Control of the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), using entomopathogenic fungi

by

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Thesis presented in fulfilment of the requirements for the degree of Master of Science at Stellenbosch University, Department of Conservation Ecology and Entomology, Faculty of AgriSciences

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April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

April 2019

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions:

My supervisors, Dr. N.F Stokwe, and Co-supervisor, Prof. A.P Malan, for their guidance, patience and constructive criticism throughout this project. Thank you for entrusting me to conduct this research project.

Hort Pome for funding of my project.

D. Odendaal for her guidance and assistance in the laboratory.

T. Platt, T.M Dlamini, K.K. Dintwe, R. Nematswerani and A. Nxitywa, for their assistance with my field work.

Prof. D.G. Nel for his help with statistical analysis of my data.

M. Isaacks and M. Jacobs for their technical assistance with hiring University vehicles for my field work.

Farmers and farm managers at Heidiland (D. Brink and C. Groenewald), Fruit-ways (D. Vermulen), ARC –Grabouw and Monteith Trust farms, for allowing me to conduct my research on their farms.

My parents, M.J Mathulwe and M.A Mathulwe; siblings, M.M Mathulwe, M.T Mathulwe, M. Mathulwe, M.M Nchabeleng, P.P Ramohlale, M. Nchabeleng; aunt, M.V Mokhachele, and cousin's, M.N Molefane, L. Morwaswi and N.P Ramaila, for their love, constant support and encouragement.

All of my friends especially D. Thobela, for their support throughout.

Department of Conservation Ecology and Entomology, Stellenbosch University.

DEDICATION

This thesis is dedicated to both of my parents, M.A. and M.J. Mathulwe. Thank you for always believing in me, supporting and encouraging me to strive to achieve better things in life.

SUMMARY

Eriosoma lanigerum (Hausmann) (Hemiptera: Aphididae), or woolly apple aphid (WAA), is a serious pest of apple trees (*Malus domestica* Borkhausen), attacking both the root system and the arboreal parts of the tree. Current management of WAA in apple orchards relies on the use of both biological and chemical control. However, biological control using the principal parasitoid of the WAA, *Aphelinus mali* (Heldemann) (Hymenoptera: Aphelinidae), was found to be ineffective. The use of chemical control has also proven to be negative, as biotypes of the WAA have developed resistance to the chemicals used.

The use of entomopathogenic fungi (EPF) has been identified as promising biological control method against a wide array of insect pests. The main aim of the current study was to conduct a survey of EPF in the local apple orchards of the Western Cape province, and to screen their ability to control the root colonies of the WAA, under optimum laboratory conditions. The above was achieved by collection of soil and WAA-infested root samples from six apple farms. The EPF were baited from collected soil samples, using susceptible insect hosts, and directly from WAA females collected from the infested root samples. Successfully isolated fungi were grown on agar plates and screened for their pathogenicity against insects. The isolated EPF were identified both morphologically and molecularly, which include *Beauveria bassiana*, *Isaria fumosorosea*, *Metarhizium brunneum*, *Metarhizium pinghaense*, *Metarhizium robertsii* and *Purpureocillium lilacinum*.

The second aim of the study was to screen the six isolated EPF for their virulence against the WAA under optimum laboratory conditions. The above was achieved by conducting screening, concentration-dose-response and exposure-time-response bioassays. *Metarhizium pinghaense* and *M. brunneum* proved to be the most effective species against the root colonies of WAA, indicating that the local isolates of EPF have potential for the biological control of the WAA.

The final aim of the current study was to test for the persistence of *M. pinghaense* and *M. brunneum* on apple bark over a period of 3 weeks under laboratory conditions, and to determine whether the fungal conidia would attach to crawlers, or the fourth-stage nymphs, of the WAA as they move up tree trunks from the roots to the aerial parts of the apple trees. The above was done by means of spraying apple bark with a standard conidial concentration of 1.0×10^7 conidia/ml of both *M. pinghaense* and *M. brunneum*, respectively. The persistence of fungal conidia on the apple bark was measured using codling moth larvae as an indicator. The results indicated *M. pinghaense* to have better persistence on the apple bark over a period of 3 weeks than did *M. brunneum*. Further analysis of persistence, whereby the root colonies of the WAA were exposed to the dried conidia of

M. pinghaense on apple bark, showed that *M. pinghaense* was capable of inducing mortality of about 39-82% in colonies of WAA under optimum laboratory conditions, when exposed for a period of 10 days.

The current study has highlighted both the diversity of soilborne EPF in the local apple orchards of the Western Cape, and their potential to be successfully integrated in managing the presence of the WAA colonies in apple orchards. The local isolate of *M. pinghaense* has shown to be the best candidate for managing WAA. Therefore, future research should focus on testing the efficacy of the local isolate of *M. pinghaense* against the WAA, under both glasshouse and field conditions.

OPSOMMING

Eriosoma lanigerum (Hausmann) (Hemiptera: Aphididae), ook bekend as die appelbloedluis (WAA), is ’n ernstige pes van appelbome (*Malus domestica* Borkhausen) wat skade veroorsaak aan die wortelstelsel, takke en blare. Die luis word tans bestuur in appelboorde deur die gebruik van chemiese en biologiese beheermiddels. Biologiese beheer deur die gebruik van die hoofparasiet van die WAA, *Aphelinus mali* (Heldemann) (Hymenoptera: Aphelinidae), is egter getoets en daar was gevind dat dit oneffektief is in die beheer van die luis. Daar was ook bewys dat die gebruik van chemiese middels oneffektief is, aangesien sommige biotipes van WAA weerstand ontwikkel het teen die chemikalieë wat gebruik word.

Die gebruik van entomopatogeniese swamme (EPF) is geïdentifiseer as ’n belowende, effektiewe metode vir die beheer van verskeie insek peste. Die huidige studie was gefokus daarop om ’n opname te doen van EPF in die plaaslike appelboorde van die Wes-Kaap provinsie en dan te toets of hul die vermoë het om wortel kolonies van WAA te beheer in optimale laboratorium omstandighede. Om dit te toets, was grond en wortels wat geïnfekteer was met WAA, versamel van ses appelplase. Die EPF was toe geïsoleer van die grondmonsters deur vatbare insekte te gebruik as lokinsekte. EPF is ook geïsoleer van die wortels deur WAA wyfies te versamel vanaf die geïnfekteerde wortelmonsters. Swamme wat suksesvol geïsoleer is, is dan gegroei op agarplate, waarna hul vermoë om insek peste te beheer getoets is. Die EPF wat geïsoleer is, is geïdentifiseer deur morfologiese en molekulêre tegnieke en sluit in *Beauveria bassiana*, *Isaria fumosorosea*, *Metarhizium brunneum*, *Metarhizium pinghaense*, *Metarhizium robertsii* en *Purpureocillium lilacinum*.

Die tweede doelwit van die studie was om die effektiwiteit van die ses geïsoleerde EPF te toets op appelbloedluise in optimale laboratorium omstandighede. Om dit te toets was die EPF aangewend teen verskillende konsentrasies en die appelbloedluise blootgestel aan die swamme vir verskillende tydperke. Die twee EPS spesies, *Metarhizium pinghaense* en *M. brunneum* was mees effektief teen die wortel kolonies van ABL, wat beteken dat die plaaslike EPS isolate potensiaal het vir die beheer van die ABL.

Die derde doelwit van die studie was om te toets of *M. pinghaense* en *M. brunneum* kan oorleef op appelboombas vir ’n periode van drie weke in optimale laboratorium omstandighede, asook om vas te stel of die swamme se spore kan vassit aan die kruipers of die vierde stadium nimfe soos wat hul op beweeg teen die boomstam vanaf die wortels na die takke. Om bogenoemde te toets, was appelboom bas gespuit deur gebruik te maak van ’n standaard spoor konsentrasie van 1.0×10^7 spore/ml van *M. pinghaense* of *M. brunneum*. Die oorlewing van die swam spore op die bas was

getoets deur kodlingmot larwes te gebruik as indikatore. Die resultate het getoon dat *M. pinghaense* langer infektief gebly het op die bas as *M. brunneum*, oor 'n tydperk van 3 weke. Verdere analises is uitgevoer waar die wortel kolonies van die WAA blootgestel is aan die gedroogde spore van *M. pinghaense* op appel bas en het getoon dat *M. pinghaense* in staat was om sterftesyfers van 39-82% te veroorsaak in WAA kolonies, na blootstelling vir 10 dae, in optimale laboratorium omstandighede.

Die huidige studie beklemtoon die diversiteit van EPF in die plaaslike appelboorde van die Wes-Kaap, asook hul potensiaal om suksesvol geïntegreer te word in die bestuur van WAA getalle in appelboorde. Die studie het getoon dat die plaaslike isolaat, *M. pinghaense*, die beste kandidaat is uit die wat getoets is, vir die beheer van WAA. Toekomstige navorsing moet dus fokus daarop om die effektiwiteit te toets van *M. pinghaense* teen WAA in beide glashuis- en veld omstandighede.

Table of Contents

TABLE OF FIGURES

LIST OF TABLES

ABBREVIATIONS

min	minutes
sec	seconds
h	hours
EPF	Entomopathogenic fungi
WAA	Woolly apple aphid
EPN	Entomopathogenic nematodes
EF	Elongation factor
ITS	Internal transcribed spacers
m	metres
ml	mili-litres
µl	Micro-litres
L	litres
ºC	Degrees Celsius
%	Percent
>	Above/greater than

CHAPTER 1

An overview of woolly apple aphid, *Eriosoma lanigerum*, and the potential of South African entomopathogenic fungi as biological control agents

ABSTRACT

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is a serious pest of apple trees (*Malus domestica* L.), that attack both the root system and arboreal parts of the trees. The pest negatively affects apple production in the Western Cape province and in other apple-producing areas globally. Feeding of the WAA colonies on the cortical tissues of the apple trees induces formation of hypertrophic galls in the inner bark of the host plant root systems, which restricts the movement of nutrients and water throughout the tree. Attacks of apple trees by WAA also disrupts normal plant growth. The current management of populations of WAA in South Africa involves the use of both chemical and biological control. However, using such methods has proven to be ineffective in controlling the pest. Entomopathogenic fungi have been used across agroecosystems to control a wide range of agricultural pest insects. This study provides a comprehensive overview of the biology of WAA in apple orchards, and of the potential of entomopathogenic fungi as possible mycoinsecticides in controlling WAA in South African apple orchards.

Key words: entomopathogenic fungi, *Eriosoma lanigerum*, biological control, apples, *Malus domestica*, mycoinsecticides, *Beauvaria*, *Metarhizium*

INTRODUCTION

Apple production is by far one of the most important agricultural practices that make a significant economic contribution to the South African agricultural sector (Von Hoesslin 1978; Schwabe 1980). Apple trees, *Malus domestica* L. (Borkhausen), are deciduous fruit crops from the family Rosaceae. The practice of commercial apple farming was first established in the country during the late 19th century by the former Prime Minister of the Cape Colony, Cecil Rhodes, in the Western Cape province (Ferree & Warrington 2003). The main apple-producing areas in South Africa include mainly the coastal areas of the country, which are found in both the Western and the Eastern Cape provinces, as well as in portions of KwaZulu-Natal. These areas have ideal climatic conditions that foster and favour the production of good-quality deciduous fruits like apples. The commercial apple production areas of the Western Cape include the Grabouw–Vyeboom area, Elgin, Ceres, Groenland, Villiersdorp, and the Langkloof Valley (Pringle 1998; Meyer & Breitenbach 2004). These production areas experience cold, wet winters and warm, dry summers. In South Africa, the main apple cultivars grown in the country include Starking, Top Red, Jonathan, Golden Delicious, Winter Pearmain, Dunn's Seedling, Granny Smith, York Imperial, and Starkrimson. Of the apple cultivars, the apple industry is dominated by mainly three cultivar types, namely Golden Delicious, Granny Smith and the three red delicious types, which are Top Red, Starking, Starkrimson and Starkspur (Von Hoesslin 1978).

Eriosoma lanigerum (Hausmann) (Hemiptera: Aphididae), also known as the woolly apple aphid (WAA), is a serious pest of apple trees in all apple-producing regions across the world, including the production areas in the Western Cape province of South Africa (Klimstra & Rock 1985; Damavandian & Pringle 2002; Short 2003; Ge *et al.* 2016). The aphid is native to North America, where it uses the American elm trees (*Ulmus americana* Chapm.) as its primary host, and apple trees as its secondary host (Mols & Boers 2001; Short 2003; Lordan *et al.* 2015). In North America, WAA uses *U. americana* as its winter host, and *M. domestica* as its summer host (Mols & Boers 2001). The pest has since spread to other apple-producing regions throughout the world (Asplach & Bus 1999; Nicholas 2000; Sandanayaka *et al.* 2003; Bus *et al.* 2008). In the absence of their primary host, the WAA develop on their secondary host plant throughout the year (Lordan *et al.* 2015). WAA was transported from North America, and distributed to other apple-producing countries globally, mainly through the export of nursery apple plants (Walker *et al.* 1988).

In South Africa, the presence of *E. lanigerum* in apple orchards was first recorded in 1895, and the aphid has since spread throughout the apple-producing areas in the Western and Eastern Cape, which are the main regions of apple production in the country (Damavandian 2000; Heunis 2001; Christians 2003). A study conducted by Heunis and Pringle (2006), researching the field biology of *E. lanigerum* and its natural enemy *Aphelinus mali* Haldeman (Hymenoptera: Aphelinidae) in apple

orchards of the Western Cape, observed that the high abundance of the presence of WAA in these production areas peaks mainly in summer.

Edaphic populations of WAA pose a major threat to apple production in South Africa, as its presence in apple orchards affects the production of apple trees. WAA may infest apple trees simultaneously, both above- and below-ground (Pringle & Heunis 2001). Underground colonies of the aphid cause great damage to trees, before the effects of arboreal colonies can be detected (Short 2003; Short & Bergh 2004). Infestations by *E. lanigerum* also negatively affect apple plants health by colonising and attacking the root systems of both young and mature trees.

This review includes a comprehensive overview of the biology of WAA, the damage to apple trees, and the current management practices for the control of WAA, including the biology and potential of entomopathogenic fungi (EPF) as possible mycoinsecticides in controlling WAA in South African apple orchards.

WOOLLY APPLE APHID

Dispersal of woolly apple aphid

WAA is dispersed and transported between orchards, during management practices, and naturally by wind (Heunis & Pringle 2006; Costa *et al.* 2014). Dispersal is mainly through the first instar nymphs, which are also known as crawlers (Mols & Boers 2001; Lordan *et al.* 2015). Crawlers are produced mainly by overwintering females that reproduce at a relatively slow rate below ground, on the roots of the tree (Nicholas 2000; Damavandian & Pringle 2007; Gresham 2013). The aphid colonies infest both the root system and the aerial parts of the apple tree, including the branches and the tree trunk (Gupta & Miles 1975). Crawler activity peaks mainly during spring and early summer, mainly when the plant roots are actively growing and taking up nitrogen (Sandanayaka & Hale 2003; Heunis & Pringle 2006; Van Zyl 2011; Gresham 2013). During these seasons, some of the crawlers from the WAA colonies present at the plant root system, migrate up the apple trees in great numbers, and colonise the aerial parts of the trees, where they initiate large colonies (Heunis & Pringle 2006; Damavandian & Pringle 2007; Gontijo 2011; Van Zyl 2011).

In South Africa, the migration of crawlers from apple tree root systems to the arboreal parts of the tree occurs all year round. However, peaks in migration are mainly observed during the months of October until December (Heunis & Pringle 2006; Pringle & Heunis 2008; Lordan *et al.* 2015). During late autumn and early winter, small portions of some of the arboreal colonies move down to the root system, where they remain until spring (Nicholas *et al.* 2003; Heunis & Pringle 2006). However, cold winter temperatures do not usually have a negative effect on the pest colonies

concerned (Heunis & Pringle 2006). The colonies that are present on the aerial parts of the trees tend to attack mainly the injured parts of the trees, such as unhealed wounds (Nicholas *et al.* 2005; Pringle & Heunis 2008; Lordan *et al.* 2015). The aphids also colonise the undamaged parts of the trees, and especially the new sprouts of the season, during spring (Van Zyl 2011; Lordan *et al*. 2015).

The root colonies of WAA are a constant source of aerial infestation, due to the upward movement of crawlers from the roots to the aerial parts of the trees (Nicholas *et al.* 2005; Damavandian & Pringle 2007; Lordan *et al.* 2015). WAA infests both the canopy and the root system of apple trees, feeding on the bark, pruning wounds and new growth, but not on the leaves (Costa *et al.* 2014; Ge *et al.* 2016). In South Africa, as in other countries, feeding on the plant root system is more severe, in comparison to the feeding on the upper parts of the trees (Klimstra & Rock 1985; Ge *et al.* 2016). The capacity of the aphid to reproduce at high rates, reaching exponential population growth, also has a devastating impact on apple trees (Goggin 2007; Gresham 2013).

Morphology

The WAA are characterised by their reddish to purple colour and short antennae, with the adults measuring an approximate body length of 1 to 2 mm (Fig.1.1A) (Nicholas 2000; Christians 2003; Gresham 2013). The aphid derives its common name from its production of waxy filamentous white exudates that have a woolly appearance and that cover the aerial colonies (Fig.1.1B) (Sandanayaka & Bus 2005). *Eriosoma lanigerum* belongs to the aphid subfamily known as Pemphiginae. Aphids belonging to this subfamily are mainly characterised by the presence of wax glands in their cuticles (Smith 1999). The presence of WAA in apple orchards is distinguished by woolly masses, consisting of a wax covering that is secreted by the epithelial cells (Shaw & Walker 1996; Smith 1999).

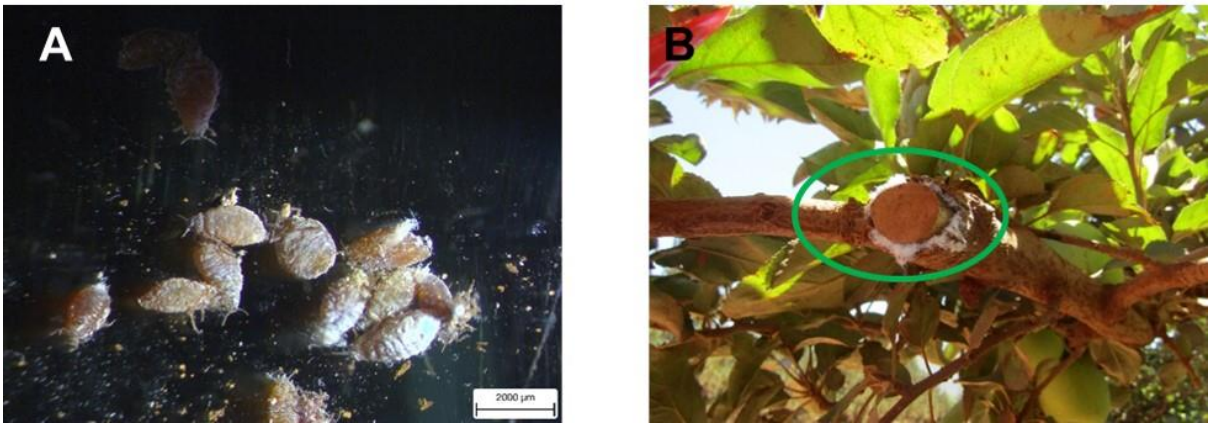

Fig. 1.1. *Eriosoma lanigerum* A) Woolly apple aphid adult females; B) arboreal colonies of WAA on a pruned wound, covered by a white waxy filamentous secretion.

