Efficacy of entomopathogenic nematodes and fungi as biological control agents of woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) under South African conditions

by

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Declaration

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Abstract

The woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) is an important pest of apples, *(Malus domestica* Borkh.). Severe infestations by WAA can lead to colonies near spurs that can deposit honeydew on fruit, which serve as a substrate for sooty mould. If not controlled, it can lead to destruction of developing buds in the leaf axils and a reduction in tree vigour or formation of galls. Increasing interest in environmentally sustainable farming has increased the demand for environmentally friendly pest control methods. Therefore the use of naturally occurring biocontrol agents and more environmental friendly methods are needed. The study aims to determine the potential of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) to control WAA.

EPNs and EPF are naturally occurring and environmentally friendly microbials that have the potential to be developed into bio-pesticides. Research on the use of EPNs and EPF as biocontrol agents against *E. lanigerum* showed that they have potential to be used to control WAA. This is the first report on the use of EPNs and EPF to control WAA in South Africa. These findings are significant, especially to those interested in integrated pest management (IPM).

The first objective of this study was to evaluate the virulence of endemic EPN species against WAA by conducting laboratory bioassays and to determine the effect of imidacloprid on the infectivity of EPNs. A total of seven entomopathogenic nematode isolates were evaluated for their potential as biological control agents for WAA in the laboratory, using a 24-well bioassay protocol screening method. From these, the two most virulent nematode isolates were selected. In all cases studied the degree of infection associated with mortality was less than 50% and the other tested nematode isolates were less successful. Although penetration, recovery of the non-feeding Infective Juveniles (IJ) to the feeding stage and development into the adult stage was observed in some cases, the nematodes failed to complete their life cycle inside the WAA.

When this failure to develop and reproduce was investigated using direct screening with the associated symbiotic bacteria and insect extract, it became evident that the WAA haemolymph contains an inhibitory factor that prevented the symbiotic EPN bacteria from growing. WAA size was also shown to have an effect on insect mortality, with higher mortality observed in adults and low mortality for intermediates, while no mortality was observed for the crawlers. The two most virulent nematode isolates, Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 and *Heterorhabditis zealandica* Poinar, 1990 were not affected by the addition of imidacloprid, leading to the conclusion that EPNs and imidacloprid could potentially be applied as tank mix.

The second objective of the study was to evaluate the use of two EPF, *Beauveria bassiana* and *Metarhizium anisopliae*, for the control of WAA in the laboratory. The five fungal isolates tested were pathogenic to WAA and they caused significant WAA mortality compared with the control. The commercial isolates *Beauveria bassiana* (Eco- Bb strain R444*)* and *Metarhizium anisopliae* (ICIPE 69) caused the highest rate of WAA mortality and were selected for further evaluation. When the effect of EPF concentration and exposure time of these two most virulent isolates were evaluated, it became evident that increased EPF concentration (1×10^7 to 1×10^{10} conidia ml^{-1}) and exposure time increased WAA mortality. This lead to the conclusion that a relatively long incubation period and high EPF concentration are needed to achieve full efficacy of the these fungal isolates for controlling WAA.

The third objective of the study was to evaluate the interaction between EPNs and EPF for the control of WAA and the effect of mulching on EPN efficacy in pot trials. Joint use of EPNs and EPF increased insect mortality when compared to treatments with only EPNs or only EPF, indicating an antagonistic effect. Combining *S. yirgalamense* with *B. bassiana* (Eco- Bb® strain R444*)* and *M. anisopliae* (ICIPE 69) provided no advantage for improved efficacy against WAA, which lead to the conclusion that *M. anisopliae* (ICIPE 69) or *B. bassiana* (Eco- Bb® strain R444) could be used for suppression of WAA. When the

environment was manipulated by adding mulches, WAA mortality was slightly higher compared to the unmulched soil. However, these differences were not statistically significant.

The fourth objective of the study was to evaluate the efficacy of *S. yirgalamense*, *B. bassiana* and *M. anisopliae* against WAA in the field. A single application of *S. yirgalamense* (80 IJs/cm^2) *B. bassiana* (Eco- Bb® strain R444*)* (1g/L) and *M. anisopliae* (ICIPE 69) (200 ml/ha) effectively controlled WAA under South African field conditions. The EPF performed better than EPNs. Based on these results the success in controlling the root colonies resulted in greatly reduced infestations of the aerial parts of the apple trees in the trial.

These studies attempted to determine if local EPNs and EPF are pathogenic to WAA and if they could be used to control it in the field. It was found that all the isolates tested were pathogenic to WAA in the laboratory and that *S. yirgalamense*, *B. bassiana and M. anisopliae* were the most effective isolates. They were able to control WAA under field conditions, although the degree of control was low. Therefore future studies should be designed to investigate the application and post-application conditions required for EPF to be effective with respect to WAA control and to ensure that the application of the fungi is economically viable. The role of the wetting agent in improving soil saturation, penetration and uptake of EPNs and EPF also requires to be investigated.

Opsomming

Appelbloedluis, *Eriosoma lanigerum*, is ’n belangrike pes van appels (*Malus domestica* Borkh.). Swaar besmettings van appelbloedluis lei tot die vorming van kolonies op lootaansluitings. Dit veroorsaak dat heuningdou die vrugte besmet wat bydra tot die groei van poeieragtige meeldou. Indien appelbloedluis nie beheer word nie, kan infestasies lei tot die vernietiging van oksellêre knoppe en ’n afname in boomgroeikrag deur die vorming van voeding galle op die wortels. ’n Toename in belangstelling in volhoubare omgewingsvriendelike boerderypraktyke het gelei tot ’n toename in die behoefte vir volhoubare omgewingsvriendelike beheer metodes. Gevolglik word meer sisteme wat natuurlik voorkom en omgewingsvriendelik is, benodig. Die doel van hierdie studie was om die potensiële bydrae van entomopatogeniese nematodes (EPNs) en entomopatogeniese fungi (EPF) tot die beheer van appelbloedluis te bepaal.

EPNs en EPF kom natuurlik in grond voor en is omgewingsvriendelike mikrobes wat oor die potensiaal beskik om as biologiese insekbeheermiddels ontwikkel te word. Navorsing op die gebruik van EPNs en EPP as biologiese beheeragente het bewys dat hulle potensiaal toon vir die beheer van appelbloedluis. Hierdie is die eerste verslag rakende die gebruik van EPNs en EPF vir die beheer van appelbloedluis in Suid-Afrika. Die bevindinge van die studie is van belang, veral vir diegene wat in geïntegreerde plaagbeheer (IPB) belang stel.

Die eerste doelwit van die studie was om die virulensie van endemiese EPN spesies teen appelbloedluis deur middel van laboratoriumtoetse te evalueer en om die uitwerking van imidaclopried op die infestasievermoë van die nematodes te bepaal. ’n Totaal van sewe EPN isolate is geëvalueer deur middel van ’n laboratorium siftings metode wat hulle potensiaal as biologiese beheeragente teen appelbloedluis toets. Hieruit is die twee mees virulente isolate geselekteer. Die graad van infestasie gekoppel aan mortaliteit, was in alle gevalle minder as 50%. Alhoewel dit waargeneem is dat die nematodes, nadat hulle die appelbloedluis penetreer het, wel ontwikkeling het tot volwasse stadia, maar daarna almal

dood gegaan het. Verdere ondersoek gedoen deur middel van direkte-toetsing met bakterieë en insekekstrak, het getoon dat 'n onderdrukkende faktor in appelbloedluis haemolymph die EPF bakterieë verhoed het om te groei. Daar is ook bewys dat die grootte en die ontwikkeling stadium van die appelbloedluis 'n effek op insekmortaliteit het. Hoër mortaliteit is in die volwasse stadium, laer mortaliteit in die intermediêre stadiums en geen mortaliteit het onder die kruipers voorgekom nie. Die twee mees virulente nematode isolate, *Steinernema yirgalamense* en *Heterorhabditis zealandica* was nie geaffekteer deur die byvoeging van imidaclopried nie, wat beteken dat EPN en imidakloried potensieel as tenkmengsels toegedien kan word.

Die tweede doelwit van hierdie studie was om die gebruik van twee EPF, *Beauveria bassiana* en *Metarhizium anisopliae,* te evalueer vir die beheer van appelbloedluis in die laboratorium. Die vyf getoetsde fungi isolate was patogenies teenoor appelbloedluis en het betekenisvolle mortaliteit veroorsaak. Die kommersiële isolate *B. bassiana* (Eco-Bb isolaat R444*)* en *M. anisopliae* (ICIPE 69), het die hoogste appelbloedluis mortaliteit veroorsaak en is geselekteer vir verdere evaluering. Laboratoriumtoetse het bewys dat 'n verhoging in EPF konsentrasie, sowel as die periode van blootstelling, 'n verhoging in appelbloedluis mortaliteit teweeg gebring het. 'n Relatiewe lang inkubasieperiode van, en hoë EPF konsentrasie is dus nodig om doeltreffende beheer van appelbloedluis te verkry.

Die derde doelwit van die studie was om die interaksie tussen EPNs en EPF vir die beheer van appelbloedluis, sowel as die effek van 'n deklaag op EPN effektiwiteit in potproewe te bepaal. Gesamentlike toediening van *S. yirgalamense* met *B. bassiana* (Eco-Bb® isolaat R444) en *M. anisopliae* (ICIPE 69) het nie effektiwiteit teenoor appelbloedluis verhoog nie, en die gevolgtrekking is gemaak dat slegs *M. anisopliae* (ICIPE 69) of *B. bassiana* (Eco-Bb® isolaat R444) aangewend kan word vir die onderdrukking van appelbloedluis. Manipulasie van die omgewing, deur die gebruik van deklae, het die mortaliteit van appelbloedluis effens verhoog in vergelyking met grond sonder deklae, maar die verskille waargeneem was nie statisties betekenisvol nie.

Die vierde doelwit van die studie was om die effektiwiteit van *S. yirgalamense*, *B. bassiana* en *M. anisopliae* teenoor appelbloedluis in veldproewe te evalueer. ’n Enkel toediening van *S. yirgalamense*, *B. bassiana* (Eco-Bb® isolaat R444*)* en *M. anisopliae* (ICIPE 69) het appelbloedluis effektief onder Suid-Afrikaanse toestande beheer. Die EPF isolate het beter gevaar as die EPNs. Hierdie studie het gewys dat suksesvolle beheer van appelbloedluis se wortelkolonies grootliks kan bygedra het tot ’n verlaging in die voorkoms van bogrondse kolonies op die appelbome.

Bogenoemde studies het gepoog om te bepaal of plaaslike EPNs en EPF patogenies is teenoor appelbloedluis en of dit gebruik kan word vir die beheer van appelbloedluis in die veld. Dit is bevind dat getoetse isolate wel patogenies was in die laboratorium teenoor appelbloedluis met *S. yirgalamense*, *B. bassiana en M. anisopliae* as die mees effektiewe isolate. Alhoewel beheer van appelbloedluis onder veldtoestande waargeneem is, was die graad van beheer relatief laag. Metodes van toediening en toedieningstoestande mag egter die resultaat beïnvloed het. Verdere studies is nodig om die toediening en na-toedieningstoestande benodig deur EFF met betrekking tot appelbloedluis beheer, te ondersoek en om te verseker dat die toediening van die fungi ekonomiese volhoubaar is. Die rol van ’n benattingsagent ter bevordering van grondversadiging en grond deurdringbaarheid, verdien verdere ondersoek. Van kern belang is egter die verdere soektog na meer virulente isolate van beide EPN en EPF as potensiële toekomstige kandidate vir die gebruik as biologiese beheer agente.

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Dedication

This dissertation is dedicated to my son, Avethandwa, with the hope that he will one day realise that education is a “weapon” to fight ignorance and poverty and a key to open doors for success.

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Literature Review

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Introduction

Apples (*Malus domestica* Borkh.) have been grown in South Africa since 1652, when Jan van Riebeeck established the first plantings of apple trees. It is currently one of the most important deciduous fruits grown in South Africa. The main apple-growing areas are in the Western Cape province, including the areas Groenland, Ceres and Villiersdorp, as well as the Langkloof in the Eastern Cape province (Meyer & Breitenbach, 2004). The apple industry, in addition to its contribution to revenue generation, also provides permanent employment and seasonal labour for harvesting and packing on a contractual basis.

The woolly apple aphid

Woolly apple aphid (WAA), *Eriosoma lanigerum* is an important pest of apples in the Western Cape province of South Africa, and in other apple-growing areas of the world. Of North American origin, (WAA) was distributed on nursery material virtually worldwide (Baker 1915; Schoene & Underhill, 1935), with it first being described by Friedrich (Hausmann) in 1802 (Baker, 1915). This insect is now regarded as a pest wherever apple is grown commercially (Walker *et al*., 1988). In South Africa, WAA was first reported by Pillans in 1894 as American blight (*Schizoneura lanigera*) or plant louse of apple. However, at the time it was already established in South Africa. Fuller, in 1904, pointed out that WAA, which was to be found throughout the country, was the worst of all the pests attacking apples at the

time. Locally, it is currently rarely found on other host plants, although it has been recorded on peach (*Prunus domestica* L.) and pear (*Pyrus communis* L.) (Myburgh *et al*., 1973; Millar, 1994).

Biology

The aphid forms densely packed colonies covered with white, waxy, filamentous secretions on the above-ground parts and on the roots of apple trees (Shaw & Walker, 1996). Favourite feeding sites are new growth shoots, branches, pruning and other wounds, leaf axils and the roots of apple trees (Mueller *et al*., 1992). Their feeding activity causes galls to form on the woody tissue, which can lead to the destruction of young lateral shoots and buds. The galls, which are ideal feeding sites for aphid offspring, frequently give rise to the formation of densely packed colonies (Heunis & Pringle, 2006). The calyces of apples may also be infested by aphid colonies, resulting in contamination with honeydew and sooty mould (Shaw & Walker, 1996).

In South Africa and in other parts of the world, in the absence of American elm, the life cycle of WAA is restricted to apple trees and is anholocyclic. The insect is active on apple tree roots throughout the year (Damavandian & Pringle, 2007). The root colonies are usually the source of above-ground infestation. In spring and summer a portion of the crawlers (first-instar nymphs) from the root populations migrate up the trunks of the trees, remaining in large numbers on the above-ground parts of the tree until autumn (Schoene & Underhill, 1935; Hoyt & Madsen, 1960; Heunis & Pringle, 2006; Damavandian & Pringle, 2007). There are two peaks of activity in subterranean WAA populations, one during early summer and one during autumn (Damavandian & Pringle, 2007). All developmental stages were recorded on the roots of apple trees throughout the year. Above the ground, aphids settle in leaf axils, or in injured bark and pruning wounds where they form typical white, woolly colonies and complete their development. There are four nymphal stages. In the Western Cape province subterranean WAA populations have approximately 18 generations per year (Damavandian & Pringle, 2007). Alate (winged) females are produced exclusively

during autumn, possibly as a response to overcrowding, but such forms do not give rise to the sexual wingless males and females (Heunis, 2001). When populations are high there is general, random movement of crawlers, some of which may be downward. There is not downward migration, which is directional (Heunis & Pringle, 2006).

Symptoms

Eriosoma lanigerum can be a mere nuisance, a detriment to apple production or a threat to the tree's survival, depending on the level of infestation and on where the colonies are located. In the field the presence of the aphid is distinguished by the production of white, woolly masses that consist of a wax covering secreted by the epithelial cells. Trees can be infested simultaneously with arboreal and edaphic colonies of WAA (Pringle & Heunis, 2001; Walsh & Riley, 1869; Baker, 1915; Pescott, 1935; Lal & Singh, 1946).

Above-ground damage by WAA includes the destruction of developing buds in the leaf axils and a reduction in tree vigour due to aphid feeding in leaf axils (Annecke & Moran, 1982; Pringle & Heunis, 2006; Pringle *et al*., 2015). In severe infestations colonies near spurs can deposit honeydew on fruit, which serve as a substrate for sooty mould. Historically, nursery trees and newly planted trees are at higher risk than are older trees, with tree mortality involved sometimes being substantial (Sherbakoff & McClintock, 1935). The insect can directly infest fruit by entering the core through the calyx (Pringle & Heunis, 2001; Essig, 1942; Madsen *et al*., 1954), with cultivars with an open calyx being particularly susceptible to such infestation. The presence of insects either in or on the fruit can present a problem for the phytosanitary protocols for import/export to some countries.

Feeding by WAA on roots causes the formation of galls (Brown *et al*., 1991). Root galls, which can develop around colonies in as short a period of time as four weeks, tend to enlarge over a number of years (Weber & Brown, 1988). Root damage also weakens the tree, negatively affecting tree health (Welty & Murphy, 2000). Subterranean colonies often remain undetected and uncontrolled due to their cryptic habit. They can cause significant

damage to a tree before above-ground symptoms are noticed. However, despite the importance of the damage caused by root-feeding WAA, such damage has been more difficult to document and quantify than has damage caused by above-ground populations, because of the difficulties that have been encountered in sampling subterranean aphids.

Management of WAA

Host plant resistance

The number of trees in an orchard infested with WAA tends to increase as the trees age (Brown, 1986). However, not all cultivars are likely to be equally infested, with some being only lightly attacked, whereas a very few are resistant to such infestation (Cummins *et al*., 1981). An important horticultural consideration in the selection of an apple rootstock is its susceptibility to WAA (Cummins, 1971; Robinson, 2003; Weibel & Häseli, 2003). The Malling-Merton series of rootstocks, parented with the apple variety Northern Spy in the breeding line (Pescott, 1935; Knight *et al*., 1962), was bred for resistance to WAA (Sen Gupta & Miles, 1975), with it still being the main control strategy that is recommended for use on edaphic populations. The rootstocks concerned may owe their resistance to a very high ratio of phenolics to α-amino nitrogen, thus making the nitrogen source unavailable to aphids (Sen Gupta & Miles, 1975). Apple germplasm is suspected to contain many different types of resistance (Sandanayaka *et al*., 2005), with some genes having already been identified and characterised (Tobutt *et al*., 2000). Some scion accessions are also resistant to WAA, with perhaps more than one resistance mechanism being involved. Many accessions slow down the growth and the reproductive rate of the aphid, which may allow *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae) to provide better biological control than it otherwise could (Sandanayaka *et al*., 2005).

Eriosoma lanigerum biotypes have, however, been found to infest resistant rootstocks in North Carolina (Dozier *et al*., 1974; Rock & Zeiger, 1974; Young *et al*., 1982; Bai *et al*., 2004), South Africa (Giliomee *et al*., 1968), and South Australia (Sen Gupta &

Miles, 1975). WAA have been shown to have very low genetic diversity, especially in growing regions outside the Elgin area. It is believed that the majority of the fruit-growing regions of South Africa are inhabited by closely-related WAA strains allowing for the resultant possibility that strains can be managed with the use of a small number of resistant rootstock lines (Timm *et al*., 2005).

Chemical control

For many decades the chemical control of above-ground infestations has been the most widely used control tactic for WAA. The necessity for, and the timing of such sprays should be based on regular monitoring (Pringle & Heunis, 2001). As different classes of chemicals have come into use the aphid has changed from a rare resident of orchards to being a severe pest in them. For example, in 1946 the use of dichlorodiphenyltrichloroethane (DDT) began to interfere with the parasitoid in Washington, USA, causing outbreaks of WAA. By 1952, *A. mali* had developed some resistance to the use of DDT in the above-mentioned state, with the extent of parasitism having increased (Johansen, 1957). In general, the use of organophosphate-based programmes for Lepidoptera have also proved themselves capable of suppressing WAA. However when the application of azinphos-methyl is replaced by the use of other control measures, outbreaks of the aphid can still occur (Holdsworth, 1970; Penman & Chapman, 1980).

Eriosoma lanigerum, which can reproduce rapidly under favourable conditions, is capable of producing many generations per year. Such a rapid regenerative rate enables the rapid establishment of populations that are resistant to a particular insecticide. In 1962 a new systemic insecticide, vamidothion, was introduced for the control of the above-ground populations of WAA (Tarr & Hyde-Wyatt, 1965) and it soon became widely used (Loubser, 1968; Swart & Flight, 1990; Swart *et al.,* 1992). Vamidothion, together with chlorpyrifos, was also used in tank mixtures (Swart *et al*., 1992). However, tolerance to vamidothion in the Elgin area was later reported by Pringle *et al*. (1994).

Chemicals that are presently registered against WAA in South Africa have short residual actions and they are often unable to penetrate the waxy filaments covering aphid colonies, leading to inadequate control. The exception is imidacloprid, a chloronicotinyl insecticide (Nauen & Elbert, 1994) that is applied to the soil, which can control populations for up to three seasons with a resultant reduction in aerial infestations (Pringle, 1998).

The parasitoid, *A. mali,* has been shown to be susceptible to organophosphates, carbaryl, pyrethroids, imidacloprid, and sulfur compounds (Cohen *et al*., 1996; Heunis & Pringle, 2003). The disruption by relatively new compounds can also lead to outbreaks of the pest. A few of the neonicotinyl insecticides, such as thiamethoxam and imidacloprid, can WAA (Beers & Himmel, 2002).

Biological control

WAA has a number of natural enemies that may contribute to population regulation, and which can be exploited for integrated control. Known predators of WAA include Syrphidae (*Syrphus opinator* Osten Sacken, *Eupeodes fumipennis* Thomson and *Eupeodes americanus* Wiedeman), Chrysopidae (*Chrysopa nigricornis* Burmeister), Coccinellidae (*Coccinella transversoguttata* Brown), lacewings, predatory hemipterans and earwigs (Walker, 1985; Mueller *et al.,* 1992; Asante, 1997; Gontijo, *et al*. 2012). Altogether, 73 species of natural enemies have been recorded for WAA worldwide, with the most common being *A. mali* (Asante, 1997). Less frequently, WAA has also been observed to be infected by the fungal pathogen *Lecanicillium* (=*Verticillium lecanii* (Zimm) (Asante, 1997) and by the nematode *Steinernema carpocapsae* (Weiser) that attacks edaphic aphid colonies (Brown *et al.,* 1992). However, only a small proportion of biological control agents target edaphic populations, with many of them having been shown to be relatively ineffective.

Damavandian (2000) sampled and screened two apple orchards (Molteno and Oak Valley) and found a number of pathogenic fungi, which exhibit potential for use in the control of soil-borne life stages of WAA. The genus *Conidiobolus* and *Hirsutella* were always active

in aphids washed from soil and infested root samples and their levels of infection differed. For instance the highest peak of fungal infection at Oak Valley occurred during August 1997 in soil samples and affected almost 50% of the subterranean *E. lanigerum* population. At Molteno the highest infection levels were during September and more than 10% of the subterranean WAA in soil samples were infected.

While little is known of the biological control of edaphic populations, the biological control of above- ground populations has been extensively studied. *Aphelinus mali*, which is native to eastern North America, can aid in the control of WAA colonies on above-ground parts of the tree. The wasp, which was first introduced into South Africa in 1920 (Lundie, 1939), has been active in local apple orchards ever since. It lays its eggs inside the aphid, where the unfertilised eggs develop into males and where the fertilised eggs develop into females (Asante & Danthanarayana, 1992; Mueller *et al*., 1992). Although the parasitoid oviposits in all instars of its host, it seems to prefer the third instar (Mueller *et al*., 1992). It is now the most commonly encountered natural enemy of the aphid in the Western Cape province (Heunis, 2001).

Improvement of biological control has been a concern in many regions of the world, because of the chronic infestations of WAA. The control that is afforded by *A. mali* often appears to be disappointing, as it seems to act too late during the early summer period to be able to control the initial infestation. Also, some of the parasitic wasps tend to enter winter dormancy as early as autumn (Heunis, 2001). The size and shape of the above-ground colonies causes *A. mali* to have a poor numerical and functional response to host density in the event of an outbreak. The percentage parasitism tends to decline as the colonies increase in size, because the parasitoids cannot penetrate to the centre of dense colonies. If WAA form large colonies quickly in spring, *A. mali* becomes increasingly less efficient the quicker the formation occurs (Mueller *et al*., 1992). However, this parasitoid appears too late in the season to prevent colony formation and bud damage (Heunis, 2001). The use of

pesticides against harmful insects and mites frequently disrupts biological control. Most natural enemies are highly sensitive to the use of such agricultural chemicals.

The response of *A. mali* to the growth of a WAA population is further diminished by the prevalence of low temperatures. The lower development threshold of *A. mali* is higher than is that of its host (Asante & Danthanarayana, 1992), with the development rate of the parasitoid peaking at higher temperatures than that of its host (Walker, 1985). Furthermore, the reproductive rate of *A. mali* is much lower than is that of WAA, especially at low temperatures (Walker, 1985). Field experiments have indicated that the development of *A. mali* tends to lag behind WAA, even in hot weather (Evenhuis, 1958; Walker 1985; Asante & Danthanarayana, 1992). This explains early-season outbreaks of the aphid, especially in Europe (Evenhuis, 1958; Bonnemaison, 1965; Fernandez *et al*., 2005). After an analysis of the simulated reproduction of both the host and the parasitoid, Walker (1985) came to believe that the summers were too brief in Wenatchee, Washington, USA for the parasitoid to be able to eliminate the host. Mols & Boers (2001) proposed introducing a strain from Nova Scotia, Canada, to the Netherlands, because the Dutch strain, which originally came from Virginia, was less well adapted to a cold climate. Asante & Danthanarayana (1992) advocated introducing a better biological control agent in northern New South Wales, Australia, due to the inability of *A. mali* to suppress aphid outbreaks. Without the presence of natural enemies, the aphid colonies in Wenatchee were found to increase steadily throughout the summer (Walker, 1985). Even when parasitoids were allowed access to the colonies, the latter still increased in size. Only predators and parasitoids together showed that they were able to reduce the size and number of aphid colonies. Predators have frequently been found to reject mummified woolly apple aphids, thus favouring the parasitoid (Walker, 1985).

Integrated management

The reduced use of broad-spectrum pesticides favours greater diversity and higher populations of arboreal predators and parasitoids. *Aphelinus mali* is a key feature of

integrated pest management (IPM) against WAA in apple. Low WAA infestation levels can be maintained by ensuring that the initial plant material is clean, by keeping severe pruning to a minimum and by the application of mulches (Damavandian, 2000; Pringle & Heunis, 2001). Taking such actions can delay and minimise WAA colony formation in the trees during summer, giving the parasitic wasp a chance to establish and eventually, to control the pest. Sprays should only be applied if the monitoring data indicate that doing so is necessary. When making a decision regarding spraying, both infestation levels and parasitoid activity should be considered (Pringle & Heunis, 2001).

Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae are used to control soilborne insect pests. The broad host range of these nematodes, and their compatibility with commercial rearing and application techniques, make them especially attractive for biocontrol. Many species are currently being marketed as inundative biological control agents of insects (Kaya & Gaugler, 1993).

Life cycle and mode of action

The infective juvenile (IJ), which is the only free-living stage of EPNs, enters the insect host through the mouth, anus or spiracles or by means of direct penetration through the cuticle. If the mode of entry is via the mouth or the anus, the nematode penetrates the gut wall to reach the haemocoel, and if it is via the spiracles, it enters the haemocoel through the tracheal wall. When the IJ reaches the haemocoel of a host it releases bacteria that multiply rapidly in the haemolymph. Usually the insect dies within 48-72 hours after infection. Even though the bacterium is primarily responsible for the mortality of most insect hosts, the nematode also produces a toxin that is lethal to the insect (Burman, 1982). Once inside the host the IJs develop into feeding third-stage juveniles that consume the bacteria and their metabolic by-products. They moult into fourth-stage juveniles and then into males and females of the first generation (Grewal & Georgis, 1999). After mating the females lay eggs

that hatch into first-stage juveniles in the host. They complete the developmental stages to become males and females of the second generation. When the food in the cadaver becomes limited the late second-stage juveniles cease feeding. They then incorporate the associated bacteria in the intestine and moult into third-stage IJs. They retain the cuticle of the second stage as a sheath, and leave the cadaver in search of a new host. In small hosts the eggs that are laid by the first-generation adult females develop directly into IJs. The cycle, from the entry of the IJ into a host, to the emergence of the IJ from the host, is temperature-dependent and it varies somewhat among different species and strains. However, on average, completion of one generation in a host takes about 7-10 days at 25 °C in *Galleria mellonella* L. (Wouts, 1979; Nguyen & Smart, 1992).

