed to control foliar insect pests. The addition of 0.03% Zeba® to nematode suspensions resulted in a more even distribution of nematodes by effectively retarding their sedimentation. The same adjuvant contributed to increasing mealybug mortality obtained at 60% and 80% RH by retarding desiccation, improving nematode mobility and extending nematode survival. Furthermore, the combined addition of both Nu-Film-P® and Zeba® substantially reduced the loss of nematodes by runoff.

To achieve the third objective of this study, the susceptibility of *Cryptolaemus montrouzieri*, a commercially available biocontrol agent aimed to control *P.* citri, to *H. zealandica* and *S. yirgalemense* and the latter's tolerance to two adjuvants, Zeba® and Nu-Film-P®; two biopesticides, Cryptogran™ and Helicovir™ and an insecticide Cyperphos 500 E.C.® was determined. The above mentioned are examples of the most common biocontrol agents and agrochemicals used in an IPM programme for citrus in South Africa. Results showed adult *C. montrouzieri* to be twice as susceptible to *S. yirgalemense* as to *H. zealandica* indicating the susceptibility of natural enemies to vary depending on the nematode−insect species interaction. Susceptibility also varies depending on the life-stage of natural enemies as beetle larvae were much more susceptible to both nematode species tested than adult beetles. Results suggest that the nematode species tested should not be applied when high numbers of *C. montrouzieri* are present on trees. Results of the nematode tolerance and persistence experiment showed *H. zealandica* to be highly compatible with all products tested. *Steinernema yirgalemense* showed sensitivity to Cryptogran™, Helicovir™ and Cyperphos 500 E.C.® after 12 h exposure and Nu-Film-P®, after 6h exposure, indicating that *S. yirgalemense* should not be tank mixed with the above-mentioned products for prolonged periods before application. A significant increase in *S. yirgalemense* mortality was also observed after 6 h exposure. Exposure to the above mentioned products did not prove to have any effect on nematode persistence as high levels of mealworm mortality was still obtained after 24 h exposure. The objective of Chapter 4 was partly met as compatibility of *S. yirgalemense* and *H. zealandica* to only some of the vast variety of biocontrol agents and agrochemicals used in IPM programmes for citrus were evaluated. The current study did, however, illustrate some of the factors that should be taken into consideration before nematodes are applied in commercial orchards in an IPM programme.

The final objective of the study was to evaluate the addition of a polymer product, Zeba®, and a surfactant, Nu-Film-P®, to nematode application suspensions to improve the ability of *H. zealandica* and *S. yirgalemense* to control *P. citri* on citrus under simulated glasshouse and semi-field trials conducted under local conditions. Results of a growth chamber assay, simulating glasshouse conditions showed *S. yirgalemense* to have greater potential for control of *P. citri* under sub-optimal field conditions than *H. zealandica.* The ability of adjuvants to improve control obtained by *S. yirgalemense* was then further investigated and results indicated adjuvants to improve both the infectivity and the survival rate of *S. yirgalemense* 2–3 h post-application. Results of the semi-field trial showed the addition of Zeba® to *S. yirgalemense* to greatly improve the ability of the latter to control *P. citri* obtaining > 50% control. To further investigate the potential of EPNs to control *P. citri,* field trials should be conducted on naturally infested citrus trees. Further, research should be directed towards improving application technology to further improve nematode mobility and prolong survival.

The conclusive results from this study indicate two indigenous EPN species tested to show potential for controlling *P. citri* under local semi-field conditions. Although only marginal, *S. yirgalemense* would be regarded as the more effective of the two species. From the results of this study, it is however of major importance that a high humidity and temperature should be maintained for at least 6 h or longer after field application either by using weather forecasting, manual manipulation through the addition of adjuvants and the correct time of day for application. To save on cost, labour and water, nematodes can be tank mixed with other biological control agents such as Crytogran™. When an insect biocontrol agent is used for the control of meallybugs, care should be taken to apply nematodes before the release of such agents or when they occur naturally in high numbers, as nematodes might also have a non-target effect, such as seen with *C. montrouzieri.* Most of the studies were done under suboptimal conditions, indicating a higher infection potential under natural *P. citri* populations in cryptic habitats in an orchard environment.

Further, research should be directed towards improving application technology to further improve nematode mobility and prolong survival. New nematode species are frequently found and the susceptibility of *P. citri* to such species should be determined to identify if new isolates hold potential for *P. citri* control. Crossbreeding with the current selected nematode species can also be undertaken to breed strains which are more tolerant to harsh above-ground conditions.

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ATTACHMENT 1: NEMATODE SPECIES AND CONCENTRATION

POLO-PC

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Input file >
input: =LC RH 100
input: *hz
input: 0 80 12
input: 5 80 39
input: 10 80 46
input: 20 80 50
input: 40 80 57
input: 80 80 73
input: *sy
input: 0 80 8
input: 5 80 30
input: 10 80 48
input: 20 80 49
input: 40 80 56
input: 80 80 69

preparation	dose	log-dose	subjects	responses	resp/subj
hz	0.00000	0.000000	80.	12.	0.150
	5.00000	0.698970	80.	39.	0.480
	10.00000	1.000000	80.	46.	0.575
	20.00000	1.301030	80.	50.	0.625
	40.00000	1.602060	80.	57.	0.713
	80.00000	1.903090	80.	73.	0.913
Sy	0.00000	0.000000	80.	08.	0.100
	5.00000	0.698970	80.	30.	0.375
	10.00000	1.000000	80.	48.	0.600
	20.00000	1.301030	80.	49.	0.613
	40.00000	1.602060	80.	56.	0.700
	80.00000	1.903090	80.	69.	0.863

Number of preparations: 2
Number of dose groups: 10
Do you want probits [Y] ? Is Natural Response a parameter [Y] ? Do you want the likelihood function to be maximized [Y] ? LD's to calculate [10 50 90] > Do you want to specify starting values of the parameters [N] ?
The probit transformation is to be used
Natural Response is a parameter
The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is (1) hz
Estimating natural response

Maximum log-likelihood -271.06892

	Parameter	standard error	t ratio
Hz	-1.1428967	.28070133	-4.0715755
NATURAL	0.15338479	.40924293E-01	3.7480131
SLOPE	1.0977079	.19500444	5.6291430

Variance-Covariance matrix

	Hz	NATURAL	SLOPE
Hz	.7879324E-01	-.4754853E-02	-.5167361E-01
NATURAL	-.4754853E-02	.1674798E-02	.1963371E-02
SLOPE	-.5167361E-01	.1963371E-02	.3802673E-01

Chi-squared goodness of fit test

Preparation	subjects	responses	expected	deviation	probability
Hz	80.	39.	36.220	2.780	.452744
	80.	46.	44.915	1.085	.561435
	80.	50.	53.740	-3.740	.671746
	80.	57.	61.778	-4.778	.772221
	80.	73.	68.348	4.652	.854350
NATURAL	80.	12.	12.271	-.271	.153385

chi-square 5.0458 degrees of freedom 3 heterogeneity 1.6819

A large chi-square indicates a poor fit of the data by the probit
analysis model. Large deviations for expected probabilities near 0 or 1

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are especially troublesome. A plot of the data should be consulted.
See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.29397 g(.95)=.53758 g(.99)=1.8108