The significance of the secreted wax

The thick filamentous waxy white mass covering colonies of the WAA provides protection against parasites and predators by acting as a feeding deterrent (Smith 1999; Moss *et al.* 2006). Other functions include waterproofing the aphid colonies during the rainy seasons, particularly for the arboreal colonies living on exposed branches (Smith 1999; Moss *et al.* 2006). The woolly mass protects the aphids, by preventing coating of the colonies with honeydew that may attract fungal pathogen attacks (Smith 1999; Short 2003). The wax coating of *E. lanigerum* is also highly reflective of ultraviolet light, and the white coating causes solar radiation to be reflected and thereby protecting the WAA colonies against solar radiation and harmful ultraviolet light (Smith 1999; Short 2003). The waxy covering also protects the arboreal colonies from some of the chemical insecticides that are commonly used to control pests (Van Zyl 2011).

Life cycle

The life cycle of *E. lanigerum* is composed of four nymphal instars and an adult stage (Fig. 2), with the aphid undergoing four moults, resulting in five apterous instars (Asante & Cairns 1995; Gontijo 2011; Costa *et al.* 2014). The first instar nymphs, which are known as crawlers, have the longest developmental period. Crawlers are the most motile instar of *E. lanigerum*, while the other remaining instars, and the adult stage, are mostly sessile once they start feeding (Asante 1994; Gontijo 2011).

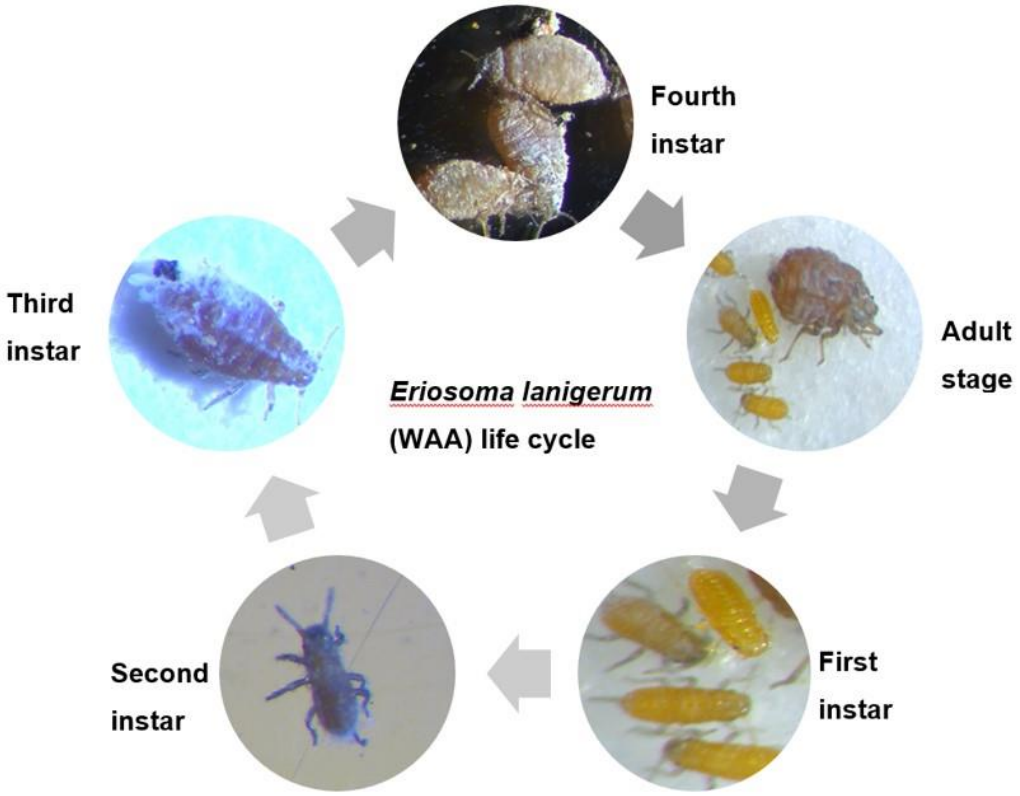

Fig.1.2. Pictorial life cycle of parthenogenetic *Eriosoma lanigerum* in South African apple orchards.

In North America, *E. lanigerum* reproduces both sexually and asexually on American elm and apple trees (Sandanayaka & Bus 2005; Gontijo 2011). The aphid has a holocyclic life cycle on American elm, where it overwinters as eggs on the primary host, and in early spring, wingless, viviparous females hatch from the eggs to feed on the developing buds of the elm tree (Craig 1953; Gontijo 2011). Winged offspring are mainly produced in response to unfavourable environmental conditions, such as poor plant quality, overcrowding on the host plant, or predation risks (Timm 2003; Le Ralec *et al.* 2010). Hatched females, which are the stem mother are also known as fundatrices (Christians 2003; Timm 2003). On the American elm, the stem mother females give birth parthenogenetically to two spring generations of wingless viviparous female aphids (Craig 1953; Timm 2003). The third generation that is produced by the aphids consists of alate viviparous females, which appear in early summer and fly to their secondary host, apple plants where they start reproducing parthenogenetically, managing to build up new colonies or populations in a relatively short period of time (Sandanayaka & Hale 2003; Timm 2003; Bhatia *et al.* 2011). In the absence of the American elm, the WAA overwinter on their secondary host plant in apple-producing regions with

their anholocyclic life cycle being restricted mainly to apple trees (Christians 2003; Timm 2003; Gresham 2013).

In South Africa, the reproduction of WAA is entirely parthenogenetic, which is a form of asexual reproduction that is found in female aphids. The growth and development of embryos takes place without fertilisation by a male aphid and the adult females give birth to live female offspring (Heunis & Pringle 2006; Van Zyl 2011). Parthenogenesis results in formation of true clones and offspring that are produced are genetically identical. In the Western Cape, the aphid can produce up to 18 generations a year (Timm 2003). Therefore, management of the aphid is challenging because of their short life cycle and extremely high reproductive rates (Dogimont *et al.* 2010).

Damage caused by the feeding of WAA colonies

Feeding of the WAA colonies upon the cortical tissues of the apple trees induces formation of hypertrophic galls in the inner bark of the host plant root systems (Sandanayaka & Hale 2003; Sandanayaka *et al*. 2003; Dardeau *et al*. 2014). Galls formed on the root system provide a fully sheltered micro-environment for the founding colonies and generations that they produce (Miles 1999; Dedryver *et al*. 2010). The formation and development of hypertrophic galls on the apple tree root system restricts water and nutrient movements and disrupt the normal plant growth and development (Brown *et al.* 1991; Damavandian 2000; Damavandian & Pringle 2007). The roots of apple trees, with galls formed by WAA, are also found to offer five times more resistance to water conduction than the non-galled roots (Brown & Schmitt 1990; Brown *et al.* 1991). Often, the formation of galls ruptures the plant tissue which provide additional feeding sites for the root colonies of WAA (Brown *et al*. 1991; Damavandian 2000; Dardeau *et al.* 2014). The ruptured plant tissue is invaded by fungal pathogens that cause fungal diseases, like canker disease in apple trees (Childs 1929; Nicholas 2000).

The galls that are created by colonies on the edaphic parts of the plant also have higher concentrations of amino acids and carbohydrates than do the non-galled parts of the trees, leading to such parts becoming readily colonised by the aphid (Mueller *et al.* 1992; Nicholas 2000). Nitrogen concentrations are also higher in the galled portions of the roots than in the non-galled roots, which causes nitrogen sinks that reduce the nitrogen concentrations in both the shoot and leaf tissues (Heunis 2001; Gontijo *et al.* 2012). Therefore, the galls on apple tree root systems tend to disrupt the nutrient balance of apple trees. Nitrogen is the major component in the nutrition of the WAA (Klingauf 1987; Miles 1999). WAA uses nitrogen in the form of amino acids (Damavandian & Pringle 2007).

High levels of root infestation impede young tree growth and usually kill off nursery plants, as root colonies cause damage to the apple tree root systems (Sandanayaka *et al.* 2003; Van Zyl 2011). Severe infestations of arboreal colonies in young apple trees can destroy bud growth on the trees (Costa *et al.* 2014). Root colonies, which have a cryptic habit occur all year round on the roots of mature trees mostly remaining undetected and therefore making it difficult to control them (Timm 2003).

Both the adults and the nymphs of the WAA cause damage to the apple tree root systems and thus affect tree vitality, which often leads to poor yields in terms of fruit quality and quantity (Brown *et al.* 1995; Dedryver *et al.* 2010; Costa *et al.* 2014). The aphid weakens apple trees in orchards by feeding on plant bark and roots, which reduces the fitness of the tree and also feeding by the aphids prevent wounds from healing (Sandanayaka *et al.* 2005). The aerial parts of apple trees, where feeding of WAA occurs, are also more sensitive to frost injury during winter than are the non-colonised parts (Van Zyl 2011). Feeding of WAA colonies on arboreal parts of apple trees where the canker disease has estabolished can spread the disease in the orchards (Van Zyl 2011). An increase in the number of WAA population outbreaks of arboreal colonies can also have severe and prolonged effects on an orchard's yield, because the presence of the aphids reduces the number of fruit buds, weakens the fruit-bearing wood, and stimulates premature defoliation of the plants (Short & Bergh 2004; Bergh & Short 2008; Gresham 2013). Therefore, heavy infestations of WAA often result in crop losses in apple orchards (Pringle & Heunis 2008).

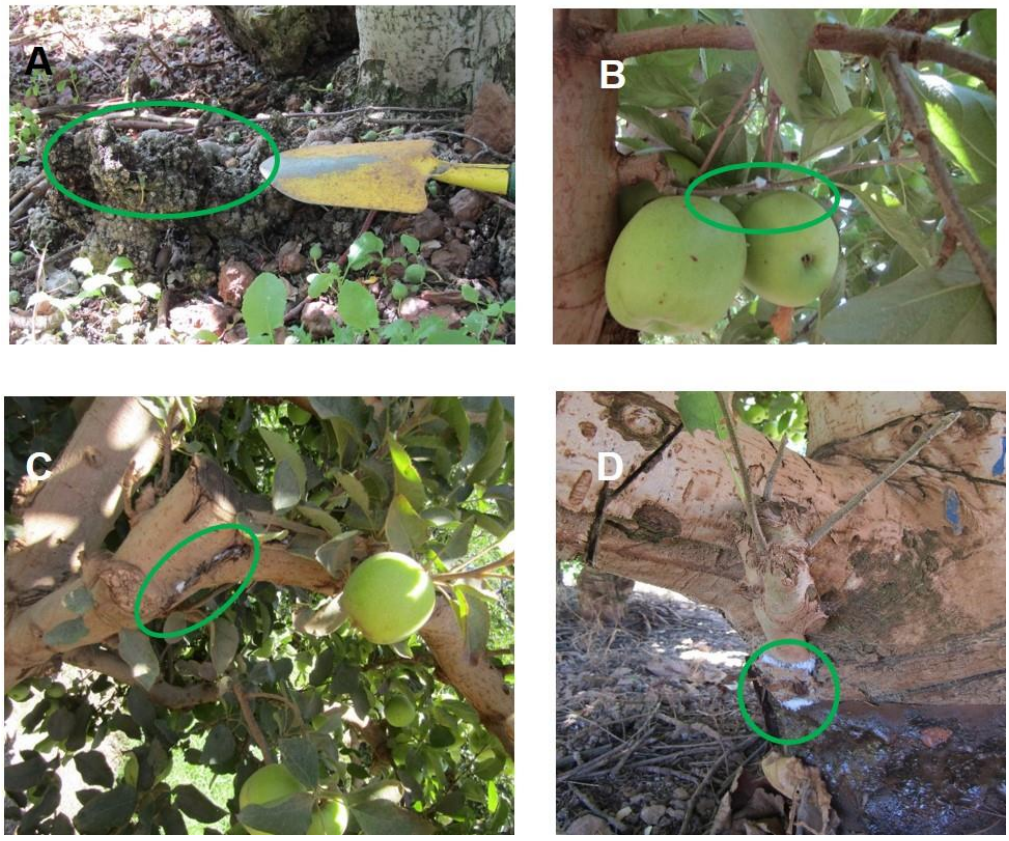

Fig. 1.3. Field damage caused by *Eriosoma lanigerum* (WAA) in apple orchards. A) Galled roots of apple trees; B) arboreal colonies of WAA on apple fruits; C, D) the colonisation of WAA on healing wounds on the tree trunk or branches.

CHEMICAL CONTROL

Over past decades, the most common method of controlling WAA infestations in apple orchards has been the application of high doses of chemical insecticides (Vega *et al.* 2009). Such application includes the use of both systematic and contact chemical insecticides (Bhatia *et al.* 2011; Van Zyl 2011). Control of WAA using contact insecticides has proven to be unsuccessful, mainly because of the protection that arboreal colonies offer to each other, due to high clustering, with protection also being provided by the waxy mass of filamentous exudates covering colonies (Heunis 2001; Christians 2003; Van Zyl 2011).

Contact insecticides also have a short residual action (Timm 2003). WAA are difficult to control with routine contact insecticide sprays, as some of the arboreal colonies tend to feed on wounds that provide some level of protection against the chemical sprays (Christians 2003). Therefore, the use of systematic chemical insecticides is mostly preferred, and is considered to be an effective method of control, because it is absorbed through the phloem sap and kills the feeding aphids (Bhatia *et al.* 2011). Systematic insecticides such as Imidacloprid damage the nervous system of insects that feed

on the phloem sap of treated plants, eventually leading to the death of the insect (Pringle *et al.* 1994; Van Zyl 2011). Therefore, systematic chemicals are the main means of chemical control for both arboreal and the underground colonies of WAA (Van Zyl 2011).

Chemical pesticides such as Vamidothion and other organophosphates have been used since the 1960s to control WAA (Damavandian 2000; Nicholas 2000; Damavandian & Pringle 2002). However, many apple growers globally have reported that the application of Vamidothion has failed to provide effective control of WAA in their orchards (Pringle *et al.* 1994; Nicholas 2000). Different biotypes of the WAA have been shown to have developed some level of resistance to the insecticide (Timm 2003). Pringle *et al.* (1994) found a Vamidothion-resistant biotype of WAA in South Africa occurring across two apple-producing areas in the Elgin and Grabouw areas. The aphid has also developed some level of resistance against a wide array of chemical insecticides that were used in the past to control the aphid pest (Christians 2003). The chemicals include chlorpyrifos and endosulfan, which have been used extensively in apple orchards where the strains of *E. lanigerum* have been shown to be resistant to Vamidothion (Pringle *et al.* 1994; Nicholas *et al.* 2003).

Chemicals like methyl-parathion, diazinon and endosulfan which have since the 1980s provided effective control of WAA infestation in apple orchards are no longer used due to the regulatory restrictions imposed on the export of treated fruits to other countries (Dedryver *et al.* 2010; Gontijo 2011; Van Zyl 2011; Costa *et al.* 2014). These chemical insecticides were considered to be the most effective in controlling arboreal colonies of WAA and in their absence additional outbreaks of the aphid have been experienced (Short 2003). The development of resistance against most chemical pesticides by WAA biotypes is thought to be the consequence of the repeated heavy application of a single class of insecticide in apple orchards (Jouanin *et al.* 1998).

An increase in the outbreaks and resistance of WAA to chemical pesticides seems to be associated mainly with the changes in pesticide programmes (Gontijo 2011; Gontijo *et al.* 2012). Presently, no pesticide is rated effective for controlling both the aerial and the edaphic colonies of WAA (Short 2003; Short & Bergh 2004). The removal of some of the chemical insecticides from the market, due to export restrictions on treated apples, has also resulted in a decline in the number of insecticides that are available for controlling and for keeping WAA at manageable levels (Beers & Himmel 2002). The high reproduction rates of the parthenogenetic females of WAA also contribute to the difficulty of controlling the aphid species using chemical control (Heunis 2001). The root colonies of WAA are mostly protected from the chemicals used, and it is therefore difficult to achieve good control with chemical insecticides (Klimstra & Rock 1985; Damavandian & Pringle 2002; Sandanayaka *et al.* 2003).

Chemical and biological control, as well as the use of resistant apple rootstocks, are presently regarded as the three main methods for managing WAA globally. The methods stated are used mainly to suppress WAA infestations of commercial apple orchards (Pringle 1998; Timm 2003; Gontijo 2011; Gresham 2013). Natural enemies play a major role in maintaining the lower population levels of WAA in most apple-growing regions (Bhatia *et al.* 2011; Gresham 2013). The use of chemicals also offers a short-term solution for the control of localised pest populations, and does not contribute to permanent pest density regulation (Stern *et al.* 1959).

BIOLOGICAL CONTROL OF THE WOOLLY APPLE APHID

Parasitoids

Parasitoids are insects that complete their larval development stage within a single host, whereby the host body provides all the nutrients that are required for the development of parasitoid larvae into an adult stage (Cross *et al.* 1999; Le Ralec *et al.* 2010). Feeding of the larvae eventually results in death of their hosts, once the parasitoid reaches maturity (Cross *et al.*1999a; Le Ralec *et al.* 2010). Parasitoids of all aphid species are endoparasitic koinobionts, meaning that the hosts are able to recover, and to continue feeding after having been parasitised, while the parasitoid continues to develop within the host (Cross *et al.* 1999a). Parasitoids normally insert their ovipositors through the insect's cuticle, depositing their eggs into their host. Thereafter, the parasitoid detaches its ovipositor and some of the aphid species either roll themselves into balls, while others show no sign of discomfort (Lundie 1924).

Aphelinus mali (Heldemann) (Hymenoptera: Aphelinidae) is a minute, solitary parasitic wasp and the principal parasitoid of WAA (Howard 1929; Shaw & Walker 1996; Mols & Boers 2001). The parasitic wasp, which is mainly active during the daytime, requires an environmental temperature of between 16 ºC and 37 ºC for activity (Nicholas *et al.* 2003). Like the WAA, *A. mali* is native to North America, where it is widely distributed in Canada and the eastern parts of the USA (Lundie 1924; Howard 1929; Short & Bergh 2004). The parasitoid, which was exported from North America in the early 20th century, was successfully introduced to many apple-growing regions globally, mainly for the biological control of *E. lanigerum* (Howard 1929; Cross *et al.* 1999a; Short 2003). In South Africa, *A. mali* was first introduced from North America to apple orchards in the Western Cape province in 1920 (Lundie 1939). Initially, the parasitoid had a high rate of success in biological control of *E. lanigerum*, to the point where the use of chemical insecticides to control the pest was no longer necessary, following the release of the parasitoid in apple orchards (Heunis 2001).

Fig. 1.4. Aerial colonies of woolly apple aphid parasitised by *Aphelinus mali*.