EPNs as biopesticides

EPNs have potential for the biological control of many economically important insect pests, such as *Planococcus citri* (Hemiptera: Pseudococcidae) (Van Niekerk & Malan, 2012), *Cydia pomonella* (Lepidoptera: Tortricidae) (De Waal *et al*., 2011), and *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) (Malan *et al*., 2011). Worldwide, more than 100 species of EPNs have been identified, of which 11 have already been commercialised. The different species of EPNs vary in the range of insects that they attack, their environmental needs, as well as in terms of their stability as commercial products. A given species of EPN might also control a particular pest more effectively than it does another species. Therefore, the insect pest concerned must be identified before selecting the EPN species most appropriate for biological control.

Although field and laboratory experiments have indicated that many insect species are susceptible to nematode infection, different nematode species and strains exhibit varying levels of infectivity to particular insects (Molyneux, 1985). It is clear that the degree of biocontrol by EPNs for a given pest depends on the species of EPN, its infectivity to the host insect, and environmental conditions.

Brown *et al*. (1992) tested *S. carpocapsae* against edaphic populations of WAA in laboratory and field studies. Nematodes were found within the body cavity of several aphids, but nematode reproduction was absent from the host. The researchers concluded that the mortality could have been caused by the infection of the aphids by the symbiotic bacteria associated with the EPNs, or by the physical damage caused. In their field studies, the researchers were able to show a significant decrease in the number of WAA colonies on roots, as the result of a broadcast spray of EPNs.

In a study undertaken in Belgium, Berkvens *et al*. (2014) tested six commercially available EPNs for their potential to colonise and parasitise WAA in multiwell bioassay plates, namely *Heterorhabditis bacteriophora Poinar*, 1979, *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, *S. carpocapsae*, *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, *Steinernema glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982, and *Steinernema kraussei* (Steiner, 1923) Travassos, 1927. Of the EPNs tested, only *S*. *carpocapsae* caused higher mortality (20-40%) than the mortality caused by the control treatment. However, the mortality observed with *S*. *carpocapsae* was later found to be a test artefact that had not been induced by nematode activity. Even though *S*. *carpocapsae* IJs were found inside the aphids concerned, the presence of the nematodes had no effect on WAA reproduction. They further demonstrated that the growth of the EPN symbiotic bacteria is inhibited by the haemolymph of WAA (Berkvens *et al*., 2014). Unfortunately, only a few studies have been undertaken to test the efficacy of South African EPN species against insect pests. The susceptibility of WAA to South African EPNs still requires investigation.

Entomopathogenic fungi

The majority of entomopathogenic fungi (EPF) are found in the order Entomophthorales, of which approximately 200 species are regarded as pathogens of insects and mites (Benny *et al*., 2014). The taxonomy of fungi has recently been adjusted according to phylogenetic studies, with the Entomophthorales being reclassified in the

subphylum Entomophthoromycotina and the classes Entomophthoromycetes, Neozygitomycetes and Basidiobolomycetes (Humber, 2012). All members of the families Entomophthoraceae and the Nezygitaceae are obligate pathogens of insects, and they are notable for the epizootics that they induce in Homoptera, Lepidoptera, Orthoptera and Diptera (Benny *et al.,* 2014). They form asexual spores or conidia, which are forcibly discharged (Hajek *et al*., 2012). The most common genera of EPF used in biological control efforts are fungi in the Hypocreales, including *Beauveria* (Balsamo) and *Metarhizium* (Metschnikoff) (Inglis *et al*., 2001).

At the species level EPF, which have both a restricted and a wide range of hosts, have evolved significant intraspecific heterogeneity with respect to host preference. Their life cycle consists of mainly two phases, a vegetative (mycelium) growth, which often forms outside the host, and a budding phase, which occurs mostly in the haemocoel of the host. EPF, which are naturally widespread, are distributed globally, with them being readily open to mass culture. Because EPF occur naturally, it is thought that they are generally environmentally friendly with low to no mammalian and residual toxicity. As a result they have been developed as microbial insecticides for controlling many major arthropod pests in agriculture, forestry and urban settings in several countries, including the United States (Goettel *et al*., 2005).

Life cycles and modes of action

Most, if not all, EPF have life cycles that synchronise with insect host stages and environmental conditions. Spore germination, which is highly dependent on moisture, probably requires the presence of free water (Newman & Carner, 1975; Shimazu, 1977), but this requirement might be met by the moisture conditions of the microclimate, in the absence of measurable precipitation (Kramer, 1980; Mullens *et al*., 1987). The conidia, upon making contact with a potential host, initiate a series of steps that could lead to a compatible (infection) or to a noncompatible (resistance) reaction. When on the surface of a suitable host, fungal conidia become attached to its cuticle where they germinate and form of a

penetrative germ tube (Bateman *et al*., 1996). Penetration of the cuticle is achieved by means of mechanical and enzymatic degradation, which allows the germ tube to grow into the haemocoel. Once in the haemocoel, growth continues by means of the formation of mycelium and hyphae that colonise the host organs and haemolymph. During the process of colonisation, the fungi produce toxins. The death of an insect is usually the result of mechanical damage that is caused by the mycelia growing inside the insect (mummification) or by the toxins released by the pathogen. After the host's death, the fungus emerges from the cadaver, with it completing its life cycle by means of sporulation on the outside of the cadaver. After the conidia are dispersed to another host the infection cycle restarts (Hajek & St Leger, 1994; Inglis *et al*., 2001).

EPF as biopesticides

The significance of fungi in regulating insect populations was noted early in recorded history by the ancient Chinese (Roberts & Humber, 1981) due to the frequency of natural epizootics, and the conspicuous symptoms that are associated with fungus-induced mortality (Steinhaus, 1964; McCoy *et al*., 1988). EPF, like other natural enemies of insects, can be employed in classical biological control, augmentation or conservation. The safety of EPF for humans, for the environment and for nontarget organisms makes for a safer alternative for IPM than is the use of chemical insecticides (Goettel & Hajek, 2000).

Although fungal pathogens have much in common with viruses, bacteria and other insect pathogenic microbes, they are unique in several different ways (Ferron, 1978). The most significant difference lies in their mode of infection. Whereas most entomopathogens infect their hosts through the gut following ingestion, fungi typically penetrate the insect cuticle, thus becoming the only major pathogens that are known to infect insects with sucking mouthparts in the orders Hemiptera and Homoptera (Roberts & Humber, 1981).

Most EPF are best used to control insect populations below a specific economic threshold, with some crop damage being regarded as acceptable, rather than for the total

eradication of a pest. Despite there being an estimated 700 species of EPF in approximately 90 genera (Roberts & Humber, 1981), most of the commercially produced fungi are species of *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria*, which are all relatively easy to mass produce. Fungal pathogens, particularly *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *Isaria fumosorosea* Wize and *Metarhizium anisopliae* (Metschnikoff) Sorokin, are currently being evaluated for use against agricultural and urban insect pests. Several species belonging to the orders Isoptera (Hussain *et al*. 2010, 2011), Lepidoptera (Goble, 2009; Hussain *et al*., 2009; Coombes, 2012), Coleoptera (Ansari *et al*., 2006), Hemiptera (Leite *et al*., 2005), and Diptera (St Leger *et al*., 1987; Goble, 2009) are susceptible to various fungal infections. This has led to a number of attempts to use EPF for pest control, with varying degrees of success.

Previous studies regarding the biocontrol of WAA have not yet considered the viability of EPF. However, a number of different insect pathogenic fungi have been recorded as infecting WAA (Damavandian, 2000). Since *B. bassiana* and *M. anisopliae* already exist in commercial formulations the efficacy/feasibility of large-scale field applications should be investigated.

The effect of combining EPNs, EPF and chemical control

Studies have indicated the tandem application of biological and chemical insecticides to achieve a greater total effect than does the sum of their individual effects, so that such an application might offer a promising approach for insect pest management in terms of different agricultural systems. In the case of nematodes that attack mainly soil-dwelling pests, joint applications with EPF would appear to be a promising approach to increasing the present levels of control. One of the few studies on interactions between fungi and nematodes has been that of Barbercheck & Kaya (1991), who investigated the interaction of *H. bacteriophora* and *B. bassiana* against *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) larvae. Their study showed that a combination of the two agents achieved higher host mortality than when they were used separately. Shapiro-Ilan *et al*., (2004) demonstrated

that combining *Heterorhabditis indica* Poinar, Karunakar & David, 1992 with *M. anisopliae* at low concentrations, or combining *S. carpocapsae* with *B. bassiana* at high concentrations, had an additive effect on the mortality of *Curculio caryae* Horn (Coleoptera: Curculionidae) larvae. Although nematode-fungus combinations generally resulted in additive effects on target mortality, in most studies the mortality that was caused by the individual agents was too high to allow for a significant improvement to be made (Koppenhöfer & Grewal, 2005). However, the interaction between entomopathogens can also lead to antagonistic effects (Shapiro-Ilan *et al*., 2004; Ansari *et al*., 2005).

On investigating the compatibility of simultaneously using EPNs with the pesticide imidacloprid (Confidor®) on the survival and infectivity of EPNs, Le Vieux and Malan (2015) found that such usage displayed no negative effect for both factors. In studies by Morales-Rodriquez and Peck (2009) on the interaction between EPN and neonicotinoid insecticides, synergies were found to be consistent across trials from glasshouse to field, specifically in relation to the white grub, *Amphimallon majale* (Razoumowsky) (Coleoptera: Scarabaeidae). In laboratory studies the effects of sublethal concentrations of imidacloprid alone or in combination with *M. anisopliae* and *B. bassiana* on the mobility, mortality and mycosis of the citrus root weevil showed that both larval mortality and mycosis increased synergistically (Quintela & McCoy, 1998).

Conclusions

From the literature reviewed and personal communications with producers and extension officers, it is evident that WAA, especially in terms of the edaphic populations, poses a major threat to apple production in South Africa. Although the use of resistant rootstocks is recommended, unfortunately local research has discovered a strain of WAA that is capable of overcoming the resistance factor in Northern Spy and related rootstocks in South Africa. Biological control with the parasitoid, *A. mali*, has also had no effect where the aphid colonies persist on the roots, as the parasitoids in question occur only above ground. In South Africa the only current control measure that is available for subterranean

populations of WAA is the soil application of imidacloprid. This, in itself, poses a major threat to the control of WAA, as no alternative options are available, should the use of the product be banned in South Africa.

Future control of edaphic populations is a research challenge that should be actively pursued. Valuable information on the effective monitoring of soil populations is available through existing local research, as the WAA populations are difficult to determine and to monitor, with them being largely responsible for the persistence of WAA in South African orchards. The use of EPNs and EPF offers potential biocontrol options that should be exploited to determine their potential for the control of edaphic WAA populations. Local research also indicates the occurrence of resident EPF, as isolated from the field-collected WAA. These soil biological control options, entailing the use of locally isolated and commercially available EPNs and EPF, have not yet been studied under local conditions.

Aims of the study

In view of the above-mentioned findings, the overall aim of this study was to evaluate the efficacy of EPN, EPF, and a combination of a nematode and fungi against arboreal and subterranean *E. lanigerum* populations in laboratory, screen house, and field trials.

The objectives of the study were therefore:

1. To evaluate the pathogenicity of EPN isolates of *E. lanigerum* in the laboratory, and to identify the most virulent isolates to be used in glasshouse and field trials.
2. To determine the efficacy of the selected EPN species, two EPF species, and a combination of a nematode and a fungal species for control of arboreal and subterranean *E. lanigerum* populations in pot trials.
3. To investigate the effect of mulching on the efficacy of EPNs, for the control of soil populations of *E. lanigerum* in pot and field trials.

4. To investigate the efficacy of the selected EPN species, two EPF, and a combination of a nematode and a fungal species, for control of subterranean *E. lanigerum* populations in field trials.

The results from this study should provide a basis for the development of an integrated management plan for WAA.

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CHAPTER 2

Laboratory bioassays on the susceptibility of woolly apple aphid, *Eriosoma lanigerum* (Hemiptera: Aphididae), to entomopathogenic nematodes

Abstract

Woolly apple aphid (WAA), *Eriosoma lanigerum*, is an important pest of apples, occurring throughout the apple-growing regions of the world. The aphid feeds on shoots, branches, wounds, and roots, causing hypertrophic gall formation, which affects the transport of water and nutrients. Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae and their symbiotic bacteria have generated extensive interest as inundative biological control agents of pest insects. With the development of the resistance of *E. lanigerum* to certain chemicals, export restrictions, and the inability of parasitoids to control the aphid successfully early in the season, it is important to consider EPN as an alternative biocontrol agent. Apple roots infested with *E. lanigerum* from an apple orchard were kept in plastic containers to maintain a steady supply of insects. Seven EPN species were tested for their pathogenicity against WAA. They included *Steinernema khoisanae*, *S. yirgalemense, S. citrae, S. feltiae, Heterorhabditis zealandica, H. bacteriophora*, and *H. safricana*. Laboratory bioassays identified *S. yirgalemense* and *H. zealandica* as being the most virulent against the subterranean stage of the WAA, with mortality rates of 48% and 38%, respectively. Studies on the effect of *E. lanigerum* size showed that the last instar is most susceptible to infection by *S. yirgalemense* and *H. zealandica,* whereas smaller instars appear to be too small for nematode penetration and infection. Increasing the exposure period of the aphids and nematode concentration had no positive effect on the *E. lanigerum* infection rate. When the ability of infective juveniles (IJs)

of *S. yirgalemense* and *H. zealandica* to tolerate exposure to imidacloprid was evaluated, both nematodes species were found to be compatible with this chemical, with no significant nematode mortality incurred. Imidacloprid also did not affect the ability of the nematodes to infect their hosts after 24 h exposure to the product. In Petri dish bioassays, the haemolymph of WAA showed an inhibitory effect on the development of the symbiotic bacteria, *Xenorhabdus indica* and *Photorhabdus zealandica*, which are respectively associated with *S. yirgalemense* and *H. zealandica*.

Introduction

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is native to North America (Beers *et al*., 2007). The aphid is an important pest of apples, *Malus domestica* L. (Rosaceae) in the Western Cape province of South Africa (Pringle & Heunis, 2001) and in other apple-growing areas of the world. WAA was most probably introduced into South Africa where it was first recorded during 1895, on infested plant material (Myburgh *et al*., 1973). In South Africa, Annecke and Moran (1982) and Fuller (1904) point out that WAA has been found all over the country and that it is the most virulent of all the pests including fungi that attack apples. Locally, it is rarely found on other host plants although it has been recorded on apple (*Malus pumilus* Miller), peach (*Prunus domestica* L.) pear *(Pyrus communis* L.) (Millar, 1994).

Apples are by far the most important export fruit in terms of volume as they constitute 30% of the total deciduous fruit crop in South Africa. They are grown in several provinces around South Africa, but the Western Cape province is the heartland of deciduous fruit (Meyer & Breitenbach, 2004). The Western Cape which is a winter rainfall area has a climate that is similar to that of the Mediterranean region, which is favourable for apple production.

The aphid forms densely packed colonies that are covered with a white, waxy, filamentous secretion, on the aerial parts and roots of apple trees (Shaw & Walker, 1996). Favourite penetration sites are new growth shoots, branches, pruning and other wounds, leaf axils and the roots of apple trees (Evenhuis, 1958; Mueller *et al*., 1988). In South Africa

WAA propagates entirely pathogenically and therefore only one aphid is needed to start a new colony (Heunis & Pringle, 2006). The feeding activity of WAA which causes galls to form on the woody tissue can destroy young lateral shoots and buds. The WAA galls which are ideal feeding sites for their offspring frequently give rise to densely packed colonies. The infestation of calyces of apples by aphid colonies can result in contamination with honeydew and sooty mould. In South Africa, Damavandian and Pringle (2007) showed a seasonal pattern in the population fluctuations of edaphic WAA. The pattern consists of two population peaks per year, one during spring and early summer (November, December) and one during autumn (February to April).

Entomopathogenic nematodes (EPN) of the families Steinernematidae Filipjef, 1934 and Heterorhabditidae Poinar, 1976 have been found to be the most successful for the biological control of insects (Kaya & Gaugler, 1993). Although field and laboratory experiments have indicated that many insect species are susceptible to nematode infection, different nematode species and strains have exhibited different levels of infectivity to particular insects (Begley, 1990). Both nematode genetic constitutions and environmental factors can interfere to determine such biological control levels.

The parasitic wasp *Aphelinus mali* (Haldeman) can aid in the control of *E. lanigerum* colonies in aerial parts of the tree. The wasp which is native to eastern North America, was introduced into South Africa in 1920 (Lundie, 1939) and it has been active in local apple orchards ever since. It lays its eggs inside the aphid where the unfertilised eggs develop into males and the fertilised eggs develop into females (Asante & Danthanarayana, 1992; Mueller *et al*., 1992). Although the parasitoid uses all instars of its host, it seems to prefer the third instar (Mueller *et al*., 1992). It is now the most commonly encountered natural enemy in the Western Cape province (Heunis, 2001). Improvement of biological control has been a concern in many regions of the world, because of the chronic infestations of *E. lanigerum*. The degree of control that is afforded by *A. mali* often appears to be disappointing, as it seems to appear too late during the early summer period to control the initial infestation. In

addition a portion of the parasitic wasps enters winter dormancy during autumn (Heunis, 2001). The size and shape of aerial colonies causes *A. mali* to have a poor numerical and functional response to host density in the event of an outbreak. The percentage of parasitism declines as the colonies increase in size, because parasitoids cannot penetrate to the centre of dense colonies. If WAA forms large colonies quickly in spring, the efficiency of *A. mali* decreases accordingly (Mueller *et al*., 1992). This parasitoid appears too late in the season to prevent colony formation and bud damage (Heunis, 2001). The use of pesticides against harmful insects and mites frequently disrupts biological control as most natural enemies on which the biological control of pest species depends, are highly sensitive to such agricultural chemicals. Biological control is becoming an important component of the integrated pest management (IPM) of WAA, especially now with the increasing restrictions being placed on the use pesticides.

The chemicals that are most commonly used against *E. lanigerum* are vamidothion, endosulfan and chlorpyrifos with recently imidacloprid also coming into common usage (Pringle, 1998; Pringle *et al*., 1994). These chemicals which tend to have a short residual action are often unable to penetrate the waxy filaments covering aphid colonies, thus leading to inadequate control of the colonies concerned. The exception is imidacloprid, which by means of soil applications can control populations for up to three years at a time (Pringle, 1998).

This study investigated the potential of native and imported EPN to control *E. lanigerum* and determined their suitability for use as part of an IPM programme directed at management of the insect. Laboratory bioassays were used to determine the efficacy of local and imported EPNs and the effect of size on nematode infectivity. They were also employed to assess rates of penetration and reproduction, the amount of time and the concentration required for maximum efficacy, and the effect of imidacloprid on the mortality of the nematodes concerned. Growth of the associated bacteria in the haemolymph as a quick method for the screening for pathogenicity was also investigated.

Materials and methods

Source of insects and nematodes

Apple roots infected with *E. lanigerum* were collected from an apple orchard on the Infruitec experimental farm in Grabouw, Western Cape province and stored in closed plastic containers (150 × 100 cm). The containers were kept in the dark and the roots were sprayed with water to keep them moist. The infested root cultures were used as a steady supply of the different *E. lanigerum* life stages.

The larvae of the greater wax moth *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were reared in the laboratory. Eggs were collected from wax paper that was used to line a glass container holding adult moths. The eggs were then transferred to a container together with a fresh diet (consisting of baby cereal, brown bread flour, yeast, wheat germ, beeswax, glycerine and honey) and kept at a temperature of 25ºC in a growth chamber (Van Zyl & Malan, 2015).

The larvae of the beetle *Tenebrio molitor* (L.) were raised on wheat bran in closed plastic containers. Apple, potato or carrot slices were laid over the surface of the colony to provide suitable humidity. To maintain a supply of mealworms in a state of dormancy, mealworms were kept at a temperature of 4ºC in the refrigerator (Van Zyl & Malan, 2015).

The local EPN species used in the current study were obtained from previous surveys (Malan *et al*., 2006; 2011) and they were maintained in the EPN collection of Stellenbosch University, while formulated *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976 were obtained from River BioScience (Table 2.1). The IJs of all nematode species were reared in last-instar larvae of *T. molitor* or *G. mellonella* at room temperature and then harvested and quantified using standard procedures described by Kaya and Stock (1997). The nematodes were stored horizontally in 150 ml distilled water in vented 500 ml culture flasks at 14ºC, which were shaken weekly for aeration with the nematodes concerned being used within one month

after harvesting. Nematodes were used at a concentration of 200 IJs / 50 µl, which was calculated as appropriate using the method developed by Glazer and Lewis (2000).

Table 2.1. *Steinernema* and *Heterorhabditis* species; isolate number, habitat, locality, Genbank accession number and associated symbiotic bacteria (*Photorhabdus* spp. or *Xenorhabdus* spp.)

Species	Isolate no.	Soil type	Locality	Genbank accession numbers	Associated symbiotic bacteria (verified by Microbiology SU)
H. bacteriophora	190-C	Citrus	Riviersonderend, Western Cape	FJ217349	*P. luminescence* subsp. *laumondi*
H. bacteriophora	-	-	e-nema, Germany	-	*P. luminescence subsp. laumondi*
H. noenieputensis	SF669	Fig tree	Noenieput, Northern Cape	JN620538	*P. luminescence* subsp. *noenieputensis*
H. bacteriophora	SF351	Grapevine	Wellington	-	*P. luminescence* subsp. *laumondi*.
H. safricana	**SF281**	**Disturbed**	**Piketberg, Western Cape**	**EF488006**	***P. luminescence* subsp. *laumondi***
H. zealandica	**SF41**	**Natural**	**Patensie, Eastern Cape**	**EU699436**	***P. zealandica***
H. zealandica	GM25	Natural	Grabouw, Western Cape	-	*Photorhabdus* sp. (unknown sp.)
S. citrae	**141-C**	**Disturbed**	**Piketberg, Western Cape**	**EU740970**	***Xenorhabdus*** sp.
S. feltiae	**-**	**-**	**e-nema, Germany**	**-**	***X. kotzodoi***
S. jeffreyense	**J194**	**Garden soil**	**Jeffrey's Bay, Eastern Cape**	**KC897093**	***X. khoisanae***
S. khoisanae	**SF80**	**Natural**	**Tulbagh, Western Cape**	**DQ314287**	***X. khoisanae***
S. sacchari	SB10	Sugar cane	Gingindlovu, KwaZulu-Natal	KC633095	*X. khoisanae*
S. yirgalamense	**157-C**	**Disturbed**	**Friedenheim, Mpumalanga**	**EU625295**	***X. indica***
Steinernema sp.	F2	Fynbos	Clanwilliam, Western Cape	KP325084	*Xenorhabdus* sp.
Steinernema sp.	WS9	Litchi	Friedenheim, Mpumalanga	-	*Xenorhabdus* sp.
Steinernema sp.	MT1-5	*Acacia mearnsii*	Piet Retief, Mpumalanga	KR527216	*Xenorhabdus* sp. (new sp.)

Bold = Screened with 24-well bioassay plates

Isolation of associated symbiotic bacteria

The symbiotic bacteria of 14 local EPNs (Table 1) were isolated from their associated EPN species. All isolates were confirmed to be either *Xenorhabdus* sp. or *Photorhabdus* sp. Infective juveniles (IJs) of the different nematodes were inoculated at a concentration of 200 IJs/insect in a Petri dish with five *Galleria* larvae. After 18 h the wax moth larvae were bent and pricked just behind the leg and a drop of haemolymph was spotted onto a Nutrient Agar Bromothymol Blue (NBTA) medium (9 g Tryptone soy broth; 12.6 g Agar No. [2] Bacteriological; 0.025 g Bromothymol blue powder; 900 ml distilled water) (Kaya & Stock, 1997; Hirao & Ehlers, 2009). A sterile loop was used to streak the haemolymph onto the agar plate. Plates were incubated in the dark at 25 ±2°C in a growth chamber for 48 h. Individual colonies were suspended in 30 ml Tryptic Soy Broth (TSB) in an Erlenmeyer flask which was left on an orbital shaker (Benchmar; Orbishaker Jr) in a growth chamber at 25 ±2°C for 48 h. Cryotubes containing 120 µl sterilised glycerol and 800 µl of the culture from the NB were stored at -80°C till required (Ferreira *et al*., 2015).

Twenty-four well bioassay protocol

Adult females of WAA were individually exposed to IJs in 24-well culture plates (Flat bottom, Nunc^{TM}, Cat. No. 144530). Filter paper discs (of 13 mm diam.) were added to 12 alternate wells in the 24-well plates used. Nematodes were inoculated onto the discs at a predetermined concentration of 200 IJs / 50 µl (Glazer & Lewis, 2000). Thereafter, a single last instar WAA was added to each of the 12 alternate wells. The cover of the bioassay plates was fitted with a piece of glass and secured with a rubber band to confine the insects to their wells. After being placed in plastic containers that were lined with moistened paper towels and closed, so as to ensure the maintenance of high humidity, the plates were incubated at 25 ± 2ºC in a growth chamber. Five plates were used for each isolate tested (*n* = 60 insects). An equal number of replicates that were used as control were treated with water only so as to determine the rate of natural death. Mortality and nematode infection

were determined after 48 h by means of gentle prodding with each cadaver being dissected with the aid of a dissecting microscope so as to confirm the extent of mortality that had occurred due to nematode infection. All bioassays and experiments were repeated on a different test date with freshly prepared nematode inoculum.

Susceptibility of WAA to infection by EPNs

The 24-well bioassay protocol was used to test seven EPN species for their pathogenicity against adult WAA (Table 2.1). The EPN species investigated included *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006, *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004*, S. citrae* Stokwe, Malan, Knoetze & Tiedt, 2001*, S. feltiae, Heterorhabditis zealandica* Poinar, 1990*, H. bacteriophora* and *H. safricana* Malan, Nguyen, De Waal & Tiedt, 2008.

Effect of WAA size and morphology on infectivity

To determine the effect of *E. lanigerum* size on nematode infectivity, they were visually categorised as adults, intermediates and crawlers. Ten aphids were placed in a 9 cm-diameter Petri dish lined with filter paper and inoculated with 2 000 IJs of either *S. yirgalamense* or *H. zealandica* with water being used for the control in 500 µl filtered water. For each size category six replicates and a control were employed. The Petri dishes used were placed in a plastic container that was lined with damp paper towelling to maintain conditions of high humidity. After two days' incubation at 25 ± 2ºC in a growth chamber, dead aphids were removed, rinsed in tap water (to remove any external nematodes) and placed in small Petri dishes containing moistened filter paper and left for another two days at 25 ± 2ºC in a growth chamber. A random sample of 10 aphids from each size category was measured using a motorised Olympus stereomicroscope, after which they were dissected to confirm nematode infection. This experiment was repeated on a different date with fresh nematode inoculum.