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

	Dose	limits	0.90	0.95	0.99
LD50 hz 1	0.99427	lower	3.28996		
		upper	19.97892		
LD90 hz 1	61.67621	lower	69.42731		
		upper	1920.45381		
LD95 hz	346.42591	lower	118.60992		
		Upper	9735.32534		

Intercepts and slopes unconstrained. Preparation is (2) sy
Estimating natural response

Maximum log-likelihood -269.02777

	Parameter	standard error	t ratio
Sy	-1.1645346	.25248904	-4.6122184
NATURA .	99753468E-01	.33426817E-01	2.9842347
SLOPE	1.0869575	.17916823	6.0666864

Variance-Covariance matrix

Sy	NATURAL	SLOPE	
Sy	.6375072E-01	-.2821232E-02	-.4270043E-01
NATURAL	-.2821232E-02	.1117352E-02	.1092048E-02
SLOPE	-.4270043E-01	.1092048E-02	.3210126E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
sy	80.	30.	32.670	-2.670	.408374
	80.	48.	41.763	6.237	.522043
	80.	49.	51.089	-2.089	.638608
	80.	56.	59.689	-3.689	.746108
	80.	69.	66.821	2.179	.835267
NATURAL	80.	08.	7.980	.020	.099753

chi-square 3.8827 degrees of freedom 3 heterogeneity 1.2942

A large chi-square indicates a poor fit of the data by the probit
analysis model. Large deviations for expected probabilities near 0 or 1
are especially troublesome. A plot of the data should be consulted.
See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.19476 g(.95)=.35615 g(.99)=1.1997

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		Dose	limits	0.90	0.95	0.99
LD50	sy	11.78612 l	ower	5.48948	3.21444	
			Upper	18.96794	22.35198	
LD90	sy	177.99068	lower	83.01214	69.01308	
			Upper	1063.60396	4785.20706	
LD95	sy	384.27	lower	145.70	116.25	
			Upper	4098.5	31024.	

Intercepts and slopes constrained (lines are the same)
Not estimating natural response

Maximum log-likelihood -540.15468

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	Parameter	standard error	t ratio
INTERCPT	-1.1545963	.17423972	-6.6264819
SLOPE	1.0922763	.12891831	8.4726237

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.3035948E-01	-.2142100E-01
SLOPE	-.2142100E-01	.1661993E-01

Testing hypothesis that slopes and intercepts are the same
chi-square .1160 degrees of freedom 2 tail probability .944
Hypothesis ACCEPTED

Chi-squared goodness of fit test

Preparation	subjects	responses	expected	deviation	probability
INTERCPT	80.	39.	35.830	3.170	.447881
	80.	46.	44.453	1.547	.555657
	80.	50.	53.252	-3.252	.665645
	80.	57.	61.319	-4.319	.766484
	80.	73.	67.963	5.037	.849543
	80.	30.	33.032	-3.032	.412905
	80.	48.	42.201	5.799	.527509
	80.	49.	51.557	-2.557	.644464
	80.	56.	60.135	-4.135	.751691
	80.	69.	67.201	1.799	.840012

chi-square 8.9700 degrees of freedom 8 heterogeneity 1.1213

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.05401 g(.95)=.08306 g(.99)=.17585

Effective Doses

		Dose	limits	0.90	0.95	0.99
LD50	INTERCPT	11.4039	lower	8.53413	7.82670	6.10548
			Upper	14.33770	15.08460	16.96135
LD90	INTERCPT	169.95746	lower	107.94569	98.94241	82.53593
			Upper	342.75264	432.37410	882.70051
LD95	INTERCPT	365.55291	lower	203.20876	181.80699	144.48930
			Upper	919.27265	1250.57356	3234.32766

Slopes constrained (lines are parallel)
Not estimating natural response

Maximum log-likelihood -540.09756

	Parameter	standard error	t ratio
Hz	-1.1354794	.18286810	-6.2092809
Sy	-1.1709727	.18092239	-6.4722375
SLOPE	1.0919594	.12885876	8.4740791

Variance-Covariance matrix

	Hz	sy	SLOPE
Hz	.3344074E-01	.2757538E-01	-.2142289E-01
sy	.2757538E-01	.3273291E-01	-.2137329E-01
SLOPE	-.2142289E-01	-.2137329E-01	.1660458E-01

Testing hypothesis that slopes are the same
chi-square .0017 degrees of freedom 1 tail probability .967
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
hz	80.	39.	36.305	2.695	.453815
	80.	46.	44.960	1.040	.561998
	80.	50.	53.738	-3.738	.671726
	80.	57.	61.738	-4.738	.771719
	80.	73.	68.287	4.713	.853588
sy	80.	30.	32.592	-2.592	.407401
	80.	48.	41.722	6.278	.521529

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80.	49.	51.091	-2.091	.638632
80.	56.	59.727	-3.727	.746587
80.	69.	66.880	2.120	.836002

chi-square 8.9136 degrees of freedom 7 heterogeneity 1.2734 P=0.74

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.06365 g(.95)=.09915 g(.99)=.21716

Effective Doses

		Dose	limits	0.90	0.95	0.99
LD50	hz	10.96112	lower	7.15510	6.27437	4.21961
			Upper	15.52608 1	6.86695	20.68034
	Sy	11.81297	lower	7.95262	7.04843	4.90817
			Upper	16.40593	17.75559	21.60773
LD90	hz	163.48605	lower	96.29501	86.58973	68.85598
			Upper	370.58102	492.87728	1262.60455
	Sy	176.19147	lower	103.35273	92.94029	74.01505
			Upper	405.50715	543.02796	1427.53903
LD95	hz	351.71206	lower	181.84914	159.80821	121.53757
			Upper	1007.90886	1463.04285	5064.04164
	Sy	379.04559	lower	194.55253	170.83534	129.83092
			Upper	1106.44336	1618.45051	5761.40879

Relative potencies

	Potency	limits	0.90	0.95	0.99
Sy	.92789	lower	.56551	.49317	.33729
		upper	1.50566	1.71466	2.43947

LC RH 100
Hz subjects 400 controls 80
log(L)=-271.1 slope=1.098+-.195 nat.resp.=.153+-.041
heterogeneity=1.68 g=.538

sy subjects 400 controls 80
log(L)=-269.0 slope=1.087+-.179 nat.resp.=.100+-.033
heterogeneity=1.29 g=.356
LD50=11.786 limits: 3.214 to 22.352
LD90=177.991 limits: 69.013 to 4785.207
LD95=384.269 limits: 116.246 to 31023.871
SAME subjects 800 controls 160
log(L)=-540.2 slope=1.092+-.129 nat.resp.=.125+-.000
Hypothesis ACCEPTED
heterogeneity=1.12 g=.083
LD50=11.404 limits: 7.827 to 15.085
LD90=169.957 limits: 98.942 to 432.374
LD95=365.553 limits: 181.807 to 1250.574

PARALLEL subjects 800 controls 160
log(L)=-540.1 slope=1.092+-.129 nat.resp.=.125+-.000
Hypothesis ACCEPTED
heterogeneity=1.27 g=.099
LD50=10.961 limits: 6.274 to 16.867
LD90=163.486 limits: 86.590 to 492.877
LD95=351.712 limits: 159.808 to 1463.043

Stop - Program terminated.