Predators

Apart from *A. mali,* WAA is known to have other natural enemies, including aphidophagous predatory insects such as ladybirds (Coleoptera: Coccinellidae), hoverflies (Diptera: Syrphidae), lacewings (Neuroptera: Hemerobiidae), and earwigs (Forficulidae: Dermaptera) (Mueller *et al.* 1988; Asante 1995; Dedryver *et al.* 2010; Lordan *et al.* 2015). In several European apple-producing countries, the European earwig, *Forficula auricularia,* has been recognised as an important predator of the aphids (Mueller *et al.* 1988; Asante 1995; Lordan *et al.* 2015). The earwig, which is known to feed on colonies of WAA during early spring, has been observed to be capable of controlling the population numbers of WAA (McLeod & Chant 1952). A single earwig was observed consuming a large number of overwintering WAA colonies in European apple orchards (Mueller *et al.* 1988; Lordan *et al.* 2015). Predation of the earwigs on WAA is considered important, as they help to control the population levels of WAA during the times when the *A. mali* parasitoid is not active (Lordan *et al.* 2015). In other apple-producing regions, *Pipiza radicum* (Diptera: Syrphidae) Walsh and Riley is also known to prey on the root colonies of the WAA (Lundie 1924).

Studies conducted in other countries, such as the USA, have shown that the larvae of three hoverfly species, namely *Heringia calcarata, Eupeodes americanus* and *Syrphus rectus*, are the main predators of WAA (Short & Bergh 2004; Bergh & Short 2008; Gresham 2013). Syriphids feeding on gall-forming aphids such as WAA belong to the Pipizine group. Their larvae can develop on both the edaphic and the arboreal colonies of WAA, and give rise to the spring adult generation (Rojo & Marcos-Garcia 1997). *Heringia calcarata* prey on both arboreal and edaphic colonies of WAA (Short 2003; Bergh & Short 2008).

Resistant rootstocks

All plant species express certain levels of resistance against attacks by pest species, including insect pests (Schuler *et al.* 1998). Plant resistance against insect pests is based mainly on various defence mechanisms, including an extensive range of toxic secondary metabolites that the plants produce (Schuler *et al.* 1998). Two other mechanisms of plant resistance against herbivore attacks include antixenosis and antibiosis (Dedryver *et al.* 2010; Dogimont *et al.* 2010). The antixenosis defence mechanism makes plants unsuitable or unappealing for colonisation by the pest and the plant is recognised as a poor host by pests. The antibiosis defence mechanism affects the pest's biotic potential or life cycle by reducing the pest's survival, growth or reproduction rates whenever it feeds on the plant (Christians 2003; Dedryver *et al.* 2010; Dogimont *et al.* 2010). The antibiosis mechanism mainly affects insects by reducing fecundity and by increasing its mortality (Dedryver *et al.* 2010; Dogimont *et al.* 2010). Therefore, only a few herbivorous insect pest species are able to attack and to feed on the individual plant species (Schuler *et al.* 1998). In many industrialised nations pest management strategies have become directed towards the use of transgenic plants expressing particular traits such as resistance to insects, fungi and viruses (Bus *et al.* 2008).

In response to WAA attacks and to the damage that the aphid causes to the root systems of apple trees as well as to the evolution of the pest's resistance to chemical insecticides, plants that are resistant to aphid attacks have been developed and cultivated (Norelli *et al.* 2003; Smith & Boyko 2007). Apple growers rely on the use of WAA-resistant rootstocks, mainly to limit the establishment of subterranean or root colonies on apple trees (Christians 2003; Sandanayaka *et al.* 2003). Using resistant apple cultivars has come to be considered the most effective, economical and environment-friendly strategy for management of this pest (Sandanayaka *et al.* 2003; Bhatia *et al.* 2011). Employing genetically resistant apple rootstocks is also thought to reduce the cost as well as the amount of time and effort that is spent controlling and protecting plants against WAA as their use has already resulted in a reduction in the use of insecticides (Jouanin *et al.* 1998; Schuler *et al.* 1998). Resistant rootstocks when grown into entire plants are thought to be resistant to colonisation by the aphid both below and above ground (Gupta & Miles 1975). Therefore, the use of resistant rootstock cultivars is considered to be the most desirable and preferred method for minimising the use of chemical insecticides (Dedryver *et al.* 2010; Bhatia *et al.* 2011).

The Northern Spy resistant rootstock from North America was the first identified rootstock that was found to have some degree of resistance against the establishment of WAA on their root system (Knight *et al.* 1962; Nicholas 2000; Christians 2003; Timm 2003). The apple cultivar's natural resistance against *E. lanigerum* has been known since the 19th century (Dedryver *et al.* 2010). However, work by Giliomee *et al.* (1968), undertaken in the Elgin area, Western Cape province, South Africa, showed the presence of biotypes or strains of WAA that were capable of attacking and

colonising apple trees derived from the resistant rootstocks of the Northern Spy cultivar. In Australia, biotypes of *E. lanigerum* were also observed to overcome the resistance of the Northern Spy-derived rootstocks and colonised apple trees derived from such rootstocks (Gupta & Miles 1975). Other series of resistant rootstocks, known as the Malling-Merton (MM) and Malling (M) series rootstocks were developed at the East Malling Research Station in England. The resistant rootstocks were mainly developed by using the Northern Spy plants as parent cultivars in breeding programmes (Rock & Zeiger 1974; Christians 2003; Dedryver *et al.* 2010). The resultant rootstocks include MM101-MM115 and those in the Merton group, namely M778, M779, M789 and M793 (Rock & Zeiger 1974; Nicholas 2000). The rootstocks specified are the most widely used resistant rootstocks globally (Nicholas 2000; Dedryver *et al.* 2010). However, biotypes of WAA in other apple-producing countries such as Australia, the USA and South Africa have been shown to overcome the resistance of resistant rootstocks like MM.111 (Gupta & Miles 1975).

Following on the above, the focus has shifted from the use of resistant rootstocks towards the use of relatively small apple trees derived from dwarf rootstocks. The method has been extensively used throughout apple-producing regions globally so as to reduce the impact that the aphid has on the apple-growing industries internationally (Simons & Chu 1985; Nicholas 2000). Dwarf rootstocks that are planted mostly in large quantities include M9, M26, M27, and P2 (Nicholas 2000). However, the use of such dwarf rootstock as M.26 has shown that the rootstock has intermediate susceptibility to WAA, resulting in the dwarf rootstocks not providing effective resistance against the WAA (Simons & Chu 1985).

ENTOMOPATHOGENIC FUNGI

Occurrence and distribution

The soil environment is an important reservoir of a large diversity of fungal species, with various ecological functions (Shah & Pell 2003; Meyling & Eilenberg 2007). Several studies have shown the presence of several EPF species in soil samples, with the species including *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* (Metch.) Sorokin (Hypocreales: Clavicipitaceae), and *Isaria* (= *Paecilomyces) fumosorosea* (Chase *et al.* 1986; Zimmermann 1986; Meyling & Eilenberg 2007). *Beauveria bassiana* and *M. anisopliae* are among the most well-studied fungal species within agroecosystems and have been used to control a number of insect pests in some countries (Chase *et al.* 1986; Leger *et al.* 1992; Wraight *et al.* 2000; Meyling & Eilenberg 2007). However, most of the soilborne EPF species have a global distribution (Meyling & Eilenberg 2007). Entomopathogenic fungal species were shown to be good candidates for the future biological control of insect pests, as entomopathogens are among the natural enemies that

cause epizootics in arthropod species in agroecosystems (Leger *et al.* 1992; Roy *et al.* 2006; Meyling & Eilenberg 2007). They cause lethal infections to their host insects and thus help to regulate pest populations. Fungi are also host-specific, pose a low risk of attacking non-target insects (Shahid *et al.* 2012).

Important EPF species with biocontrol potential are found mainly across two fungal divisions: Ascomycota and Entomophthoromycota. The fungal division Ascomycota contains Hypocreales fungal species, including the *M. anisopliae* complex and the *Beauveria* species, of which some are commercially produced and used globally for biological control against a variety of agricultural pests in agroecosystems (De Faria & Wraight 2007; Quesada-Moraga *et al.* 2007; Hatting *et al*. 2018). Fungal species belonging to the *M. anisopliae* species complex, including the species *Metarhizium brunneum* Petch, *Metarhizium robertsii* (Metchnikoff) Sorokin, *Metarhizium pinghaense* Chen & Guo, and *Metarhizium anisopliae* (Metchn.) Sorokin, are cosmopolitan soil-dwelling entomopathogens that have been well studied in terms of biological control (Rehner & Kepler 2017). Such commercially developed products as ICIPE69, developed from a *M. anisopliae* fungal isolate, have been used for the management of insect pests like thrips and whiteflies in South Africa (Hatting *et al.* 2018).

Beauveria contains 18 of the well-known species, of which four are found in South Africa: *Beauveria bassiana* (Bals.-Criv.) Vuill, *Beauveria brongniartii* (Sacc.) Petch, *Beauveria pseudobassiana* Rehner & Humber, and *Beauveria caledonica* Bissett & Widden (De Faria & Wraight 2007; Abaajeh & Nchu 2015). Some of the fungal isolates are currently commercialised for use against pests in South Africa (Hatting *et al.* 2018). However, entomopathogenic fungal species in the Entomophthoromycota division have not been as well assessed as biological control agents as those belonging to the Ascomycota division. However, studies have shown that some of the fungal isolates in this division, like *Pandora* (=*Erynia*) *neoaphidis* (Remaud. & Hennebert) Humber, are capable of causing increased mortalities of such pests as cereal aphids, relative to their natural predators and parasitoids (Hatting *et al.* 1999, 2000).

Life cycles of the majority of the entomopathogenic fungal species usually synchronise with the life cycle stages of their hosts, as well as with the prevailing environmental conditions (Shah & Pell 2003). The fungal species are known to have two main life cycle stages including mainly a mycelium or vegetative growth stage which forms on the outer surface of the host insect and a budding phase that occurs inside the host's haemocoel (Inglis *et al.* 2001). Moisture and temperature are the main important abiotic factors affecting EPF, because certain fungal entomopathogens require moisture from their surroundings to germinate and to penetrate their insect hosts (Cross *et al.* 1999b; Hallsworth & Magan 1999; Wraight *et al.* 2000). Most of the EPF prefer optimal growth temperatures of between 25 ºC and 35 ºC (Abaajeh & Nchu 2015). Conidiospores produced by some of the fungal pathogens also have an airborne distributive phase, enabling the spores to spread among different

hosts. The insect hosts infect each other and in addition the spores can tolerate harsh environmental conditions (Alves *et al.* 1998; Barta 2010; Burges 2012).

Mode of action

The proteinaceous cuticle of insects acts as an effective barrier against most microbes, including fungal species (Leger *et al.* 1992; Kang *et al.* 1999). However, some species of EPF are adapted to infect a variety of insect pest species including aphids, lepidopterous insects and thrips which are mostly of great concern within agricultural ecosystems globally (Roberts & Humber 1981). Unlike other disease-causing pathogenic microorganisms, such as bacteria, EPF species have a unique mechanism of attacking their host, mainly through the cuticle, with them not needing first to be ingested by the host (Leger *et al.* 1992; Cross *et al.* 1999b; Pedrini *et al.* 2007). A combination of enzymatic degradation and mechanical forces or pressures produced by the fungus are thought to be involved in overcoming the barrier presented by the insect's cuticle (Leger *et al.* 1986; Charnley & Leger 1991; Kang *et al.* 1999). Entomopathogenic fungi are known to produce a variety of insect cuticle-degrading enzymes including chitinase, lipase and protease (Leger *et al.* 1986a, b; Schrank & Vainstein 2010).

To achieve infection, infective fungal spores land directly on the cuticle of a suitable host and adhere or attach themselves to the cuticle mostly through secretion of adhesive mucilage (Charnley & Leger 1991; Roy *et al.* 2006; Shahid *et al.* 2012). After adhesion, the next stage of virulence of the conidia strains is the release of an enzyme that hydrolyses the epicuticle of the host, so as to allow the fungal spore to germinate and penetrate the host cuticle (Roy *et al.* 2006; Vega *et al.* 2009; Burges 2012; Shahid *et al.* 2012). The fungal conidia form structures including a germ tube, an appressorium and penetration pegs that assist the fungus to penetrate the cuticle (Bateman *et al.* 1996; Shah & Pell 2003). Once past the insect cuticle, the EPF germ tube multiplies as a progression of single- or multi-celled structures that grow into mycelia (Roy *et al.* 2006). The fungi invade and colonise the insect's body and the circulatory system known as the haemolymph, resulting in the death of the insect within 3 to 7 days after infection (Shah & Pell 2003; Roy *et al.* 2006; Burges 2012; Shahid *et al.* 2012). The host's death mostly results from hyphal bodies which are also known as mycelia, spreading throughout the insect body and depleting their host nutrients thereby causing the death of the host as a result of its physiological starvation (Barta 2010; Shahid *et al.* 2012). During the process, the fungus also produces many infective conidia for further transmission, and also resting structure for persistence (Roy *et al.* 2006).

Fungal insect hosts also die, either because of the production of toxins by the internally growing fungi, or due to the depletion of the insect's metabolites and the destruction of the host's vital tissues and organs (Rath 2000; Roy *et al.* 2006; Shahid *et al.* 2012). The toxins or metabolites produced by

the EPF include beauvericin and destruxins, which mostly weaken the host's defence systems while also attacking the Malpighian tubules, thus affecting the host's excretory systems and resulting in feeding and mobility difficulties (Kershaw *et al.* 1999; Schrank & Vainstein 2010). Following the host's death, the fungal mycelium then emerges from the insect's cadaver and completes its life cycle by means of sporulation (Cross *et al.* 1999b; Barta 2010). Once they are present within a specific environment, the fungal conidia spread throughout the environment in which the insect host is present (Shah & Pell 2003).

Entomopathogenic fungi belonging to the order entomophthorales have biotrophic associations with their insect hosts, and their conidia are discharged and transmitted to other insects while the initial host is still alive (Shah & Pell 2003). The conidia of such hyphomycetes fungi as *Beauveria* are passively dispersed from the cadavers of infected insects. Hymphocetes, which are mostly known as opportunistic fungal pathogens that have a hemibiotrophic association with their host, tend to infect many different insect orders (Roberts 1981; Shah & Pell 2003). The circulation, or movement, of hyphomycetes fungi within their host insect is carried out by means of blastospores, which are yeast like cells that resemble fungal hyphal bodies. They have both a parasitic phase and a saprophytic phase after the death of their insect host, with such death being primarily associated with the overwhelming production of toxins (Cross *et al.* 1999b; Shah & Pell 2003; Schrank & Vainstein 2010).

Previous research undertaken using entomopathogenic fungi

The fungal isolate *I. fumosorosea* has previously been successfully used in biological control of such hemipteran species as whiteflies and leafhoppers (Zimmermann 2008). Tounou *et al.* (2003) observed that *I. fumosorosea* resulted in 97 % mortality when used against *Empoasca decipiens* Paoli (Auchenorrhyncha: Cicadellidae), being green leafhoppers, that cause significant damage to greenhouse crops such as cucumbers and green beans in European countries. The isolate has also been used successfully in the biological control of the Russian wheat aphid, *Diuraphis noxia*, which is a serious and widespread pest of barley and wheat (Vandenberg *et al*. 1998). Lacey *et al.* (2011) also showed that the use of *I. fumosorosea* and *M. anisopliae* resulted in the successful control of the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae), which is a serious pest of potatoes in Mexico, the USA and New Zealand where it causes a disease known as zebra chip. Use of the two fungal isolates during the study was observed to reduce the symptoms of zebra chip disease significantly as well as to reduce the amount of plant damage incurred.

Lopez *et al.* (2014) also showed that *P. lilacinum* has negative effects on the reproduction of cotton aphids, *Aphis gossypii* Glover, when it is present as an endophyte of the plant under both greenhouse and field conditions. *Purpureocillium lilacinum* is mainly known as a nematophagous

fungi that is used in the biocontrol of root-knot and reniform nematodes such as *Meloidogyne incognita* and *Rotylenchulus reniformis* (Kiewnick *et al.* 2011; Munawar *et al.* 2011; Chaudhary & Kaul 2012).

In South Africa, Stokwe (2016) explored the efficacy of entomopathogenic nematodes and fungi as biological control agents of *E. lanigerum,* under South African conditions. The results indicated that the use of commercial fungal isolates derived from *B. bassiana* and *M. anisopliae,* have the potential for use against this particular pest species in both laboratory and field trials in apple orchards. Goble (2009) investigated the use of EPF to control three insect pest species of citrus in South Africa, namely false codling moth *(Thaumatotibia leucotreta*), Mediterranean fruit fly (*Ceratitis capitate*) and Natal fruit fly (*C. rosa*). The study found that the fungal isolates derived from *Beauveria* and *Metarhizium* species were capable of inducing mortality in the pest species, and thus indicated the potential of EPF use against agricultural insect pests.

Similarly, Coombes (2012) investigating the use of EPF for the control of the soilborne life stages of false codling moth*,* also showed that the application of EPF isolates *Metarhizium* and *Beauveria*, were capable of causing fungal infection of the soil-dwelling stages of the false codling moth. Damavandian (2000) showed that the pathogenic association existed between the root colonies of WAA and some of the soilborne EPF, with the results of the study concerned showing that the root colonies were infected by a wide range of EPF.

Entomopathogenic fungi as mycoinsecticides

A major limiting factor affecting agricultural production yield involves the amount of damage that is done to cultivated crops by aggressive insect pests (Vega *et al.* 2009). EPF have potential as biological control agents of such sap-sucking insects as the WAA, which cannot easily be controlled using chemical pesticides and other biological control means (Leger *et al.* 1992; Cross *et al.* 1999b; Pu *et al.* 2005). As fungal pathogens play an important role in pest insect population regulation, they are generally considered as very promising biological control agents (Charnley & Leger 1991; St Leger *et al.* 1996; Gilbert & Gill 2010). Fungal pathogens which are important natural biological control agents against many insects and other arthropods, frequently cause epizootics that reduce host populations (Leger *et al.* 1992; Roy *et al.* 2006; Vega *et al.* 2009; Shahid *et al.* 2012).

The development of resistance to chemical insecticides by pests, and the concerns expressed over the deleterious effects of chemicals on the environment have spurred on the drive to develop microbial agents for the control of insect pests (Inglis *et al.* 2001). As apple orchards provide long-term, stable habitats to the populations of various soilborne EPF, such populations are likely to be high in the soil of apple orchards (Cross *et al.* 1999b).

The use of such EPF as Hyphomycetes and Entomopathorales as mycoinsecticides for insect pests is also considered as an environmentally acceptable alternative to the employment of chemical pesticides, as they pose less harm to the environment (St Leger *et al.* 1996; Shah & Pell 2003). EPF have long been used as biological control agents for controlling many pest species, with various levels of success (Shah & Pell 2003). EPF cause mycosis in many different taxa of arthropods and in almost every order of insects, whose every life stage they are capable of infecting (Shah & Pell 2003; Shahid *et al.* 2012). EPF like *M. anisopliae*, *B. bassiana* and *Nomuraea rileyi* (Deuteromycotina: Hyphomycetes) are among some of the currently commercially available strains of fungi that are used as mycoinsecticides (Leger *et al.* 1992; Pedrini *et al.* 2007).

The use of indigenous strains of EPF against crop-damaging insects across agricultural ecosystems is considered as the preferred method in contrast to others because the fungi are already adapted to the local climatic conditions (Abaajeh & Nchu 2015). *Beauveria* (Ascomycota: Hypocreales) and *Metarhizium* (Ascomycota: Hypocreales) are also considered as the cosmopolitan genera of soilborne EPF. The *Beauveria* fungal species are capable of infecting approximately 700 arthropod species, with the *Metarhizium* species also having a wide range of insect hosts and being capable of infecting over 200 different insect species of which most are agricultural crop pests (Abaajah & Nchu 2015). Different species of EPF are identified mostly on the basis of the fungal growth observed on the outside of the insect cadaver (Vega *et al.* 2009; Barta 2010).