Assessment of nematode penetration and reproduction

Steinernema yirgalamense and *S. feltiae* were used to inoculate 20 adult aphids placed in a 9-mm Petri dish lined with filter paper, together with 4 000 IJs / 1 ml of distilled water. The Petri dishes were placed in a plastic container lined with wet filter paper towels so as to maintain a high humidity level and incubated at 25 ± 2ºC in a growth chamber. The dead aphids were then rinsed in tap water so as to remove any surface nematodes, whereupon they were moved to nematode-free Petri dishes and incubated at 25 ± 2ºC in a growth chamber. Every two days, 10 aphids were randomly selected from each treatment and dissected under the microscope with the nematode development being noted in each instance. Individual aphids were assessed for colour change, infection and stage of nematode development. The first-generation males and females were counted so as to determine the penetration rate of the nematodes. Five Petri dishes with insect cadavers were placed on modified White traps (Woodring & Kaya, 1988).

Effect of incubation time and nematode concentration on mortality

The aim of the experiment was to determine the effect of the concentration of *S. yirgalamense*, *S. feltiae* and *H. zealandica* and the amount of time to be used in further laboratory tests. Petri dishes (of 9-cm diam.), each containing 20 adult aphids were inoculated with concentrations of 200, 143, 102, 73, 52 and 0 IJ/insect. After application the Petri dishes were placed in plastic containers lined with damp paper towels to maintain conditions of high humidity and they were then incubated at 25 ± 2ºC in a growth chamber. Five replicates were used for each treatment (concentration/exposure period) and for the control (only water) employed in each exposure period for a total of 30 replicates. Every 12 h the mortality for each treatment was noted. After 72 h the experiment was terminated and infection confirmed by means of dissection.

Direct screening using bacteria and insect extract

Seven bacterial isolates, from the same nematodes used in the 24-well bioassay, were isolated from their respective nematodes and their growth on the insect host extract was examined as described by Berkvens *et al*. (2014). Additionally a further five bacterial symbionts that were not screened against WAA by means of the traditional method were isolated from their respective nematodes and tested for bacterial growth inhibition. The nematodes involved included *H. noenieputensis*, *H. bacteriophora* (190-C) and *Steinernema* spp. (WS9; F2, M-T 1-5). The bacterial cultures were verified and identified by sequencing 16S rRNA gene (Table 3) (Ferreira *et al*., 2013a, b; 2014; 2015) and stored in five glycerol cryotubes each at -80°C. In a laminar flow a flamed metal loop was used to scrape the surface and to streak the bacteria onto an NBTA plate. The agar plates, sealed with Parafilm were incubated in the dark for 48 h at 25°C. Single colonies of bacteria were selected and streaked onto new plates of nutrient agar. After obtaining pure colonies a single colony was taken with a sterile loop and inoculated in TSB in an Erlenmeyer flask, which was plugged with non-absorbent cotton wool. The flask was then placed on an orbital shaker for 2 days at 25°C.

Insect extracts were prepared by means of sterilising 100 mg codling moth, *Galleria* larvae and approximately 1000 adult female WAA in 96% alcohol, after which the insects were separately crushed in Eppendorf tubes filled with 1 ml potassium phosphate buffer (PB). The extracts were then centrifuged at 12×10^3 rpm for 90 sec. A sterile 100 ml syringe was used to filter sterilise the supernatant of each tube through a membrane with a pore size of 0.24 µm.

Using a sterile glass spreader, 50 µl of 48 h-old TSB bacterial culture from the Erlenmeyer flask were evenly distributed onto an NBTA plate. Four filter paper discs (of 3 mm diam.) were placed in a diamond configuration on the inoculated medium. Each middle disc was then spotted with 10 µl PB, which served as the control. The other three discs were each spotted with 10 µl of one of the insect extracts. Each aphid extract was consequently

tested on four NBTA plates, using the same bacteria. The NBTA plates concerned were then incubated in a growth chamber at 25 ± 2°C. Bacterial proliferation or inhibition around the filter discs was scored after 2-4 days post treatment. Bacteria were regarded as being inhibited if the extract showed a transparent circle of nonbacterial growth around the spotted disc.

Effect of imidacloprid on nematode infectivity and survival

Imidacloprid, which is an insecticide that is commonly used for *E. lanigerum* control was evaluated for its effect on *S. yirgalamense* and *H. zealandica* IJs in the laboratory. Nematode suspensions containing 4 000 IJs/ml were prepared for each nematode species tested. Imidacloprid formulation was prepared at twice the label rate (12ml/1L). One millilitre of nematode suspension and 1 ml of insecticide formulation was added to a Petri dish that was then kept in a growth chamber at 25 ± 2ºC. Five Petri dishes were prepared for each nematode species. The nematode survival rate obtained was compared to that of nematodes in water only in five control dishes. To estimate nematode survival, 10 µl samples were collected up until the time that 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.

To determine whether the nematodes were still virulent after exposure to the insecticide, an additional 5 ml of the treatment was prepared and kept in the same growth chamber. After 24 h, each treatment was diluted in 1 L distilled water. Nematodes were allowed to settle to the bottom of the measuring cylinders, after which excess fluid was siphoned off to 10 ml. Five Petri dishes per treatment and five control dishes each containing 10 mealworm larvae, were prepared for each nematode species. Treatment dishes were inoculated with a concentration of 100 IJs/insect. The control dishes received water only. After 48 h, insect mortality was assessed. The experiment was repeated on a different test date.

Data analysis

All statistical analysis was performed using the data analysis software system STATISTICA 11.0 (StatSoft Inc., 2008). Data were analysed using a one-way ANOVA with post-hoc comparisons of means, using Bonferroni's method, or by means of the use of a bootstrap multiple comparison method for any residuals that were not normally distributed (Efron & Tibshirani, 1993). Significant differences were determined on a 95% probability level.

Results

Susceptibility of WAA to infection by EPNs

Significant differences in susceptibility to infection were detected among the different nematode isolates 48 h after treatment ($F_{(6.62)} = 23.91$, $p < 0.001$). Adult WAA was susceptible to infection by all EPN species tested. *Steinernema yirgalemense* caused significantly greater infection than did the other nematode species tested (Fig. 2.1). Infection caused by *H. zealandica* (38.3 ± 2.5%) and *S. feltiae* (35 ± 4.3%) was significantly higher compared to that of all other nematode species tested except for *S. yirgalemense (*48.3 ± 2.7%). Larval infection caused by *S. khoisanae*, *H. bacteriophora, H. safricana* and *S. citrae* (mean infection 14.2 ± 3.5%, 10.8 ± 3.7%, 10.8 ± 2.5% and 10.8 ± 3.1%, respectively) was not significantly different from the mortality observed in the control (Fig. 2.1).

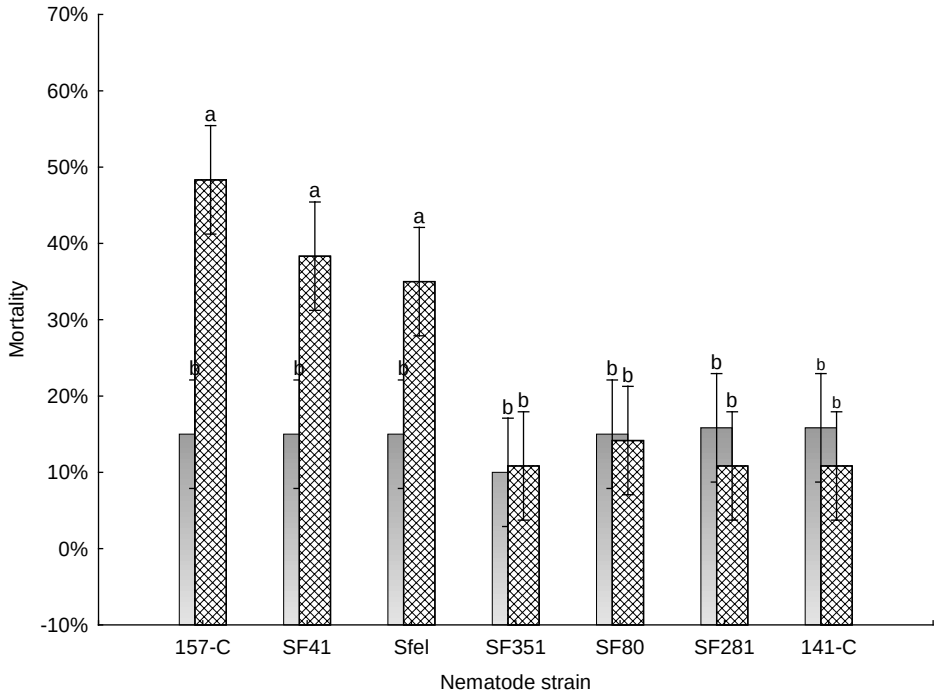


Fig. 2.1. Mean percentage (95% confidence interval) mortality for adult females of *Eriosoma lanigerum,* induced by seven species of entomopathogenic nematodes (▨) (*Steinernema yirgalemense* (157-C)*, Heterorhabditis zealandica* (SF41)*, Steinernema feltiae* (Sfel)*, S. khoisanae* SF80, *H. bacteriophora* (SF351)*, H. safricana* (SF281) and *S. citrae (*141-C*)* at a concentration of 200 IJs/insect and a control with water only (▩), after a period of 48 h in multiwell bioassay plates (one-way ANOVA; $F_{(6.62)} = 23.91$, $\rho < 0.001$). Different letters above bars indicate significant differences.

Effect of WAA size and morphology on infectivity

A significant interaction effect on infectivity between nematode species and size (Table 2.2) of WAA 48 h after exposure to the two nematode species was detected ($F_{(2,24)} = 4.54$, $p = 0.021$) (Fig. 2.2). This interaction effect was a result of the differences in infectivity caused by the two nematode isolates on the adult stage of WAA. The highest infection (58.33% ± 4.6%) was caused by *S. yirgalemense* on the adult WAA, and no infection by *S. yirgalemense* being observed on the crawler stage. However, no significant difference was found in the infection caused by *S. yirgalemense* between crawlers and intermediates. In the case of *H. zealandica*, the highest infectivity (38.3% ± 5.0%) was observed for the adult stage followed by 8.3% ± 5% for the intermediates whereas no infectivity was observed on the crawlers.

Table 2.2. *Eriosoma lanigerum* were grouped into three visual size classes, adults, intermediates and crawlers, based on mean length and width (range ± S.D.).

Size (*n* = 10)	Length	Width	% infected *S. yirgalemense*	% infected *H. zealandica*
Adults	1890 ± 127 (1757–2111)	1041± 68 (881–1142)	58.0	38.0
Intermediates	1070 ± 360 (117–1340)	554 ± 37 (473–611)	16.7	8.3
Crawlers	618 ± 105 (488–794)	236 ± 17 (202–258)	0	0
S. yirgalemense	*635 ± 36 (548–692)	*29 ± 2.2 (24–33)	–	–
H. zealandica	*685 (570–740)	*27 (22–30)	–	–

*Malan *et al*., 2006.

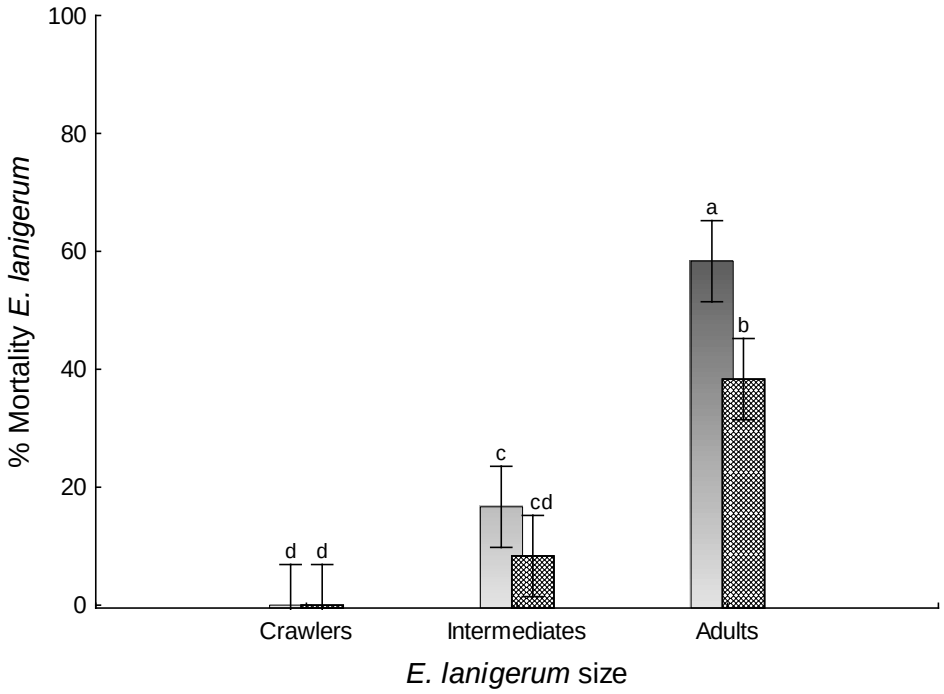


Fig. 2.2. Mean percent mortality (95% confidence interval) for different stages of *Eriosoma lanigerum,* using *Steinernema yirgalemense* ( ) and *Heterorhabditis zealandica* ( ), at a concentration of 200 IJs/insect, after a period of 48 h in multiwell bioassay plates (one-way ANOVA; $F_{(2,24)}$ = 4.54, p = 0.021). Means with the same letter above bars are not significantly different.

Assessment of EPN penetration and reproduction

Changes in infected WAA and nematode development were documented for 8 days after exposure to EPNs (Table 2.3). Several dead WAA from the inoculated dishes were found to contain live nematodes. Crushing of the aphids in water, revealed nematodes swimming in their internal organs. Neither the native *S. yirgalamense*, nor the imported *S. feltiae* successfully developed and propagated in the adult WAA. Dead aphids contained dead, fully developed adult nematodes of both sexes, 8 days post infection. No development and propagation of the nematodes inside WAA bodies was observed.

Table 2.3: Observations of the development of *Steinernema yirgalemense* and *S. feltiae*.

Nematode species	No. of days	Stage of nematode development
S. yirgalamense	2	Infective juveniles
	4	Infective juveniles
	6	First-generation adults
	8	First-generation adults (dead)
S. feltiae	2	IJ and first generation
	4	IJ and first generation
	6	First-generation adults
	8	First-generation adults (dead)

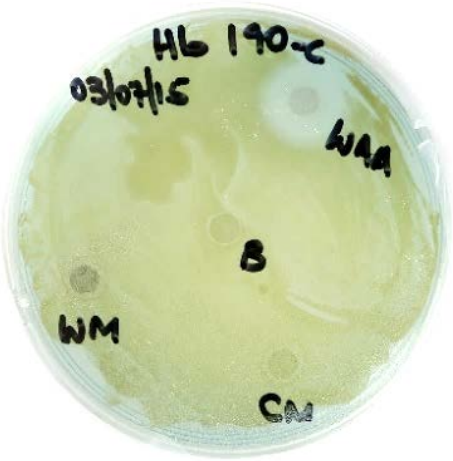
Effect of incubation time and nematode concentration on mortality

No effect on the infectivity of last-instar WAA was observed, despite using different nematode concentrations and exposure time intervals. The lethal dose and time could not be calculated, as the slope for the concentration-mortality did not change over time, even in response to prolonged exposure time (data not shown).

Direct screening using bacteria and insect extract

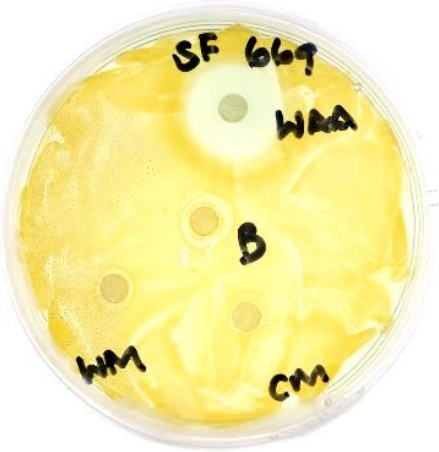
A total of 14 nematode-associated bacteria were tested for growth inhibition (Table 1), using the spotting technique of Berkvens *et al*. (2014). The bacteria tested included the associated bacteria of the seven nematode isolates (Table 1) used in the 24-well bioassay screening (except for SF351). The bacterial growth assays confirmed inhibition of the associated bacterial symbionts of six *Heterorhabditis* isolates including the imported *H. bacteriophora* from e-nema German, two local *H. bacteriophora* and two endemic Heterorhabditis, viz. *H. noenieputensis and H. safricana.* A transparent circle of nonbacterial growth was observed around the WAA spotted disc (Fig. 2.3), in comparison to the

appearance of the phosphate buffer control and to that of the codling moth and wax moth spotted discs, which showed no circle of nongrowth of the bacteria (Fig. 2.3).



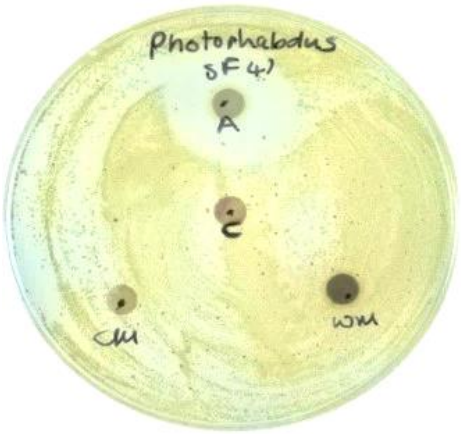
P. luminescence subsp. *laumondi*
(*H. bacteriophora* 190-C)

P. luminescence subsp. *laumondi*
(*H. bacteriophora* e-nema)

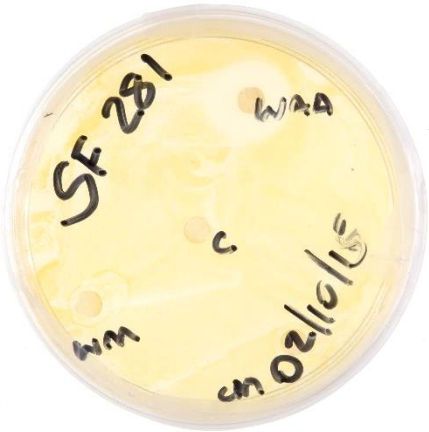


P. luminescence subsp. *noenieputensis*
(*H. noenieputensis* SF669)

Photorhabdus zealandica.
(*H. zealandica* GM25)

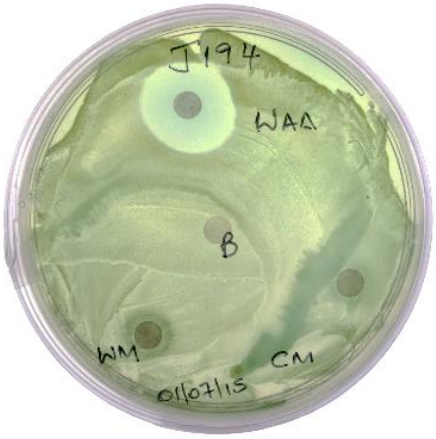


P. luminescence subsp. *zealandica*
(*H. zealandica* SF41)



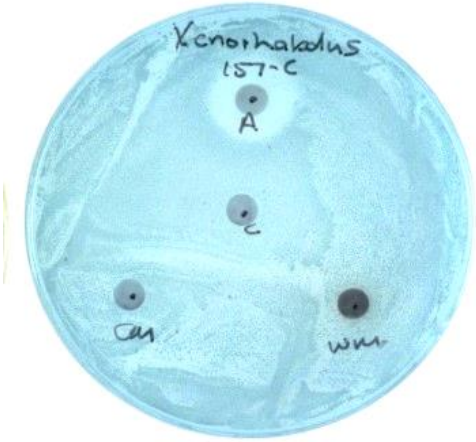
Photorhabdus sp.
(*H. safricana* SF281)

X. kozodoi
(*S. feltiae* e-nema)

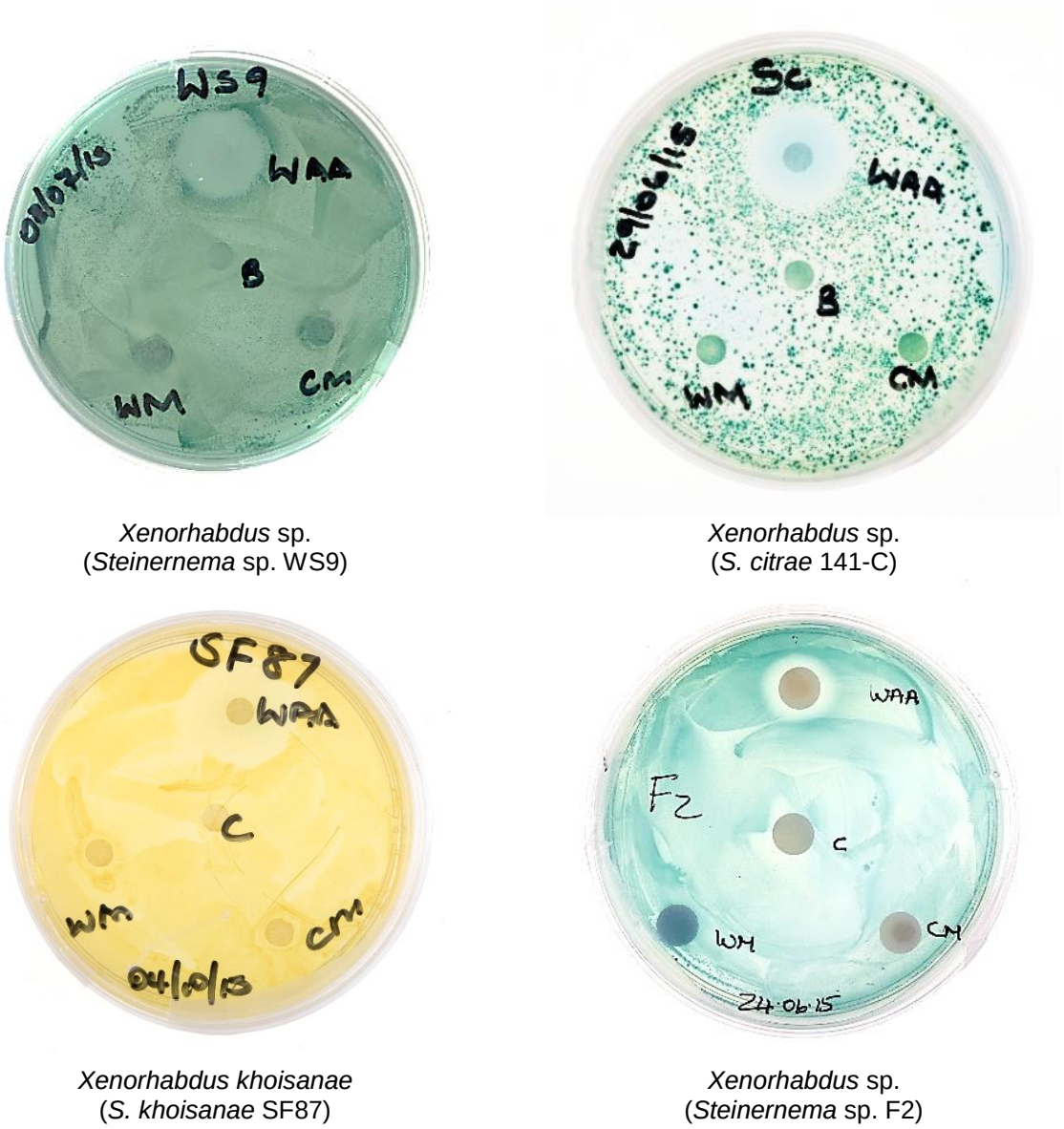


Xenorhabdus sp.
(*S. jeffreyense* J194)

Xenorhabdus sp.
(*S. saccari* SB10)



X. indica
(*S. yirgalemense* 157-C)



Xenorhabdus sp.
(*Steinernema* sp. WS9)

Xenorhabdus sp.
(*S. citrae* 141-C)

Xenorhabdus khoisanae
(*S. khoisanae* SF87)

Xenorhabdus sp.
(*Steinernema* sp. F2)

Fig. 2.3: Growth of *Xenorhabdus and Photorhabdus* spp. (full-plate inoculation of Nutrient Agar Bromothymol Blue (NBTA) plates) challenged with *Cydia pomonella* (CM), *Galleria mellonella* (WM), and *Eriosoma lanigerum* (WAA), with the centre disc spotted with phosphate buffer (B) as a control (C).

Effect of imidacloprid on nematode infectivity and survival

No significant interaction effect in the infectivity of *S. yirgalemense* was found between treatment (i.e. water and imidacloprid) and time of exposure ($F_{(3,72)}$ = 0.500, P = 0.683) (Fig. 2.4). However, the mortality of *S. yirgalemense* due to imidacloprid was highest at 24 h after exposure, although it was not significantly different from that which was caused

by water for the same duration of exposure. In addition, no significant difference was found in the mortality of *S. yirgalemense* due to imidacloprid for between 0 and 12 h exposure. The level of *S. yirgalemense* mortality due to the presence of water was significantly higher after 24 h exposure than it was at the start (0 h) of the experiment. No significant difference in *S. yirgalemense* mortality was observed over time, compared to the control.

Imidacloprid also proved to have no effect on *H. zealandica* (Fig. 2.5), with there being no significant interaction effect on mortality due to treatment and the elapse of time ($F_{(3,72)}$ = 2.258, p = 0.856). However, a significant difference on mortality due to imidacloprid was found to occur for between 12 and 24 h of exposure compared to exposure at 0 h. However, the level of mortality at 6 h exposure did not differ significantly from that at all other time intervals. The level of mortality due to the presence of water was not significantly different between 6 h and 24 h after exposure. However, mortality due to the presence of water at 0 h was significantly lower than that which was observed between 12 and 24 h. Control mortality (1.2, 8.8, 14.0 and 18% over 0, 6, 12 and 24 h, respectively) and mortality in the case of the imidacloprid only treatments (1.0, 10.2, 17.8 and 18% over 0, 6, 12 and 24 h, respectively) was low (Fig. 2.5).

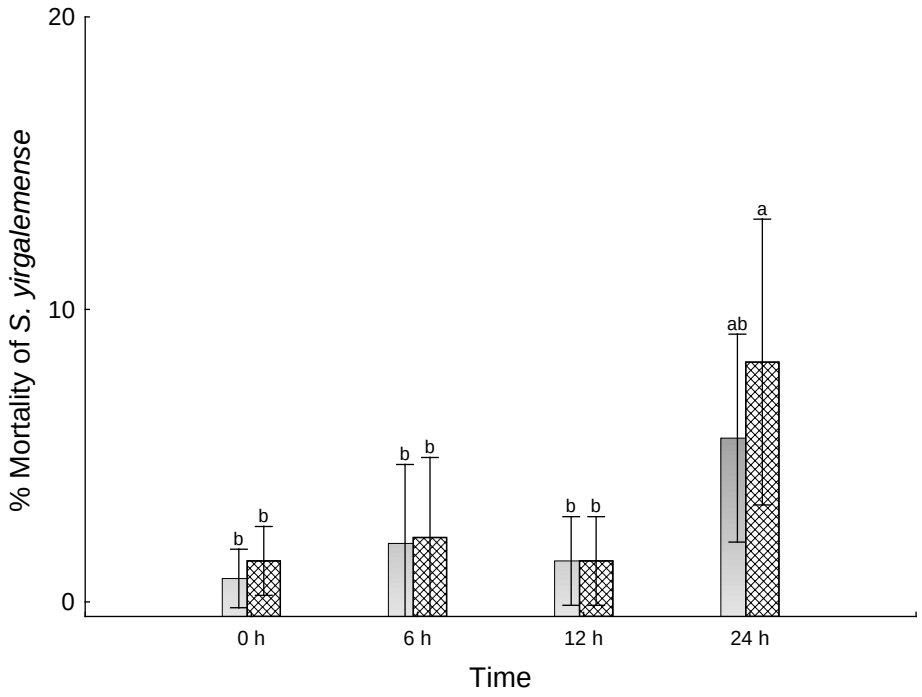


Fig. 2.4: Mean percentage (95% confidence) mortality *Steinernema yirgalemense* after exposure to water only ( ) and imidacloprid over time (one-way ANOVA; $F_{(3,72)} = 0.500$, P = 0.683). Means with the same letter above bars () are not significantly different.