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ATTACHMENT 2: HUMIDITY AND CONCENTRATION

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Input file >
input: =Dose
input: *60
input: 0 80 6
input: 5 80 7
input: 10 80 8
input: 20 80 14
input: 40 80 13
input: 80 80 29
input: *80
input: 0 80 6
input: 5 80 9
input: 10 80 14
input: 20 80 20
input: 40 80 19
input: 80 80 33
input: *100
input: 0 80 12
input: 5 80 39
input: 10 80 46
input: 20 80 50
input: 40 80 57
input: 80 80 73

preparation	dose	log-dose	subjects	responses	resp/subj
60	.00000	.000000	80.	6.	.075
	5.00000	.698970	80.	7.	.087
	10.00000	1.000000	80.	8.	.100
	20.00000	1.301030	80.	14.	.175
	40.00000	1.602060	80.	13.	.163
	80.00000	1.903090	80.	29.	.363
80	.00000	.000000	80.	6.	.075
	5.00000	.698970	80.	9.	.113
	10.00000	1.000000	80.	14.	.175
	20.00000	1.301030	80.	20.	.250
	40.00000	1.602060	80.	19.	.238
	80.00000	1.903090	80.	33.	.413
100	.00000	.000000	80.	12.	.150
	5.00000	.698970	80.	39.	.488
	10.00000	1.000000	80.	46.	.575
	20.00000	1.301030	80.	50.	.625
	40.00000	1.602060	80.	57.	.713
	80.00000	1.903090	80.	73.	.913

Number of preparations: 3
Number of dose groups: 15
Do you want probits [Y] ? Is Natural Response a parameter [Y] ? Do you want the likelihood function to be maximized [Y] ? LD's to calculate [10 50 90] > Do you want to specify starting values of the parameters [N] ?
The probit transformation is to be used
Natural Response is a parameter
The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is (1) 60
Estimating natural response

Maximum log-likelihood -197.26380

	parameter	standard error	t ratio
60	-3.5605307	1.2700900	-2.8033689
NATURAL	.80603320E-01	.27082484E-01	2.9762160
SLOPE	1.5661605	.69055010	2.2679897

Variance-Covariance matrix

	60	NATURAL	SLOPE
60	1.613129	-.2554623E-01	-.8708154
NATURAL	-.2554623E-01	.7334609E-03	.1305469E-01
SLOPE	-.8708154	.1305469E-01	.4768594

Chi-squared goodness of fit test

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preparation	subjects	responses	expected	deviation	probability
60	80.	7.	6.951	.049	.086887
	80.	8.	8.144	-.144	.101801
	80.	14.	11.148	2.852	.139344
	80.	13.	17.226	-4.226	.215319
	80.	29.	27.114	1.886	.338919
NATURAL	80.	6.	6.448	-.448	.080603

chi-square 2.4047 degrees of freedom 3 heterogeneity .80

Index of significance for potency estimation:
g(.90)=.52598 g(.95)=.74682 g(.99)=1.2899

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

Effective Doses

	dose	limits	0.90	0.95	0.99
LD50 60	187.67813				
LD90 60	1235.06462				

Intercepts and slopes unconstrained. Preparation is (2) 80
Estimating natural response

Maximum log-likelihood -230.67955

	parameter	standard error	t ratio
80	-2.2961786	.49362713	-4.6516458
NATURAL	.74380769E-01	.28671934E-01	2.5942013
SLOPE	.98792823	.28407699	3.4776778

Variance-Covariance matrix

	80	NATURAL	SLOPE
80	.2436677	-.8874560E-02	-.1354646
NATURAL	-.8874560E-02	.8220798E-03	.3989294E-02
SLOPE	-.1354646	.3989294E-02	.8069973E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
80	80.	9.	9.962	-.962	.124527
	80.	14.	13.014	.986	.162679
	80.	20.	17.505	2.495	.218817
	80.	19.	23.558	-4.558	.294476
	80.	33.	31.030	1.970	.387872
NATURAL	80.	6.	5.950	.050	.074381

chi-square 2.1052 degrees of freedom 3 heterogeneity .70

Index of significance for potency estimation:
g(.90)=.22371 g(.95)=.31763 g(.99)=.54860

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

	dose	limits	0.90	0.95	0.99
LD50 80	210.97754	lower	111.92819	102.27743	
		upper	936.75193	1736.69377	
LD90 80	4182.6	lower	940.39	780.16	
		upper	.24508E+06	.14434E+07	

Intercepts and slopes unconstrained. Preparation is (3) 100
Estimating natural response

Maximum log-likelihood -271.06892

	parameter	standard error	t ratio
100	-1.1428967	.28070133	-4.0715755
NATURAL	.15338479	.40924293E-01	3.7480131
SLOPE	1.0977079	.19500444	5.6291430

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Variance-Covariance matrix

	100	NATURAL	SLOPE
100	.7879324E-01	-.4754853E-02	-.5167361E-01
NATURAL	-.4754853E-02	.1674798E-02	.1963371E-02
SLOPE	-.5167361E-01	.1963371E-02	.3802673E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
100	80.	39.	36.220	2.780	.452744
	80.	46.	44.915	1.085	.561435
	80.	50.	53.740	-3.740	.671746
	80.	57.	61.778	-4.778	.772221
	80.	73.	68.348	4.652	.854350
NATURAL	80.	12.	12.271	-.271	.153385

chi-square 5.0458 degrees of freedom 3 heterogeneity 1.6819

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.29397 g(.95)=.53758 g(.99)=1.8108

"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

	dose	limits	0.90	0.95	0.99
LD50 100	10.99427	lower	3.28996		
		upper	19.97892		
LD90 100	161.676	lower	69.42731		
		upper	1920.45381		

Intercepts and slopes constrained (lines are the same)
Not estimating natural response

Maximum log-likelihood -801.06468

	parameter	standard error	t ratio
INTERCPT	-1.8927069	.19230633	-9.8421454
SLOPE	.94382729	.12773141	7.3891557

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.3698173E-01	-.2373183E-01
SLOPE	-.2373183E-01	.1631531E-01

Testing hypothesis that slopes and intercepts are the same
chi-square 204.10 degrees of freedom 4 tail probability .000
Hypothesis REJECTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
INTERCPT	80.	7.	14.450	-7.450	.180623
	80.	8.	19.051	-11.051	.238134
	80.	14.	25.064	-11.064	.313305
	80.	13.	32.319	-19.319	.403988
	80.	29.	40.396	-11.396	.504955
	80.	9.	14.006	-5.006	.175077
	80.	14.	18.638	-4.638	.232977
	80.	20.	24.693	-4.693	.308657
	80.	19.	31.996	-12.996	.399954
	80.	33.	40.128	-7.128	.501604
	80.	39.	19.639	19.361	.245486
	80.	46.	23.876	22.124	.298445
	80.	50.	29.413	20.587	.367665
	80.	57.	36.094	20.906	.451169
	80.	73.	43.531	29.469	.544144

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chi-square 205.52 degrees of freedom 13 heterogeneity 15.809

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.90808 g(.95)=1.3514 g(.99)=2.6273

"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	INTERCPT	101.24020				
LD90	INTERCPT	2307.68878				