CONCLUSIONS

Based on the information obtained through the literature review and by way of communication with various farmers around the apple-producing areas of the Western Cape province, the presence of *E. lanigerum* in apple orchards can be seen to be of great concern to the apple-producing industry of South Africa. The pest species poses a great threat not only to the production output as it affects the vitality of apple trees by means of attacking both the aerial and the root systems of the trees, as well as affecting the quality of the apple fruit produced. The management of WAA through such methods as the use of apple cultivars like Northern Spy, derived mainly from resistant apple rootstocks, the use of chemical insecticides and the use of biological control using natural predators and parasitoids has proven to be relatively ineffective in controlling the WAA. Biological control using the parasitic wasp *A. mali*, has also not been very effective in controlling the presence of WAA in South African apple orchards, since the edaphic colonies of the WAA tend to occur in protective environments that protect them against the particular parasitoid mentioned, therefore resulting in the parasitoid only effectively controlling aerial WAA colonies.

Research conducted in previous studies has indicated the potential of soilborne EPF isolates in controlling the various insect pest species across agricultural ecosystems. Exploring the use of

indigenous soil fungi as possible mycoinsecticides for use against WAA in apple orchards is therefore of importance. Research focusing on the effect that the local EPF isolates have when used against WAA should enable the discovery of a possible EPF isolate that might have some level of virulence when tested against the WAA.

AIM OF THE STUDY

The main aim of the current study was to investigate the potential of local EPF isolates as biological control agents against WAA.

The objectives of the study included the following:

1. To survey for the presence of EPF in local apple orchards, and to identify isolates to species level using molecular techniques;

2. To screen selected identified EPF isolates for their virulence against WAA under laboratory conditions; and

3. To apply the most virulent EPF isolate to apple bark, so as to be able to determine the degree of persistence and contact infection attained under laboratory conditions.

The chapters of the present study have been written as separate publishable papers, and, therefore, some repetitions, in the different chapters, have been unavoidable. The chapters are written according to the format of the journal *African Entomology.*

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CHAPTER 2

A survey of entomopathogenic fungi in *Eriosoma lanigerum*-infested apple orchards of the Western Cape province of South Africa

ABSTRACT

Eriosoma lanigerum (Hausmann) (Hemiptera: Aphididae), woolly apple aphid (WAA), is an important crop pest that attacks apple trees globally, resulting in significant economic losses. WAA is a pest of serious concern to the apple-producing areas of South Africa as it has become widespread, with it currently being considered as the worst of all pests attacking apple trees. The aphid has developed some level of resistance against several chemical insecticides used for its control. The main aim of this study was to survey entomopathogenic fungi (EPF) in the local apple orchards of the Western Cape province, and to screen them for their ability to cause WAA mortality under optimum laboratory conditions. Soil and infested root samples were collected across six apple orchards. EPF were baited from the soil and root samples; and the isolated EPF were identified to species level. The soil samples were baited using *Galleria mellonella* and *Tenebrio molitor*, and then grown on Saboraud dextrose agar plates supplemented with yeast to obtain clean fungal colonies. Isolated EPF strains were tested for pathogenicity against insects in a screening bioassay and pathogenic EPF were morphologically and molecularly identified. Phylogenetic trees of collected EPF were constructed, using the internal transcribed spacer (ITS) and the elongation factor (EF) gene regions. Six species of EPF were isolated and identified from the soil samples. Based on the results from the screening bioassay, the EPF isolates have shown potential for successful use in management of WAA.

Key words: Biological control, entomopathogenic fungi, pathogenicity, virulence, apple orchards

INTRODUCTION

Apple, *Malus domestica* (Borkhausen), production is one the most economically important horticultural crop production practices in temperate regions across the world (Harris *et al.* 2002; Stoeckli *et al.* 2008). However, apple trees in apple orchards are mostly subjected to attack by several insect pests that negatively affect productivity, resulting in significant crop damage (Beers *et al.* 2003). *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) or woolly apple aphid (WAA) is a major crop pest that attacks apple trees in apple-producing regions globally, resulting in significant economic losses to the apple-producing industry (Blackman & Eastop 2000; Saeid & Ateyyat 2014). It is native to North America, having initially been described by Friedrich Hausmann in 1802 (Brown *et al.* 1991), with the record of its presence in South African apple orchards in 1895 having been reported by Lounsbury (Myburgh *et al.* 1973; Pringle *et al.* 1994).

WAA is of serious concern to the main apple production areas of South Africa, as it has become widespread within apple orchards, with it being considered as one of the main insect pests that attack apple trees (Damavandian 2000; Heunis & Pringle 2003). Infestation of apple trees by root-based colonies of WAA induces formation of hypertrophic galls on the root system, with the presence of galls impeding the flow of plant nutrients and water throughout the plant (Brown *et al.* 1995). The root colonies of WAA are the main source of arboreal infestation, as the first instar nymphs which are also known as crawlers, continuously migrate from the roots to the arboreal parts of the plant (Walker 1985; Pringle *et al.* 1994; Mols & Boers 2001; Lordan *et al.* 2015). Crawlers are produced by adult female aphids which reproduce parthenogenetically and overwinter on the roots (Thwaite & Bower 1983; Pringle *et al.* 1994).

Arboreal colonies of WAA form densely packed colonies, covered by a white, waxy filamentous secretion which protects the colonies from unfavourable environmental conditions, attacks by parasites and predators, and from chemical pesticides/insecticides (Smith 1999; Moss *et al.* 2006). Arboreal colonies mostly infest and develop on vulnerable parts of the apple trees such as on pruning cuts and spits that have resulted from heavy cropping (Nicholas *et al.* 2005). In cases of severe infestation, the presence of the WAA can result in yearly reduction of the quality and quantity of the fruit that is produced by each apple tree concerned. This is due to the destruction of buds, shoots and fruit-bearing wood, which eventually affects production capacity (Childs 1929; Brown & Schmitt 1990; Brown *et al.* 1995).

Current management of population levels of WAA in South African apple orchards, and across the world, generally relies on the combined use of biological and/or chemical control with other alternative methods like use of resistant apple cultivars (Stern *et al.* 1959; Pringle 1998; Timm 2003; Stoeckli *et al.* 2008; Gontijo 2011; Gresham 2013). For biological control, during the 20th century the main parasitoid of WAA, *Aphelinus mali* (Heldemann) (Hymenoptera: Aphelinidae) was successfully

introduced from North America into apple orchards globally. In the South African apple-growing areas, the parasitoid was introduced in 1920 (Lundie 1939; Hagen & Van den Bosch 1968; Cross *et al.*1999b; Short 2003; Dedryver *et al.* 2010). Initially following its release, the parasitoid had high rates of success in controlling the population levels of WAA in apple orchards. The parasitoid prefers parasitizing the third instar nymphs of WAA, and overwinter as larvae within the aphid (Gurney 1926; Mueller *et al.* 1992; Cross *et al.* 1999b; Heunis 2001). However*, A. mali* parasitizes and controls mainly the arboreal colonies of WAA, and not the root colonies, which contributes to the current failure of the parasitoid to control the WAA (Heunis 2001). Hence, the parasitoid alone was found to be ineffective at controlling and at preventing outbreaks of WAA (Walker 1985; Heunis & Pringle 2006).

The most common method of controlling WAA infestation in apple orchards has, for the past several decades, been the application of high doses of chemical insecticides (Vega *et al.* 2009; Bhatia *et al.* 2011). Such chemical pesticides as Vamidothion, and other organophosphates, have been used since the 1960s to control WAA in apple orchards (Damavandian 2000; Nicholas 2000; Damavandian & Pringle 2002; Christians 2003). However, many apple growers globally have reported that Vamidothion failed to provide effective control of WAA in their orchards, as different biotypes of WAA have been shown to have developed some level of resistance to the insecticide (Pringle *et al.* 1994; Nicholas 2000; Timm 2003). Pringle *et al.* (1994) also found a Vamidothion-resistant biotype of WAA in South Africa, across two apple-producing areas in the Elgin and Grabouw area. The aphid has also developed some level of resistance against a wide array of chemical insecticides that were used in the past to control it (Christians 2003).

The development of resistance against chemical insecticides by pests such as WAA, as well as concerns regarding the deleterious effects of chemicals on the environment, have provided a strong drive for the development of microbial agents to be used in controlling insect pests (Inglis *et al.* 2001). Entomopathogenic fungi (EPF) have the potential to be used for biological control of sap-sucking insects such as the WAA, which cannot easily be controlled using chemical pesticides and other biological control means (Leger *et al.* 1992; Cross *et al.* 1999a; Pu *et al.* 2005; Darbro *et al.* 2011). The potential of EPF for biological control was first noticed during the 19th century by Metchnikoff, in 1879, and by Krassilstschik, in 1888, when they mass-produced *Metarhizium anisopliae* (Metchn.) Sorokin, testing the fungus for control of the wheat cockchafer, *Anisoplia austriaca*, as well as for the control of the sugar beet curculionid, *Cleonus punctiventris* (Gillespie & Moorhouse 1989).

The fungal division Ascomycota contains the Hypocreales fungal species including the ‘PARBE’ clade of the *M. anisopliae* complex and the *Beauveria* species, of which some are commercially produced and used globally for biological control against a variety of agricultural pests in agroecosystems (De Faria & Wraight 2007; Quesada-Moraga 2007; Hatting *et al*. 2018). Fungal species belonging to the *M. anisopliae* species complex, including the species *Metarhizium*

brunneum Petch, *Metarhizium robertsii* (Metchnikoff) Sorokin, *Metarhizium pinghaense* Chen & Guo, and *M. anisopliae* are cosmopolitan soil-dwelling entomopathogens that have been well studied in terms of biological control (Rehner & Kepler 2017). Entomopathogenic fungi cause mycosis in many different taxa of arthropods and in almost every order of insects, with them having the ability to infect all the life stages of insects (Dedryver *et al.* 2010; Shahid *et al.* 2012).

Beauveria bassiana (Bals.-Criv.) Vuill., which is one of the most extensively studied EPF has received much developmental attention as a microbial insecticide that can be employed for commercial purposes over other EPF (Bartlett & Jaronski 1988; De Faria & Wraight 2007; Ortiz-Urquiza *et al.* 2010; Lacey 2016). Mycoinsecticides developed from strains of *M. anisopliae, Isaria fumosorosea* (Wize) A.H.S. Br. & G. Sm. (formerly known as *Paecilomyces fumosoroseus*), and *Beauveria brongniartii* (Saccardo) Petch, are also commercially available and are used to control a variety of crop pests in agricultural environments (Shi & Feng 2004; De Faria & Wraight 2007; Lacey 2016). Such commercially developed products as Real Metarhizium 69 (Meta 69 strain ICIPE69), developed from a *M. anisopliae*, have been used in South Africa for management of such insect pests as thrips and whiteflies (Hatting *et al.* 2018). The use of indigenous strains of EPF, across agricultural ecosystems, against crop-damaging insects is also considered as a preferred method, because the fungi concerned are already adapted to the local climatic conditions (Abaajeh & Nchu 2015).

The current study addressed the following question: Can local EPF isolates with a potential to control WAA be successfully isolated from the soil environment of the local apple orchards of the Western Cape? It was thus predicted that EPF capable of causing epizootics in populations of WAA will be successfully isolated from the soil environment in apple orchards. The aim of the study was to survey for EPF in the local apple orchards of the Western Cape province. The fungal isolates were screened for their virulence against WAA. The above was accomplished by means of the collection of soil and WAA-infested root samples from various apple orchards. EPF were trapped from the soil samples using susceptible host insects, and directly from the WAA females. All isolated fungi were identified to species level, using both morphological and molecular techniques.

MATERIALS AND METHODS

Study site

The current study was conducted in the Grabouw and Vyeboom areas located in the Western Cape province, South Africa. The six farms used as sampling sites included three Fruitways farms: Eikenhof (34º7’47.75”S 19º2’52.95”E), Glen Elgin (34º8’50.05”S 19º2’24.30”E) and Graymead (34º1’48.29”S 19º7’18.09”E). The other three farms surveyed were the Agricultural Research Council

(ARC) experimental farm in Grabouw (34º08'24.4"S 19º01'14.4"E), Monteith Trust farm (34º15'45.8"S 19º03'06.1"E) and Heideland farm (34º11'19.9"S 19º02'17.3"E) (Fig. 2.1).

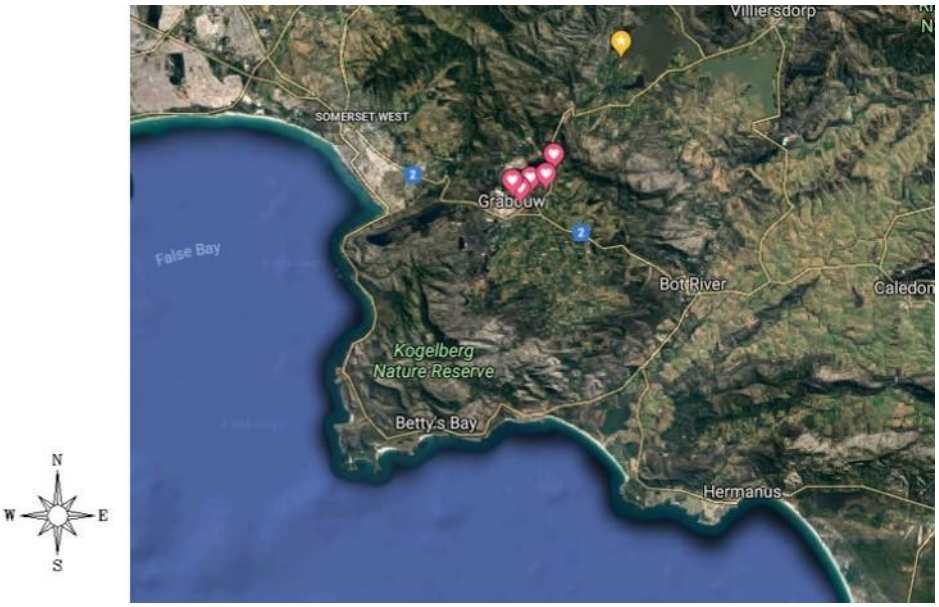


Fig. 2.1. Map showing study sites.

Collection of soil samples

A total of 48 soil samples, comprised of 240 soil subsamples, were collected across six apple farms. At each farm, two apple orchards infested with the WAA were selected and from each orchard four soil samples were collected from each orchard. Each apple orchard was divided into four equal quadrants and from each quadrant five apple trees were randomly selected and soil subsamples collected from a depth of 15 cm under each tree canopy, using a small hand trowel shovel (Fig. 2.2). The five soil subsamples collected from each quadrat were kept in clear labelled plastic bags and transported to the laboratory in a cooler box. The subsamples were mixed in the laboratory to form a single soil sample (Fig. 2.4A). From each apple farm, a total of eight soil samples were collected and the location of each soil sample was recorded.

Fig. 2.2. Soil-sampling process employed across various apple orchards.

Origin of bait insects

Larvae of both *Galleria mellonella* L. (Lepidoptera: Phyralidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae) were reared in the laboratory. The *G. mellonella* eggs were collected from a piece of wax paper that was placed in a glass container holding the adult moths and these eggs were transferred to containers containing a fresh diet. The diet consisted of baby cereal, brown bread flower, beeswax, honey, glycerine, yeast and wheat germ. The glass containers with the eggs were then kept at a controlled temperature of ± 25 ºC in a growth chamber (Van Zyl & Malan 2015). The *T. molitor* larvae were cultured according to the technique of Van Zyl & Malan (2015), using bran and carrots or apples.

Collection of root samples

The root sample collection method developed by Damavandian & Pringle (2002) was initially used to collect root samples. However, the method of using a soil auger to collect the infested root samples proved to be challenging, mainly due to the rockiness of the apple orchards' soil and the thickness of the galled roots. An alternative method of using a pick mattock was therefore used to collect the root samples. Five apple trees per orchard were randomly selected from the two already selected orchards and galled roots were collected at a distance of 0.5 m from the tree trunk (Fig 2.3). In the laboratory, each root sample was stored in a plastic container, fitted with moist paper towels, and kept in the dark at a controlled room temperature of ± 25 ºC. Distilled water was used to spray the root samples to keep them moist. The infested roots were used to supply the different life stages of WAA (Fig. 2.6A). To ensure a steady supply of WAA colonies in the laboratory during the experimental trials, root samples were collected every two weeks.

Fig. 2.3. A) The presence of root colonies of woolly apple aphid in the apple orchards, B) the galled roots of apple trees infested with the root colonies of woolly apple aphid.

Preparation of the agar plates and slants

Sabouraud dextrose agar plates (9-mm-diam.) with 1 g of yeast (SDAY) were prepared (Meyling 2007; Inglis *et al.* 2012). After 30 min of cooling, 200 µl of Penicillin-Streptomycin was added. Agar slants for storage of cultures were prepared, using SDAY in 28-ml-wide-mouthed McCartney glass bottles, by pumping 10 ml of the cooled SDAY into the 28-ml autoclaved bottles, using a bottle-top dispenser (Vitlab). The bottles were then closed, autoclaved and positioned in a slanting position for their contents to cool down and solidify. Cultures were stored at -6 °C.

Baiting and isolation of fungi and EPN from the soil samples

In the laboratory, each soil sample (comprised of five subsoil samples) was mixed and sieved through a mesh sieve (4 mm) to remove the rock and leaf material (Fig. 2.4 A, B) and transferred to 1-L containers, which were closed with lids. For aeration, the 1-L containers were filled with soil samples leaving 10 cm space on top. All soil samples were baited for EPF after collection, and in the case of the soil samples that were dry, distilled water was used to moisten the soil to maintain enough humidity during the baiting process (Meyling & Eilenberg 2007; Goble 2009). Ten *Galleria* and ten mealworm larvae were added to the surface of the soil samples in the plastic containers, which were then closed and incubated in the dark, at a controlled room temperature of ± 25 ºC (Fig. 2.4 A-D) (Zimmermann 1986; Meyling 2007).

Fig. 2.4. Baiting of fungi from collected soil samples. A) Mixing of soil subsamples, B) sifting of soil samples, C) baiting with larvae of *Galleria mellonella* and *Tenebrio molitor*, and D) mycosis on the larvae.

To ensure that the insects penetrated the soil, the containers were inverted daily (Goble 2009). After 7 and 14 days the dead larvae were removed (Fig. 2.4D). The dead larvae were then surface sterilised by first being dipped in distilled water to remove the soil and then dipped first in 70% ethanol and then in distilled water. To isolate the EPF, each dead larva with symptoms of fungal infection, characterised by fungal growth or by hardening of the cadaver was placed on a Petri dish fitted with a filter paper that was moistened with distilled water (Fig. 2.5) (Goble 2009). The Petri dishes were then placed in 2-L plastic containers with paper towels that were moistened using distilled water. The plastic containers were closed and incubated in a controlled room temperature of ± 25 ºC, in the dark.

Every four days, the dead or mycosed insects were removed, and the fungi were scraped from the surface of the larvae cadaver and placed on SDAY plates. The fungal cultures on the agar plates were then incubated at a controlled temperature of ± 25 ºC in a growth chamber and checked for fungal growth every four days. The fast-growing saprophytic fungi were discarded, and the contaminated plates were re-plated. Fungal plates were grouped according to morphological characteristics.