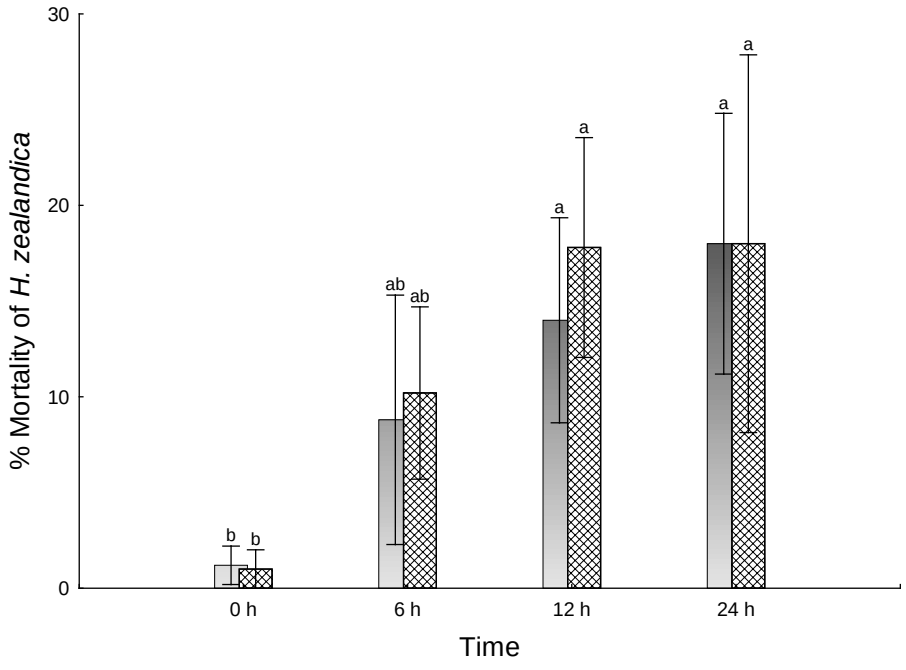


Fig. 2.5: Mean percentage (95% confidence) mortality *Heterorhabditis zealandica* after exposure to water only ( ) and to imidacloprid ( ) over time (one-way ANOVA; $F_{(3,72)} = 2.258$, p = 0.856). Means with the same letter above bars are not significantly different.

Imidacloprid exposure also did not affect the infectivity of EPNs (data not shown). These results suggest that Imidacloprid is compatible with *S. yirgalamense* and with *H. zealandica* and that it can be successfully used with their applications as it does not have a significant effect on the survival and infectivity of the nematodes concerned.

Discussion

EPNs have been successfully used for the control of a wide range of soil stages of pest insects. In South Africa, the WAA attacks both the aerial parts and the roots of apple trees. Damavandian and Pringle (2007), in studying the field biology of the WAA under South African conditions, found that annual migration from the roots to the aerial parts of the tree leads to the replenishment of the greatly redused winter population. Effective control of the subterranean root population would lead to reduced damage to the roots, as well as to less damage to leaf axils and young growth in the aerial parts of the tree during the summer months.

The current study has shown that *S. yirgalamense* has the greatest infection rate (48%) for the adult soil stage of WAA of all seven nematode isolates tested. *S*uccessful nematode infection and mortality of WAA with treatments of *S. yirgalamense, H. zealandica* and the commercial nematode, *S. feltiae* were obtained. However, in all cases studied the degree of infection associated with mortality was <50%. The other nematode isolates tested were less successful, although in some cases penetration, recovery from the non-feeding IJ to the feeding stage and development into the adult stage was also observed.

In a previous study, Brown *et al*. (1992) applied *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982 against edaphic populations of WAA in both laboratory and field studies. Despite nematodes being found within the body cavity of several aphids, no nematode reproduction was found to have occurred in the host. The researchers concerned concluded that the WAA mortality might have been caused by the infection of the aphids, either as a result of the symbiotic bacteria associated with the EPNs, or as a result of

the physical damage caused by the nematodes themselves. In field studies, Brown *et al*. (1992) were able to show a significant decrease in the number of WAA colonies on roots in response to the application of EPNs.

Results from the current study are in contrast to those that were obtained with a recent study undertaken by Berkvens *et al*. (2014) who found no control of the aerial adult stages of WAA, together with no recovery of IJs to the feeding stage after penetration of the body cavity of adult WAAs in laboratory studies. The researchers tested six commercial EPN species including *S. feltiae*. However, in the current study, nematodes were clearly responsible for the WAA mortality involved, and they did enter the aphid's body cavity where they recovered to the feeding stages and developed into adults. The use of these nematodes to control WAA might well therefore be highly relevant since the present investigation showed that both adult and intermediate stages of WAA from apple tree roots can be killed, with nematodes developing up to the adult stages under laboratory conditions.

The bioassays undertaken on the effect of WAA size on infectivity indicated no mortality of WAA crawlers caused by *S. yirgalamense* and *H. zealandica*. The small size of crawlers (0.6 mm) can possibly limit penetration, attractiveness and recognition. Adults (± 1.8 mm) were found to be the most susceptible to infection. The intermediates were also susceptible to infection, although at lower rates than were the adults and only in the case of *S. yirgalemense*. WAA belong to the Hemimetabola group of insects that are characterised by gradual and usually incomplete metamorphosis, with no evidence of a change occurring in the physiological status of the different soil stages involved. In the case of mealybugs (Pseudococcidae) size was also shown to have an effect on insect mortality (Stokwe & Malan, 2015). In a study by Bastidas *et al*. (2014) insects smaller than 5 mm were regarded as micro-insects. The results of their study indicated that invasion decreased in tandem with a decrease in insect size. The different sizes of WAA used in the present study ranged from 0.6 to 1.9 mm, indicating them to be much smaller than the micro-insects used by Bastidas *et al*. (2014), with the crawlers concerned being shorter than both of the nematode species

studied. Therefore, it is not surprising that none of the crawlers were infected with nematodes.

To complete their life cycle, EPNs must invade and kill the host, the invader nematodes have to develop into adult stages and produce offspring, and new nematodes need to become IJs, and to emerge from the cadavers in search of a new host. Poor host attraction and penetration capability may be responsible for limitation of the increase in mortality in response to increasing nematode concentrations. The results obtained in the current study suggest that EPNs do invade and kill WAA, but that their life cycle cannot be completed. This could be related to the insect size and its defence mechanism.

The inability of *S. yirgalamense* to complete its reproductive cycle within WAA limits its effectiveness for use as a biological control agent against this pest species. Determining the factors responsible for the reproductive failure of this nematode might provide greater insight into what constitutes an acceptable host for a given nematode. The consistent mortality response of *E. lanigerum* to *S. yirgalemense*, *H. zealandica* and *S. feltiae* IJs indicates that only a small number of IJs were able to penetrate the host. Although the life cycle of *Steinernema* has been studied in model organisms like *G. mellonella* and other insects, only a few studies (Schroeder, 1987; LeBeck *et al*., 1993) have focused their efforts on investigating how this process changes when the potential hosts are too small to support nematode populations. *Steinernema* does kill small hosts, with their efficiency in controlling thrips and other small insects being the result of inundative releases (Ebssa *et al*., 2001; Premachandra *et al*., 2003; Bastidas *et al*., 2014; Stokwe & Malan, 2015), or of high-frequency applications (Belay *et al*., 2005).

The NBTA plate screening of associated bacterial symbionts of the nematode isolates clearly demonstrated that all 14 tested symbiotic bacteria were unable to grow in the WAA haemolymph. The isolates involved included four *Photorhabdus* isolates, of which two were from the *Heterorhabditis* genus (*H. noenieputensis* and *H. safricana*) and indigenous to South Africa and 10 *Xenorhabdus* isolates of which six were from indigenous species (*S.*

khoisanae, S. sacchari, S. jeffreyense, S. citrae and two undescribed species). This inability of the symbiotic bacteria to grow in the haemolymph of the WAA implies that they are unable to reproduce and to convert the inside of the insect into a food source for the nematodes, while simultaneously producing endotoxins to kill the WAA effectively. These results correlate with the poor efficiency of EPNs to kill WAA, and it also explains why still living, yet successfully colonised WAA were observed. However, as no bacterial development was noted within the WAA haemolymph, it can be concluded that the most probable cause of death would have been the lack of a bacterial food source that would have enabled nematode development to take place in the WAA cadaver. Berkvens *et al*. (2014) indicate that the EPNs that they studied failed to control the aerial adult WAAs, ascribing such failure to the lack of bacterial development taking place. However, their non-recovery of IJs from the body of the host led them to speculate that the antibacterial activity found to have occurred in the haemolymph of the WAAs was the cause of the unsuccessful colonising of the host nematode recovery and development. They also suggested the necessity of testing the edaphic populations from other regions of the world.

The relationship between the bacterium and EPNs is well-known to be highly specific with the latter only maintaining mutual associations with their cognate bacteria or with very closely related strains (Eleftherianos *et al*., 2010). Phylogenetic analysis assigned the bacteria isolated from the *Heterorhabditis* and *Steinernema* nematodes, respectively, into the *Photorhabdus* or *Xenorhabdus* genus. EPNs depend on their associated bacteria to a greater or lesser extent for the death of the host, and for their nutrition (Han & Ehlers, 2000; Ciche *et al*., 2006). Although most EPNs highly depend on their symbiotic associate bacteria for their success in killing the host, some species such as *S. carpocapsae* are able to kill their host in the absence of such bacteria (Chiche *et al*., 2006). In the current study, *S. yirgalemense, S. feltiae* and *H. zealandica* were able to penetrate the host, where they developed into adults and killed off the adult WAA, without the assistance of the associated bacteria. Therefore, the failure of the nematodes to reproduce and their death can be

ascribed to the failure of the bacteria to digest the host tissue and thereby to provide suitable nutrient conditions for nematode growth (Burnell & Stock, 2000) and development, thus leading to the starvation and death of the adults concerned.

The toxicity of imidacloprid to natural enemies is a key factor to its acceptability in pome fruit IPM. IJs can tolerate short-term exposure (2-24 h) to many chemical and biological insecticides, fungicides, herbicides, fertilisers and growth regulators that can be tank-mixed and applied together (Krishnayya & Grewal, 2002; De Nardo & Grewal, 2003; Van Niekerk & Malan, 2014). However, generalisations cannot be made, because the nematode's susceptibility depends on several factors, including the species, strain, agrochemical formulation and application dose (Grewal *et al*., 2002). Previous laboratory studies with other steinernematid nematodes (excluding *S. scapterisci*) have demonstrated compatibility with pesticides for example, imidacloprid being synergistic with *S. glaseri* (Steiner, 1929) Wouts, Mracek, Gerdin & Bedding, 1982 or *H. bacteriophora* against white grubs (Koppenhöfer *et al*., 2000). The results of the effect of imidacloprid on the infectivity and survival of *S. yirgalamense* and *H. zealandica* tests suggest that imidacloprid does not affect nematodes at the rates used, making its use compatible with the biological control of WAA and other pests in the orchard. Although this study did not directly test tank-mixing effects, the potential for tank-mixing does seem to exist in respect to the use of Imidacloprid.

In summary, the results for this study demonstrate that some species and strains of EPNs have an effect on WAA mortality under laboratory conditions, although they have no effect on the crawler stage, which is regarded as too small for nematode penetration. Higher concentrations of nematodes and the lengthening of exposure time have no effect on WAA mortality. This study confirms the failure of the nematode symbiotic bacteria to develop to any degree in the haemolymph of the WAA, which leads to the death of the developing nematodes. Previous research (Damavandian, 2000) suggests that a number of different insect pathogenic fungi have been recorded as infecting WAA. Future research should be aimed at the effect of entomopathogenic fungi *Beauveria bassiana* (Eco-Bb® strain R444*)*

and *Metarhizium anisopliae* (ICIPE 69) available as commercial products individually or in combination with EPNs as possible biocontrol agents.

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CHAPTER 3

The use of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, for the control of *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae)

Abstract

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) is an important pest of apples in the Western Cape province of South Africa and in other apple-growing areas of the world. WAA has the potential for rapid genetic change, enabling it to complete multiple generations per year. As different classes of chemicals have come into use, the aphid has changed from a rare resident of orchards to being a severe pest. Although biological control of the aerial populations has been extensively studied, little is known of the natural mortality factors that regulate edaphic populations. In this study, the pathogenicity of five isolates of entomopathogenic fungi, including three *Beauveria bassiana* and two *Metarhizium anisopliae* isolates, were tested against adult female WAA in laboratory bioassays. *Steinernema yirgalamense* (80 IJs/cm^2) *B. bassiana* (Eco- Bb® strain R444) (1g/L) and *M. anisopliae* (ICIPE 69) (200 ml/ha) were applied using a single-dose bioassay. The mortality of WAA was determined after 7 days. All five fungal isolates caused significantly higher mortality of WAA compared to the control treatment, and there were significant differences in mortality amongst the different fungal isolates. The commercial isolates *M. anisopliae* (ICIPE 69) (74.2% ± 3.7%) and *B. bassiana* (Eco-Bb strain R444) (70% ± 6.9%) were the most pathogenic. The LT_{50} for Eco-Bb strain R444 and ICIPE 69 were found to be 57.7 and 45.4 h, respectively.

Introduction

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), which is native to eastern North America, has become a cosmopolitan pest of apples. Feeding on tree bark and roots, it reduces vegetative, and hence production capacity (Brown, 1986; Brown *et al.*, 1995; Pringle & Heunis, 2001). Their feeding activity causes hypertrophic gall formation on the roots and limbs of trees (Brown *et al.*, 1991; Heunis & Pringle, 2003. During spring and summer the crawlers move up the trunks of the trees to settle in leaf axils, pruning wounds and injured bark where they complete their development (Damavandian & Pringle, 2007). They remain there as adults with their parthenogenetic reproduction giving rise to colonies where the original crawlers settled. These aphids produce white waxy filaments, making the colonies in which they live highly visible. Both features (woolly protective layer and hidden life forms) make them particularly difficult to control (Beers *et al*., 2007). WAA control in South Africa is currently a combination of resistant rootstocks, chemical and biological control (Gilomee *et al*., 1968; Heunis & Pringle, 2003; Pringle, 1998; Walker, 1985).

The hypocrealean entomopathogens are ubiquitous microorganisms attacking various arthropods by means of causing acute mycoses (Hajek & St Leger, 1994). They can spread fast horizontally among host populations via aerially produced conidia, infecting their host by means of penetration of the cuticle with germ hyphae. After crossing the insect integument, they grow within the internal fluids, depleting the nutrients of degraded proteins and fat bodies, and producing toxins that kill the host. After the host's death, the mycelium grows throughout the cadaver and protrudes outside completing the life cycle by forming abundant conidia (Hajek & St Leger, 1994). Many strains of entomopathogenic fungi have been isolated and tested on different insect pests in a variety of cropping systems (Legaspi *et al.*, 2000; Leland *et al*., 2005; Pu *et al*., 2005), To date, several fungal strains have been successfully licensed for commercial use against whiteflies, aphids, thrips and numerous

other pests (Shah & Pell, 2003). However, the development of microbial agents against WAA has so far drawn only marginal attention.

Damavandian (2000) sampled and screened various apple orchards and surrounding refugia for fungal isolates that exhibit potential for use in the control of the soilborne life stages of WAA. No reports have as yet been written on the evaluation of indigenous pathogenic fungi isolates against WAA. This chapter is aimed at: (1) screening of indigenous isolates, *M. anisopliae* and *B. bassiana*, and the commercial formulation of *M. anisopliae* and *B. bassiana* for use against WAA under laboratory conditions and (2) determining of the lethal dose and time of the most promising isolates.

Materials and methods

Origin of WAA

Apple roots infected with WAA were collected from an apple orchard in Grabouw and maintained in the laboratory in plastic containers in the dark at 25°C. The roots were periodically sprayed with distilled water to keep the relative humidity (RH) high. Only adult or last-stage WAA nymphs were used in the experiments. The aphids were gently removed by encouraging them to withdraw their feeding tube, by means of gentle prodding with the bristles of a fine brush. In the case of the experimental treatment of the leaves, apple branches with WAA colonies were collected from an apple orchard at Grabouw experimental station.

Origin of EPF

Commercial isolates *Beauveria bassiana* (Eco-Bb strain R444) and *Metarhizium anisopliae* (ICIPE 69) were obtained from the manufacturers concerned, being Plant Health Products, KwaZulu-Natal, South Africa and Real IPM, South Africa, respectively. They were used following the instructions given on the label. *Beauveria bassiana* isolates PPRI 6756 and PPRI 6383 that had previously been isolated (Damavandian, 2000) from infected WAA

were retrieved from the South African National Collection of Fungi at the Plant Protection Research Institute, Agricultural Research Council situated in Pretoria, South Africa. One additional *Metarhizium* isolate (R4 T_1-T_4), isolated from soil samples collected in an apple orchard using *Tenebrio molitor* (Lepidoptera: Pyralidae) was also tested.

Fig. 3.1. Cultures of two of the investigated isolates. A. *Beauveria bassiana* (PPRI 6756) and B. *Metarhizium anisopliae* (R4 T1-T4).

Conidia preparation

Fungi were cultivated on a selective medium consisting of Sabouraud Dextrose Agar (SDA) (60 g Sabouraud Dextrose Agar, 1 ml Diodine, 50 mg Chloramphenicol, 50 mg Rifampicin, 1 l distilled water) to encourage fungal growth (Meyling, 2007). Five Petri dishes (90-mm-diam.) of each culture were incubated for 21 days at 25 ±2°C with a photoperiod of 16:8 (L:D) h. Conidia of 15-day-old cultures were harvested and suspended in 50 ml sterile distilled water with a drop of Tween™ 20 (Difco™). The bottles were closed and vortexed for approximately two minutes to produce a homogenous suspension prior to the determination of the concentration and viability of the suspensions (Goettel & Inglis, 1997).

The concentration of the suspension was determined using a heamocytometer. Prior to use, the chamber and cover slips were thoroughly rinsed with 70% ethanol. Two counts were made for each replicate using a 1/100 dilution (Goettel & Inglis, 1997). The average

count was used in further calculations. The concentration of the original suspension was then determined using the following formula (Goettel & Inglis, 1997): Conidia.ml^{-1} = df × d × c, where: d f = dilution factor; d = dilution; c = average number of conidia counted.

A conidial concentration of 1 × 10^{8} conidia.ml^{-1} was used as a single dose for the maximum challenge bioassay. This concentration was chosen based on previous bioassays that were conducted by researchers in the field of EPF (Kanga *et al*., 2003; Rodriguez *et al*., 2009; Shaw *et al.*, 2002).

Viability of spores

A 100 µl conidial suspension of each isolate was spread onto SDA plate in a laminar flow cabinet. The plates were allowed to dry in the cabinet for ten minutes. A clean, sterile cover slip was placed in the centre of each plate which was subsequently incubated at 26°C in complete darkness. Percentage germination was determined by means of counting 100 spores using a compound microscope under 40 × magnification after 24 h (Ekesi *et al*., 2002). A spore was considered to have germinated if its germ tube became longer than the spore diameter. Only fungal cultures in which more than 90% of the conidia germinated were used in the bioassay. Each plate served as a replicate and there were four replicates per isolate.

Pathogenicity test for virulence

A dipping bioassay was used to assess the virulence of each of the five EPF isolates in order to identify the most virulent strains for further evaluation (Abaajeh, 2014). A group of 10 adult WAA was dipped in a 3-ml conidial suspension for 30 s in a Petri dish at a concentration of 1 × 10^{8} conidia.ml^{-1}.The treated larvae were placed in a Petri dish, sealed with Parafilm and incubated at 25 ±2°C in the dark. The control WAA was dipped in sterile distilled water containing a drop of 0.05% Tween^{TM} 20. The treatments were replicated six times. After the dead insects were surface-sterilised in 70% ethanol, they were transferred

onto SDA (Abaajeh, 2014). Mortality was recorded at seven days post-treatment. The experiment was repeated on a different test date with a fresh batch of conidia.

Leaf exposure

Adult WAA collected from the leaf axils in an apple orchard formed the basis of the establishment of WAA colonies on twigs, which were kept at room temperature in a closed plastic container lined with moist filter paper. Leaves were placed separately in plastic containers. About 20 adult WAA were placed on a twig, where they were given two days in which to settle. Six replicates were used for each treatment with there being a total of 36 containers (n = 720 insects). The twigs with aphids attached were dipped in a 10 ml fungal solution at a concentration of 1×10^{8} conidia.ml^{-1} for about 30 seconds. The control treatment received water only. Observations of mortality were carried out seven days post-treatment. Aphid cadavers were sterilised in 70% ethanol and then transferred onto solid SDA plates and observed for mycosis to confirm fungus as causal agent of insect mortality. The experiment was repeated on a different test date with a fresh batch of conidia.

Fig. 3.2. Experimental set-up of *Eriosoma lanigerum* colonies established on an apple twig

Root exposure

WAA were established on pieces of apple roots collected from the field. A piece of moist filter paper was placed in the bottom of the container. Roots were placed separately in plastic containers. About 20 adult WAA were placed on a piece of root where they were given two days in which to settle. Six replicates were used for each treatment with there being a total of 36 containers (n = 720 insects). The roots, with aphids attached were dipped in a solution of the five different EPF at a concentration of 1×10^8 conidia.ml^{-1} for about 30 seconds. Observations of mortality were carried out daily for seven days after treatment. Aphids that died during this period were surface-sterilised in 70% ethanol and transferred onto SDA so as to enable the observation of mycosis. The experiment was repeated on a different test date with a fresh batch of conidia.

Fig. 3.3. Experimental set-up for *Eriosoma lanigerum* colonies established on a piece of apple root.

Concentration and time exposure

To determine the effect of EPF concentration and exposure time, the most virulent isolates, Eco-Bb strain R444 and ICIPE 69 were used. Concentrations of 1×10^7, 1×10^8, 1×10^9 and 1×10^{10} conidia ml^{-1} were applied to adult WAA at four different time periods,

being 12, 48, 72 and 96 h after the start of the investigation, following the same procedure as described above for the pathogenicity test. The WAA were placed in a Petri dish (9-cm-diam.). Twenty WAA were used for each concentration of each EPF isolate with the control being treated with distilled water containing a drop of 0.05% Tween^{TM} 20. Treated Petri dishes were sealed with Parafilm and incubated at 25°C. All treatments and their control were replicated six times with 20 insects per replicate. Dead insects, after being surface-sterilised in 70% ethanol were transferred onto SDA so as to enable the observation of mycosis.

Statistical analysis

For screening trial results, the mortality percentage data were subjected to analysis of variance (ANOVA). All the analyses conducted were executed using the data analysis software system STATISTICA 11.0 (StatSoft Inc., 2008). Probit analysis was conducted using Polo and Software NCSS 2007 ver. 07.1.1 (Hintze, 2007).

Results

Viability of spores

Viability tests showed that more than 90% of the spores of all the isolates germinated within 24 h under laboratory conditions.

Pathogenicity test on virulence

All five tested fungi isolates were pathogenic to WAA which resulted in the WAA mortality being significantly higher than was the mortality experienced with the control ($F_{5,30}$ = 28.43, P<0.01). The mortality induced by the fungi (Fig. 3.4) after seven days of incubation varied from 47.5 ± 3.8% (PPRI 6756) to 74.2 ± 3.7% (ICIPE 69) (Fig. 3.5). The most virulent isolates Eco-Bb strain R444 and ICIPE 69 were ultimately able to kill >70% of the initial WAA individuals seven days after inoculation, with no significant differences being found between the two isolates.

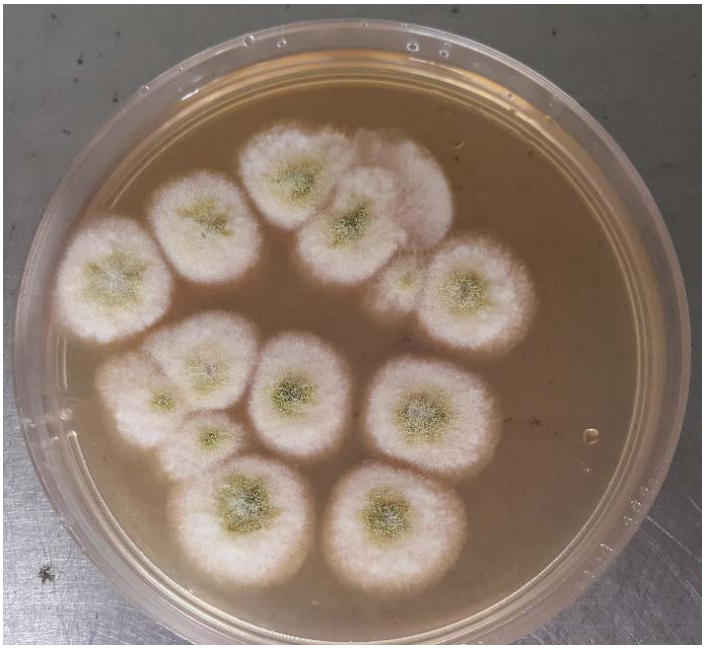


Fig. 3.4. *Eriosoma lanigerum* cadavers showing signs of fungal mycosis, after incubation on SDA plates post treatment with *Metarhizium anisopliae*.

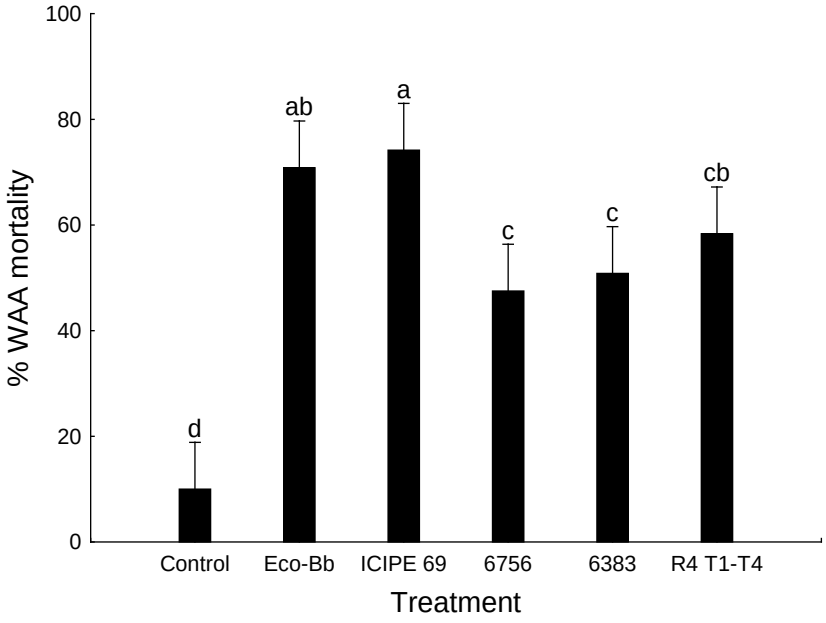


Fig. 3.5. Mean mortality (95% confidence limit) of adult *Eriosoma lanigerum* (WAA) exposed to *Beauveria bassiana* (Eco-Bb strain R444), *Metarhizium anisopliae* (ICIPE 69), PPRI 6756 (*B. bassiana*) and PPRI 6383 (*B. bassiana)* and R_4 T_1-T_4 (*M. anisopliae*) at conidial concentrations of 1 × 10^8 conidia ml^{-1} after seven days (one-way ANOVA; $F_{5,30} = 28.43$, $P < 0.01$). Different letters above bars are indicative of a significant difference.