Slopes constrained (lines are parallel)
Not estimating natural response

Maximum log-likelihood -699.69344

	parameter	standard error	t ratio
60	-2.8288186	.25244076	-11.205871
80	-2.5012009	.23777702	-10.519102
100	-1.1747611	.19767272	-5.9429603
SLOPE	1.1223924	.14118727	7.9496711

Variance-Covariance matrix

	60	80	100	SLOPE
60	.6372633E-01	.4892229E-01	.4093377E-01	-.3169856E-01
80	.4892229E-01	.5653791E-01	.3972835E-01	-.3076510E-01
100	.4093377E-01	.3972835E-01	.3907450E-01	-.2574147E-01
SLOPE	-.3169856E-01	-.3076510E-01	-.2574147E-01	.1993385E-01

Testing hypothesis that slopes are the same
chi-square 1.3623 degrees of freedom 2 tail probability .506
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
60	80.	7.	7.953	-.953	.099416
	80.	8.	9.682	-1.682	.121024
	80.	14.	12.742	1.258	.159276
	80.	13.	17.580	-4.580	.219750
	80.	29.	24.411	4.589	.305135
80	80.	9.	9.136	-.136	.114200
	80.	14.	12.169	1.831	.152111
	80.	20.	16.980	3.020	.212254
	80.	19.	23.797	-4.797	.297463
	80.	33.	32.422	.578	.405277
100	80.	39.	35.853	3.147	.448158
	80.	46.	44.721	1.279	.559013
	80.	50.	53.746	-3.746	.671828
	80.	57.	61.949	-4.949	.774362
	80.	73.	68.607	4.393	.857589

chi-square 10.8872 degrees of freedom 11 heterogeneity .99 P=0.55

Index of significance for potency estimation:
g(.90)=.04281 g(.95)=.06079 g(.99)=.10499

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	60	331.39600	lower	198.05400	181.89582	155.43030
			upper	671.60705	796.65469	1167.82703
	80	169.22128	lower	113.12176	105.75669	93.32246
			upper	291.50546	332.10822	444.27327
	100	11.13418	lower	8.12221	7.56747	6.49520
			upper	14.51290	15.22977	16.72318

LD90	60	4593.6	lower	1882.5	1636.6	1272.2
			upper	17175.	23908.	50798.
	80	2345.6	lower	1076.8	953.22	765.67

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```
                                upper      7444.1       9949.3     19278.
     100        154.33401       lower      100.79321    94.13762    83.13540
                                upper      284.27627    330.58366   465.16286

Relative potencies
              potency    limits      0.90          0.95         0.99
80            1.95836    lower     1.18198      1.07312       .88528
                         upper     3.41268      3.84464      4.94533
100          29.76385    lower    15.79106     14.22721     11.73938
                         upper    71.45934     88.37483    142.34995

 Dose
   60        subjects 400   controls 80
     log(L)=-197.3   slope=1.566+-.691   nat.resp.=.081+-.027
     heterogeneity=.80   g=.747
   80        subjects 400   controls 80
     log(L)=-230.7   slope=.988+-.284   nat.resp.=.074+-.029
     heterogeneity=.70   g=.318
     LD50=210.978   limits: 102.277 to 1736.694
     LD90=4182.610   limits: 780.158 to 1443376.977
   100       subjects 400   controls 80
     log(L)=-271.1   slope=1.098+-.195   nat.resp.=.153+-.041
     heterogeneity=1.68   g=.538
   SAME      subjects 1200   controls 240
     log(L)=-801.1   slope=.944+-.128   nat.resp.=.100+-.000
     Hypothesis REJECTED
     heterogeneity=15.81   g=1.351
   PARALLEL   subjects 1200   controls 240
     log(L)=-699.7   slope=1.122+-.141   nat.resp.=.100+-.000
     Hypothesis ACCEPTED
     heterogeneity=.99   g=.061
     LD50=331.396   limits: 181.896 to 796.655
     LD90=4593.575   limits: 1636.632 to 23907.929

Stop - Program terminated.
```

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ATTACHMENT 3: NEMATODE SPECIES AND WATER ACTIVITY

```
POLO-PC
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Input file >
input: =aw
input: * 157-C
input: 0 479 8
input: 0.8 40 0
input: 0.9 40 2
input: 0.91 40 1
input: 0.92 40 4
input: 0.93 40 5
input: 0.94 40 7
input: 0.95 40 14
input: 0.96 40 10
input: 0.97 40 24
input: 0.98 40 30
input: 0.99 40 35
input: 1.00 40 38
input: * SF41
input: 0 479 13
input: 0.8 40 1
input: 0.9 40 0
input: 0.91 40 0
input: 0.92 40 0
input: 0.93 40 2
input: 0.94 40 2
input: 0.95 40 1
input: 0.96 40 10
input: 0.97 40 17
input: 0.98 40 24
input: 0.99 40 31
input: 1.00 40 32
```

preparation	dose	log-dose	subjects	responses	resp/subj
157-C	.00000	.000000	479.	8.	.017
	.80000	-.096910	40.	0.	.000
	.90000	-.045757	40.	2.	.050
	.91000	-.040959	40.	1.	.025
	.92000	-.036212	40.	4.	.100
	.93000	-.031517	40.	5.	.125
	.94000	-.026872	40.	7.	.175
	.95000	-.022276	40.	14.	.350
	.96000	-.017729	40.	10.	.250
	.97000	-.013228	40.	24.	.600
	.98000	-.008774	40.	30.	.750
	.99000	-.004365	40.	35.	.875
	1.00000	.000000	40.	38.	.950
SF41	.00000	.000000	479.	13.	.027
	.80000	-.096910	40.	1.	.025
	.90000	-.045757	40.	0.	.000
	.91000	-.040959	40.	0.	.000
	.92000	-.036212	40.	0.	.000
	.93000	-.031517	40.	2.	.050
	.94000	-.026872	40.	2.	.050
	.95000	-.022276	40.	1.	.025
	.96000	-.017729	40.	10.	.250
	.97000	-.013228	40.	17.	.425
	.98000	-.008774	40.	24.	.600
	.99000	-.004365	40.	31.	.775
	1.00000	.000000	40.	32.	.800

```
Number of preparations:  2
Number of dose groups:  24
Do you want probits [Y] ? Is Natural Response a parameter [Y] ? Do you want the likelihood function to be maximized [Y] ? LD's to
calculate [10 50 90] > Do you want to specify starting values of the parameters [N] ?
The probit transformation is to be used
Natural Response is a parameter
The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is ( 1) 157-C
Estimating natural response