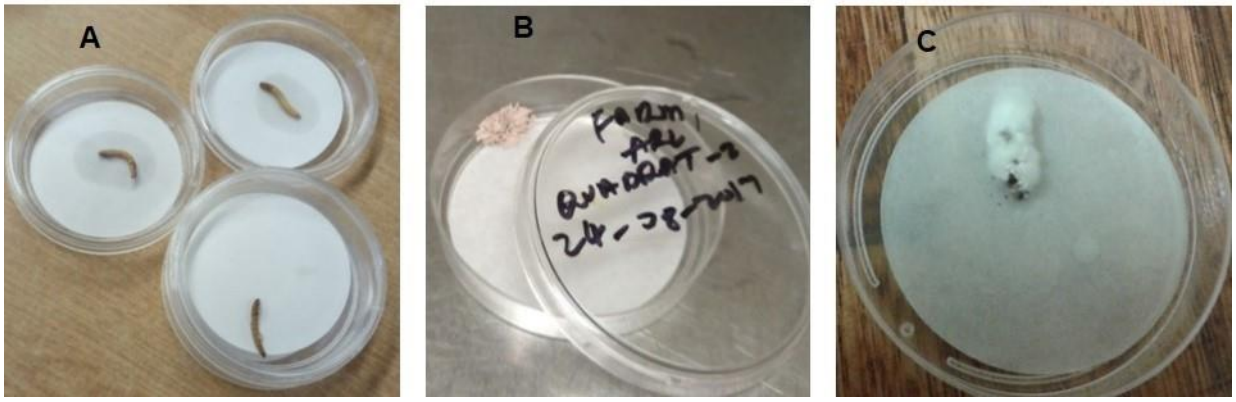


Fig. 2.5. Pictures showing the process followed for isolation of entomopathogenic fungi.

Trapping and isolation of EPF from the WAA on the root samples

Entomopathogenic fungi were trapped from the root samples by first collecting ten WAA individual insects from the collected WAA-infested apple plant roots, with the aid of a microscope using forceps. Each of the ten aphids was surface-sterilised with 70 % ethanol and plated on SDAY medium. The plates were incubated at a controlled temperature of ± 25ºC and after four days each plate was checked for mycosis. Fungi growing on plates with mycosis were harvested and transferred onto the SDAY Petri dishes. The potential EPF arising from the isolation procedure were collected and grouped together based on their morphological characteristics.

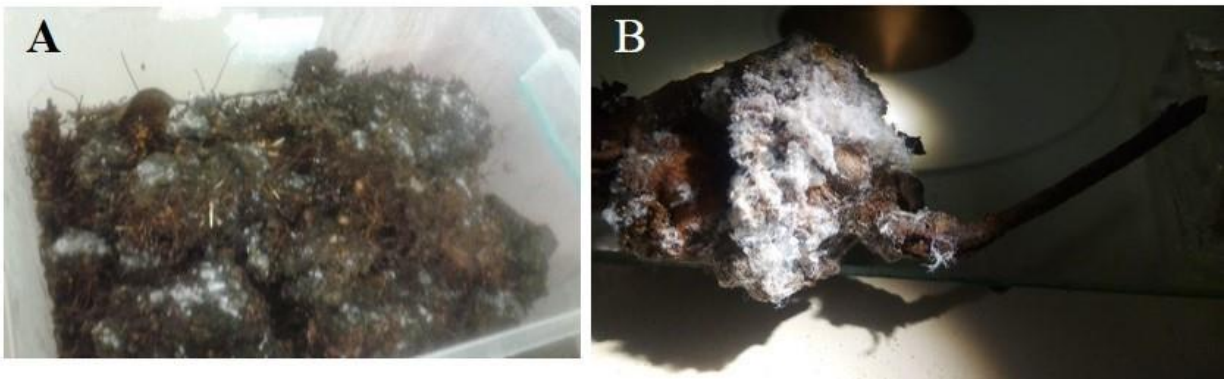


Fig. 2.6. Infested root samples used as a WAA colony in the laboratory.

Screening for detection of virulent EPF

To determine the insect pathogenicity of the isolated fungi, one fungal agar plate was selected from each of the morphologically similar groups of fungal culture plates. The fungal conidia were scraped from the growth medium plates using a dissecting scalpel sterilised with 99 % ethanol and subjected to the flame of a Bunsen burner. The fungal spores were then transferred into autoclaved 1.5-ml microcentrifuge tubes, containing 1.0 ml sterilised distilled water and a single drop of 0.05 % Tween 20. The microcentrifuge tubes were then closed and vortex-mixed for 60 sec to produce a

homogenous conidial suspension (Goble 2009). Five last instar larvae of *G. mellonella* and five last instar larvae of *T. molitor* were dipped in each conidial solution for a period of 5 sec. The larvae of of both *G. mellonella* and *T. molitor* were used as indicator insects to test for the pathogenicity of the collected fungal isolates against insects in general.The dipped larvae were then transferred to 90-mm-diam. Petri dishes, each fitted with a piece of filter paper moistened with distilled water. The Petri dishes were placed in 2-L plastic containers that were then closed with a lid, and incubated at a controlled room temperature in the dark. After two to three days, the Petri dishes were checked for dead larvae which were removed from the Petri dishes and surface-sterilised for mycosis to develop.

The larval cadavers were surface-sterilised using a procedure whereby each larva was first dipped in 70 % ethanol, thereafter in distilled water, followed by being dipped in 5 % bleach and three times in distilled water to conclude the process, in three separate Petri dishes (Lacey & Solter 2012). Each cadaver was then placed on a paper towel and positioned on a SDAY plate which was sealed with Parafilm and incubated at room temperature for a period of three to four days to verify the death of the larvae by mycosis (Fig 2.7). A total of 12 morphologically different EPF isolates with a mortality rate of > 90 % were selected for further analysis. Agar slants were then used to store the fungal isolates that were found to be entomopathogenic, at 4 °C. The larval mortality of *G. mellonella* and *T. molitor* resulting from the process was recorded for post-treatment with the conidial suspension.

Fig 2.7. Results from the quick-screening process for the collected virulent strains of isolated EPF.

DNA extraction

DNA was extracted from the 12 selected morphologically different EPF isolates using the Zymo Research Quick-DNA fungal / bacterial miniprep kit (Zymo Research Corporation), according to the protocol supplied. To initiate the process, fungal conidia were scraped off the fungal culture plates. About 50-100 mg of fungal conidia from each of the EPF isolates were transferred into a Zymo Research (ZR) BashingBead™ lysis tube (0.1 and 0.5 mm) with 750 µl of lysis solution being added

to the tube (Fig 2.8). Following the above, the lysis tubes for each fungal isolate were then secured in a Bead beater fitted within a 2-ml tube holder assembly and processed at a frequency of 30.0 hertz per sec for 5 min.

The ZR BashingBead™ lysis tubes were then centrifuged in a microcentrifuge at 10 000 × g for 1 min. Following the above, 400 µl supernatant of each fungal isolate was transferred into a Zymo-Spin™ IV spin filter, in which it was centrifuged at 7 000 × g for 1 min. Prior to centrifugation, the base of each Zymo-spin IV spin filter was snapped off. Following centrifugation, the spin filter was removed from the collection tube and 1200 µl of Genomic lysis buffer was added to the filtrate in the collection tube. For each fungal isolate 800 µl of the mixture was then transferred to a Zymo-Spin™ IIC column in a collection tube in which it was centrifuged at 1 000 × g for 1 min with the flow through the collection tube being discarded. The remaining 400 µl of the mixture in the collection tube was then transferred again into the same Zymo-Spin™ IIC column in a collection tube, in which it was centrifuged at 1 000 × g for 1 min, and the flow through the collection tube was also discarded.

DNA (200 µl) prewash buffer was then added to the Zymo-Spin™ IIC column and placed in a new collection tube for each isolate and centrifuged at 1 000 × g for 1 min. A 500 µl DNA wash buffer was added to the column along with the collection tube, which was then centrifuged at 1 000 × g for 1 min. Following the above, the Zymo-Spin™ IIC column was then transferred to a clean, sterilised 1.5-ml microcentrifuge tube with 100 µl of DNA elution buffer being added directly to the column matrix, which was then centrifuged at 10 000 × g for a period of 30 sec to elute the DNA.

The extract within each of the 1.5-ml microcentrifuge tubes was measured for the concentration of DNA present. The ND-1 000 software was used on a computer connected to a spectrophotometer, prior to the nucleic acid measurement being taken on the spectrophotometer pedestal, on which the ultrapure DNA sample for each fungal isolate was placed, the pedestal was wiped using a soft paper towel. A drop of 2 µl distilled DNA-free water was used to calibrate the ND-1000 program to zero. After the pedestal was wiped, the process was repeated. After calibration, the ultrapure DNA of each fungal isolate was measured by means of extracting and placing 2 µl of the DNA extract from each of the 1.5-ml microcentrifuge tubes onto the pedestal of the spectrophotometer. The spectrophotometer was then closed so as to ensure the formation of a column of the sample, after which the nucleic acid concentrations for each sample was measured by means of clicking ‘Measure’ on the ND-1000 program on the computer. After the measurement of each sample, the pedestal was wiped off using a soft tissue.

Fig. 2.8. DNA extraction process of virulent EPF strains.

Polymerase chain reaction

The polymerase chain reaction (PCR) process was undertaken for the molecular identification process for the selected EPF strains, using the KAPA2G ReadyMix PCR Kit. The DNA extract of each of the selected EPF from the DNA extraction process was used for the process. Internal transcribed spacer (ITS) primers, ITS 1 [forward primer (5'-TCCGTAGGTGAACCTGCGG-3')] and ITS 4 [reverse primer (5'-CTCCTCCGCTTATTGATATGC-3')], and the elongation factor-1α gene (EP) sequencing primers, as well as EF1F [forward primer (5'-GTCGGTGGTATCGACAAGCGT-3')] and EF2R [reverse primer (5'-AGCATGTTGTCGCCGTTGAAG-3')], were used for the PCR procedure. The PCR thermocycle conditions consisted of the initial denaturing step that was undertaken at 95ºC for 3 min, followed by 36 cycles that were performed at 95ºC for 20 sec, at 48ºC for 20 sec and at 72ºC for 30 sec. The reaction was completed with a final extension for 5 min at 72ºC, after which cooling off took place with the substance being held at 4 ºC for the ITS primers. The same procedure was used when using the EF primers EF1F and EF2R. The thermocyclic conditions were adapted from Abaajeh & Nchu (2015). The PCR thermocyclic conditions consisted of the first denaturing step being undertaken at 94 ºC for 10 min, followed by 36 cycles of 94 ºC for 30 sec, at 56 ºC for 30 sec (for the ITS primers), or at 53 ºC for 30 sec (for the EF primers) and then at 72 ºC for 1 min. The PCR reaction was completed with a final extension at 72 ºC for 7 min, after which cooling off took place, with the substance being held at 4 ºC.

The PCR products were visualised on an agarose gel using ethidium bromide. The unpurified PCR products were then sent to the Central Analytical Facility (CAF), DNA Sequencing Unit, Department of Genetics at Stellenbosch University, for the post-PCR clean-up and sequencing reaction. DNA sequences were aligned and edited using the software program, CLC Main Workbench, version 6. Sequences were blasted against the NCBI (National Centre for Biotechnology Information) GenBank database for species identification.

Phylogenetic analysis

For the construction of the phylogenetic trees, several sequences of the ITS and EF gene regions, with similar identity and closely related species to those that were collected in apple orchards, were downloaded from the NCBI GenBank database for comparison. The evolutionary history of the fungal isolates was inferred using the maximum parsimony method based on the Jukes-Cantor model. Distance analysis was done using neighbour joining and the strength was calculated using a 1000 bootstrap repetitions (Abaajeh & Nchu 2015). The analysis of the ITS region involved 11 nucleotide sequences, whereas the EF tree analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. The evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

RESULTS

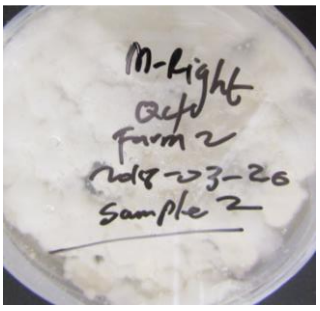
Molecular identification of the isolated soil EPF, using ITS primers, indicated that the 12 selected morphologically different indigenous isolates belonged to four different EPF species. The isolated EPF species included three isolates of *I. fumosorosea*, one isolate of *Purpureocillium lilacinum* (Thom), one isolate of *B. bassiana* and seven isolates belonging to the *M. anisopliae* complex. The EF gene region identified the species as falling in the PARBE clade of the *M. anisopliae* complex, using the ITS primers. From the EF region, it was identified that from the seven isolates two of the isolates were *M. robertsii,* one isolate was *M. pinghaense* and four of the isolates were *M. brunneum*. Molecular identification of fungi isolated directly from the adult female WAA, using the ITS1 and ITS4 primers showed that two saprophytic fungal isolates, *Fusarium tricinctum* (Corda) Sacc. and *Fusarium babinda* (Summerell), were isolated (Fig.2.9). Generally, fungal isolates in the *Metarhizium anisopliae* complex were found to be the most prevalent isolates obtained from the soil environment across the six surveyed farms.

Metarhizium pinghaense

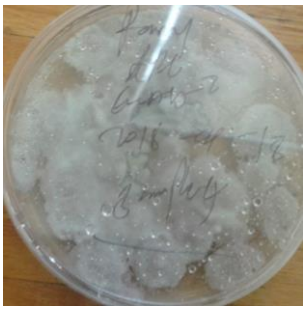
Metarhizium brunneum

Isaria fumosorosea

Metarhizium robertsii



Beauveria bassiana



Purpureocillium lilacinum

Fig. 2.9. Morphological growth, on agar plates, of entomopathogenic fungi isolated from the collected soil samples.

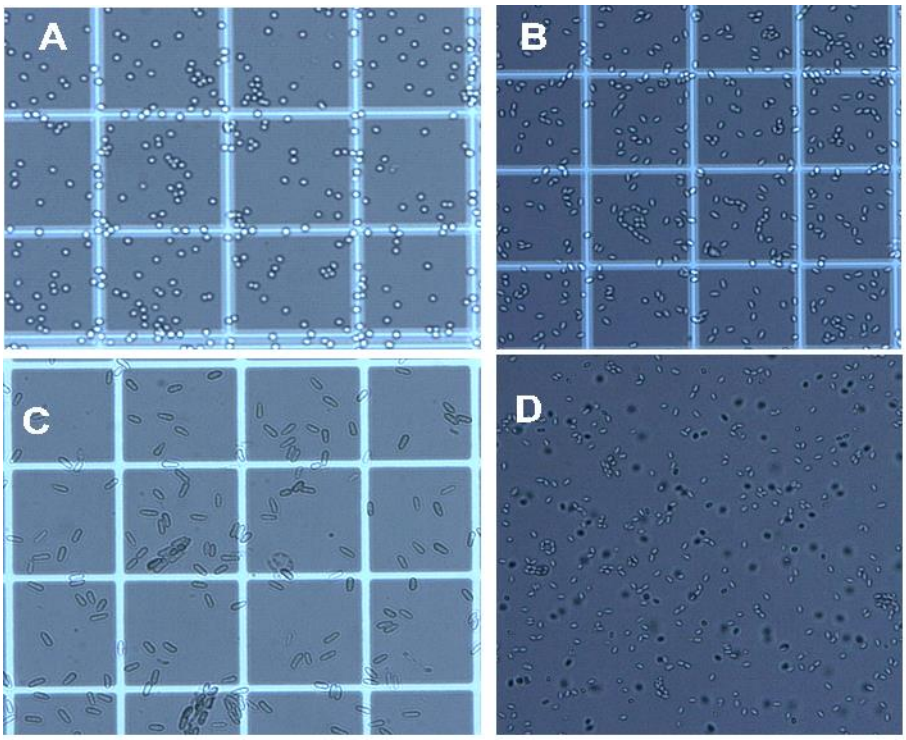


Fig. 2.10. Microscopic fungal conidia of (A) Beauveria bassiana, (B) Isaria fumosorosea, (C) Metarhizium anisopliae species complex, and (D) Purpureocillium lilacinum.

Table 2.1 below shows the information related to the various fungal isolates that were collected across the six farms in the Grabouw and Vyeboom areas in terms of the farm where each isolate was collected, the number of isolates that were collected from each farm, the order or class of the isolate and the name of the family to which the isolates belonged. The collected species names are indicated. One fungal isolate which is not known to be an entomopathogenic fungi, namely *Aspergillus tamarii* (Kita) Centralbl. Bakteriol., was also collected during the survey.

Table 2.1. Fungal isolates collected during a survey of entomopathogenic fungi across six apple farms in the Western Cape province, South Africa.

Farm	Number of isolates collected	Isolate	Class/ Order	Family	Species name
ARC – Grabouw	5	1ARC	Hypocreales	Cordycipitaceae	*Beauveria bassiana*
		6ARC	Hypocreales	Cordycipitaceae	*Isaria fumosorosea (Paecelomyces fumosoroseus)*
		4ARC	Hypocreales	Ophiocordy-cipitaceae	*Purpureocillium lilacinum (Paecilomyces lilacinus)*
		12ARC	Hypocreales	Clavicipitaceae	*Metarhizium robertsii*
		_	Eurotiales	Trichocomacea	*Aspergillus tamarii*
Heideland	2	8HEID	Hypocreales	Clavicipitaceae	*Metarhizium brunneum*
		5HEID	Hypocreales	Clavicipitaceae	*M. pinghaense*
Fruitways – Graymead	2	3GREY	Hypocreales	Clavicipitaceae	*M. brunneum*
		6GRAY	Hypocreales	Clavicipitaceae	*M. robertsii*
Fruitways – Glen Elgin	1	3GLEN	Hypocreales	Clavicipitaceae	*M. brunneum*
Fruitways – Eikenhof	3	6EIKEN	Hypocreales	Clavicipitaceae	*M. robertsii*
		3EIKEN	Hypocreales	Clavicipitaceae	*M. brunneum*
		12EIKEN	Hypocreales	Clavicipitaceae	*M. pinghaense*
Montheith Trust farm	1	2MONT	Hypocreales	Cordycipitaceae	*B. bassiana*

The tree with the highest likelihood for the ITS gene region is shown below (Fig. 2.13). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown alongside the branches concerned (Felsenstein 1985). A total of 485 positions were present in the final dataset. No difference was observed in the phylogeny between the fungal isolate sequences collected from the various apple orchards (*I. fumosorosea, B. bassiana and* P. *lilacinum*) and those that were downloaded from the NCBI GenBank database for the phylogenetic tree based on the ITS region. A 100 % similarity was observed between the isolates from each group. A difference between the out-group, *M. acridum* (Driver & Milner) J.F. Bisch., Rehner and Humber, and the other three groups, *I. fumosorosea, B. bassiana and P. lilacinum*, was observed. The phylogenetic tree shows that the *I. fumosorosea* group forms a monophyletic group

with the *B. bassiana* group, indicating that the two groups evolved from the same, or a common, ancestor. A 96 % in common ancestry between *I. fumosorosea* and *B. bassiana* isolates was observed. These two groups also form a paraphyletic group with the *P. lilacinum* fungal group (Fig. 2.11).