Leaf exposure

All five EPF isolates were found to infect WAA and to have caused significantly higher mortality compared to the control seven days post inoculation ($F_{3,30} = 13.1$, $P < 0.05$). The presence of mycelia on all the fungal-inoculated, surface-sterilised WAA cadavers indicated that the dead aphids had been colonised by the fungi. On average, the commercial isolates of *B. bassiana* and *M. anisopliae* performed better (60 ± 7.3% and 65 ± 13.8% respectively) than did the non-commercial isolates, PPRI 6756, PPRI 6383 and R_4 T_1-T_4, 40.8 ± 11.6%, 42.5 ± 9.4% and 50 ± 8.9%, respectively (Fig. 3.6).

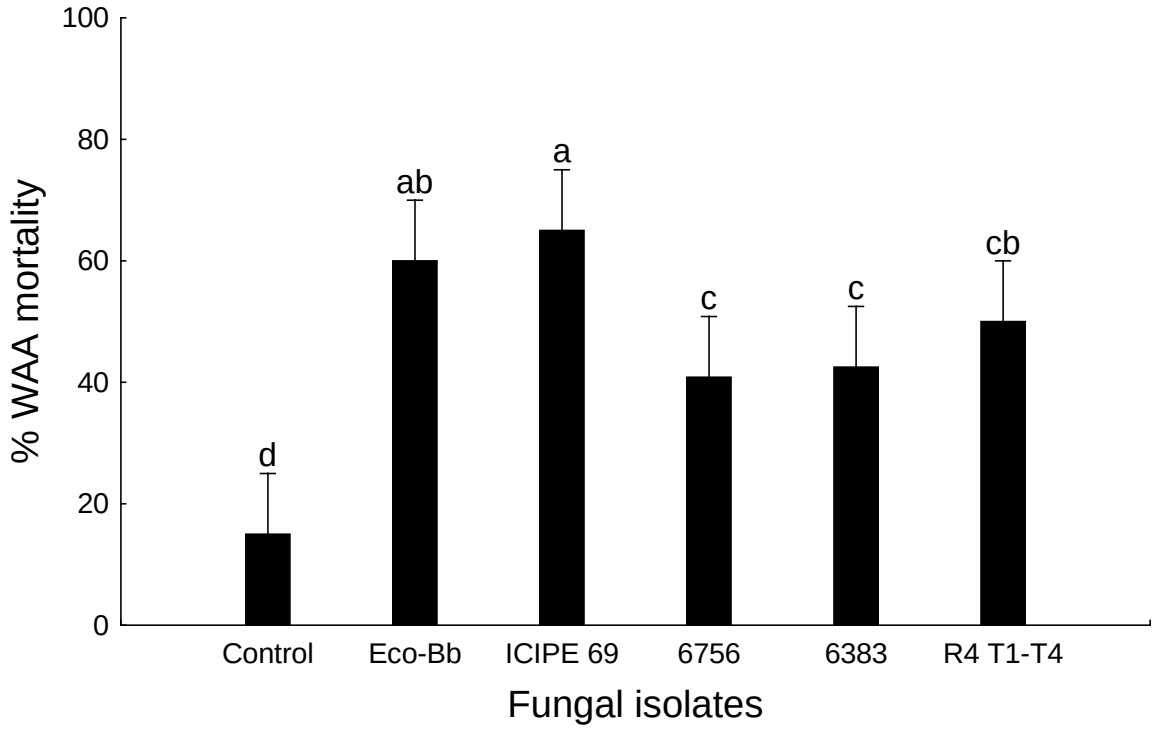


Fig. 3.6. Mean mortality (95% confidence limit) of adult *Eriosoma lanigerum* (WAA) treated with *Beauveria bassiana* (Eco-Bb strain R444*), Metarhizium anisopliae* (ICIPE 69), PPRI 6756 (*B. bassiana*) and PPRI 6383 (*B. bassiana*) and R_4 T_1-T_4 (*M. anisopliae*) after treatment with a concentration of 1×10^8 conidia ml^{-1} on apple twigs (one-way ANOVA; $F_{3,30} = 13.1$, $P < 0.05$). Bars with the same letter indicate that the difference is not significant.

Root exposure

Results from the roots bioassay showed that all the EPF isolates infected the WAA ($F_{5,30}$ = 9.4, P < 0.05) with varied degrees of mortality (36.7 ± 10.8% – 53.3 ± 9.8%) occurring among the fungus-treated WAA (Fig. 3.7). However, the commercial isolates ICIPE 69 and Eco-Bb strain R444 were the most pathogenic causing 55 ± 13.0% and 53.3 ± 9.8% mortalities, respectively. The least pathogenic strains were found to be PPRI 6756 and R4 T1-T4, which had induced 36.7 ± 10.8% and 40 ± 7.4% mortality, respectively, among the adult WAA seven days after treatment (Fig. 3.7).

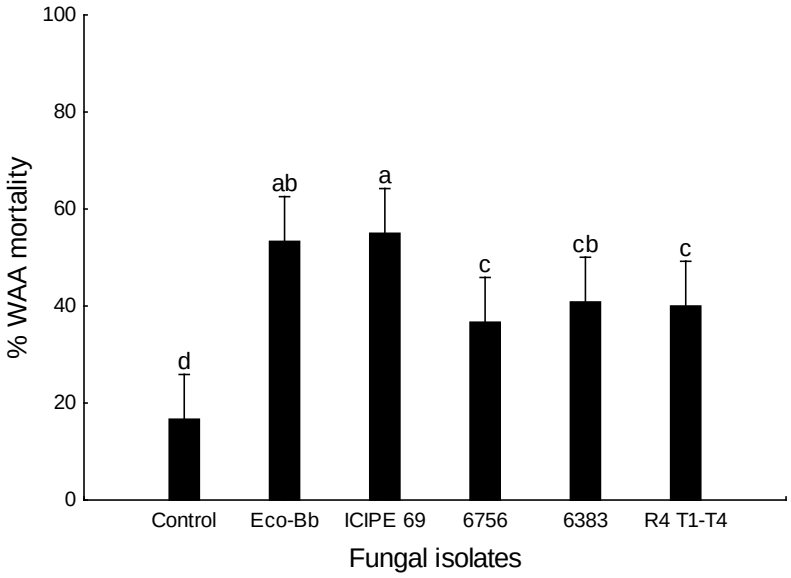
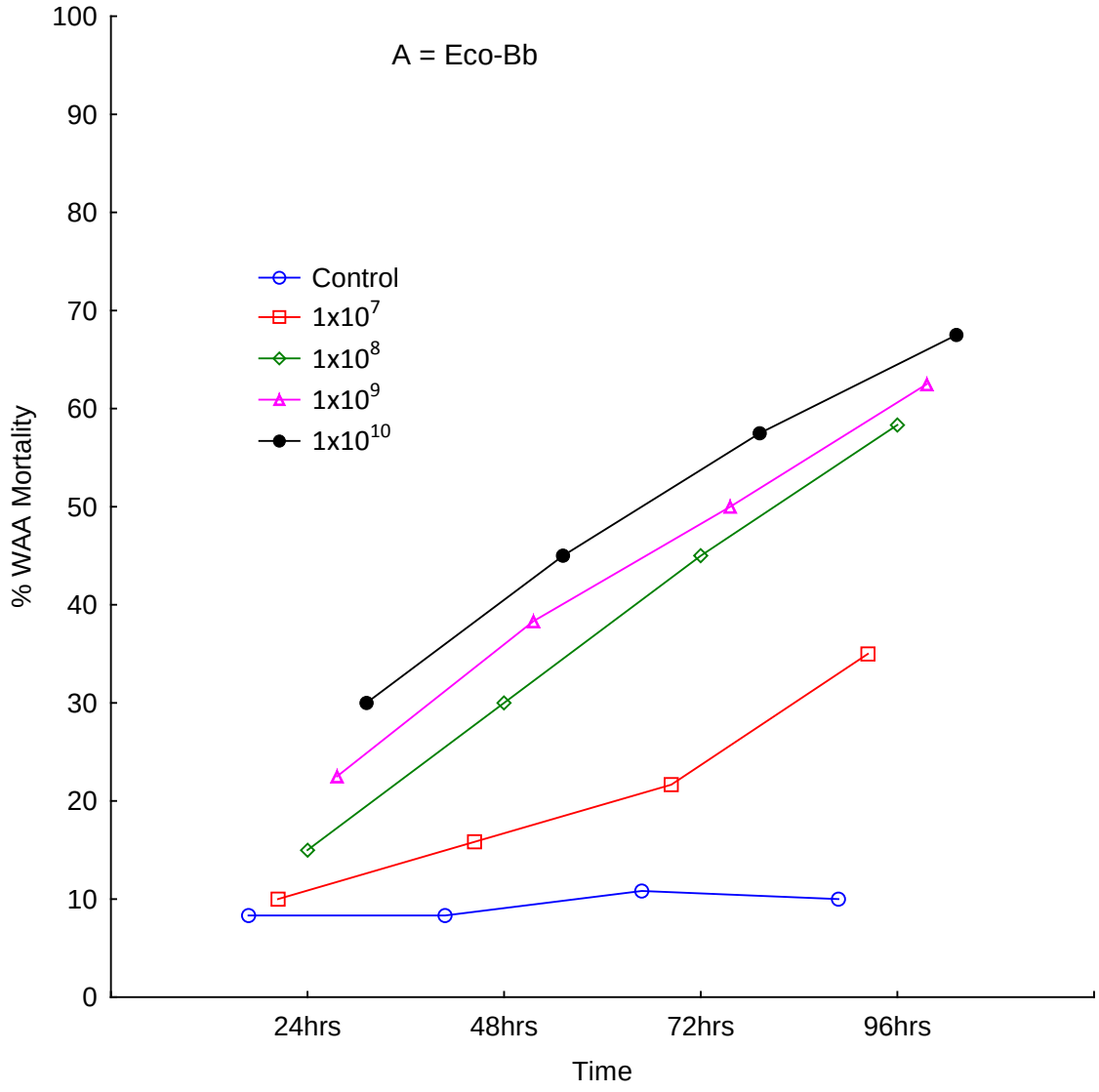


Fig. 3.7. Mean mortality (95% confidence limit) adult *E. lanigerum* (WAA) treated with *Beauveria bassiana* (Eco-Bb strain R444*), Metarhizium anisopliae* (ICIPE 69), PPRI 6756 (*B. bassiana*) and PPRI 6383 (*B. bassiana*), and R_4 T_1-T_4 (*M. anisopliae*) at a concentration of 1 × 10^8 conidia ml^{-1} on pieces of apple root (one-way ANOVA; $F_{5,30}$ = 9.4, P < 0.05). Bars with the same letter indicate that the difference is not significant.

Concentration and exposure time

Concentration and exposure-time-response trials of WAA adults exposed to four conidial concentrations, at four different exposure times, showed a positive relationship. An increase in the total percentage of mycosis was observed with an increase in exposure time and concentration. Figure 3.8A shows the mean percentage mortality caused by Eco-Bb strain R444 at different rates of conidial concentration and for different exposure periods.

The results show certain levels of variability in virulence with no significant differences being detected among the different conidial concentrations and for the different exposure periods ($F_{9,80}$ = 0.43, P = 0.91). Mortality ranged from 10 ± 5.53% (1×10^7 conidia ml^{-1}; 24 h) to 67.5 ± 5.53% (1×10^{10} conidia ml^{-1}; 96 h). The same trend was observed for ICIPE 69 ($F_{9,80}$ = 0.62, P = 0.78) where the lowest mortality of 11.7 ± 4.84% was recorded at a concentration of 1×10^7 conidia ml^{-1} and for an exposure time of 24 h whereas the highest mortality of 68.3 ± 4.84% was recorded after 96 h at a concentration of 1×10^{10} conidia ml^{-1} (Fig. 3.8B.)



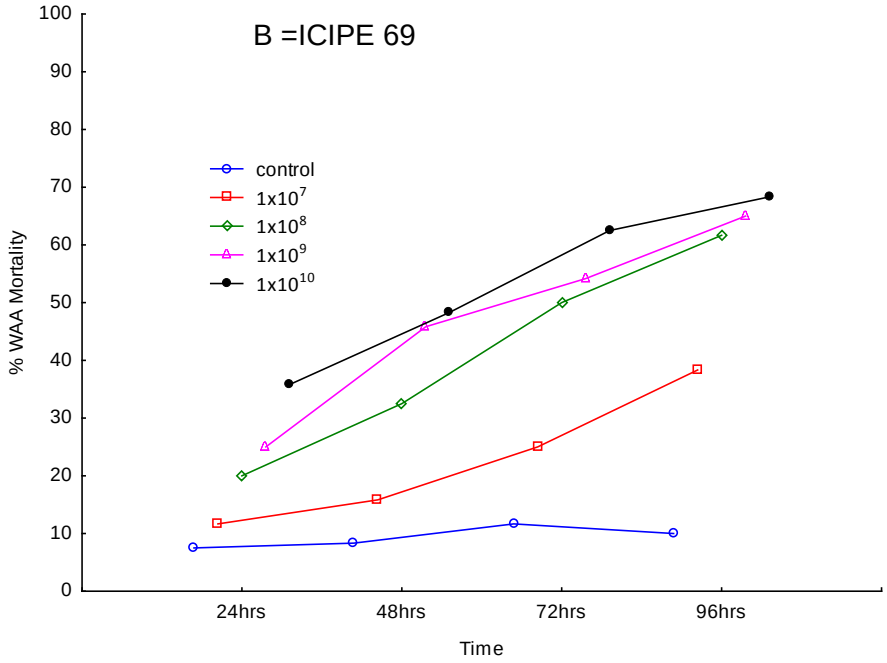


Fig. 3.8. Mean percentage mortality (95% confidence level) of *Eriosoma lanigerum* (WAA) adults at different levels of conidia concentration after 24, 48, 72 and 96 h of exposure, (A) *Beauveria bassiana* (Eco-Bb strain R444) (one-way ANOVA; $F_{(9,80)}$= 0.43, P = 0.91) and (B) *Metarhizium anisopliae* (ICIPE 69) (one-way ANOVA; $F_{(9,80)}$ = 0.62, P = 0.78).

The LT_{50} and LT_{90} were determined (Table 3.1) using Probit analysis. The analysis indicated that at the higher concentration of 1×10^{10} conidia.ml^{-1}, an exposure time of 57.7 h and of 364.1 h was required to obtain an LT_{50} and LT_{90} respectively for Eco-Bb strain R444 (Table 3.1). In the case of ICIPE 69 it was indicated that at the higher concentration of 1×10^{10} conidia.ml^{-1}, an exposure time of 45.4 and 359.1 h was required to obtain an LT_{50} and LT_{90}, respectively (Table 3.1).

Table 3.1. LC_{50}, LC_{90}, LT_{50} and LT_{90} estimated for Eriosoma lanigerum (WAA) after treatment with *Beauveria bassiana* Eco-Bb strain R444*)* and *Metarhizium anisopliae* (ICIPE 69) at a concentration of 1×10^{10} conidia ml^{-1} over 96 h under laboratory conditions.

Fungal isolate	**LC_{50}**	**LC_{90}**	**LT_{50} (h)**	**LT_{90} (h)**
Metarhizium anisopliae (ICIPE 69)	1×10^{8}	1×10^{14}	45.4	359.2
Beauveria bassiana (Eco-Bb strain R444)	1×10^{8}	1×10^{14}	57.7	364.1

Discussion

Bioassays are the starting point of any biological investigation where virulence is of importance. This allows for various measures including the lethal time and concentration values to be determined. As the virulence of a fungus can change depending on age, growth conditions (e.g. temperature and humidity) and the media on which it is cultured, it is important to ensure that the factors in question remain constant throughout the experiment, so as to allow for the drawing of accurate comparisons (Goble *et al.,* 2011). Although EPF have great promise for use as biological control agents against different insects, their infectivity varies significantly depending on the fungus species and on the developmental stage of the insects concerned (Samson, 1981). In most cases, fungal isolates are pathogenic to their original host or to a closely related species (Poprawski *et al*., 1985; Samuels *et al*., 1989). However, in the present instance the isolates originating from WAA (PPRI 6756 and PPRI 6383) were less pathogenic than the commercial ones (ICIPE 69 and Eco-Bb strain R444). Taking into account the results presented here, the screening of potential isolates should not be limited to those isolated from the original host. *Metarhizium anisopliae* and *B. bassiana* are ubiquitous pathogens that have been recorded on many hosts and that can therefore be tested against insects that are not associated with them in nature, enabling them to be developed as biopesticides.

Despite all the fungal isolates tested in the current study being pathogenic to adult WAA, the degree of virulence found between the different fungal isolates varied. The variations might have been due to fungus-specific characteristics. Such variations have already been reported with different host species, including coleopterans (Ansari *et al*., 2004; Ferron *et al*., 1975) with the result that there is a marked need for strain selection.

The WAA dipped in fungal suspension were more susceptible than were those that were sprayed on either leaves or roots. The higher mortality of WAA treated by means of the dipping method might be due to the fact that the larvae tend to collect high quantities of conidia during immersion (Boucias *et al*., 1988; Fernandez *et al*., 2001). EPF could offer the

possibility of great potential for the control of WAA considering the aggregating behaviour of the insect concerned. Fungus-infected insects could spread the pathogen among healthy insects thereby initiating epizootics.

When a particular insect pest control programme is considered to be worth using, the dose and time of exposure of the host to the insect fungus as well as the amount of time that is taken to kill the host are important parameters for evaluating its virulence. Fungi that require a relatively short exposure period and that are capable of killing off the host quickly are key to practical application. It is therefore important that the activity of selected fungus isolates should be screened against the particular target host during the initial stage. The existence of a pathogenic relationship might intimate the virulence of the species and the concentration of fungus required to kill at least 50% or more of the insect pests involved.

In the present study, the degree of mortality caused by each fungus was low at relatively low conidial concentrations and exposure times, and increased with the heightening of the conidial concentration and exposure time; revealing the existence of a dose and time-dependent relationship. The relationship in question has been reported in a number of other studies (Anand *et al.*, 2009; Begemann, 2008; Coombes *et al*., 2015; Hafez *et al.*, 1997). Anand and Tiwary (2009) observed the highest mortality of 2nd instar larvae of *Spodoptera litura* (Fabricius) at the highest conidial concentration of fungal isolates. The median lethal time (LT_{50}) of *Spodoptera littoralis* (Boisduval) showed prolongation with a decrease in the tested concentrations of fungi, *M. anisopliae* (Abou-Bakar, 1997). Similarly, Amer *et al.* (2008) found that the mortality of *S. littoralis* larvae increased with raised conidial concentrations and with the amount of time that elapsed after treatment.

Eco-Bb R444 and ICIPE 69 were found not to vary significantly in their effect against WAA and in the case of both a relatively long period of incubation was required to achieve full effectiveness of the tested fungal isolates before the killing of the WAA involved was achieved. Although there was indication that the fungi were capable of killing WAA after 24 h incubation, the number of dead aphids continued to increase with the elapsing of longer

periods of time. The doses obtained in this study are quite high in comparison with those used in other studies. For example, the LD_{50} of *Metarhizium* spp. in *Locusta migratoria migratorioides* (Reiche & Fairmaire) varied from 60 to 300 conidia per grasshopper (Nowierski *et al*., 1996). In his concentration-response trials against *Thaumatotibia leucotreta* (Meyrick), Goble (2009) also revealed a dose-dependent relationship with the two isolates, G 14 2 B5 and G Moss R10, involved being found to induce mycosis in 50% of the test population (LC_{50}) at concentrations of 8.8×10^5 and 6.8×10^5 conidia.ml^{-1}, respectively. Relatively steep slopes of these isolates were also observed, indicating that the target insects became more vulnerable over a given time period to increasing concentrations of conidia. On investigating eight different EPF isolates, Coombes (2012) discovered that only three (G 11 3 L6, FCM Ar 23 B3 and G Ar 17 B3) showed potential, performing well in dose-response assays with relatively low LC_{50} and LC_{90} values being coupled with low false codling moth, *T. leucotreta* eclosion percentages on exposure to a concentration of 1×10^6 conidia.ml^{-1}.

Although in the case of the current study, a relatively large number of conidia were required to cause 50% mortality of the WAA, the study nevertheless provides data indicating that the fungus strains in question as well as others do offer an alternative method for controlling WAA. Although the results obtained in this study suggest that entomopathogenic fungi, particularly *M. anisopliae* ICIPE 69 and *B. bassiana* R444 are effective against WAA, for effective control to be obtained with their use, sufficient concentrations and exposure times of the pathogens are required.

The results reported on this chapter should provide insights into whether or not EPF can be used to control WAA and opens up the possibility for including indigenous fungal strains in the control of WAA in South Africa. The results indicate that such fungi have the potential to control the pest, but further work under more realistic semi-field conditions is still required to ensure the efficacy of the isolates concerned. If successful, the use of the fungi

isolates might result in a reduction in the use of chemicals and consequently a reduction in the chemical residues on produce as well as a weakening of insecticide resistance.

The commercial isolates ICIPE 69 and Eco-Bb strain R444 were selected for further evaluation, based on the promising results that were obtained with them against WAA in the pathogenicity test described above. Based on the data presented in this study, WAA adults proved to be more susceptible to the commercial isolates than they were to the non-commercial ones, making the former potentially good candidates for further studies. This study has indicated ample opportunity for proceeding with investigations into the use of EPF for the control of WAA. As the most virulent two strains in this study are commercially available, they have the potential for use as a cost-effective and relatively straightforward means of effective control of WAA.

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CHAPTER 4

Interaction between entomopathogenic nematodes and fungi for the control of woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) and the effect of mulching in pot trials

Abstract

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is a pest of apples worldwide. In South Africa it attacks both the roots and the aerial parts of apple trees throughout the year. Entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF), alone and in combination, were evaluated for their potential to control woolly apple aphid, *E. lanigerum*, in pot trials. Local isolates of *Steinernema yirgalamense* and *Heterorhabditis zealandica* were reared *in vivo* in the laboratory and a commercial formulation of *Beauveria bassiana* (Eco-Bb® strain R444) and of *Metarhizium anisopliae* (ICIPE 69) were used. In the aerial WAA colonies, a significant reduction of 45.7% to 58.9% of WAA was observed in the application of fungi, and in the application of fungi combined with nematode treatments, compared to the control mortality. The results of the nematode-only treatment (29.2%) did not differ significantly from the results obtained with the control. In root colonies a significant reduction of 38.0% to 62.9% was recorded in the nematode and fungi individually, and in the fungi and nematode treatments combined, which differed significantly from the results that were obtained with the control treatment. The potential of using *S. yirgalamense* and *H. zealandica* together with two different mulches (pinewood shavings and apple wood chips) to control *E. lanigerum* was evaluated. Combining nematodes with different mulches did not enhance their efficacy, compared to treatments with nematodes alone.

Introduction

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is an important pest of apples in the Western Cape province of South Africa (Damavandian & Pringle, 2007) and in other apple-growing areas of the world, including the USA (Beers *et al*., 2007, 2010), Spain (Lordan *et al*., 2015), New Zealand (Sandanayaka *et al.*, 2005) and Australia (Asante, 1999). The aphid, which is of North American origin was most probably introduced into South Africa on infested plant material (Pillans, 1894).

The aphid forms densely packed colonies covered with a white, waxy, filamentous secretion on the aerial parts and roots of apple trees (Pringle, 1998; Shaw & Walker, 1996; Heunis & Pringle, 2006). Their feeding activity causes galls to form on the woody tissue and on young lateral shoots, where buds can be destroyed. These galls which are ideal feeding sites for the aphid offspring often give rise to the formation of densely packed colonies. The calyces of apples can also become infested by aphid colonies, resulting in contamination of fruit with honeydew and sooty mould (Shaw & Walker, 1996; Heunis & Pringle, 2006). The widespread employment of chemical insecticides for the control of this pest has caused such problems as insecticide resistance along with the contamination of fruit with chemical residues (Pringle *et al*., 1994). Thus, there is a growing interest in using pathogenic control agents as an alternative to chemical methods of control.

Entomopathogenic nematodes (EPNs) offer an environmentally safe alternative to chemical insecticides that are still the prevailing method employed for *E. lanigerum* control. However, not all tests that have investigated the use of EPNs against *E. lanigerum* have been successful (Berkvens *et al*., 2014). Thus, the efficacy of using EPNs for *E. lanigerum* control is considered as perhaps being capable of improvement through combining them with entomopathogenic fungi (EPF). The use of EPF for the control of agricultural pests has a relatively long history when compared to that of EPNs. Although the first reports of applications of fungi against insect pests appeared as long ago as the late 1800s, the commercialisation of products based on fungi began more recently, in the 1960s (Lacey &

Goettel, 1995). Interest in the use of EPNs in pest control programmes is now on the increase, although as with all biological control agents they are subject to certain restrictions. Of the few studies carried out on interactions between fungi and nematodes, Barbercheck and Kaya's (1990) investigation into the interaction of *Heterorhabditis bacteriophora* Poinar, 1976 and *Beauveria bassiana* (Balsamo.) Vuillemin, 1912 (Hypocreales: Cordicipitaceae), against *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae shows that a combination of the two agents causes a synergistic effect, resulting in higher host mortality than when they are used separately. Shapiro-Ilan *et al.* (2004) demonstrate that combining *Heterorhabditis indica* Karunaker & David, 1992 with *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae), 1883 at low concentrations or combining *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982 together with *B. bassiana* at high concentrations, has an additive effect on the mortality of *Curculio caryae* (Horn, 1873) (Coleoptera: Curculionidae) larvae. Although nematode-fungus combinations have generally been found to result in additive effects on target mortality, in most studies done so far the mortality caused by the individual agent was too high to allow for significant improvement (Koppenhöfer & Grewal, 2005). However, interaction between entomopathogens can also lead to antagonistic effects (Shapiro-Ilan *et al*., 2004).

Persistence is an important contributor to the success of a biological control agent against the soil-dwelling life stages of pests. With *E. lanigerum*, which is present in the soil throughout the year (Damavandian & Pringle, 2007), entomopathogens that are capable of remaining viable within the soil for an extended period might prove to be more economical than those that only remain viable for a short period. Not only have mulching and crop residue facilitated the prolonged survival of EPNs in cropping systems, but they have also enhanced their insect parasitic activity (Stewart *et al*., 1998; Sweeney *et al*., 1998; Shapiro *et al*., 1999; Wilson *et al.*, 1999; De Waal *et al.*, 2011). Other advantages of mulching include the limiting of soil erosion, the reducing of water loss by evaporation, the moderating of diurnal fluctuations in soil temperature and the increasing of the organic matter content,

porosity, water retention and nutrient availability of soil (Novak *et al*., 2000; Mathews *et al*., 2002).