Maximum log-likelihood  -226.73861
```

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	parameter	standard error	t ratio
157-C	1.3745393	.16055818	8.5610049
NATURAL	.17675088E-01	.58870151E-02	3.0023854
SLOPE	86.322522	8.7915866	9.8187649

Variance-Covariance matrix

	157-C	NATURAL	SLOPE
157-C	.2577893E-01	.1428742E-03	1.209453
NATURAL	.1428742E-03	.3465695E-04	.1521257E-01
SLOPE	1.209453	.1521257E-01	77.29199

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
157-C	40.	0.	.707	-.707	.017675
	40.	2.	.904	1.096	.022593
	40.	1.	1.310	-.310	.032747
	40.	4.	2.276	1.724	.056909
	40.	5.	4.209	.791	.105236
	40.	7.	7.477	-.477	.186925
	40.	14.	12.169	1.831	.304223
	40.	10.	17.920	-7.920	.448007
	40.	24.	23.968	.032	.599192
	40.	30.	29.447	.553	.736180
	40.	35.	33.745	1.255	.843616
	40.	38.	36.674	1.326	.916859
NATURAL	479.	8.	8.466	-.466	.017675

chi-square 11.422 degrees of freedom 10 heterogeneity 1.1422

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.03892 g(.95)=.05882 g(.99)=.11900

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	157-C	.96400	lower	.95978	.95875	.95626
			upper	.96819	.96920	.97163
LD90	157-C	.99752	lower	.99077	.98947	.98677
			upper	1.00706	1.00987	1.01757

Intercepts and slopes unconstrained. Preparation is (2) SF41
Estimating natural response

Maximum log-likelihood -209.55138

	parameter	standard error	t ratio
SF41	1.1046974	.16100618	6.8612110
NATURAL	.22134769E-01	.56695920E-02	3.9041204
SLOPE	110.59393	12.265746	9.0164861

Variance-Covariance matrix

	SF41	NATURAL	SLOPE
SF41	.2592299E-01	.3914324E-04	1.610869
NATURAL	.3914324E-04	.3214427E-04	.1059598E-01
SLOPE	1.610869 .	1059598E-01	150.4485

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
SF41	40.	1.	.885	.115	.022135
	40.	0.	.887	-.887	.022172
	40.	0.	.897	-.897	.022435
	40.	0.	.958	-.958	.023958
	40.	2.	1.223	.777	.030579
	40.	2.	2.095	-.095	.052387
	40.	1.	4.292	-3.292	.107291
	40.	10.	8.552	1.448	.213796
	40.	17.	14.969	2.031	.374236
	40.	24.	22.533	1.467	.563323
	40.	31.	29.557	1.443	.738931
	40.	32.	34.733	-2.733	.868335
NATURAL	479.	13.	10.603	2.397	.022135

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chi-square 9.5923 degrees of freedom 10 heterogeneity .96

Index of significance for potency estimation:
g(.90)=.03328 g(.95)=.04725 g(.99)=.08161

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	SF41	.97726	lower	.97414	.97353	.97230
			upper	.98051	.98117	.98254
LD90	SF41	1.00369	lower	.99840	.99754	.99597
			upper	1.01097	1.01271	1.01658

Intercepts and slopes constrained (lines are the same)
Not estimating natural response

Maximum log-likelihood -449.35444

	parameter	standard error	t ratio
INTERCPT	1.1992357	.11049444	10.853358
SLOPE	93.510821	6.8016466	13.748262

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.1220902E-01	.6300366
SLOPE	.6300366	46.26240

Testing hypothesis that slopes and intercepts are the same
chi-square 26.1289 degrees of freedom 2 tail probability .000
Hypothesis REJECTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
INTERCPT	40.	0.	.707	-.707	.017675
	40.	2.	.748	1.252	.018693
	40.	1.	.874	.126	.021859
	40.	4.	1.272	2.728	.031793
	40.	5.	2.288	2.712	.057200
	40.	7.	4.420	2.580	.110495
	40.	14.	8.109	5.891	.202734
	40.	10.	13.409	-3.409	.335224
	40.	24.	19.762	4.238	.494047
	40.	30.	26.152	3.848	.653803
	40.	35.	31.574	3.426	.789342
	40.	38.	35.473	2.527	.886818
	40.	1.	.885	.115	.022135
	40.	0.	.926	-.926	.023148
	40.	0.	1.052	-1.052	.026299
	40.	0.	1.448	-1.448	.036188
	40.	2.	2.459	-.459	.061481
	40.	2.	4.581	-2.581	.114534
	40.	1.	8.254	-7.254	.206354
	40.	10.	13.530	-3.530	.338242
	40.	17.	19.854	-2.854	.496344
	40.	24.	26.215	-2.215	.655375
	40.	31.	31.612	-.612	.790298
	40.	32.	35.493	-3.493	.887332

chi-square 46.651 degrees of freedom 22 heterogeneity 2.1205

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.03308 g(.95)=.04825 g(.99)=.08914

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	INTERCPT	.97090	lower	.96729	.96653	.96487
			upper	.97465	.97548	.97732
LD90	INTERCPT	1.00203	lower	.99585	.99476	.99265
			upper	1.01055	1.01277	1.01812

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Slopes constrained (lines are parallel)
Not estimating natural response

Maximum log-likelihood -437.71462

	parameter	standard error	t ratio
157-C	1.5275841	.13704873	11.146284
SF41	.93853294	.12116207	7.7460956
SLOPE	95.982655	6.9842984	13.742634

Variance-Covariance matrix

	157-C	SF41	SLOPE
157-C	.1878235E-01	.8987063E-02	.7540917
SF41	.8987063E-02	.1468025E-01	.5813520
SLOPE	.7540917	.5813520	48.78042

Testing hypothesis that slopes are the same
chi-square 2.8492 degrees of freedom 1 tail probability .091
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
157-C	40.	0.	.707	-.707	.017675
	40.	2.	.789	1.211	.019728
	40.	1.	1.026	-.026	.025646
	40.	4.	1.717	2.283	.042919
	40.	5.	3.345	1.655	.083619
	40.	7.	6.462	.538	.161560
	40.	14.	11.345	2.655	.283634
	40.	10.	17.639	-7.639	.440964
	40.	24.	24.352	-.352	.608797
	40.	30.	30.313	-.313	.757824
	40.	35.	34.743	.257	.868572
	40.	38.	37.512	.488	.937811
SF41	40.	1.	.885	.115	.022135
	40.	0.	.896	-.896	.022405
	40.	0.	.939	-.939	.023486
	40.	0.	1.104	-1.104	.027598
	40.	2.	1.608	.392	.040190
	40.	2.	2.858	-.858	.071446
	40.	1.	5.389	-4.389	.134731
	40.	10.	9.596	.404	.239901
	40.	17.	15.368	1.632	.384204
	40.	24.	21.944	2.056	.548611
	40.	31.	28.200	2.800	.705002
	40.	32.	33.195	-1.195	.829866

chi-square 23.126 degrees of freedom 21 heterogeneity 1.1012 P=0.662303

A large chi-square indicates a poor fit of the data by the probit
analysis model. Large deviations for expected probabilities near 0 or 1
are especially troublesome. A plot of the data should be consulted.
See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.01727 g(.95)=.02522 g(.99)=.04674

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	157-C	.96402	lower	.96045	.95968	.95804
			upper	.96756	.96832	.96993
	SF41	.97774	lower	.97401	.97324	.97161
			upper	.98165	.98250	.98437
LD90	157-C	.99412	lower	.98926	.98834	.98652
			upper	1.00004	1.00146	1.00469
	SF41	1.00826	lower	1.00273	1.00170	.99965
			upper	1.01510	1.01674	1.02052

Relative potencies

	potency	limits	0.90	0.95	0.99
SF41	.98597	lower	.98057	.97939	.97684
		upper	.99119	.99228	.99460

aw
157-C subjects 480 controls 479
log(L)=-226.7 slope=86.323+-8.792 nat.resp.=.018+-.006

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```
    heterogeneity=1.14   g=.059
    LD50=.964   limits: .959 to .969
    LD90=.998   limits: .989 to 1.010
  SF41       subjects 480   controls 479
    log(L)=-209.6   slope=110.594+-12.266   nat.resp.=.022+-.006
    heterogeneity=.96   g=.047
    LD50=.977   limits: .974 to .981
    LD90=1.004   limits: .998 to 1.013
  SAME       subjects 960   controls 958
    log(L)=-449.4   slope=93.511+-6.802   nat.resp.=.022+-.000
    Hypothesis REJECTED
    heterogeneity=2.12   g=.048
    LD50=.971   limits: .967 to .975
    LD90=1.002   limits: .995 to 1.013
  PARALLEL   subjects 960   controls 958
    log(L)=-437.7   slope=95.983+-6.984   nat.resp.=.022+-.000
    Hypothesis ACCEPTED
    heterogeneity=1.10   g=.025
    LD50=.964   limits: .960 to .968
    LD90=.994   limits: .988 to 1.001

Stop - Program terminated.
```