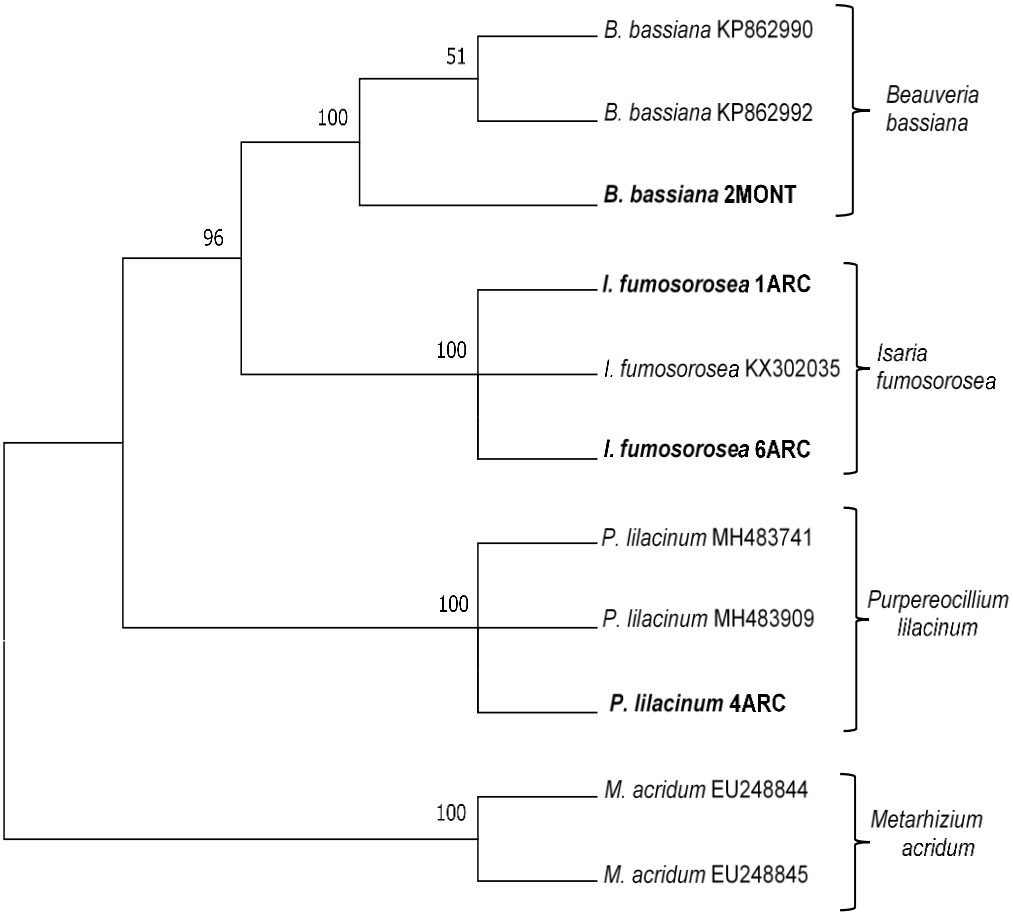


Fig. 2.11. A phylogenetic tree based on the internal transcribed spacer (ITS) region, with *Metarhizium acridum* used as the out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (using 1000 replicates) are shown alongside the branches.

For the EF region, a tree with the highest likelihood is shown below (Fig. 2.12). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (with 1000 replicates) is shown alongside the branches (Felsenstein 1985). A total of 313 positions existed in the final dataset. The phylogenetic relationship between the *M. robertsii* isolates collected from the apple orchards and those that were downloaded from the NCBI GenBank showed a similarity of 79 %, with the *M. pinghaense* isolates showing 64 %, and the *M. brunneum* isolate showing 73 %, similarity. The isolates *M. pinghaense*, *M. robertsii* and *M. brunneum* were shown to form a

monophyletic grouping, indicating evolution from a recent common ancestor. All of the isolates were shown to differ from *Metarhizium lepidiotae* (Driver & Milner) J.F. Bisch. Rehner and Humber, which was used as the out-group (Fig. 2.12).

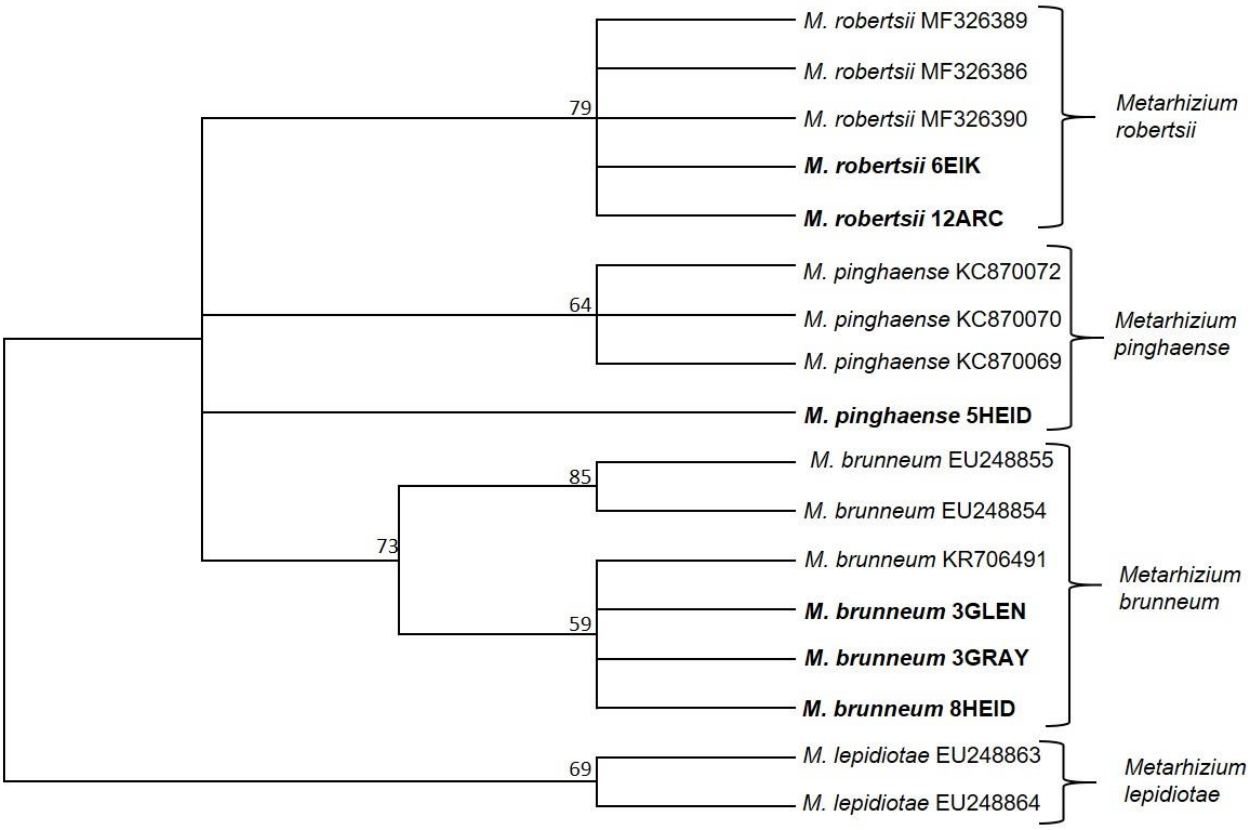


Fig 2.12. A phylogenetic tree based on the elongation factor (EF) gene region, with *Metarhizium lepidiotae* used as the out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (using 1000 replicates) are shown alongside the branches.

DISCUSSION

The soil environment harbours a large biodiversity of fungal species, having different ecological functions within their immediate environment (Shah & Pell 2003; Meyling & Eilenberg 2007). In this study, a survey of EPF in local apple orchards of the Western Cape province was done and collected EPF isolates were screened for their potential ability to infect *E. lanigerum*. The process involved collecting both soil and WAA-infested root samples across six apple farms, trapping or isolating EPF from both the root and soil samples and identifying the isolated EPF using both morphological and molecular techniques.

From the study, six different EPF species were obtained across six apple farms. Collected EPF were all hypocrealean fungal species, which are among the well-known EPF species that are common components of the soil environment microbiota with a cosmopolitan distribution (Chandler *et al.* 1997; Zimmermann 2007). These EPF isolates were obtained mainly from collected soil

samples rather than from the WAA females that were collected from the root samples. *Fusarium tricinctum* and *F. babinda* are among some of the recorded saprophytic fungi that have a pathogenic effect on the root colonies of WAA (Damavandian 2000). Previously, Stokwe (2016) found that one of the EPF isolates that was successfully isolated during the study, namely *P. lilacinum*, was also isolated from soil samples that were collected in apple orchards. Similar observations to those that were made in the current study were made in a study conducted by Abaajeh & Nchu (2015), in which fungal isolates belonging to six entomopathogenic fungal species were also successfully isolated from soil samples collected in the Western Cape province, using similar baiting techniques, where collected EPF species included both *M. robertsii* and *P. lilacinum*.

Among the collected EPF isolates were *I. fumosorosea, B. bassiana* and *M. anisopliae* species complex isolates, which are well-known to cause mycosis within various taxa of arthropod, and in virtually all insect orders (Chase *et al.* 1986; Zimmermann 1986; Meyling & Eilenberg 2007). *Purpureocillium lilacinum*, when previously used against the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), was observed to have a negative effect on the reproduction of this aphid species when present as an endophyte of the plants (Lopez *et al.* 2014). The use of *P. lilacinum* isolate collected during the current study will enable the testing of the efficacy of the fungal isolate when it is used in managing WAA.

Strains of almost all of the collected EPF species have also been commercially developed as mycoinsectides for biological use against various insect pest species across agroecosystems (De Faria & Wraight 2007; Asensio *et al.* 2003; Shi & Feng 2004). Some of the fungal isolates are currently commercialised for use against other insect pests in South Africa (Hatting *et al.* 2018). Three of the isolated *M. anisopliae* species complex isolates collected during the survey, namely *M. robertsii*, *M. pinghaense* and *M. brunneum*, are known to be of particular interest as entomopathogens in agricultural ecosystems (Rehner & Kepler 2017; Hatting *et al.* 2018). In South Africa, five commercial products containing *B. bassiana* are currently registered for the management of insect pests, with the products including BB plus WP® product that is registered for use against both aphids and spider mites (Hatting *et al.* 2018). All isolates collected during the current study are also known to affect all the life stages of their host, as they produce toxic metabolites such as Beauvericin, Oosporein and insecticidal cyclic peptides, known as destruxins (Inglis *et al.* 2001; Zimmermann 2007; Dedryver *et al.* 2010; Shahid *et al.* 2012). Further analysis, involving testing of the efficacy of the six locally isolated EFP strains against WAA should provide an opportunity to test the success of local EPF isolates, when used in the biological control of insect pests.

The use of the ITS and EF gene regions enabled the molecular identification of the collected fungal isolates, by means of the amplification of the fungal DNA. The analysis of phylogenetic relationships among collected fungal isolates showed that some of the collected fungal isolates had 100 % similarity to those collected from other countries. *Isaria fumosorosea* isolate from the ARC

farm in Grabouw had 100 % similarity to the isolate collected in China, KX302035 isolate, with the *B. bassiana* isolate also having 100 % similarity to an isolate collected in Turkey (KP862992 isolate). *Purpureocillium lilacinum* showed similarities to isolates collected in China (MH483909 and MH483741 isolates). *Metarhizium robertsii* isolates from this survey showed 75 % similarity to isolates collected in the USA (MF326386 and MF326389 isolates). *Metharhizium pinghaense* showed 55 % similarity to isolates collected in India (KC870070 isolate), and the *M. brunneum* isolates showed 65 % similarity to an isolate previously collected in Switzerland.

The results obtained in the current study provide an insight into the diversity of EPF species across the agricultural lands of the Western Cape province, South Africa. The analysis of phylogenetic relationships has also provided insights into how similar or different the local isolates of EPF are, relative to the other isolates collected in other countries as well as into the evolutionary relationship between the collected isolates. The results of the present study will allow for further analysis of the effect that EPF have on WAA, which will also provide insights into determining which of the isolated EPF have a relatively high level of virulence, and which have a potential for successful use in the management of WAA.

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CHAPTER 3

Laboratory bioassays of woolly apple aphid, *Eriosoma lanigerum* (Hemiptera: Aphididae), for susceptibility to entomopathogenic fungi

ABSTRACT

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hemiptera: Aphididae), is a serious pest affecting apple production on a global scale. Feeding of the edaphic WAA colonies on the roots of apple trees induces the formation of hypertrophic galls, which restrict the movement of water and nutrients, which has a negative effect on plant growth and production. Entomopathogenic fungi (EPF) have been identified as promising biological control agents against a wide array of insect pests, particularly sap-sucking insects. EPF have also been described as an alternative means of controlling insect pests to using chemicals. The aim of the current study was to screen six locally isolated EPF species: *Beauveria bassiana, Isaria fumosorosea, Metarhizium brunneum, Metarhizium pinghaense, Metarhizium robertsii* and *Purpureocillium lilacinum* for their virulence against the WAA under optimum laboratory conditions. The study investigated the susceptibility of female WAA to the six species of EPF isolated in apple orchards using screening bioassays, concentration–dose-response and exposure–time-response bioassays. The results obtained indicate *M. brunneum* and *M. pinghaense* to be most effective against WAA. Locally isolated EPF offer the potential for use as biological control agents against both root and aerial colonies of WAA in the apple orchards of the Western Cape province.

Key words: *Beauveria bassiana*, *Metarhizium*, woolly apple aphid, biocontrol, entomopathogenic fungi

INTRODUCTION

Woolly apple aphid (WAA), *Eriosoma lanigerum* L. (Hausmann) (Hemiptera: Aphididae), is a serious pest of *Malus domestica* (Borkhausen), in all apple-producing regions across the world, including the production areas in the Western Cape province of South Africa (Klimstra & Rock 1985; Damavandian & Pringle 2002; Lavandero *et al.* 2009; Ge *et al.* 2016). The presence of edaphic populations of WAA in apple orchards poses a great threat to apple production in South Africa. Feeding of the edaphic colonies on the roots of apple trees induces formation of hypertrophic galls, which restrict the movement of water and nutrients from the roots to the arboreal parts of the host plants, which affects the plant growth and development (Brown *et al.* 1991; Damavandian 2000; Damavandian & Pringle 2007; Dardeau *et al*. 2014). WAA-formed galls also provide a sheltered micro-environment to founding colonies on the apple roots, and the generations that they produce (Miles 1999; Wool 2004; Dedryver *et al*. 2010). Hypertrophic galls on the root system rupture plant tissue, which is readily invaded by fungal pathogens that cause fungal diseases, like canker disease on infested trees (Brown *et al*. 1991; Damavandian 2000; Nicholas *et al.* 2005; Dardeau *et al.* 2014).

Edaphic colonies of WAA in South African apple orchards reproduce entirely parthenogenetically, with the females on the plant root system reproducing asexually, and giving birth to live offspring (Heunis & Pringle 2006; Lavandero *et al.* 2009; Van Zyl 2011). The edaphic colonies are the source of arboreal colonies of WAA, which attack the aerial parts of apple trees, resulting in aerial infestations. Arboreal colonies of WAA infest mainly unhealed wounds and new growth (Nicholas *et al.* 2005; Pringle & Heunis 2008; Van Zyl 2011; Lordan *et al.* 2015). Infestations by arboreal colonies have a prolonged effect on orchard yield, because the colonisation of WAA reduces fruit buds, weakens fruit-bearing wood and stimulates premature defoliation of apple trees (Short & Bergh 2004; Bergh & Short 2008; Gresham 2013). Infestations of WAA colonies present on the root system of apple trees are usually severe, relative to the infestations that occur on the arboreal parts of the trees (Klimstra & Rock 1985; Sandanayaka *et al.* 2003; Van Zyl 2011; Ge *et al.* 2016). Therefore, management of edaphic colonies is important.

Entomopathogenic fungi (EPF) are promising biological control agents against a wide range of insect pests, particularly sap-sucking insects (Chandler *et al.* 1997; Wraight *et al.* 1998). They cause damaging epizootics in populations of insect pests of crops. The success of EPF in controlling pests is dependent on its ability to penetrate the insect cuticle, which acts as the main barrier protecting insects against external threats, such as invasion by microorganisms, like fungal species, by means of cuticle penetration resulting in diseases (Leger *et al.* 1991).

The advantage of EPF as a biocontrol agent is that they are able to initiate diseases in their host through direct contact and penetration of the epicuticle, without first having to be ingested to initiate infection (Wraight *et al.* 1998; Goettel *et al.* 2005). To penetrate the host cuticle successfully,

EPF use both mechanical and biochemical action, including the production of several cuticle-degrading enzymes, like chitinases, lipids and proteases which can degrade the chitin, protein and lipid layers of the cuticle (Khan *et al.* 2012).

Beauvaria and *Metarhizium* fungal species are the cosmopolitan, soil-inhabiting EPF that are used in biological control of insect pests. Some of these fungal isolates have been successfully registered for commercial use against a wide range of pests (Shah & Pell 2003; Hatting *et al*. 2018). *Purpureocillium lilacinum* Thom (Hypocreales: Ophiocordycipitaceae) is mainly known as a nematophagous soil-borne fungi that is primarily used in biological control against the root-knot and reniform nematodes, like *Meloidogyne incognita* (Kofoid & White) Chitwood (Tylenchida: Heteroderidae) and *Rotylenchulus reniformis* Linford & Oliveira (Tylenchida: Hoplolaimidae) (Kiewnick *et al.* 2011; Munawar *et al.* 2011; Chaudhary & Kaul 2012). A study conducted by Lopez *et al.* (2014), showed that *P. lilacinum* has negative effects on the reproduction of cotton aphids, *Aphis gossypii* Glover, when present as an endophyte of the plant, under both greenhouse and field conditions. *Isaria fumosorosea* Wize (= *Paecelomyces fumosoroseus*) (Hypocreales: Cordycipitaceae) is a promising biological control agent with a wide range of arthropod hosts including whiteflies, aphids and diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) (Altre *et al.* 1999; Zimmermann 2007, 2008). *Isaria fumosorosea* is a fast-growing fungus that initially produces white colonies, which change over time to pink shades with cylindrical or fusiform conidia (Zimmermann 2008). Like other fungal pathogens, this EPF produces metabolites or toxins, like Beauvericin, which paralyses host cells (Hajek & St. Leger 1994; Zimmermann 2007).

Stokwe (2016) explored the efficacy of entomopathogenic nematodes (EPNs) and fungi as biological control agents of *E. lanigerum,* under South African conditions. Results from the study indicate that EPN cannot be used as a biocontrol agent against WAA, as the associated bacteria do not grow in the haemocoel of WAA (Stokwe & Malan 2016, 2017), however the use of commercial fungal isolates, derived from *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin, showed the potential for use against this particular pest species, under both laboratory and field conditions. No previous research has been conducted in testing the potential of locally isolated fungi from apple orchards, for the control of WAA.

The current study investigated the potential of six locally isolated EPF species as biological control agents against the female WAA. The above was accomplished by screening of the different EPF species for their virulence against WAA, under laboratory conditions. The efficacy of the isolates was further investigated by means of conducting concentration–dose and exposure–time-response bioassays.