Damavandian (2000) sampled and screened various apple orchards and surrounding refugia for fungal isolates that exhibited potential for use in the control of the soilborne life stages of WAA. The genera *Conidiobolus* and *Hirsutella* were continuously active in aphids washed from the soil, with differences being observed in the levels of infection of infested root samples. For instance, the highest peak of fungal infection in soil samples collected from Oak Valley occurred during August 1997, with almost 50% of the subterranean *E. lanigerum* population being found to be affected. In Molteno, the highest infection levels were found to occur during September, at which stage over 10% of the subterranean WAA in soil samples were found to be infected (Damavandian & Pringle, 2007).

The objectives of the present study were: (1) to evaluate the efficacy of the use of an EPN, two entomopathogenic fungal species, and a combination of the nematode with the fungal species against aerial and subterranean *E. lanigerum* populations in pot trials under controlled conditions and (2) to investigate the effect of mulching on the efficacy of EPNs under controlled conditions.

Materials and methods

Entomopathogenic nematodes

Following the methods described by Kaya & Stock (1997), last-instar larvae of *Tenebrio molitor* (L.) (Coleoptera: Pyralidae) were used for the *in vivo* culture of nematodes. Larvae were inoculated with infective juveniles (IJs) of either *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 or *Heterorhabditis zealandica* Poinar, 1976 and subsequently incubated at 25 ± 2°C in a growth chamber. The two species in question were chosen due to them both having shown some promise of *E. lanigerum* control in the laboratory bioassays previously undertaken (Chapter 2). Infected *Tenebrio* larvae were then placed in White traps (Kaya & Stock, 1997) with the IJs involved being collected

three days after their emergence, whereupon they were stored at 14°C in culture flasks. A final concentration of 80 IJs/cm^2 was used in all the experiments and the concentration of nematodes was calculated using the method developed by Glazer & Lewis (2000).

Entomopathogenic fungi

Commercial isolates *B. bassiana* (Eco-Bb® strain R444) and *M. anisopliae* (ICIPE 69) were obtained from the manufacturers, Plant Health Products, KwaZulu-Natal, South Africa and Real IPM, Grabouw, South Africa, respectively. The concentrations of applications used were in accordance with the instructions given on the label.

Trees and experiential conditions

The experiments were conducted on potted apple trees (*Malus domestica*) in a screen house. Granny Smith trees on M 109 rootstock or M 109 rootstocks alone were planted in 2-L plastic pots in sterile potting soil under screen house conditions. All the standard nutrient and cultural practices were applied, with the pots being watered every third day. WAA-infested roots were collected from the ARC experimental farm in Grabouw, in the Western Cape province, South Africa. After the shoots of the trees involved had grown approximately 15 cm long, they were infected by means of burying WAA-infested roots in the soil of the potted plants. A month later, the aphids had moved up the shoots, where they had become sufficiently well-established to be able to form shoot colonies.

Fig. 4.1. Potted apple plants infested with woolly apple aphid, *Eriosoma lanigerum*.

Bioassay protocol

The experiment was conducted in the months of April and May under natural conditions, in a screen house. During these months the average relative humidity had a daytime mean of 31% and a night-time mean of 94%, while the average temperature had a daytime mean of 26°C and a night-time mean of 9°C. The treatments applied were: 1) control with water only; 2) *S. yirgalemense*; 3) *B. bassiana*; 4) *M. anisopliae*; 5) *S. yirgalemense* + *B. bassiana* and 6) *S. yirgalemense* + *M. anisopliae*. The application was performed during late evening, with the EPNs being applied at a concentration of 80 IJs/cm^2, while *B. bassiana* and *M. anisopliae* were applied at their recommended dose of 1 g/l and 200 ml/ha, respectively. For each treatment there were six replicates, which were arranged in a complete randomised design.

Control of aerial colonies

Trees with visible WAA colonies were selected. On each tree, three shoot colonies were randomly tagged and all other aphids were removed so as to reduce the pest-induced pressure. Treatments were applied with a handgun sprayer to the point of run-off. The control trees were treated with water alone. Live and dead *E. lanigerum* counts were made 7

days after treatment. *Eriosoma lanigerum* cadavers with symptoms of EPN infection were dissected to confirm that the mortality involved had resulted from EPN infection. *Eriosoma lanigerum* cadavers with symptoms of fungal infection were surface-sterilised in 70% ethanol and were transferred onto Sabouraud Dextrose Agar (SDA) (60 g Sabouraud Dextrose Agar, 1 ml Diodine, 50 mg Chloramphenicol, 50 mg Rifampicin, 1 l distilled water) plates to stimulate sporulation. The experiment was repeated on a different date using fresh EPN and EPF inoculum.

Control of root colonies

Root colonies developed naturally and the trees with visible root colonies were selected. A little of the surface soil around the potted trees was scraped away, so as to confirm visually that all pots used in the trial were infected. The trees were distributed into four replicate blocks, with the treatments involved being randomly assigned within each block. Entomopathogens were applied in 500 ml of EPN or fungi solution or in 500 ml of a combination of EPN and fungi, which was applied to the soil. The volume used completely saturated the soil in the pots without loss of any of the solution. The control trees were treated with water only. The trees were watered every third day and care was taken to avoid overwatering so that the applications were not washed out of the pots. After days all the trees were lifted out of the pots and all the soil still clinging to the roots was gently washed away through a sieve with 200-µm pores, according to the technique used by Damavandian & Pringle (2007). The sieve was mounted in a rectangular stainless-steel container. This container was filled with a solution of 600 g of sugar per L of water with a higher density than that of pure water, causing the aphids to float which facilitated counting them. The container and sieve were then placed under a microscope where the aphids were counted. WAA cadavers with symptoms of EPN infection were dissected to confirm that their mortality was due to nematode infection. WAA cadavers with symptoms of fungal infection were surface-sterilised in 70% ethanol and transferred onto SDA plates to stimulate sporulation. The experiment was repeated on a different date using fresh inoculum

Effect of mulching

The effect of mulching on the efficacy of *S. yirgalemense* and *H. zealandica* was investigated. This test was conducted on potted apple trees in a screen house. M 109 rootstocks were planted individually in 2-L plastic pots in a mixture of sterile plotting soil under screen house conditions. The trees were infested by taking WAA-infested roots from the orchards and burying them in the potted plant soil. After two months during which the root colonies were allowed to develop naturally, the trees with visible root colonies were selected. Two types of mulch consisting respectively of pine wood (*Pinus radiata* D. Don shavings) chips, and of apple wood chips (*Malus domestica* (Borkh.) were evaluated. Six replicate pots of each mulch type were used for each nematode species, with non-mulched bare ground and an untreated control making up a total of 48 potted trees in all. The trees were then distributed into four replicate blocks, with the treatments being randomly assigned within each block. The appropriate mulch specific to the trial being conducted was applied on top of the soil to a depth of 5 cm. The trees were watered three days before each application. All nematode and water applications were made using calibrated shoulder pump sprayers. The experiment was repeated on a different date using fresh EPN and EPF inoculum.

Data analysis

All analysis was carried out using the data analysis software system STATISTICA 11.0 (StatSoft Inc., 2008). The results were analysed using analysis of variance (ANOVA) followed by Tukey's post hoc test, if a significant difference was reported as a result of the ANOVA.

Results

Control of aerial colonies

Significant differences ($F_{(5.30)}$ = 18.23, $p < 0.001$) were recorded among the different treatments (Fig. 4.2). After 7 days had elapsed, differences in larval mortality were observed for the entomopathogens inoculated individually and in combination (Fig. 4.2.). *Steinernema yirgalamense* was found to have caused 29% ± 3.6% mortality of WAA with no significant difference ($p = 0.644$) between it and the control mortality. *Beauveria bassiana* was found to have caused 45.7% ± 3.6% ($p < 0.005$) mortality, while *M. anisopliae* was found to have caused 46% ± 3.6% mortality ($p < 0.005$), with no significant difference being found in the effect of the two fungi ($p = 1$). When 157-C was inoculated in combination with *B. bassiana*, the insect mortality experienced increased to 55% ± 3.6%. When 157-C was inoculated together with *M. anisopliae*, the insect mortality increased to 58% ± 3.6%.

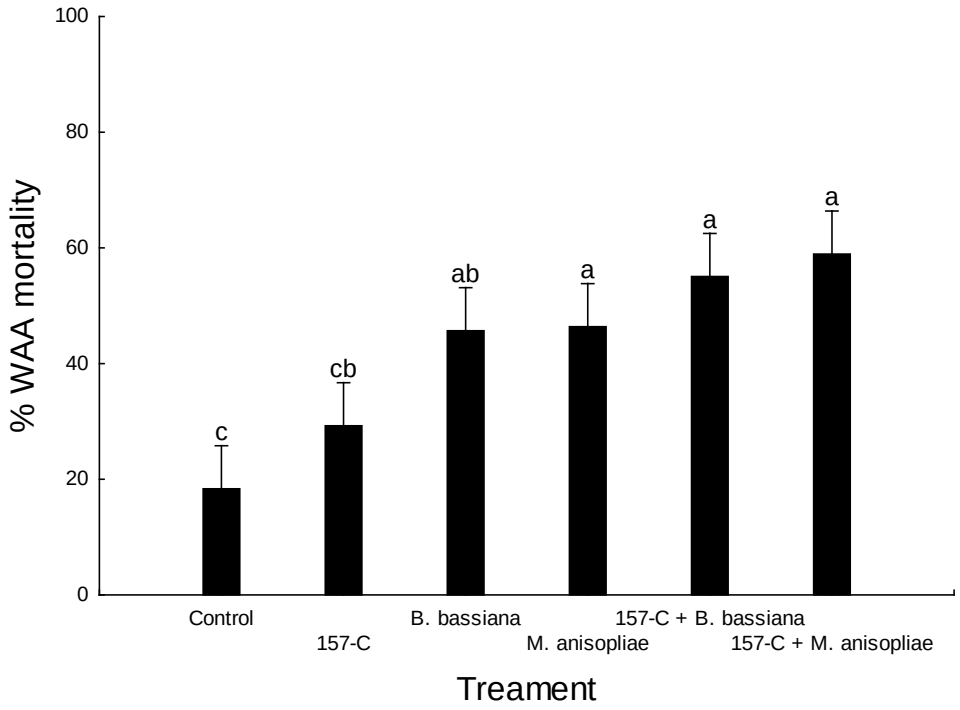

Fig. 4.2. Percentage mortality (95% confidence limit) of aerial colonies of *Eriosoma lanigerum* (WAA) exposed *to Steinernema yirgalamense* (157-C) at a concentration of 80 IJs/cm^2, *Beauveria bassiana* Eco-Bb® strain R444 (1g/L), *Metarhizium anisopliae* ICIPE 69 (0.2 l/ha) or their combination on mortality seven days after application (one- way ANOVA: $F_{(5.30)} = 18.23$, $p < 0.001$). Different letters above bars indicate significant differences.

Control of root colonies

Significant differences ($F_{(5.30)} = 17.68$, $p < 0.001$) were recorded among the nematode, fungi, and fungi and nematode treatments compared to the untreated control (Fig. 4.3). Seven days post-treatment, the following reduction was found: 38% ± 4.2% in the nematode only ($p = 0.0047$); 46% ± 4.2% in the *B. bassiana* ($p < 0.005$); 48% ± 4.2% in the *M. anisopliae* ($p < 0.005$); 59% ± 4.2% in the nematode and *B. bassiana* ($p < 0.005$); and 63% ± 4.2% in the nematode and *M. anisopliae* treatment ($p < 0.005$). All treatments differed significantly from the control treatment, while only the combination of nematode and *B. bassiana* ($p = 0.017$) and of nematode and *M. anisopliae* ($p = 0.004$) differed significantly from the nematode only treatment.

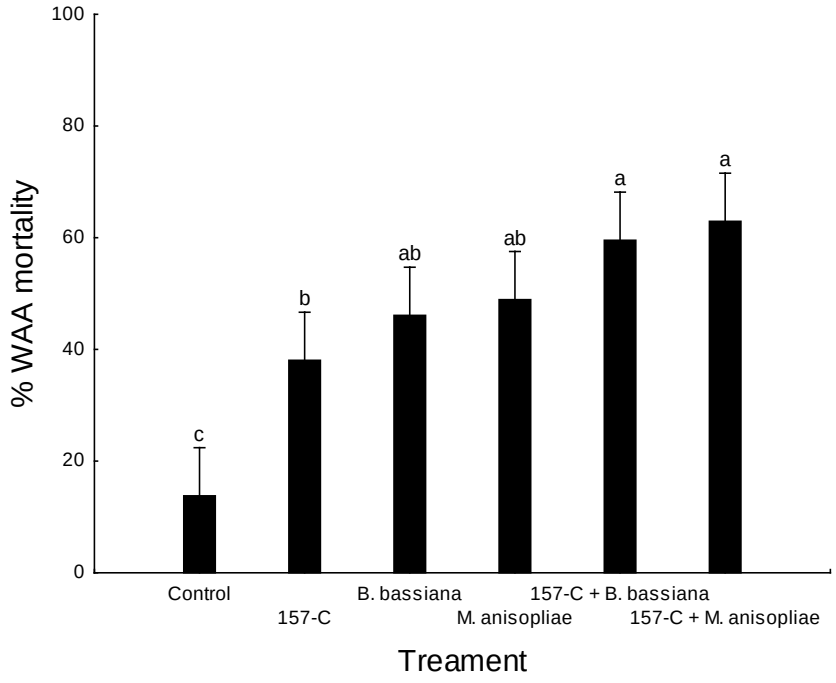

Fig. 4.3. Percentage mortality (95% confidence limit) of root colonies of *E. lanigerum* (WAA) exposed *to Steinernema yirgalamense* (157-C) at a concentration of 80 IJ/cm^2, *Beauveria bassiana* Eco-Bb® strain R444 (1g/l), *Metarhizium anisopliae* ICIPE 69 (0.2 l/ha) or their combination on mortality seven days after application (one-way ANOVA: $F_{(5.30)}$ = 17.68, p < 0.001). Different letters above bars indicate significant differences.

Effect of mulching

When using *S. yirgalemense,* significant differences ($F_{(3.20)}$ = 8.98, p < 0.001) were recorded among the different treatments (Fig. 4.4). The addition of both mulch types caused a significant difference in *E. lanigerum* mortality compared to that in the control. The application of IJs of *S. yirgalamense* to apple or pine wood mulch was found to have led to higher control (44.2% ± 4.8% or 48.2% ± 4.8%) than when it was applied to the bare soil (34.1% ± 4.8%), when it was evaluated seven days after application. The mortality rate of *E. lanigerum* was higher with the pine wood shavings and apple wood chips mulch treatments with it being significantly higher than it was in the control treatment. Significant differences were observed for treatments with *H. zealandica* in the case of the use of both types of mulch ($F_{(3.20)}$ = 13.01, p < 0.001) compared with the untreated control in which larval mortality did not exceed 25%. When evaluated seven days after application, the IJs of *H. zealandica* applied to apple wood or pine wood mulch were found to have led to higher

control (41.2% ± 3.48% or 47.0% ± 3.48%) than when they were applied to the bare soil (32.6% ± 3.48%), (Fig. 4.5). However, no significant differences were detected between the bare ground and mulch treatments.

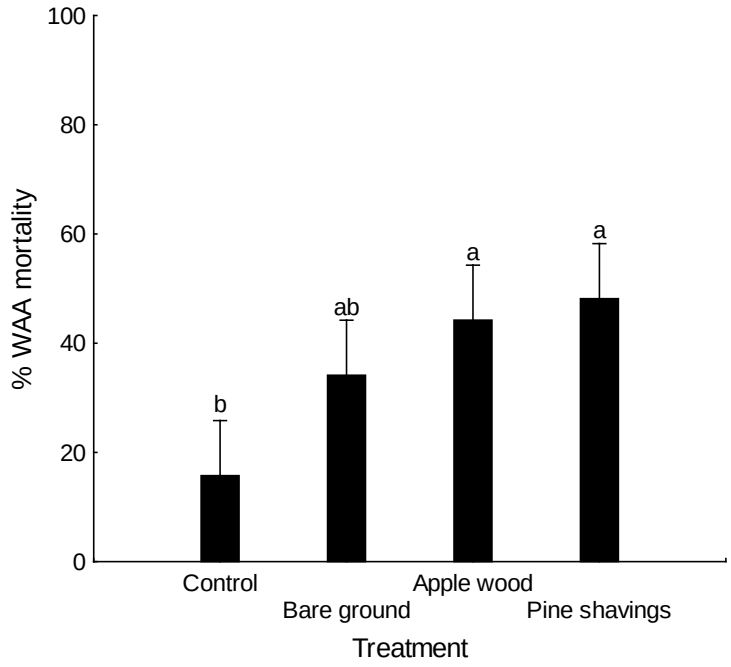

Fig. 4.4. Percentage mortality (95% confidence limit) of *Eriosoma lanigerum* (WAA) seven days after application of *Steinernema yirgalamense* (157-C) at a concentration of 80 IJs/cm^2 to pots with bare ground and to pots containing apple wood or pine shavings as mulch (one-way ANOVA; $F_{(3.20)}$ = 8.98, < 0.001). Different letters above bars indicate significant differences.

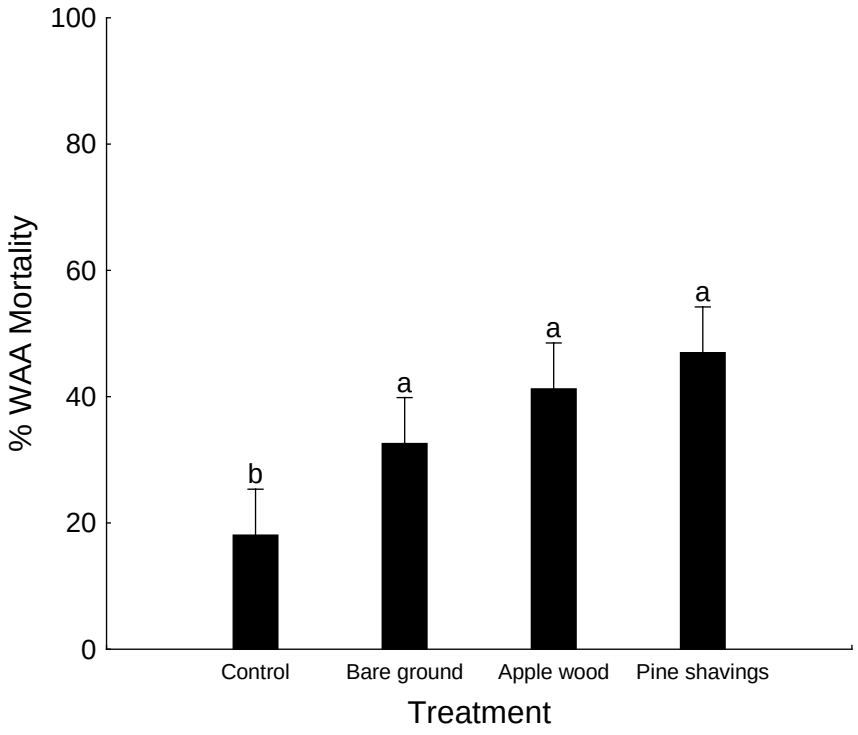

Fig. 4.5. Percentage mortality (95% confidence limit) of *Eriosoma lanigerum* (WAA) seven days after application of *Heterorhabditis zealandica* (80 IJs/cm^2) to pots with bare ground and to pots with apple wood or pine wood shavings as mulch (one-way ANOVA: $F_{(3.20)} = 13.01$, $p < 0.001$). Different letters above bars indicate significant differences.

Discussion

Successful WAA reduction was obtained with the commercial fungi Eco-Bb® strain R444 of *B. bassiana* and ICIPE 69 of *M. anisopliae*. The fungi were tested as was recorded in Chapter 3 with a control rate of >70% of adult WAA under laboratory conditions, where they performed better than the local fungal isolates. Eco-Bb® is registered for the control of whitefly and red spider mite (Plant Health Products, South Africa), while the virulence of ICIPE 69 has been tested against fruit fly species, *Ceratitis capitata* (Weidemann), *Ceratitis cosyra* (Walker) and *Ceratitis rosa* var. *fasciventris* (Bezzi) (Diptera: Tephritidae) as well as against flower thrips, *Megalurothrips sjostedti* (Trybom) (Thysanoptera: Thripidae) with good control being achieved in all cases (Ekesi *et al*., 1999, 2002). The effectiveness of the fungi might be explained by the fact that EPF conidia tend to disperse passively where there is

water in the soil (Storey & Gardner, 1988), and where they do not have to move far to contact their target host.

EPNs are predominantly isolated from soil habitats. Unsurprisingly, many subterranean insects have received attention in relation to control efforts, using EPNs that have been exerted towards them. Examples of research that has been conducted in this regard and into the practical use of EPNs for the control of *E. lanigerum* larvae are those undertaken by Berkvens *et al*. (2014) and Brown *et al*. (1992). Homoptera and Hemiptera generally are considered unlikely candidates for control using EPNs because their sucking mouthparts effectively block this portal of entry for the nematodes (Eidt & Thurston, 1995). The infectivity of *H. bacteriophora*, *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, *S. carpocapsae*, *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, *Steinernema glaseri* (Steiner 1929) and *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 for *E. lanigerum* was tested in laboratory trials conducted by Berkvens *et al.* (2014). Only *S. carpocapsae* caused higher mortality (20–40%) than did the control treatment. However, the mortality that was observed with *S. carpocapsae* was found to be a test artefact and not induced by its specific entomopathogenic activity. These results are contrary to those that were obtained in a study by Brown *et al.* (1992) who on testing *S. carpocapsae* against edaphic populations of the WAAs under laboratory conditions found that the nematodes in question were capable of killing the insect concerned.

Despite a demand for effective microbial sprays against foliar pests, EPNs are still rarely used against above-ground pests (Arthurs *et al*., 2004). Attempts to control foliage-feeding pests with nematodes from the mid-1950s to the early 1980s were disappointing with commercial interest likely being discouraged by low host mortality or by the inability to reduce foliage damage adequately (Begley, 1990; Kaya, 1985). Failures can mostly be attributed to EPN sensitivity to abiotic factors, including ultraviolet (UV) radiation, desiccation and extreme temperatures (Mason *et al*., 1998; Smits, 1996). In the case of EPF, Jaronski (2010) reports that once spores are applied to foliage, their levels decline, with the rate of

decline being affected by a number of factors, including: sunlight; rain; temperature; humidity; leaf surface chemistry; and phylloplane microbiota.

Subsequently, the successful control of above-ground insect pests using EPNs and EPF remains a challenge when considering unfavourable aerial conditions. Such lack of effective control was evident in the current study as well where poor performance of the entomopathogens was observed when they were applied to aerial colonies of WAA. However, growing restrictions on the use of chemical insecticides coupled with the increasing availability of nematode-based products during the intervening years has renewed interest in using nematodes in above-ground habitats.

Suggestions have been made regarding the minimisation of the negative environmental effects of foliar EPN application with some tests having demonstrated the potential of aerial application with certain EPN species against particular insect pests (De Waal, 2008; Lello *et al*., 1996). Beck *et al*. (2013) also demonstrated that three spreading agents (Silwet L-77, SBPI and Addit) increased the EPN deposition on leaves and that relative EPN deposition roughly doubled when one of the spreading agents was added to the EPN suspension. In the case of EPF, the evaluation of a number of UV protectants has led to the identification of a few with practical potential. Inglis *et al*. (1995) identified a number of water-soluble and oil-soluble UV protectants in laboratory tests. When the candidates were tested outdoors, however, the degree of protection was found to be greatly reduced for all protectants, but a quantitative indication was obtained of the potential of photo protection under realistic outdoor conditions (25–37%).

Use of EPNs and EPF in the soil presents a different situation to their use in foliar applications. They have been found to be most efficacious in habitats that provide protection from environmental extremes, especially in soil which is their natural habitat. The use and application of EPNs has traditionally been focused on the control of soil-dwelling pests and/or the soil life stages of insects, in relation to which they are considered to be most virulent (Wilson & Gaugler, 2004). Although soil is the natural habitat for EPNs, and with it

being a more suitable target habitat than elsewhere, many previous attempts to control insects in the soil have failed (Kung *et al*., 1990; Shapiro-Ilan *et al*., 2006). For example, Goble *et al*. (2011) showed that the native isolates of EPF were not able to induce high mortality in the subterranean life stages of *Ceratitis capitata* (Wiedemann) and *Ceratitis rosa* (Karsch) (Diptera: Tephritidae) when they were exposed to sand treated with conidia suspensions. As with aerial application, many such factors as temperature, relative humidity, UV radiation, pH and soil moisture require consideration, as they have varying effects on the pathogenicity and survival of different EPN species (Kung *et al*., 1991; Shapiro-Ilan *et al*., 2006). Equally, different soil textures (sand, sandy loam, clay loam and clay) have varying effects on the pathogenicity, movement and survival of different EPN species (Kung *et al*., 1990; Shapiro-Ilan *et al*., 2006). Of the factors mentioned, humidity is considered to be the most important as low humidity (<90%) prevents the successful germination, infection and sporulation of the fungus (Hesketh *et al.*, 2010). However, other factors such as the temperature, can influence the rate at which infection and hence death occurs (Hesketh *et al.*, 2010).

The presence of another pathogen might make the target insects more susceptible to a fungal entomopathogen. Synergistic effects resulting from combining EPNs with other entomopathogens have been reported in a number of studies (Koppenhöfer & Kaya, 1997; Thurston *et al*., 1993, 1994). The joint use of biological control agents, or of biological control agents and chemical agents can result in a wide range of interactions. Koppenhofer and Grewal (2005) state that an additive effect occurs when there is a combination of two agents acting independently of each other (i.e. when there is no interaction between the two), while synergistic or antagonistic effects are achieved when the interaction between agents renders the combination more or less effective for pest control than it is in the case of an additive effect. In the current study, the results showed a positive effect on the percentage of insect mortality attained when *S. yirgalamense* was combined with either *B. bassiana* or *M. anisopliae*. The combination of the nematode with the fungal isolates slightly increased the

resultant insect mortality when compared to the values that were seen for the nematode or fungus alone, indicating that an antagonistic effect was attained. Similarly to the current results, various studies have also indicated less than synergistic interactions occurring between EPNs and chemical or other entomopathogenic agents (Brinkman & Gardner, 2000; Koppenhöfer & Kaya, 1997). Antagonism between EPNs and other pathogens has also been attributed to the occurrence of indirect interactions relating to competition for the same host resources (Kaya, 2002). However, such indirect interactions are generally associated with the inhibition of nematode development and with reproduction occurring within the host (Kaya, 2002) and not with the ability to kill the insect as such.

In the current study, the combination of EPNs and EPF in simultaneous applications was found not to improve WAA control. Such a finding is in agreement with Hurpin & Robert's (1968) statement that the simultaneous use of two entomopathogenic organisms for the microbial control of grubs has no value. Increased additive or synergistic effects might, however, have been attained had the pathogens been sequentially applied (Koppenhöfer & Kaya, 1997). However, in some studies the simultaneous application of nematodes with another control agent led to an observation of synergy, or best results (Barbercheck & Kaya, 1990). The conclusion is drawn that the pathogen combinations investigated are not likely to improve the suppression of WAA beyond what can be expected from the single application of the pathogen with the greatest virulence.