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ATTACHMENT 4: NEMATODE SPECIES AND EXPOSURE TIME

POLO-PC
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Input file >
input: =Dose
input: *SF41
input: 0 60 3
input: 30 60 6
input: 60 60 10
input: 120 60 8
input: 240 60 16
input: 480 60 23
input: *157-C
input: 0 60 6
input: 30 60 6
input: 60 60 14
input: 120 60 21
input: 240 60 33
input: 480 60 32

preparation	dose	log-dose	subjects	responses	resp/subj
SF41	.00000	.000000	60.	3.	.050
	30.00000	1.477121	60.	6.	.100
	60.00000	1.778151	60.	10.	.167
	120.00000	2.079181	60.	8.	.133
	240.00000	2.380211	60.	16.	.267
	480.00000	2.681241	60.	23.	.383
157-C	.00000	.000000	60.	6.	.100
	30.00000	1.477121	60.	6.	.100
	60.00000	1.778151	60.	14.	.233
	120.00000	2.079181	60.	21.	.350
	240.00000	2.380211	60.	33.	.550
	480.00000	2.681241	60.	32.	.533

Number of preparations: 2
Number of dose groups: 10
Do you want probits [Y] ? Is Natural Response a parameter [Y] ? Do you want the likelihood function to be maximized [Y] ? LD's to calculate [10 50 90] > Do you want to specify starting values of the parameters [N] ?
The probit transformation is to be used
Natural Response is a parameter
The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is (1) SF41
Estimating natural response

Maximum log-likelihood -157.73426

	parameter	standard error	t ratio
SF41	-3.2613345	.91102029	-3.5798703
NATURAL	.54021375E-01	.30757593E-01	1.7563590
SLOPE	1.0516660	.36188049	2.9061141

Variance-Covariance matrix

	SF41	NATURAL	SLOPE
SF41	.8299580	-.1841782E-01	-.3256642
NATURAL	-.1841782E-01	.9460296E-03	.6464328E-02
SLOPE	-.3256642	.6464328E-02	.1309575

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
SF41	60.	6.	5.729	.271	.095482
	60.	10.	7.899	2.101	.131653
	60.	8.	11.258	-3.258	.187639
	60.	16.	15.966	.034	.266092
	60.	23.	21.938	1.062	.365629
NATURAL	60.	3.	3.241	-.241	.054021

chi-square 1.9186 degrees of freedom 3 heterogeneity .64

Index of significance for potency estimation:
g(.90)=.32035 g(.95)=.45485 g(.99)=.78561

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"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	SF41	1262.2	lower	649.66	593.81	
			upper	9303.8	28426.	
LD90	SF41	20879.	lower	4302.5	3575.9	
			upper	.54148E+07	.13953E+09	

Intercepts and slopes unconstrained. Preparation is (2) 157-C
Estimating natural response

Maximum log-likelihood -195.74749

	parameter	standard error	t ratio
157-C	-3.4054967	.60541367	-5.6250741
NATURAL	.84878216E-01	.31478095E-01	2.6964216
SLOPE	1.3373221	.25514219	5.2414778

Variance-Covariance matrix

	157-C	NATURAL	SLOPE
157-C	.3665257	-.8006957E-02	-.1516046
NATURAL	-.8006957E-02	.9908705E-03	.2564467E-02
SLOPE	-.1516046	.2564467E-02	.6509754E-01

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
157-C	60.	6.	9.284	-3.284	.154741
	60.	14.	13.443	.557	.224054
	60.	21.	19.698	1.302	.328299
	60.	33.	27.715	5.285	.461914
	60.	32.	36.472	-4.472	.607867
NATURAL	60.	6.	5.093	.907	.084878

chi-square 4.9805 degrees of freedom 3 heterogeneity 1.6602

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.33468 g(.95)=.61203 g(.99)=2.0616

"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	157-C	351.96903	lower	191.46603		
			upper	1071.17572		
LD90	157-C	3197.4	lower	1057.3		
			upper	.14739E+06		

Intercepts and slopes constrained (lines are the same)
Not estimating natural response

Maximum log-likelihood -360.21435

	parameter	standard error	t ratio
INTERCPT	-3.3012504	.42301875	-7.8040285
SLOPE	1.1817570	.18399130	6.4228957

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.1789449	-.7675935E-01
SLOPE	-.7675935E-01	.3385280E-01

Testing hypothesis that slopes and intercepts are the same

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chi-square 13.4652 degrees of freedom 2 tail probability .001
Hypothesis REJECTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
INTERCPT					
	60.	6.	6.641	-.641	.110681
	60.	10.	9.774	.226	.162892
	60.	8.	14.553	-6.553	.242545
	60.	16.	20.986	-4.986	.349759
	60.	23.	28.625	-5.625	.477087
	60.	6.	8.381	-2.381	.139690
	60.	14.	11.412	2.588	.190197
	60.	21.	16.035	4.965	.267252
	60.	33.	22.258	10.742	.370970
	60.	32.	29.649	2.351	.494144

chi-square 20.126 degrees of freedom 8 heterogeneity 2.5157

A large chi-square indicates a poor fit of the data by the probit
analysis model. Large deviations for expected probabilities near 0 or 1
are especially troublesome. A plot of the data should be consulted.
See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.21087 g(.95)=.32428 g(.99)=.68657

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	INTERCPT	621.59916	lower	371.75670	339.03651	
			upper	1985.69018	3644.99274	
LD90	INTERCPT	7550.2	lower	2242.5	1854.7	
			upper	.18446E+06	.10800E+07	

Slopes constrained (lines are parallel)
Not estimating natural response

Maximum log-likelihood -353.75224

	parameter	standard error	t ratio
SF41	-3.6827227	.46462760	-7.9261814
157-C	-3.1722443	.43615567	-7.2731928
SLOPE	1.2327198	.19188434	6.4242856

Variance-Covariance matrix

	SF41	157-C	SLOPE
SF41	.2158788	.1924159	-.8663002E-01
157-C	.1924159	.1902318	-.8178085E-01
SLOPE	-.8663002E-01	-.8178085E-01	.3681960E-01

Testing hypothesis that slopes are the same
chi-square .5410 degrees of freedom 1 tail probability .462
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
SF41					
	60.	6.	5.019	.981	.083642
	60.	10.	7.102	2.898	.118359
	60.	8.	10.701	-2.701	.178348
	60.	16.	16.128	-.128	.268808
	60.	23.	23.271	-.271	.387857
157-C	60.	6.	9.940	-3.940	.165673
	60.	14.	14.069	-.069	.234476
	60.	21.	19.983	1.017	.333057
	60.	33.	27.379	5.621	.456324
	60.	32.	35.451	-3.451	.590843

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chi-square 7.2807 degrees of freedom 7 heterogeneity 1.0401 P=0.99

A large chi-square indicates a poor fit of the data by the probit analysis model. Large deviations for expected probabilities near 0 or 1 are especially troublesome. A plot of the data should be consulted. See D. J. Finney, "Probit Analysis" (1972), pages 70-75.