MATERIALS AND METHODS

Preparation of conidial suspensions

The following EPF species, which were previously isolated from apple orchards, were used: *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae), *Isaria fumosorosea* Wize (= *Paecelomyces fumosoroseus*) (Hypocreales: Cordycipitaceae), *Purpureocillium lilacinum* (= *Paecilomyces lilacinus*) Thom (Hypocreales: Ophiocordycipitaceae), and species of the *Metarhizium* species complex (Hypocreales: Clavicipitaceae), including *Metarhizium brunneum* Petch, *Metarhizium pinghaense* Chen & Guo and *Metarhizium robertsii* (Metschn.) Sorokin (Chapter 2). McCartney (28 ml) wide-mouth glass bottles, containing 20 ml of distilled water, were autoclaved at 120°C for 121 min. Fungal conidia were harvested from 2-3-week- old surface cultures, grown on Saboraud Dextrose Agar (SDA) plates, by means of scraping with a sterile blade, using 99 % ethanol and flaming with a Bunsen burner. Collected fungal spores in the sterile distilled water were supplemented with 0.05 % Tween 20. The bottles containing conidia were sealed, and vortex-mixed for 2 min to produce a homogenous suspension. The conidial suspension was poured into a sterilised 100-ml glass beaker, through an organza fabric, to remove the fungal hyphae and mycelium, before being poured back into the McCartney bottle, vortex-mixed for 2 min, and used as the conidial stock for serial dilutions.

Conidial concentration dilutions

One ml of the 20 ml homogenous conidial suspension, of each fungal strain, was pipetted into a bottle (28-ml McCartney) containing 9 ml of sterilised distilled water, and vortex-mixed for 2 min. To determine the conidial concentrations, a Neubauer haemocytometer was used, following serial dilution in sterile distilled water, and conidial suspensions were used within 3 h of enumeration (Coombes 2012; Chapter 2). The conidial suspension (200 µl) was loaded on the Neubauer haemocytometer, under cover slips on both sides of the haemocytometer. The number of spores were counted using a compound microscope on both ends of the haemocytometer. Following the above procedure, the following formula was used to determine the conidial concentrations, whereby the total number of spores counted on each sides of the haemocytometer were added together and an average was calculated, which was then multiplied by 5×10^4, to give a concentration of 1×10^7 conidia/ml (Inglis *et al.* 2012).

$$\text{Average} = \frac{\text{total } 1 + \text{total } 2}{2}$$

$$(\text{Average})(5 \times 10^4)$$

The following formula was used to calculate the exact volume of the conidial suspension, and that of sterilised distilled water, which must be added together to make a conidial suspension of 10 ml, with a conidial concentration of 1 x 10^7 conidia/ml.

$$C_1V_1=C_2V_2;\ V_1 = \frac{C_2V_2}{C_1}$$

where: C1 = initial concentration; V1 = required volume of conidial suspension stock to be added to the distilled water, or the required volume for the dilution of the original aliquot; C2 = desired concentration; and V2 = desired final volume of inoculum.

Viability test of fungal conidia

The viability of conidia was determined by spread-plating 100 µl of the diluted conidial suspension, with a concentration of 1 × 10^7 conidia/ml, on three SDA plates per isolate, under sterile conditions. Each plate, after being sealed with Parafilm, was incubated in a growth chamber at 25 °C ± 2 °C (Fig. 3.1). The percentage of conidia germination was determined, after 24 h, from 100-spore counts on each plate by means of a compound microscope at 40 × magnification. Spores that developed a germ tube twice the length of the spore were counted as viable, whereas those without a germ tube were counted as non-viable spores (Ekesi *et al.* 2002; Inglis *et al.* 2012). The average number for both dead and living spores for the three plates determined the viability of the fungal spores. Only fungal cultures with >80 % viability were used in the bioassays.

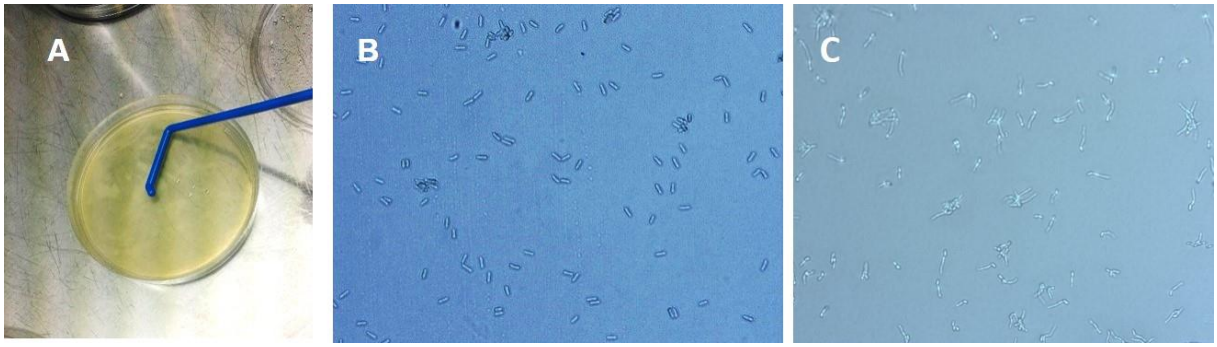

Fig. 3.1. Viability check of conidial suspension using the spread-plating method: (A) Spread-plating of conidial suspension on Saboraud dextrose agar growth-medium plate, (B) fungal conidia before germination, and (C) germination of fungal conidia on SDA growth plate, after 24 hours.

Inoculation protocol

Adult WAA were used to assess the virulence of the six EPF, so as to enable the identification of the most virulent EPF species. All isolates were tested at a standard concentration of 1 × 10^7 conidia/ml in sterile distilled water and 0.05 % Tween 20. The insects were dipped in 10 ml of aqueous conidia

formulation for 30 sec. The controls were treated with sterile distilled water containing 0.05 % Tween 20 only. A total of 60 WAA were used to test the virulence of each of the six fungal isolates.

Screening using 24-well bioassay plates

Initially, using the inoculation protocol, 24-well bioassay plates fitted with filter papers moistened using 10 µl sterilised distilled water were used as the test arena. Each WAA was placed in an alternative well, with 12 WAA insects per plate, incubated at 25 °C ± 2 °C in a growth chamber (Fig. 3.2A). The 24-well bioassay plates were placed in a 2-L plastic container, moved to a growth chamber at a controlled temperature of 25 °C ± 2 °C. The mortality of WAA insects was recorded 5 days post-treatment with the six fungal isolates. To determine whether insect mortality resulted from exposure to the fungal isolates, dead insects were surface sterilised using 70 % ethanol, placed on SDA plates and incubated at 25 °C ± 2 °C (Coombes 2012) (Fig 3.3C- D). After 5-7 days, following death, mycosis on the insect cadavers and growth of fungi on the SDA plates was checked with the aid of a dissection microscope.

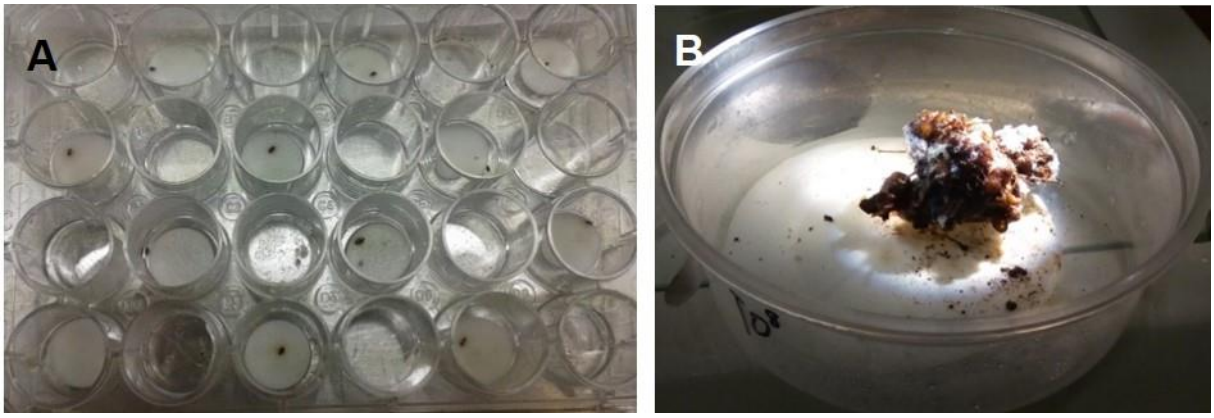

Fig. 3.2. *Eriosoma lanigerum* screening bioassay using 24-well plates (A) and (B) In situ screening on roots.

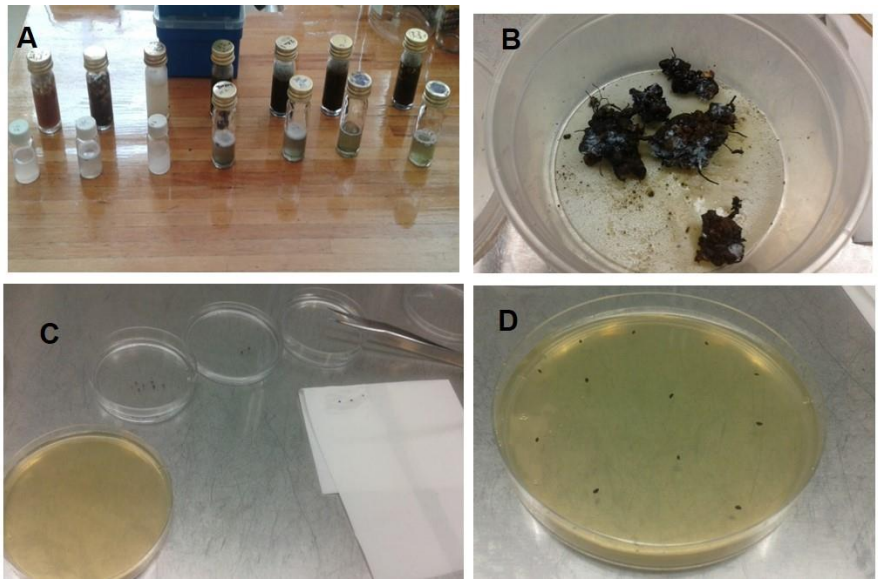

Fig. 3.3. Pictures showing: (A) serial dilutions process of the seven fungal isolates, (B) *Eriosoma lanigerum* (WAA) root colonies dipped in fungal suspensions, (C) surface- sterilisation of dead adults of WAA, dipped at different concentrations, and (D) the plating of sterilised WAA insects on Saboraud dextrose agar plates.

In situ screening on roots

The number of WAA (10 females) on a piece of root was counted. The root with the WAA was dipped in 2 ml of the aqueous conidia suspension for a period of 30 sec, after which it was transferred to a plastic container fitted with a piece of filter paper, moistened with sterilised distilled water (Fig. 3.2B and 3.3B). The treatment was repeated six times for each EPF isolate, using 60 WAA per treatment for each fungal isolate. The plastic containers were placed in a growth chamber at a controlled temperature of 25 ± 2°C. The mortality of WAA was recorded 5 days post-treatment for each of the six fungal isolates. Each dead insect was then surface-sterilised using 70 % ethanol, after which it was transferred to an SDA plate, which was incubated at 25 ± 2 °C for another 5-7 days, and then examined for mycosis using a dissection microscope (Coombes 2012).

Concentration–dose-response bioassays

The two most promising fungal isolates*, M. brunneum* and *M. pinghaense*, based on the screening bioassays, were used for the concentration–dose-response bioassays. Conidial suspensions were prepared following the same procedure detailed above. Five different concentrations (1×10^0, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/ml) were tested. A total of 60 adults of WAA, on six pieces of root, were dipped in each concentration of the two fungal species for 30 sec, and the controls were treated with sterile distilled water containing 0.05 % Tween 20 only. The dipped piece of root was placed in a plastic container fitted with a piece of filter paper, moistened with sterilised distilled

water, and incubated at 25°C ± 2°C in a growth chamber. The mortality of WAA was assessed daily up to 5 days post-treatment for each conidial concentration. To determine the cause of mortality, dead WAA were surface-sterilised using 70 % ethanol, placed on SDA plates, and incubated at 25 °C ± 2 °C in a growth chamber. After 7 days, mycosis was determined with the aid of a dissection microscope.

Exposure–time-response bioassays

The two most promising isolates, *M. brunneum* and *M. pinghaense*, based on the previous bioassays, were used for the exposure–time-response bioassays. Conidial suspensions were prepared following the procedure described above. Five different inoculation periods were tested (1, 2, 3, 4, and 5 days), at a concentration of 1×10^7 conidia/ml for each isolate. The same inoculation and experimental procedure outlined above was followed.

Data analysis

Analysis of the results was done using the statistical software, STATISTICA Version 13.3 (TIBCO Soft Inc. 2016). Data was analysed using mainly a one-way ANOVA (analysis of variance) comparison of means using LSD (least significant difference) tests and post-hoc tests (Games-Howell post-hoc test), at 95 % confidence intervals. For the exposure–time-response bioassays, a Probit analysis was conducted to calculate the LT_{50} and LT_{90} values.

RESULTS

Screening using 24-well bioassay plates

The use of the 24-well bioassay plate method for screening proved to be ineffective in testing fungal pathogenicity against WAA. The majority of the WAA adult females used for the trials died 3-4 days after incubation, without showing any sign of mycosis.

In situ screening on roots

The results from the screening of *E. lanigerum* dipped in a concentration of 1×10^7 conidia/ml of each fungal isolate showed that there is a significant difference (ANOVA, $F_{(6, 77)} = 13.59$; $\rho < 0.01$) in the percentage mortality of WAA between the different treatments. The highest percentage mortality was obtained for *M. pinghaense* (70.83 ± 5.14 %), followed by *M. robertsii* (67.50 ± 5.38 %), *M. brunneum* (65.00 ± 5.71 %) and *I. fumosorosea* (61.67 ± 6.94 %), with no significant difference between each other ($\rho > 0.05$). *Beauvaria bassiana* (36.67 % ± 5.12 %), *P. lilacinum* (29.17 ± 5.43 %), and the control treatment (22.50 ± 54.46 %) also did not differ significantly ($\rho > 0.05$) from each other (Fig. 3.4).

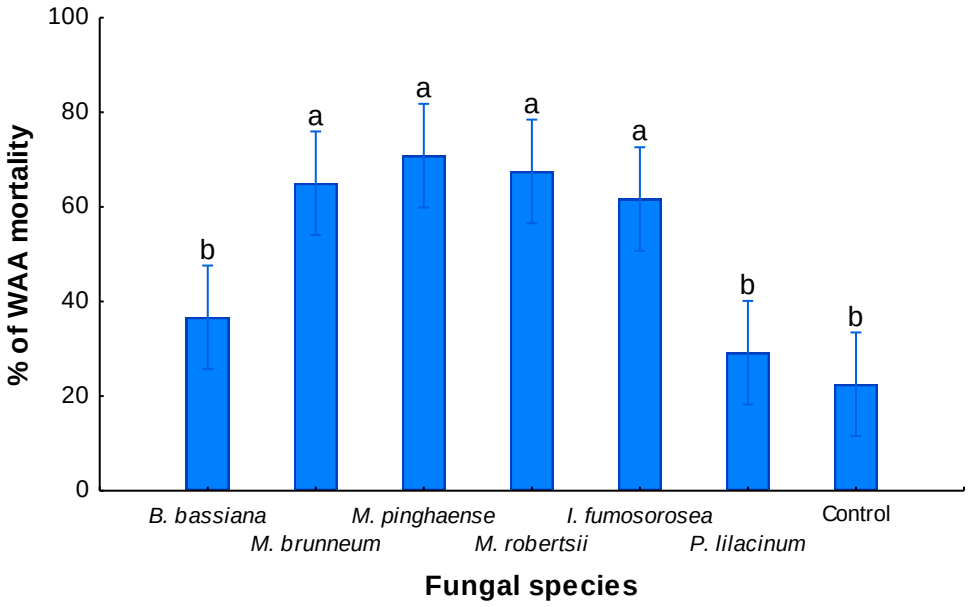

Fig. 3.4. Average percentage mortality (95 % confidence interval) of the adult females of *Eriosoma lanigerum* (WAA), as induced by six species of entomopathogenic fungi, and the control treatment with distilled water, 5 days post-treatment (one-way ANOVA, $F_{(6, 77)} = 13.59$; $\rho < 0.01$). Different letters indicate a significant difference between the fungal treatments ($\rho < 0.05$).

Further analysis to determine which of the dead WAA had a mortality induced either by the fungal treatment, or due to natural causes, was by examining for mycosis 5-7 days post-death. Mycosis showed a significant difference ($F_{(6, 77)} = 9.32$, $\rho < 0.01$) between the treatments concerned (Fig. 3.5). *Beauvaria bassiana* (22.50 ± 4.94 %), *M. robertsii* (26.67 ± 4.14 %), *P. lilacinum* (9.17 ± 2.60 %) did not differ significantly ($\rho > 0.05$) from the control (15.83 ± 4.68 %). The highest mycosis was induced by *M. brunneum* (44.17 ± 5.29 %) and *M. pinghaense* (45.83 ± 4.52 %), which did not differ significantly from each other. *Isaria fumosorosea* (31.67 ± 4.74 %) and *B. brunneum* did not differ significantly ($\rho < 0.05$) from each other.

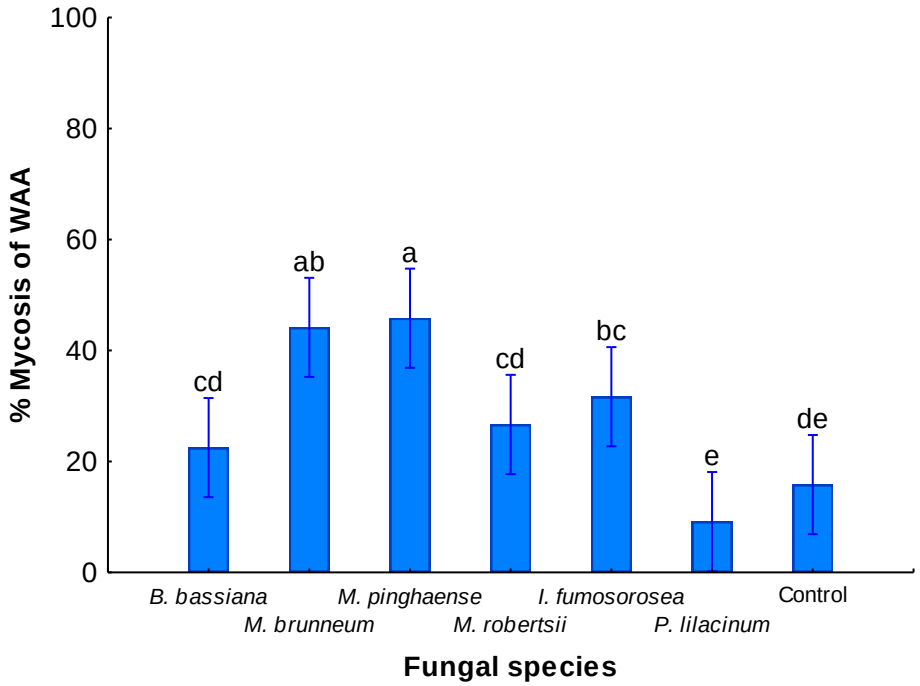

Fig. 3.5. Average percentage of mycosis (95 % confidence interval) of *Eriosoma lanigerum* (WAA), induced by six species of entomopathogenic fungi and the control, 7 days after death (one-way ANOVA; $F_{(6, 77)} = 9.32$, $\rho < 0.01$). Different letters indicate a significant difference between the fungal treatments ($\rho < 0.05$).