The intended role of the mulch treatment in this study was to produce a habitat with different soil habitat than that of bare soil and the hypothesis was drawn that the efficacy of *S. yirgalamense* would increase in mulched plants. Webster (1973) proposes the manipulation of habitats in which EPNs can be applied to favour IJ survival and infectivity. Environmental manipulation of the orchard agroecosystem by means of combining irrigation and mulches has the potential to extend the survival of the IJs involved by means of maintaining the moisture levels that are required for their optimum activity. Such mulches as woodchips can also provide an attractive habitat for overwintering larvae, especially in

orchards where smooth-barked trees provide few alternative sites for hibernacula (Lacey *et al.,* 2006). Even though no significant differences were detected that could be associated with the addition of the mulches, the mulches used seemed to retain part of the nematode population and to provide appropriate conditions for the nematodes to act on the soil stages of WAA as was shown by the increase in WAA mortality in the mulched plants compared to the mortality that was achieved with those without mulch. De Waal *et al*. (2011) demonstrated that mulches can be used to manipulate an orchard agroecosystem to the advantage of *H. zealandica* against codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Five different mulch types (pine wood *Pinus radiata* D. Don. chips and shavings; wheat straw *Triticum aestivum* L.; blackwood *Acacia mearnsii* De Wild and apple wood chips *Malus domestica* Borkh) were tested, using three different methods (cages, strips and the addition of uncontained larvae to the mulch). The highest level of codling moth mortality was obtained when using pine wood shavings as compared to using any of the other mulch types.

In the current study, attempts were made to improve EPN infectivity by means of adding two types of mulches (pine wood, and apple wood chips). However, the addition of different mulches to the EPN solution did not significantly increase control levels of the WAA. Hence, the findings are consistent with those of Sweeny *et al*. (1998) who reported that various mulches (e.g. bark, peat and hay) had negligible or minor effects on the persistence of *Steinernema* spp. and on the infection of the cone maggot, *Strobilomyia neanthracina* Michelsen. Even though the effect of mulching was slight in the case of the WAA, it poses an attractive alternative to other insects and biological control combinations.

Metarhizium anisopliae was the most effective fungus against WAA, giving relatively good control in the absence of *S. yirgalamense*. Combining *S. yirgalamense* with *B. bassiana* and *M. anisopliae* provided no advantage for improved efficacy against the WAA. Hence, if *M. anisopliae* is also the most pathogenic fungus under field conditions, it could be used alone for the suppression of WAA, making such an approach more desirable than

would be the use of two biological control agents in combination. Taking into account the results presented here, EPF seems to be the better treatment for the control of *E. lanigerum* than are EPNs. If future field work confirms the findings noted here, *B. bassiana* and *M. anisopliae* could become important biological control agents of WAA in apple orchards. Although its use depends on further work in the field, if such use proves to be successful the fungi might contribute to the development of sustainable production systems as a result of the possibility of reducing the use of chemical insecticides and consequently reducing the amount of chemical residues on produce. Previous studies indicate that in most cases it is possible to find local isolates, with higher pathogenicity for specific insect control than that of the commercial isolates that were used in this study. The surveying and screening of more local pathogenic isolates with high mortality against WAA should therefore be conducted in future.

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CHAPTER 5

Field efficacy of *Steinernema yirgalamense*, *Beauveria bassiana* and *Metarhizium anisopliae* against woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae)

Abstract

Apples, *Malus domestica* Borkh. are one of the most important deciduous fruits grown in South Africa and are by far the most important export fruit in terms of volume. Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann), is a detrimental pest in apple production. The efficacy of a local isolate of an entomopathogenic nematode (EPN), *Steinernema yirgalamense*, and two commercial entomopathogenic fungal products of *Beauveria bassiana* and *Metarhizium anisopliae* against WAA, was evaluated in an apple orchard in Grabouw, Western Cape province. Treatments were applied using a handheld sprayer at a radius of 30 cm around the trunk of each tree. Soil samples were collected were taken before application, as well as 7, 14, 21 and 90 days post-treatment. The highest rate of mortality was caused by *B. bassiana* (Eco-Bb® strain R444*)* and *M. anisopliae* (ICIPE 69). *Steinernema yirgalamense* caused a significant decrease in populations compared to those that were present in the control, but not to the extent of the two fungal isolates. Above ground, WAA colony size was reduced significantly from the control. The EPF isolates performed better than the EPN. The success that was attained in controlling the root colonies resulted in greatly reduced infestations of the aerial parts of the apple trees.

Introduction

The woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is an important insect that infests apple orchards in South Africa and it is considered to be critical to the economics of the apple industry worldwide (Bus *et al*., 2007). WAA forms densely packed colonies, that are covered with a white, waxy, filamentous secretion and which feeds on the roots and on the canopy of apple trees (Gurney, 1926; Lloyd, 1961). Their feeding causes large galls on the roots and on the aerial parts of the trees (Brown *et al*., 1991; Damavandian & Pringle, 2007), which can restrict sap flow and rupture plant tissues, thereby providing further feeding sites and allowing for the invasion of fungal diseases (Weber & Brown, 1988). The calyces of apples may also be infested by aphid colonies, resulting in contamination with honeydew and sooty mould (Heunis & Pringle, 2006; Shaw & Walker, 1996).

Biological control by the parasitoid, *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae), has been found to play a significant role in reducing WAA populations above ground (Mols, 1996). However, this parasitoid is relatively ineffective because it appears late in the season. This late appearance of parasitism might be caused by the applications of chlorpyrifos, vamidothion, prothiofos and encapsulated parathion early in the season as Heunis and Pringle (2003) have shown such substances to be extremely toxic to *A. mali*.

Entomopathogenic nematodes (EPNs) in the Steinernematidae and Heterorhabditidae (Order: Rhabditida) families are lethal parasites associated with symbiotic bacteria in the family Enterobacteriaceae. Steinernematids are associated with *Xenorhabdus* spp., whereas heterorhabditids are associated with *Photorhabdus* spp. (Boemare, 2002; Forst & Clarke, 2002). The only free-living stage of nematode is the infective juvenile (IJ), which is capable of actively seeking out hosts (Kaya & Gaugler, 1993). Following entry through natural body openings, and in some cases through the cuticle, the IJs release symbiotic bacteria into the insect haemocoel (Poinar, 1990). The bacteria then multiply rapidly, killing the host within 1-4 days. Nematodes complete 1-3 generations within the host after which free-living IJs

emerge to seek a new host. Thus, EPNs offer an environmentally safe alternative that is compatible with integrated pest management programmes (IPM).

Pringle *et al*. (1994) reported tolerance of WAA to vamidothion in the Grabouw area, following on which chlorpyrifos and endosulfan were used with unsatisfactory results for control of both root and aerial populations. In South Africa at present the only effective control measure that is available for subterranean populations of *E. lanigerum* is a soil application of imidacloprid. Pringle (1998) showed that a single application can control the subterranean population of WAA for at least three seasons. However, as such treatment is highly disruptive to soil biota it should be avoided.

Soil-inhabiting entomopathogenic fungi (EPF) are an important and widespread component of most terrestrial ecosystems, playing a key role in regulating some soil-dwelling insect populations (Meyling & Eilenberg, 2007; Quesada-Moraga *et al.,* 2007). The use of EPF has been investigated for the control of a wide range of orchard pests (Alves *et al*., 2005; Castrillo *et al.*, 2005; Cross *et al.,* 1999; Dolinski & Lacey, 2007; Lacey & Shapiro-Ilan, 2008; Puterka, 1999). The use of EPF in the form of mycoinsecticide has many desirable traits, with EPF leaving no toxic residues on crops, and generally being harmless to beneficial and other non target insects (Goettel, 1995; Zimmermann, 2007a,b). EPF also pose minimal risks to humans and animals with no damage being caused to the environment because ground water and riparian habitats are not contaminated by them, as occurs with pesticides (Zimmerman, 2007a). Their host-specific nature enhances their potential role in IPM, as it preserves natural enemies, which then help to enhance the overall regulation of the pests involved (Inglis *et al*., 2001).

A study by Damavandian (2000) revealed the existence of a number of EPF feeding on WAA in two apple orchards (Molteno and Oak Valley, South Africa). The genus *Conidiobolus* and *Hirsutella* were consistently active in aphids washed from the soil and infested root samples, although the levels of infection differed. Therefore, the purpose of the

present study was to evaluate the field efficacy of three soil-applied entomopathogens on WAA.

Materials and Methods

Origin of entomopathogenic nematodes and fungi

IJs of a local isolate of *Steinernema yirgalamense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 (157-C) were used in all experiments (Malan *et al*., 2011). Following the methods described by Kaya and Stock (1997), last-instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) were used to multiply the number of nematodes. *Galleria* larvae were inoculated with IJs, and subsequently incubated at 25 ± 2°C in a growth chamber. Infected *Galleria* larvae were then placed in White traps to harvest the nematodes. From days one to three, IJs were collected on emergence.

Commercial isolates *Beauveria bassiana* Balsamo (Vuillemin) (Eco-Bb® strain R444) and *Metarhizium anisopliae* Metchnikoff (Sorokin) (ICIPE 69) were obtained from the manufacturers, Plant Health Products, KwaZulu-Natal, South Africa and Real IPM, Grabouw, South Africa, respectively and used following the instructions given on the label. EPNs were applied at a concentration of 80 IJs/cm^2, with *B. bassiana* and *M. anisopliae* being applied at their recommended dose of 1g/L and 200 ml/ha, respectively with the concentration of nematodes being calculated by means of the method developed by Glazer & Lewis (2000).

Baseline populations of entomopathogenic nematodes and fungi

Baiting procedure

One week before the applications were made, pre-treatment soil samples were taken from the area to determine the baseline populations of EPNs and fungi. Three subsamples of soil were taken by means of a shovel, from each plot down to a depth of 15 cm. The subsamples taken from each plot (containing four trees) were placed in a plastic bag and thoroughly mixed. In the laboratory each soil sample was sieved through a metal sieve with

a mesh size of 4 mm and then transferred to 400-ml plastic containers which were closed by means of lids. If the soil samples were too dry they were moistened with distilled water to maintain humidity during baiting. Ten last-instar larvae of *T. molitor* were placed on the surface of each soil sample. All soil samples were incubated at 25 ±2°C in the dark. The samples were checked for dead larvae every 3-4 days for three weeks. All dead larvae were surface-sterilised with 70% ethanol prior to incubation, so as to prevent opportunistic external saprophytic fungi from growing on the cadavers. Larvae with symptoms of EPN infection (amounting to changes in their pigmentation) were individually kept in White traps to allow for IJ emergence. Cadavers with symptoms of fungal infection (being the hardening of the cadaver or the emergence of conidiophores) were placed on appropriate media to isolate the fungal cultures.

Isolation and identification of EPNs

Molecular techniques were used to identify the EPNs found in the samples. DNA was extracted from single females, using a modification of a method reported by Nguyen *et al*. (2007). The nematode was placed in 30 µl lysis buffer (50 mM $MgCl_2$, 10 mM DTT, 4.5% Tween-20, 0.1% gelatine and 1 µl of proteinase K, at 60 µg m^{-1}) on the side of an Eppendorf tube, where it was cut into pieces with the aid of the sharp side of a sterile insulin needle. The tube was immediately stored at -80°C for at least 15 min. The tube was then incubated at 65°C for 1 h and then at 95°C for 10 min in order to lyse the cells completely, as well as so as to digest the proteins. The tube was cooled on ice and centrifuged at 8 000 rpm at 10°C for 2 min. Twenty µl of the supernatant containing the DNA were collected and stored at -80°C for further use.

Two PCR primers were used to amplify the Internal Transcribed Spacer (ITS) rDNA regions, including ITS1, 5.8S and ITS2 ribosomal genes, as well as short parts of the 18S and 28S ribosomal genes. The 18S primer (5'-TTGATTACGTCCCTGCCCTTT-3') and 28S primer (5'-TTTCACTCGCCGTTACTAAGG-3') have been described by Vrain *et al*. (1992) for purposes of amplification of the ITS regions.

The cycling conditions were as follows: denaturation at 94°C for 20 seconds; annealing at 50°C for 30 seconds; and extension at 72°C for 45 seconds, repeated for 35 cycles. A 2-min-long incubation period at 72°C followed the last cycle in order to allow for the completion of any partially synthesised strands. The PCR product was then run on 1.5 % agarose gel in a 1 × TBE buffer, and visualised by means of ethidium bromide staining.

Post-PCR purification was undertaken using the NucleoFast Purification System (Macherey-Nagel, Waltham, Massachusetts, USA). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by electrophoresis on the 3730 × 1 DNA Analyser (Applied Biosystems, Foster City, California, USA) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University). Sequence assembly and editing was performed on the CLC Main Workbench ver. 7.6.2 (http://www.clcbio.com).

Isolation and identification of EPF

Fungal isolations were made, using selective media from Meyling & Eilenberg (2007) with the following composition and preparation: 60 g SDA (Sabouraud Dextrose Agar, Merck) supplemented with 1 ml Dodine, 50 mg/L Chloramphenicol and 50 mg/L Rifampicin. Plates were incubated at 25 ±2°C in the dark. All potential EPF were identified microscopically and selected strains were sent to the Mycology Unit at the Plant Protection Research Institute (PPRI) in Pretoria, South Africa, for morphological and molecular verification.

Immersion bioassays

A dipping bioassay was used to assess the pathogenicity of the nine fungal isolates collected from the sampled soils in order to ascertain whether or not they were pathogenic to WAA and codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) larvae. All isolates were tested at a standard concentration of 1.0×10^8 conidia. ml^{-1} in distilled water containing a drop of Tween 20. Insects were dipped in 2 ml of aqueous conidia formulation for 30 sec. Controls were treated with sterile distilled water containing a drop of Tween 20. Ten treated

insects were placed in 9-cm-diameter Petri dishes and incubated at 25 ±2°C. In order to determine the cause of mortality, dead insects were surface-sterilised in 70% ethanol and transferred onto SDA to observe mycosis.

Field application of EPF to the soil

This experiment was conducted on a 33 year old naturally infested orchard planted with Granny Smith, Top Red and Golden Delicious trees on an Agricultural Research Council (ARC) Experimental Farm in Grabouw, Western Cape province. None of the plots had been treated with insecticides during the previous year. Only rows planted with Golden Delicious were selected as treatment rows. The two edge rows were skipped and for each treatment, four adjacent trees were selected as one replicate, with four replicates per block (amounting to 16 trees per block). For each treatment two blocks were used (32 trees per block). The treatments were applied to the eight blocks in a complete randomized design. At least one untreated row was left between each treated row, with two untreated trees being left between each treated block of trees (Appendix 1). On 17 November 2014, single applications of the treatments in 2 L water were applied using a handheld sprayer in a radius of 30 cm around the trunk of each tree.

Pre-treatment samples were taken from all trees 7 days before the trial began with post-treatment samples taken after 7, 14, 21 and 90 days, using a soil auger with a hollow bit of 5 cm internal diameter and with cores being drilled to a depth of 15 cm, according to the procedure described by Damavandian & Pringle (2002). On each post-treatment sampling date, cores were taken from the eight treatment trees at a distance of 30 cm from the stem. On each post-treatment sampling date soil samples were taken from a different tree in the treatment block, so as to minimise the destructive nature of sampling (Damavandian & Pringle, 2002). The samples were placed in plastic packets and in the laboratory each sample was rinsed through a sieve with a mesh size of 200 µ (Damavandian & Pringle, 2002). The technique is described here in brief. The sieve was placed in a trough containing a solution of 600 g sugar per L water which served to float the aphids to the

surface where they could be counted using a stereoscopic microscope. All developmental stages were counted and classified as alive, dead or dry. Those that were dead were still in good condition and were assumed to have been killed during sampling, transporting or washing. Therefore, both alive and dead aphids were included in the data analysis, whereas the dry aphids were excluded. The larvae that were found to be dead in nematode treatments were dissected so as to determine the cause of death. Larvae killed in the fungal treatments were kept individually in 30 cc plastic cups and when conidiation was observed, a sample was taken and examined under a microscope to verify that the larva concerned had died from a fungal infection.

Monitoring of WAA on shoots

The impact of the three treatments (*S. yirgalamense, B. bassiana* and *M. anisopliae*) on the aerial populations of WAA was assessed. When WAA colonies started developing on the aerial parts of the plant they were counted in the leaf axils on the northern half of each of 25 trees per plot up to chest height.

Data analysis

All data were analysed using a two-way analysis of variance (ANOVA) using STATISTICA 11.0 (StatSoft Inc., 2008). Differences were considered to be significant if the probability levels were less than 0.05. For each plot, the effectiveness of the treatment was based on the average number of WAA per soil core and comparing these to the pretreatment WAA count.

Results

EPN infections were recognized by the change in colour undergone by the *T. molitor* cadavers soon after death (Fig. 1A). EPF infections were recognized by the hardening of the *T. molitor* cadaver or by the emergence of conidiophores (Fig. 5.1B).

Fig. 5.1. Cadavers of *T. molitor* in soil showing symptoms of infection by: (A) EPNs and (B) EPF.

Identification of EPNs

EPNs were found in only one pre-sample of the treatment trees in the apple orchard in Grabouw. The generated sequence (Genbank number pending) were identified with the Nucleotide Blast option from Genbank. A 99% similarity and coverage were obtained, similar to those of *H. bacteriophora* Poinar 1975 strain 89-C (EU15293) from Genbank. The measurements of the body length, 578 (523-665) µm, of the IJ confirmed the molecular identification.

Identification of EPF

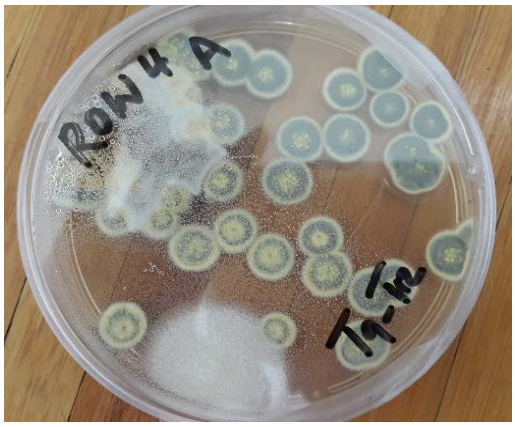
In total, 31 indigenous isolates belonging to 9 fungal species were obtained from pre-sampled soils using the insect bait technique. The most prevalent species collected from the soil using *T. molitor* was *M. anisopliae* (8 isolates), followed by *Aspergillus flavus* Link (5 isolates), *Penicillium expansum group* Link (4 isolates), *Paecilomyces lilacinus* (Thom) Samson (4 isolates), *Clonostachys rosea* f. *rosea* (Link) Schroers, Samuels, Seifert & Gams (3 isolates), *Talaromyces flavus* (Klöcker) Stolk & Samson (3 isolates), *Clonostachys rosea* f. *catenulata* (Gilman & Abbott) Schroers (2 isolates), while *Penicillium chrysogenum* Thom and *Penicillium* cf. *janthinellum* Biourge were only isolated once from the soil samples taken (Table 5.1).

Table 5.1. The list of fungal species isolated from soil samples collected in the experimental orchard.

Fungus species	No. of isolates (N=31)	Fungi	WAA dipping test	CM dipping test
Metarhizium anisopliae	8	Insect pathogen	+	+
Clonostachys rosea f. *rosea*	3	Opportunistic pathogen	_	+
Clonostachys rosea f. *catenulata*	2	Opportunistic pathogen	_	+
Talaromyces flavus	3	Secondary Colonizer	_	+
Penicillium expansum group	4	Opportunistic pathogen	_	+
Penicillium cf. *janthinellum*	1	Opportunistic pathogen	_	+
Penicillium chrysogenum	1	Opportunistic pathogen	_	+
Aspergillus flavus	5	Insect pathogen	+	+
Paecilomyces lilacinus	4	Opportunistic pathogen	_	+

WAA= Woolly apple aphid

CM= Codling moth

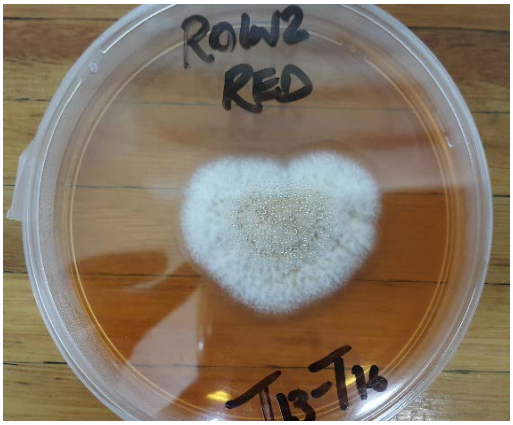


Penicillium cf. *janthinellum*

Clonostachys rosea f. *rosea*

Clonostachys rosea f. *rosea*

Penicillium expansum group



Aspergillus flavus

Talaromyces flavus

Metarhizium anisopliae

Clonostachys rosea f. *catenulate*

Paecilomyces lilacinus

Penicillium chrysogenum

Fig. 5.2. Culture morphology of some of the fungi isolated from the soil samples.

Immersion bioassays

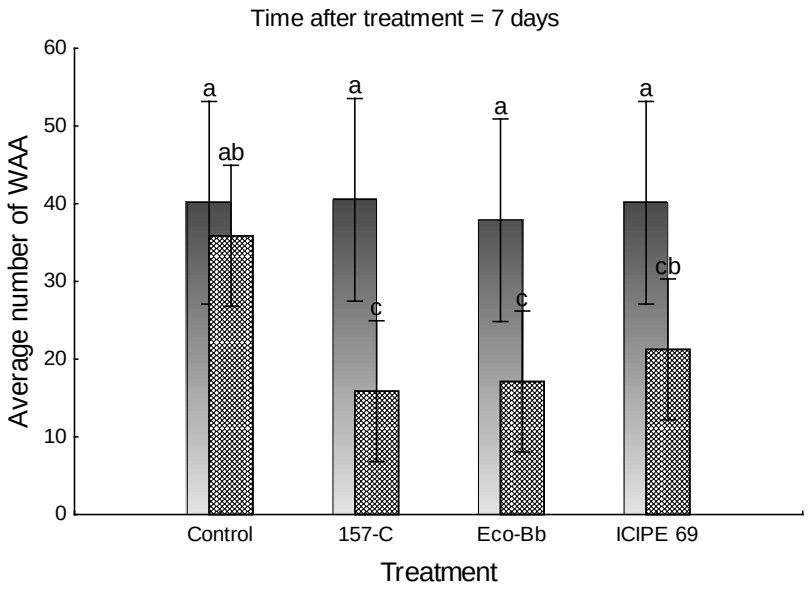
Results from the immersion bioassay showed that only two (*M. anisopliae* and *A. flavus*) of the nine fungal isolates were pathogenic to WAA while in the case of codling moth, all tested fungal isolates killed the larvae concerned (Table 5.1).

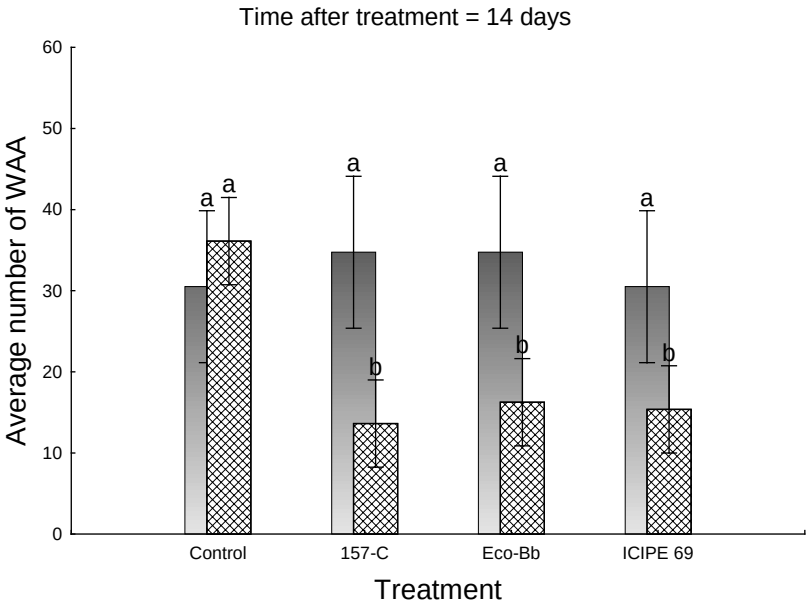
Field application of EPF to the soil

All three treatments: *S. yirgalamense*, *B. bassiana* and *M. anisopliae* reduced the population density of the aphids up to three months after application. The underground WAA populations were lower in the post treatment samples in the case of *S. yirgalamense*, *B. bassiana* and *M. anisopliae* than they were in the pre-treatment samples (Table. 5.2.). In the control treatment, no significant differences were observed in WAA populations between the pre-treatment and post treatment samples, except on the last sampling date (Fig. 5.3).

Table 5.2. Average number of *Eriosoma lanigerum* per soil core sample recorded from untreated trees (control) and from trees treated with *S. yirgalamense* (157-C), *B. bassiana* (Eco-Bb® strain) and *M. anisopliae* (ICIPE 69) over a 7, 14, 21 and 90-day period post treatment. With F-values (degrees of freedom) and probability levels for differences between treatments (P).

No. of days after application	Control	157-C	Eco-Bb	ICIPE 69	$F_{(3.28)}$	P
7	35.88	15.88	17.13	21.25	3.067	0.044
14	36.13	13.63	16.25	2.63	5.667	< 0.005
21	35.05	13.13	14.00	16.50	11.951	< 0.005
90	20.12	14.50	16.38	19.88	0.187	0.905





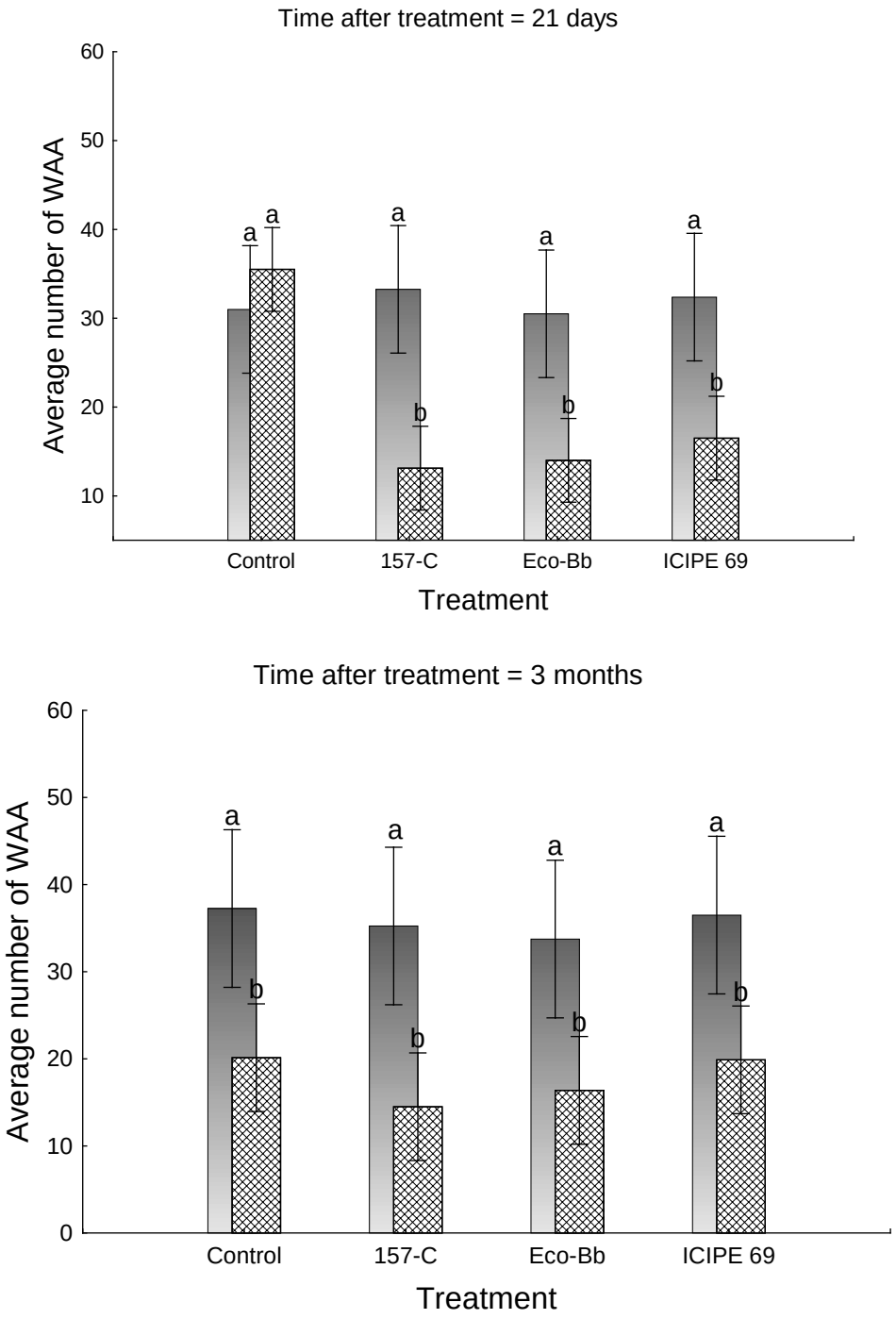


Fig. 5.3. Average number of *Eriosoma lanigerum* per soil core sample recorded from trees before (▬) and after treatment (▩) with water only, *S. yirgalamense* (157-C) (80 IJs/cm$^{2)}$, *B. bassiana* (Eco-Bb® strain R444) (1g/L) and *M. anisopliae* (ICIPE 69) (200 ml/ha) over a 7-day period, a 14-day period, a 21-day period and a 90-day period. Bars with the same letter are not significantly different.