Index of significance for potency estimation:
g(.90)=.09046 g(.95)=.14091 g(.99)=.30863

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	SF41	971.57758	lower	594.05448	537.26555	429.97635
			upper	2125.04554	2837.69276	8093.28771
	157-C	374.42953	lower	261.01870	241.13863	200.17508
			upper	631.15261	758.62785	1444.82123
LD90	SF41	10644.	lower	4086.0	3422.2	2376.2
			upper	59691.	.11725E+06	.14896E+07
	157-C	4101.9	lower	1847.6	1593.8	1175.7
			upper	17227.	30209.	.25022E+06

Relative potencies

	potency	limits	0.90	0.95	0.99
157-C	2.59482	lower	1.54269	1.35847	.97920
		upper	4.96718	6.13490	12.28782

Dose
SF41 subjects 300 controls 60
log(L)=-157.7 slope=1.052+-.362 nat.resp.=.054+-.031
heterogeneity=.64 g=.455
LD50=1262.154 limits: 593.808 to 28425.877
LD90=20878.739 limits: 3575.863 to 139530209.327
157-C subjects 300 controls 60
log(L)=-195.7 slope=1.337+-.255 nat.resp.=.085+-.031
heterogeneity=1.66 g=.612
SAME subjects 600 controls 120
log(L)=-360.2 slope=1.182+-.184 nat.resp.=.075+-.000
Hypothesis REJECTED
heterogeneity=2.52 g=.324
LD50=621.599 limits: 339.037 to 3644.993
LD90=7550.164 limits: 1854.661 to 1080018.048
PARALLEL subjects 600 controls 120
log(L)=-353.8 slope=1.233+-.192 nat.resp.=.075+-.000
Hypothesis ACCEPTED
heterogeneity=1.04 g=.141
LD50=971.578 limits: 537.266 to 2837.693
LD90=10643.648 limits: 3422.240 to 117251.097

Stop - Program terminated.

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ATTACHMENT 5: EXPOSURE TIME AND TEMPERATURE

POLO-PC
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Input file >
input: =temp
input: *25
input: 0 60 3
input: 30 60 6
input: 60 60 10
input: 120 60 8
input: 240 60 16
input: 480 60 23
input: *20
input: 0 60 2
input: 30 60 5
input: 60 60 6
input: 120 60 9
input: 240 60 14
input: 480 60 23
input: *15
input: 0 60 6
input: 30 60 5
input: 60 60 5
input: 120 60 11
input: 240 60 12
input: 480 60 14

preparation	dose	log-dose	subjects	responses	resp/subj
25	.00000	.000000	60.	3.	.050
	30.00000	1.477121	60.	6.	.100
	60.00000	1.778151	60.	10.	.167
	120.00000	2.079181	60.	8.	.133
	240.00000	2.380211	60.	16.	.267
	480.00000	2.681241	60.	23.	.383
20	.00000	.000000	60.	2.	.033
	30.00000	1.477121	60.	5.	.083
	60.00000	1.778151	60.	6.	.100
	120.00000	2.079181	60.	9.	.150
	240.00000	2.380211	60.	14.	.233
	480.00000	2.681241	60.	23.	.383
15	.00000	.000000	60.	6.	.100
	30.00000	1.477121	60.	5.	.083
	60.00000	1.778151	60.	5.	.083
	120.00000	2.079181	60.	11.	.183
	240.00000	2.380211	60.	12.	.200
	480.00000	2.681241	60.	14.	.233

Number of preparations: 3
Number of dose groups: 15
Do you want probits [Y] ? Is Natural Response a parameter [Y] ? Do you want the likelihood function to be maximized [Y] ? LD's to calculate [10 50 90] > Do you want to specify starting values of the parameters [N] ?
The probit transformation is to be used
Natural Response is a parameter
The parameters are to be estimated by maximizing the likelihood function

Intercepts and slopes unconstrained. Preparation is (1) 25
Estimating natural response

Maximum log-likelihood -157.73426

	parameter	standard error	t ratio
25	-3.2613345	.91102029	-3.5798703
NATURAL	.54021375E-01	.30757593E-01	1.7563590
SLOPE	1.0516660	.36188049	2.9061141

Variance-Covariance matrix

	25	NATURAL	SLOPE
25	.8299580	-.1841782E-01	-.3256642
NATURAL	-.1841782E-01	.9460296E-03	.6464328E-02
SLOPE	-.3256642	.6464328E-02	.1309575

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Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
25	60.	6.	5.729	.271	.095482
	60.	10.	7.899	2.101	.131653
	60.	8.	11.258	-3.258	.187639
	60.	16.	15.966	.034	.266092
	60.	23.	21.938	1.062	.365629
NATURAL	60.	3.	3.241	-.241	.054021

chi-square 1.9186 degrees of freedom 3 heterogeneity .64

Index of significance for potency estimation:
g(.90)=.32035 g(.95)=.45485 g(.99)=.78561

"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	25	1262.2	lower	649.66	593.81	
			upper	9303.8	28426.	
LD90	25	20879.	lower	4302.5	3575.9	
			upper	.54148E+07	.13953E+09	

Intercepts and slopes unconstrained. Preparation is (2) 20
Estimating natural response

Maximum log-likelihood -143.61504

	parameter	standard error	t ratio
20	-3.6441135	.96760292	-3.7661249
NATURAL	.37927993E-01	.26238542E-01	1.4455069
SLOPE	1.2059273	.38517831	3.1308288

Variance-Covariance matrix

	20	NATURAL	SLOPE
20	.9362554	-.1707699E-01	-.3691482
NATURAL	-.1707699E-01	.6884611E-03	.6183794E-02
SLOPE	-.3691482	.6183794E-02	.1483623

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
20	60.	5.	4.079	.921	.067987
	60.	6.	6.134	-.134	.102227
	60.	9.	9.654	-.654	.160897
	60.	14.	14.948	-.948	.249140
	60.	23.	21.939	1.061	.365643
NATURAL	60.	2.	2.276	-.276	.037928

chi-square .4748 degrees of freedom 3 heterogeneity .16

Index of significance for potency estimation:
g(.90)=.27602 g(.95)=.39190 g(.99)=.67689

"With almost all good sets of data, g will be substantially smaller than 1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

We will use only the probabilities for which g is less than 0.5

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	20	1051.56235	lower	606.75632	562.54142	
			upper	4651.42368	9580.79329	
LD90	20	12149.	lower	3274.8	2796.6	
			upper	.74328E+06	.60273E+07	

Intercepts and slopes unconstrained. Preparation is (3) 15
Estimating natural response