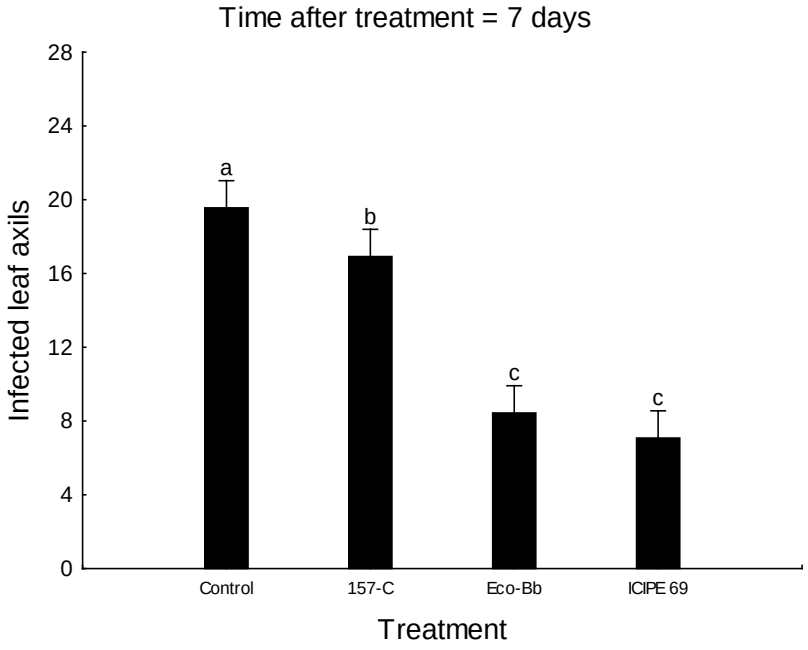
Monitoring of WAA on shoots

The number of leaf axils per half-tree infested with WAA was consistently lower on the trees treated with either S. *yirgalamense, B. bassiana* or *M. anisopliae* than they were on the

control trees (Table 5.3). These differences were significant on all sampling dates concerned (Fig. 5.4).

Table 5.3. Average number of leaf axils per half tree, infested with *Eriosoma lanigerum* in trees treated with *Steinernema yirgalamense, Beauveria bassiana* (Eco-Bb® strain R444*), Metarhizium anisopliae* (ICIPE 69) and untreated control trees, with F-values (degrees of freedom) and probability levels for differences between treatments (P).

Sampling date	Control	157-C	Eco-Bb	ICIPE 69	F (3.96)	P
01 Dec 2014	19.56	16.92	8.44	7.08	69.027	< 0.005
15 Dec 2014	20.24	13.44	8.28	6.12	55.893	< 0.005
16 Feb 2015	21.60	12.68	7.12	5.56	68.013	< 0.005



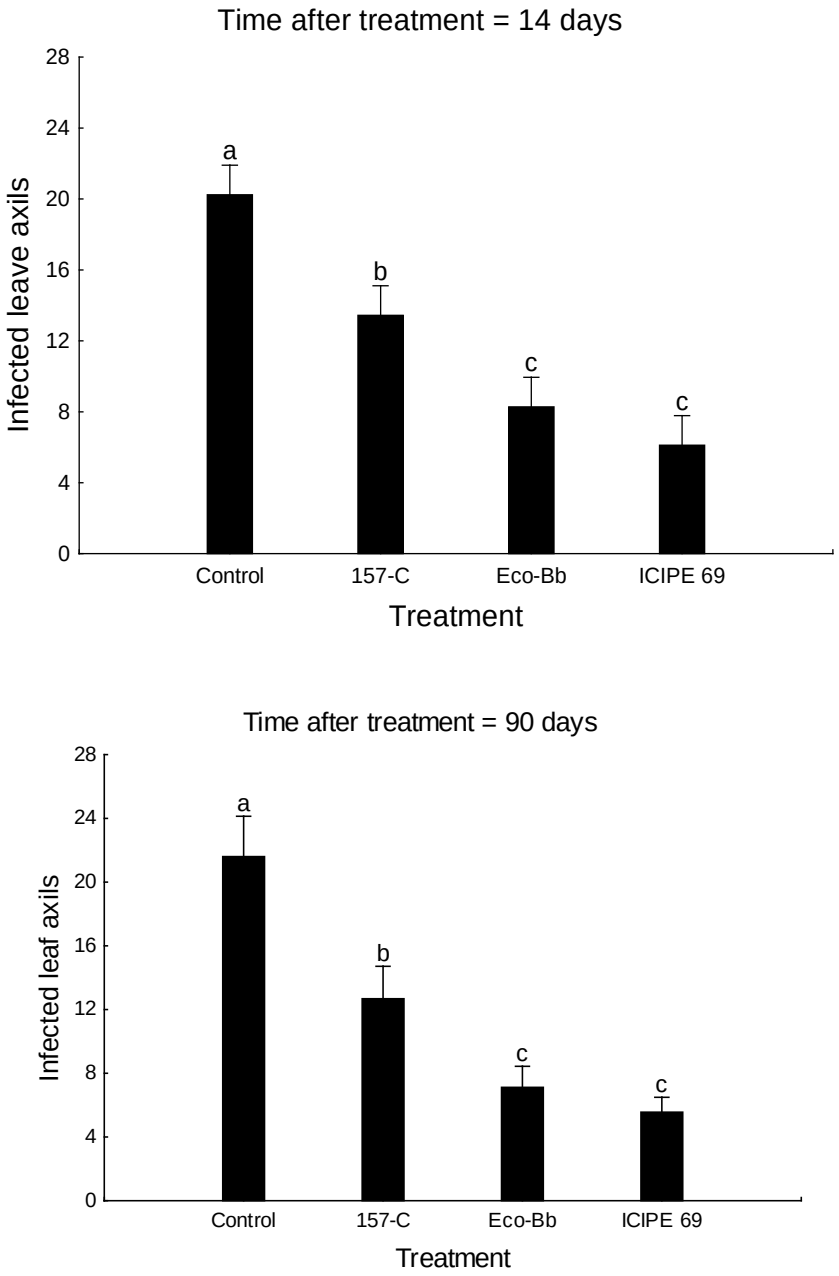


Fig. 5.4. Average number of infected leaf axils per half-tree infested with *Eriosoma lanigerum* in trees treated with *Steinernema yirgalamense* (157-C), *Beauveria bassiana* (Eco- Bb® strain R444), *Metarhizium anisopliae* (ICIPE 69) and untreated control trees, at three sampling dates. Bars with the same letter are not significantly different.

Discussion

The apple block has a long-term root infestation that provided inoculum of aerial colonies annually. Pringle (1998) showed that an imidacloprid soil drench applied during early summer provided WAA control for up to three years. However, there might be some control measurements that are more acceptable from the environmental point of view. For

example, Van Jaarsveld (2003) showed, in laboratory trials, that Biostart 2000®, consisting of a mixture of three *Bacillus* bacteria, greatly reduced the numbers of WAA on the roots of potted apple trees. In addition, Damavandian (2000) recorded a number of EPF infecting subterranean *E. lanigerum.* As part of the present trials, laboratory experiments confirmed that *S. yirgalamense*, *B. bassiana* (Eco- Bb® strain R444) and *M. anisopliae* (ICIPE 69) were capable of reducing WAA (Chapters 2 and 3). The susceptibility of insects to entomopathogens, as demonstrated in the laboratory is generally admitted not to relate to the infection rates obtained in fields (Barta, 2010). In this study, a field trial was carried out to verify WAA susceptibility to the entomopathogens in the host's natural environment.

The natural occurrence of EPF in the soil is influenced by a complicated set of abiotic and biotic factors including: soil structure (Jaronski,, 2007); organic matter in the soil (Quesada-Moraga *et al.,* 2007); ultra-violet light (UV-B) (Braga *et al.,* 2001); cultural practices; pesticide applications; tillage regimes; crop cover and rotations; fertilizers (Filho *et al.,* 2001; Jaronski, 2007; Meyling & Eilenberg, 2007); and insect hosts. Organically farmed soils have been shown to be a more suitable habitat for EPF than cultivated, conventionally farmed soils (Goble, 2009; Klingen *et al.,* 2002). In other studies, the use of compost teas (and manure) or organic fertilizers have been shown to have a positive effect on the occurrence of EPF species because an increased carbon load in the soil is favourable to soil-inhabiting insects, which themselves, are potential hosts for fungi (Ali-Shtayeh *et al.*, 2002; Klingen *et al.*,2002).

The presence of insecticides and especially fungicides might have an indirect killing effect on EPF (Khalil *et al.,* 1985; Klingen *et al.,* 2002; Meyling & Eilenberg, 2007). For example, use of the broad-spectrum fungicide azoxystrobin and the organophosphate insecticide methidathion has been shown to result in lower incidences of fungal isolates because they negatively affect EPF *in vitro* (Jaronski, 2007; Puterka, 1999).

In the baseline samples in the present study, fungal strains belonging to nine different species were obtained using the insect bait technique with *M*. *anisopliae* being the most

abundant species, representing 25.8% of the soil samples obtained. In a previous study carried out by Abaajeh (2014), 39 EPF isolates belonging to six different EPF species were recovered from soil samples collected from 10 areas in the Western Cape, of which most were *M. robertsii* J.F. Bisch., Rehner & Humber, 2009 (20 isolates), followed by *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (8 isolates), *Fusarium oxysporum* Schlecht. (5 isolates), *Aspergillus flavus* (3 isolates) and *Fusarium polyphialidicum* Marasas, Nelson, Toussoun & van Wyk 1986 (2 isolates), with one being *Beauveria pseudo-bassiana* Rehner & Humber, 2011 isolate.

Metarhizium anisopliae is a widely distributed soil-inhabiting fungus that is parasitic to insects (Maniania, 1991). The first use of *M. anisopliae* as a microbial agent against insects occurred in 1879, when Elie Metchnikoff used it in experimental tests to control the wheat grain beetle, *Anisoplia austriaca* (Coleoptera: Scarabaeidae) with it later being used to control the sugar beet curculio, *Cleonus punctiventris* (Coleoptera: Curculionidae) (Hall & Papierok, 1982). *Metarhizium anisopliae* is categorized as a green muscardine fungus due to the green colour of its sporulating colonies. The species have a wide range of virulence being known to infect more than 200 different insect species, of which many are major agricultural pests, such as sugarcane stem-borers, scarab grubs and grasshoppers (Bidochka & Small, 2005). *Metarhizium anisopliae* has been used to control a variety of insect species, including plant-feeding members of the Hemiptera (Shah & Pell, 2003; Zimmermann, 2007a).

The second most common potential pathogen that was isolated was *Aspergillus flavus* (Table 5.1), which was isolated from 16.1% of the soil samples. *Aspergillus* spp. are generally regarded as opportunistic pathogens that require wounds or otherwise weakened hosts for colonization (Raper & Fennell, 1965). *Aspergillus flavus* causes diseases of such agronomically important crops as corn and peanuts, with it being second only to *Aspergillus fumigatus* as the cause of human invasive aspergillosis, and with it being the *Aspergillus* species that is most frequently reported to infect insects. *A. flavus*, however, also has limited

parasitic abilities and, in some cases, it can directly invade seeds and colonize living tissues (Payne, 1998).

Paecilomyces lilacinus was isolated from 12.9 % of the soil samples (Table 5.1). Being a nematode egg-pathogenic fungus, it is one of the most widely tested soil Hyphomycetes for the biological control of plant-parasitic nematodes (Atkins *et al.*, 2005). *Paecilomyces lilacinus* strain-251 is currently licensed by biotech companies in Germany and South Africa for mass production and plant parasitic nematode control (Brückner, 2004). It has been shown to reduce *Meloidogyne incognita* (Kofoid & White) Chitwood (Tylenchida: Meloidogynidae) soil and root populations significantly, resulting in increased yield of tomato (Lara *et al*., 1996). Siddiqui *et al*. (2000) reported reduction of *Meloidogyne javanica* (Treub) Chitwood (Tylenchida: Meloidogynidae) infection on tomato by *P. lilacinus*.

Clonostachys rosea f. *catenulata* (syn. *Gliocladium catenulatum*) is a mycoparasite that is capable of antagonizing a range of fungal plant pathogens, such as *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Fusarium* spp., and *Pythium* spp. (Chatterton & Punja, 2009; McQuilken *et al*., 2001). It has been widely used as a biological control agent (Chatterton *et al*., 2008; Rahman & Punja, 2007). *Clonostachys rosea* f. *catenulata* was isolated from 16.1 % of the soil samples (Table 5.1).

Talaromyces flavus was isolated from 9.7% of the soil samples (Table 5.1). It is an antagonistic fungus that has been used for the biological control of such soilborne pathogens as *Verticillium dahliae*, *V. albo-atrum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Marois *et al.*, 1984; Punja, 2001). Marois *et al*. (1984) also reported that this fungus grows on the rhizosphere of greenhouse cucumber, cotton, and eggplant and that it inhibits germination of the microsclerotia of *V. dahliae*. *Talaromyces flavus* decreased the incidence of Verticillium wilt and increased the yield of eggplant, in England (Marois *et al.*, 1982) whereas Fahima and Henis (1997) found that *T. flavus* decreased Verticillium wilt on eggplant by 77%.

Penicillium expansum, which is one of the most common foodborne fungi on fruit was isolated from 12.9% of the soil samples obtained (Table 5.1). *Penicillium expansum* is often found on rotting apples, pears, and cherries, but it is also common on walnuts, pecans, hazelnuts, and acorns (Filtenborg *et al.*, 1996). High spore densities in the air are probably caused by the growth of the mould in high concentration in rotten organic material in orchards.

Clonostachys rosea f. *rosea* was isolated from 16.1% of the soil samples obtained (Table 5.1). Being also known as *Gliocladium roseum*, it belongs to the fungal family Bionectriaceae (Blaxter *et al.*, 1992). It colonises living plants as an endophyte and digests materials in the soil as a saprophyte, while it is also known to be a parasite of other fungi and nematodes (Toledo *et al*., 2006). It produces a wide range of volatile organic compounds that are toxic to other microbes (Stinson *et al*., 2003). As a result, it is of great interest as a biological control agent. *Clonostachys rosea* infects nematodes via conidia that are capable of attaching themselves to the nematode cuticle, and of producing germ tubes that penetrate the host cuticle and that kill the host (Zhang *et al.*, 2008).

Penicillium chrysogenum is a commonly occurring mould in such indoor environments as those that are characterised by dust, indoor air, and damp building materials (Chang *et al*., 1995; Hunter & Lea, 1995). It was isolated from 3.2% of the soil samples obtained (Table 5.1). Furthermore, *P. chrysogenum* is frequently identified as a food spoilage agent with it having gained much attention for its use in the production of the antibiotic penicillin (Samson *et al*., 2010). *Penicillium* cf. *janthinellum*, which is among the most important pathogenic fungi that are of economic significance to plants was isolated from 3.2% of the soil samples obtained (Table 5). *Penicillium* is a diverse genus that occurs worldwide with its species playing important roles as the decomposers of organic materials and with it causing destructive rot in the food industry in relation to which they are known to produce a wide range of mycotoxins.

In our tests, WAA populations from each sampling date and among the different treatments varied, but the data showed, in general that the application of *S. yirgalamense*, *B. bassiana* (Eco-Bb® strain R444*)* and *M. anisopliae* (ICIPE 69) reduced the size of WAA populations compared with that of the control. After treatment, the size of WAA populations declined until the termination of the experiment. All counts from 7-90 days were lower than was the pre-spray count. The highest decline in the size of WAA populations was caused by *B. bassiana* (Eco-Bb® strain R444*)*, followed by *M. anisopliae* (ICIPE 69). The potential of fungi to control WAA has been identified by Damavandian (2000). The mortality and population size decrease observed might have been due to the production of toxins by the fungi, with, for example Samuels *et al*. (1988) reporting that secondary metabolites produced by *M. anisopliae* act on insect tissues including the midgut.

Steinernema yirgalamense caused a significant decrease in the size of populations when compared to the control, but not to the same extent as that of the two fungal isolates. Georgis and Gaugler (1991) summarized the findings of 82 nematode field trials against Japanese beetle larvae showing that successful control occurred with *H. bacteriophora* when the following conditions were met: the applications were made in autumn; the soil temperatures were >20 °C; the soil type was silty clay; the irrigation frequency was at 1-4 day intervals and the depth was <10 mm. They concluded that most test failures could be explained on the basis of unsuitable nematode species or on the basis of the prevailing environmental conditions.

Metarhizium anisopliae has been suggested as being better adapted to fluctuating environmental conditions and it has been said to require relatively little movement through the target insect to maintain fungal titre and virulence (Bidochka *et al*., 1998; Quesada-Moraga *et al*., 2007; Vänninen *et al*., 2000). The reduction in the size of WAA aerial colonies in fungal treatments indicates their potential to manage WAA.

A single application of EPNs and EPF applied to the roots effectively controlled WAA aerial colonies throughout the 90-day trial period. The fungi treatments appeared to control

WAA better than did the nematodes and the water only control. Although a few individual colonies became established in the treated trees, it did not lead to the usual seasonal increase in infestation, as occurred with the control treatments. The entomopathogens concerned might play a strategic role in the management of WAA aerial colonies.

The trial undertaken in the current study demonstrates that *S. yirgalamense*, Eco-Bb® strain R444 and ICIPE 69 can be used to control WAA on trees that are 33 years old. It is possible that the entomopathogens might be able to control WAA in even older trees, proving that such physical obstructions as severe root galling from past infestations do not prevent adequate soil penetration and saturation of the root zone. In the present study, owing to the hardness of the soil at depths greater than 15 cm, it was thought that most woolly aphid root colonies were likely to occur in the top 15 cm of the soil. Whether the *S. yirgalamense*, *B. bassiana* (Eco-Bb® strain R444*)* and *M. anisopliae* (ICIPE 69) treatment's success on older trees depends on the addition of a soil-wetting agent is still unproven. The limited trial time did not allow for the investigation of the role of the wetting agent in improving soil saturation, penetration and uptake in woolly aphid control and this requires further investigation.

In summary, the current study shows that *S. yirgalamense*, *B. bassiana* (Eco-Bb® strain R444*)* and *M. anisopliae* (ICIPE 69) are relatively effective against WAA under South African field conditions with *S. yirgalamense* being the least effective of the three. The control of the root colonies was reflected in the greatly reduced infestations of the aerial parts of the apple trees concerned. In short, the study achieved its aim of assessing the field efficacy of *S. yirgalamense*, *B. bassiana* and *M. anisopliae* against WAA.

Further work is, however, still required to fine-tune the application and the post-application conditions that are required for EPF in regard to WAA control, as well as to ensure that the application of the fungi is economically viable. Combined applications of EPF and sub lethal dosages of synthetic insecticides have been proposed as a strategy that can possibly be employed for improving the efficacy of the biocontrol agents. Such a

proposal has been made by many different researchers who have obtained good results for a range of subterranean and foliar crop pests as well as for vectors of human diseases. The use of chemicals at doses 10% below the recommended rate should contribute to a significant reduction in pesticide inputs. With the use of imidacloprid and fipronil at 1% and 10% of the recommended rates, respectively, with *M. anisopliae*, Shah *et al.* (2006) obtained similar control to that achieved with the recommended rate of chemical alone for the control of black vine weevil, *Otiorhynchus sulcatus* larvae, in a range of soil-less plant-growing media.

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CHAPTER 6

General Discussion

The woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is an important pest of apples, *Malus domestica* Borkh (Rosaceae), in the Western Cape province of South Africa. Its infestation of both the aerial parts and the roots of trees results in the destruction of developing buds in leaf axils and in the formation of galls on roots. Imidacloprid is currently registered for the control of WAA in South Africa. With chemical residue restrictions being placed on exported apples and the ability of WAA to develop resistance to chemical insecticides, it has become important to identify alternate control options as part of an integrated pest management (IPM) programme to control the WAA below economic threshold. One such alternate control strategy against WAA is the use of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) to control subterranean colonies. However, such WAA colonies are currently not the target of any other biological control agent.

This is the first report undertaken on the use of EPNs to control WAA in South Africa. This research is significant, especially for those who are interested in the field of IPM. Unfortunately, the EPNs tested in the current study did not yield the expected results. Only three species, of the seven EPN species and isolates screened, differed significantly from the control, with mortality < 50% (Chapter 2). At first, the mortality rate was ascribed to the size of the WAA, which was regarded as perhaps having been responsible for the poor performance of the EPNs involved, as they are defined as micro-insects. However, upon dissection of the dead WAA, nematodes that had developed into adults (recovered infective juveniles) were discovered inside the insect, which was proof that the EPNs had been capable of penetrating the WAA, killing them and developing into adults. Even more

surprising was the discovery of EPNs inside live WAA. Adult WAA exposed to EPNs showed no visual effects (e.g. colour change), further adding to the suspicion that the development of the EPN symbiotic bacteria did not occur normally within the haemocoel of the adult WAA. Indeed, the direct screening of the EPN symbiotic bacteria which was performed on WAA extract confirmed this suspicion. All screened bacteria isolates that were isolated from 14 species and isolates of EPNs from local and two imported species did not grow on the WAA extract. Previous studies showed that the nematode on its own is able to kill the insect host without the bacteria being present. However, the bacteria are of crucial importance for creating an environment in the insect host haemocoel that is conducive to its own survival and to the exponential growth and reproduction potential of the nematode. The mortality of WAA found in the current study could be ascribed to the nematode alone, without the assistance of the symbiotic bacteria.

To improve EPN efficacy, an increasing EPN concentration, exposure time and using different mulches were applied, without any success. This contributed to the drawing of the final conclusion made in this regard, namely that EPNs are not good biocontrol agents against WAA. Although the addition of adjuvants was not tested, at this stage it is not contemplated that such testing would in fact have made any difference. Even though EPNs manage to invade and sometimes to kill WAA, the fact that they neither develop nor reproduce inside the WAA renders them ineffective and therefore not economically viable, so that there is no potential for persistent effect in a soil environment. This study has played an important role in confirming that local EPNs are ineffective for the control of WAA, and in realising that future research should therefore concentrate on investigating other biological control agents. However, it is suggested that, should there be newly identified indigenous EPN isolates, these too must be tested using both fast screening and direct bacterial screening methods in the hope of finding a bacterial isolate that can overcome the antibacterial effect that is present in the haemocoel of WAA. The potential of the antibacterial

properties of the haemolymph of WAA as an antibacterial agent, with future application properties in agriculture and the pharmaceutical industry is recognised.

Currently, EPF are used worldwide as pest control agents. Commercial and locally isolated EPF were tested both in laboratory and field-based studies, in keeping with the belief that field-based studies are extremely important for determining whether or not such isolates are capable of performing effectively in a fully operational apple orchard, where various biotic and abiotic interactions can affect fungal efficacy either positively or negatively. The EPF isolates that were tested in the current study provided some level of WAA control, with even better control being recorded for the commercial isolates compared to the indigenous isolates in laboratory bioassays. The results obtained were in contrast with those of previous work done on the control of false codling moth, using locally isolated and the same commercial fungi that were used in the present study, where it was found that local isolates gave better performance than the commercial formulations used. Although even a small level of control in the adult stage is certainly beneficial, the aim of applying EPF to the soil was to reduce arboreal and subterranean colonies substantially. The better control provided by commercial EPF in relation to both the arboreal and subterranean colonies of WAA resulted in a reduction in the size of the WAA colonies concerned. With a reduction in the size of aerial colonies, the proportion of fruit becoming infested should therefore be reduced.

The results obtained in this study suggest that EPF research is a worthwhile investment and that such organisms are capable of performing effectively against WAA. It is therefore likely that the integration of EPF into WAA management should be able to complement currently employed control measures. The timing of EPF application is therefore likely to be of importance and such application should be directed at controlling subterranean colonies before migration occurs to the aerial parts of the plants. Applying EPF to an orchard utilising incompatible chemicals would be futile as no benefit would be derived from them. Therefore, compatibility will become an important issue if the fungi are to be successfully

integrated into current WAA management. It should, however, be kept in mind, that not only do such EPF isolates need to be compatible with other WAA control options, but also with other chemicals and control agents that are used in apple orchards. Further studies should focus on the testing of chemicals that are either applied using the same equipment as that which is used to apply EPF or on chemicals that are applied directly to the soil (e.g. fertilisers), as it is in such areas that the interaction between chemical and fungus is likely to be greatest. Even though the EPF performed well in the field trials the addition of adjuvants is likely improve their performance, so it is recommended that further studies should investigate whether this is indeed so.

Of the nine fungal isolates collected from an apple orchard in the Grabouw region, Western Cape, two were identified as showing potential against WAA. At this stage however, it would be premature to state that use of the isolates will undoubtedly be beneficial in respect of integration into WAA control measures as much research is still needed in this specific area. Such issues as cost-effective mass production and formulation methods, non-target effects and virulence towards other apple pests will need to be evaluated once a suitable EPF candidate is identified. The expectation that such EPF isolates will be able to be successfully integrated into WAA control practices is high. However, their compatibility might not necessarily be of major concern as similarly to the possibility of the fungi having some level of tolerance to the environmental conditions experienced in an apple orchard, they might also have some level of tolerance towards the chemicals that are commonly applied in the orchard. The use of such EPF might complement use of the available control strategies.

Usually with a combination of a variety of control tactics a high level of insect control is normally achieved, such was not the case in the current study, where combining the EPNs and EPF did not improve the results obtained. However, undertaking more studies is believed still to be required, with future research being aimed at also looking at the effect of combining EPF with other alternate control strategies as those employing granuloviruses.

Future research should aim to investigate the effect of soil type and moisture on both fungal efficacy and persistence. The compatibility of the effective EPN isolates (Eco-Bb® strain R444 and ICIPE 69) with commonly used agrochemicals particularly those applied directly to the soil should be investigated in future studies. Persistence over a longer timeframe than that which was employed in the current study, of the isolates in their formulated state in both the presence and the absence of organic matter should be investigated More surveys in apple orchards should also be conducted, so as to obtain more indigenous EPF isolates with high efficacy against WAA.