Maximum log-likelihood -146.09095

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	parameter	standard error	t ratio
15	-3.6920337	1.2984044	-2.8435159
NATURAL	.85652172E-01	.28557461E- 01	2.9992923
SLOPE	1.0385861	.50414203	2.0601061

Variance-Covariance matrix

	15	NATURAL	SLOPE
15	1.685854	-.2021814E-01	-.6454223
NATURAL	-.2021814E-01	.8155286E-03	.6466465E-02
SLOPE	-.6454223	.6466465E-02	.2541592

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
15	60.	5.	5.988	-.988	.099795
	60.	5.	6.922	-1.922	.115368
	60.	11.	8.578	2.422	.142967
	60.	12.	11.242	.758	.187361
	60.	14.	15.130	-1.130	.252169
NATURAL	60.	6.	5.139	.861	.085652

chi-square 1.9157 degrees of freedom 3 heterogeneity .64

Index of significance for potency estimation:
g(.90)=.63749 g(.95)=.90514 g(.99)=1.5633

"With almost all good sets of data, g will be substantially smaller than
1.0, and seldom greater than 0.4."
- D. J. Finney, "Probit Analysis" (1972), page 79.

Effective Doses

	dose	limits	0.90	0.95	0.99
LD50 15	3588.10742				
LD90 15	61490.				

Intercepts and slopes constrained (lines are the same)
Not estimating natural response

Maximum log-likelihood -451.54449

	parameter	standard error	t ratio
INTERCPT	-3.4791330	.44702945	-7.7827825
SLOPE	1.0910916	.18944917	5.7592840

Variance-Covariance matrix

	INTERCPT	SLOPE
INTERCPT	.1998353	-.8375724E-01
SLOPE	-.8375724E-01	.3589099E-01

Testing hypothesis that slopes and intercepts are the same
chi-square 8.2085 degrees of freedom 4 tail probability .084
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
INTERCPT	60.	6.	4.996	1.004	.083270
	60.	10.	6.755	3.245	.112579
	60.	8.	9.657	-1.657	.160948
	60.	16.	13.961	2.039	.232679
	60.	23.	19.696	3.304	.328268
	60.	5.	4.060	.940	.067674
	60.	6.	5.849	.151	.097481
	60.	9.	8.800	.200	.146674
	60.	14.	13.178	.822	.219625
	60.	23.	19.010	3.990	.316840
	60.	5.	6.835	-1.835	.113923
	60.	5.	8.535	-3.535	.142251
	60.	11.	11.340	-.340	.189004
	60.	12.	15.500	-3.500	.258336
	60.	14.	21.044	-7.044	.350729

chi-square 12.0359 degrees of freedom 13 heterogeneity .93

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Index of significance for potency estimation:
g(.90)=.08157 g(.95)=.11581 g(.99)=.20003

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	INTERCPT	1544.08714	lower	953.00426	887.47187	782.63911
			upper	3549.11075	4499.60296	8154.03219
LD90	INTERCPT	23080.	lower	8013.7	6882.7	5280.5
			upper	.15245E+06	.26326E+06	.10442E+07

Slopes constrained (lines are parallel)
Not estimating natural response

Maximum log-likelihood -447.52290

	parameter	standard error	t ratio
25	-3.4059262	.45863725	-7.4261874
20	-3.4320159	.45449521	-7.5512698
15	-3.8738596	.49082141	-7.8926051
SLOPE	1.1142345	.19220919	5.7969886

Variance-Covariance matrix

	25	20	15	SLOPE
25	.2103481	.1972520	.2070301	-.8569445E-01
20	.1972520	.2065659	.2054460	-.8503879E-01
15	.2070301	.2054460	.2409057	-.8925427E-01
SLOPE	-.8569445E-01	-.8503879E-01	-.8925427E-01	.3694437E-01

Testing hypothesis that slopes are the same
chi-square .1653 degrees of freedom 2 tail probability .921
Hypothesis ACCEPTED

Chi-squared goodness of fit test

preparation	subjects	responses	expected	deviation	probability
25	60.	6.	5.466	.534	.091102
	60.	10.	7.619	2.381	.126984
	60.	8.	11.075	-3.075	.184591
	60.	16.	16.039	-.039	.267321
	60.	23.	22.416	.584	.373601
20	60.	5.	4.414	.586	.073560
	60.	6.	6.514	-.514	.108569
	60.	9.	9.916	-.916	.165265
	60.	14.	14.844	-.844	.247394
	60.	23.	21.229	1.771	.353821
15	60.	5.	5.849	-.849	.097484
	60.	5.	6.741	-1.741	.112357
	60.	11.	8.415	2.585	.140253
	60.	12.	11.223	.777	.187055
	60.	14.	15.438	-1.438	.257294

chi-square 4.3178 degrees of freedom 11 heterogeneity .39 P=0,04

Index of significance for potency estimation:
g(.90)=.08051 g(.95)=.11431 g(.99)=.19744

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	25	1139.56967	lower	691.65936	639.74833	555.23388
			upper	2527.46304	3146.03577	5391.56285
	20	1202.69595	lower	723.79912	669.13970	580.66069
			upper	2735.25139	3433.29585	6016.59402
	15	2997.1	lower	1448.3	1292.2	1050.2
			upper	9512.9	13058.	28449.
LD90	25	16103.	lower	5843.2	5040.6	3890.7
			upper	94987.	.15795E+06	.56545E+06
	20	16995.	lower	6065.8	5221.4	4016.0
			upper	.10363E+06	.17404E+06	.63930E+06
	15	42351.	lower	12463.	10410.	7581.6
			upper	.35099E+06	.64122E+06	.28959E+07

Relative potencies

	potency	limits	0.90	0.95	0.99
20	.94751	lower	.55237	.49102	.37983
		upper	1.59857	1.78420	2.25594

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15            .38022   lower      .17368      .14415      .09412
                       upper      .73055      .82746     1.06463



 temp
   25        subjects 300   controls 60
    log(L)=-157.7   slope=1.052+-.362   nat.resp.=.054+-.031
    heterogeneity=.64   g=.455
    LD50=1262.154   limits: 593.808 to 28425.877
    LD90=20878.739   limits: 3575.863 to 139530209.327
   20         subjects 300   controls 60
    log(L)=-143.6   slope=1.206+-.385   nat.resp.=.038+-.026
    heterogeneity=.16   g=.392
    LD50=1051.562   limits: 562.541 to 9580.793
    LD90=12149.138   limits: 2796.585 to 6027302.576
   15         subjects 300   controls 60
    log(L)=-146.1   slope=1.039+-.504   nat.resp.=.086+-.029
    heterogeneity=.64   g=.905
   SAME       subjects 900   controls 180
    log(L)=-451.5   slope=1.091+-.189   nat.resp.=.061+-.000
    Hypothesis ACCEPTED
    heterogeneity=.93   g=.116
    LD50=1544.087   limits: 887.472 to 4499.603
    LD90=23079.740   limits: 6882.663 to 263260.249
   PARALLEL   subjects 900   controls 180
    log(L)=-447.5   slope=1.114+-.192   nat.resp.=.061+-.000
    Hypothesis ACCEPTED
    heterogeneity=.39   g=.114
    LD50=1139.570   limits: 639.748 to 3146.036
    LD90=16102.901   limits: 5040.645 to 157945.094

Stop - Program terminated.
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