The visual representation of mortality induced by fungal treatments, as observed by mycosis of WAA, is indicated in Fig. 3.6, with the growth of fungal isolates when the dead WAA was placed on an SDA medium being visualised in Fig. 3.7.

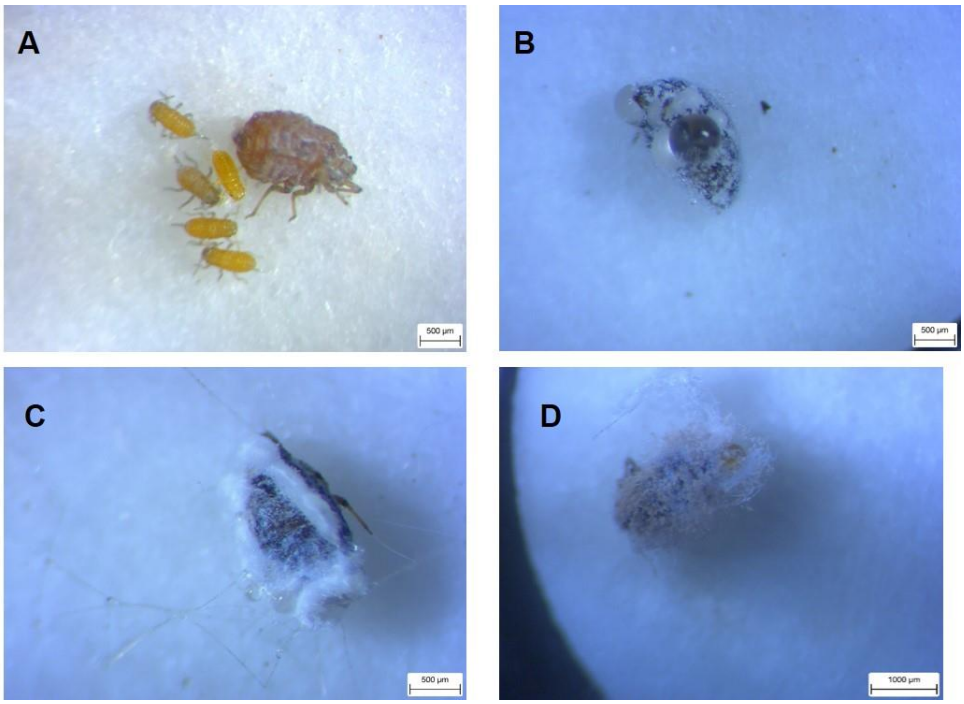

Fig. 3.6. (A) Healthy *Eriosoma lanigerum* and dipped specimens, (B) *Beauveria bassiana*, (C) *Metarhizium* species complex, and (D) *Isaria fumosorosea*.

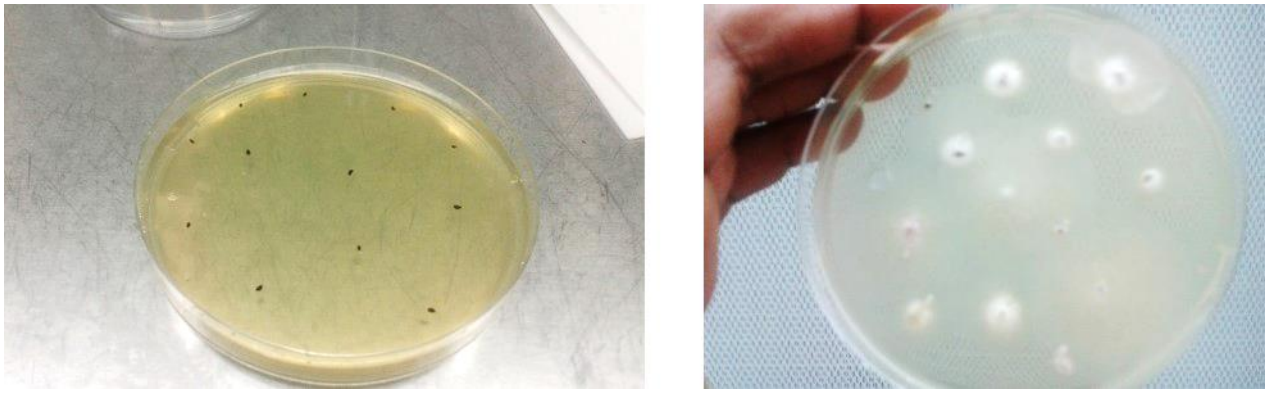

Fig. 3.7. Mycosis of *Eriosoma lanigerum* on Saboraud dextrose agar plates, following surface sterilisation of dead dipped females for each fungal isolate.

Concentration–dose-response bioassays

The results showed a significant difference (ANOVA, $F_{(4,125)} = 17.43$, $\rho < 0.01$) in the average percentage mortality of WAA between the control and the five concentrations of *M. brunneum* treatments. A significant difference in mortality was observed between the conidial concentrations of 1×10^5 and 1×10^4 conidia/ml. A conidial concentration of 1×10^5 conidia/ml showed a higher mortality of WAA, relative to the other three concentrations, 5 days post exposure to *M. brunneum*,

with the control treatment showing a lower percentage in mortality. The concentration–dose bioassay showed a positive correlation between the percentage of WAA mortality and the number of days of exposure to the four different concentrations of *M. brunneum*, as the mortality increased over time. However, an increase in mortality was also observed for the control group treatment, across the 5-day period (Fig. 3.8).

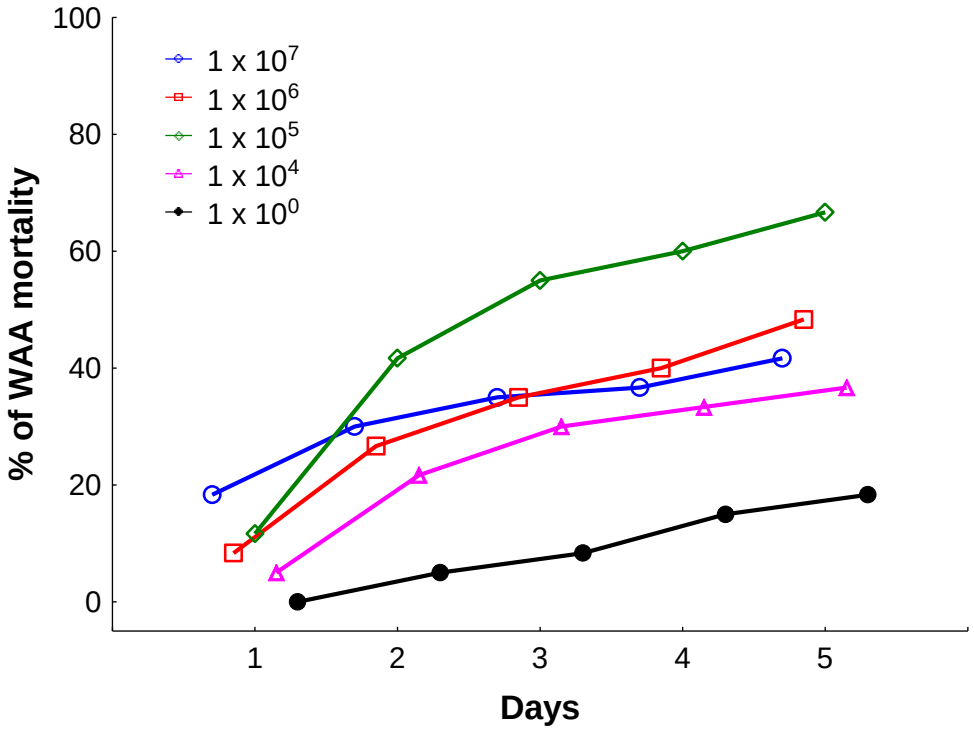

Fig. 3.8. Average percentage of mortality of *Eriosoma lanigerum* (WAA), following exposure to four different conidial concentrations of *Metarhizium brunneum* and a control treatment, daily, up to 5 days post-exposure.

To verify the cause of mortality through mycosis, a positive relationship was indicated between the average percentage of WAA that died due to infection by *M. brunneum* and the amount of time of exposure that elapsed after the treatment. As the number of days of exposure increased, the average percentage in mortality also increased. The highest percentage of mortality due to the fungus was observed at a concentration of 1×10^7 conidia/ml (38.33 % ± 5.43 %), with the lowest percentage being observed at 1×10^4 conidia/ml (18.33 ± 4.01 %), after five days of exposure to the fungal pathogen. No significant difference was observed, per day, between the treatments, in terms of the average percentage of mycosis of the dead insects (ANOVA, $F_{(16, 125)} = 0.27$, $\rho = 0.99$) (Fig. 3.9).

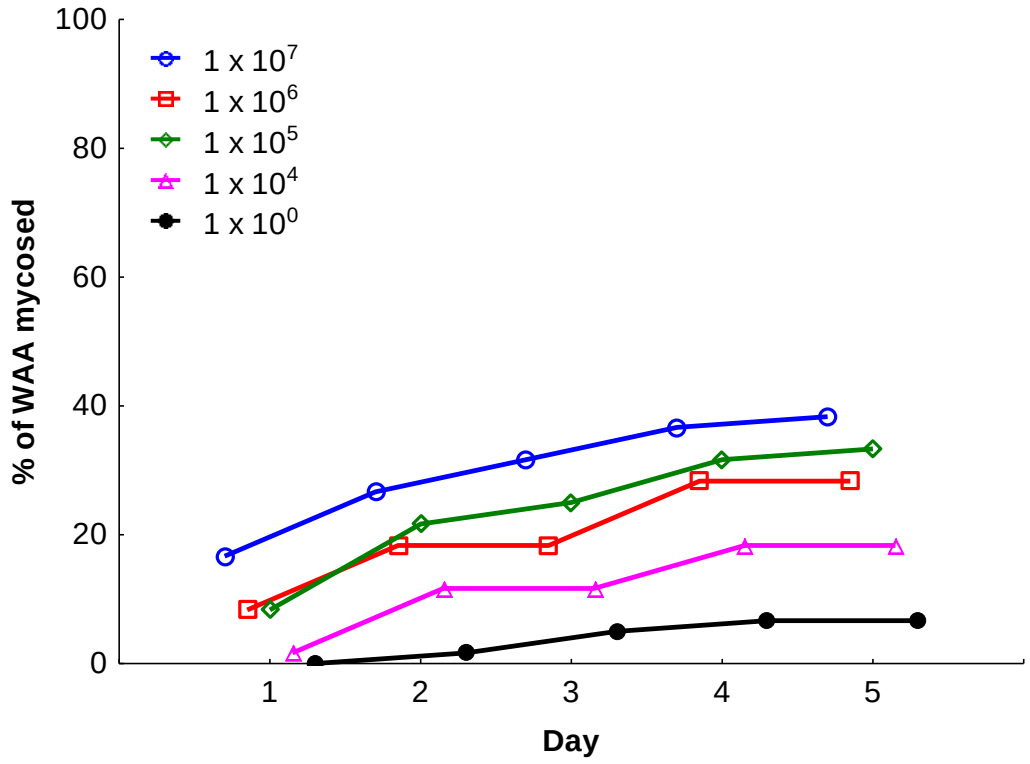

Fig. 3.9. Average percentage of mycosis of the adult females of *Eriosoma lanigerum* (WAA) post-death, following exposure to four different conidial concentrations of *Metarhizium brunneum*, and a control treatment of water only, daily up to 5 days after death.

Concentration–dose-response bioassays using *M. pinghaense* against the adult females of *E. lanigerum* in five different treatments showed no significant difference in percentage mortality between the different concentrations for the different exposure times (ANOVA, $F_{(16,125)} = 0.49$, $\rho = 0.95$). The average percentage in mortality ranged between 18 % ± 3.07 %, at the lowest concentration of 1×10^4 conidia/ml after 1 day of exposure to the fungal pathogen, and 65 % ± 9.92 % at the highest conidial concentration of 1×10^7, 5 days post-exposure (Fig. 3.10).

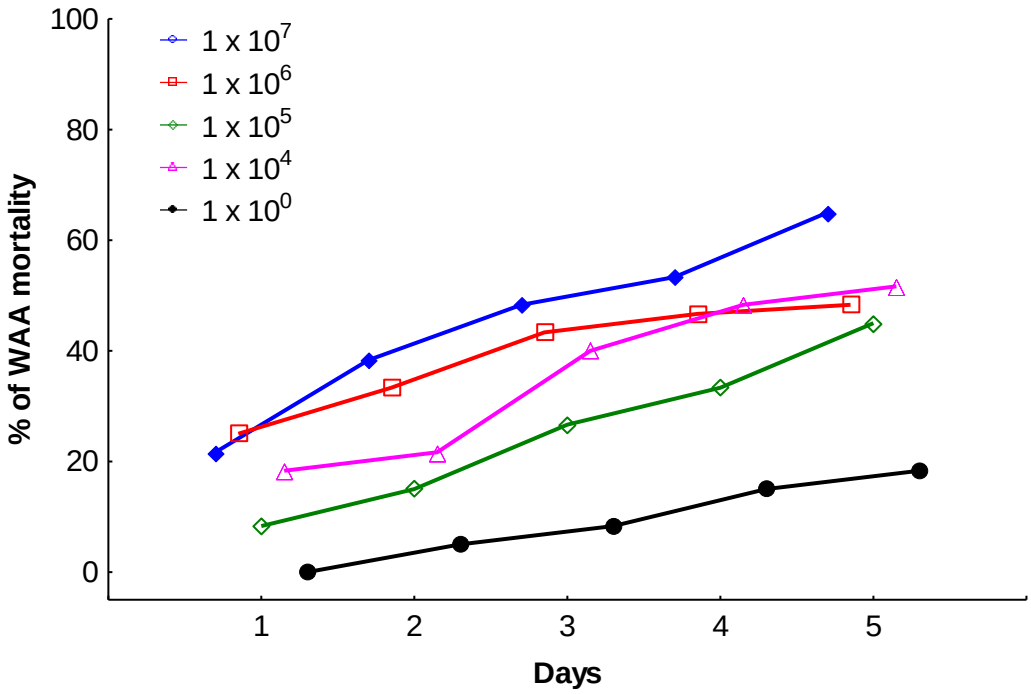

Fig. 3.10. Average percentage of mortality of the adult females of *Eriosoma lanigerum* (WAA), following exposure to four different conidial concentrations of *Metarhizium pinghaense*, and a control treatment, daily up to 5 days post-exposure.

A similar pattern was observed when evaluating mycosis of the insect's post-death. No significant difference in mycosis of the dead insects was observed between the four different *M. pinghaense* conidial concentrations. The average percentage of mycosis ranged between 13.33 % ± 4.22 %, at the lowest concentration of 1×10^4, after 1 day of exposure to the fungal pathogen, and 51.67 % ± 8.33 % at the highest conidial concentration of 1×10^7, 5 days post- exposure (Fig. 3.11).

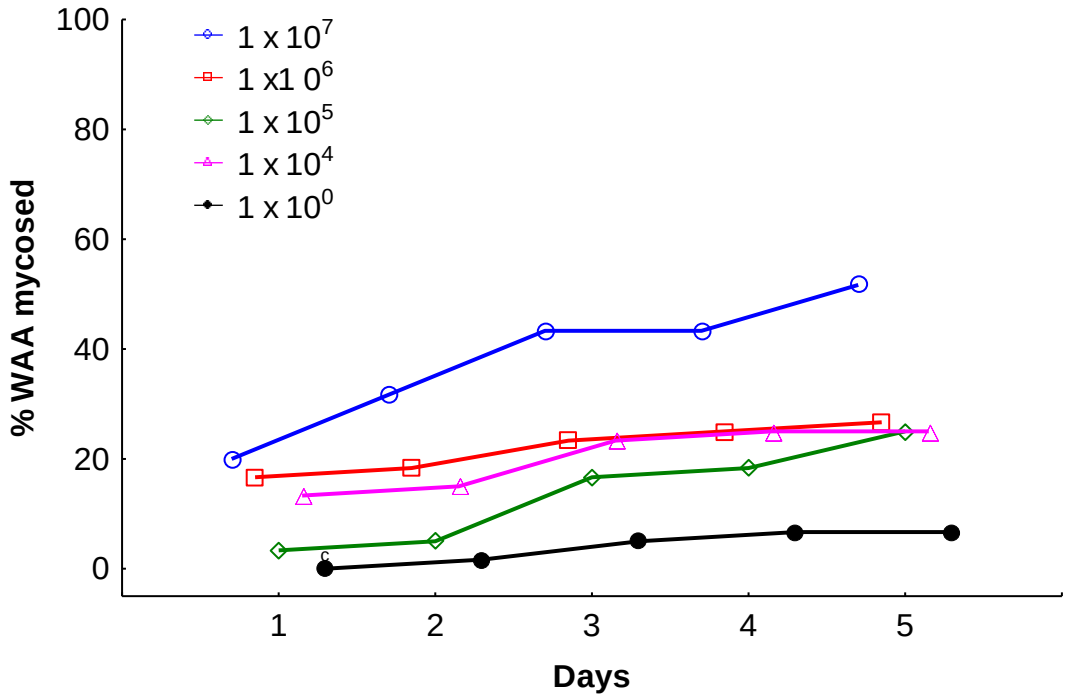

Fig. 3.11. Average percentage of mycosis of the adult females of *Eriosoma lanigerum* (WAA) post-death, following exposure to four different conidial concentrations of *Metarhizium pinghaense*, and a control treatment, daily, up to 5 days after death.

Mycosis of the dead female adults of WAA, following exposure to five treatments of *M. pinghaense*, showed a significant difference in the number of insects killed by infection of *M. pinghaense*, observed through mycosis (ANOVA, $F_{(4,\ 125)} = 20.63$, $\rho < 0.01$). The conidial concentration dose of 1×10^7 conidia/ml differed significantly to the other three conidial concentrations, and the control treatment. A significant difference was observed between the control treatment and the four conidial concentrations ($\rho < 0.05$), whereas no significant difference was observed between the conidial concentrations of 1×10^6, 1×10^5, and 1×10^4 conidia/ml ($\rho > 0.05$) (Fig. 3.11).

Exposure–time-response bioassays

Exposure–time-response bioassays were conducted using a probit analysis, to calculate the LT_{50} and LT_{90} values (the median lethal time) at a standard concentration of 1.0×10^7 conidia/ml for M. brunneum and M. pinghaense (Table 3.1). The results obtained showed that an exposure time of 9.43 days and 199.56 days was required to obtain LT_{50} and LT_{90} for M. brunneum, respectively (Fig. 3.12.). For M. pinghaense, an exposure time of 4.77 days and 52.73 days was required to obtain LT_{50} and LT_{90}, respectively (Fig. 3.13).

Table 3.1. Estimated LT_{50} and LT_{90} values for *Eriosoma lanigerum*, following treatment with *Metarhizium brunneum* and *Metarhizium pinghaense* at a standard concentration of 1×10^7 conidia/ml, over a period of 5 days under laboratory conditions.

Fungal isolate	LT_{50} (days)	LT_{90} (days)
Metarhizium brunneum	9.43	199.56
Metarhizium pinghaense	4.77	52.73

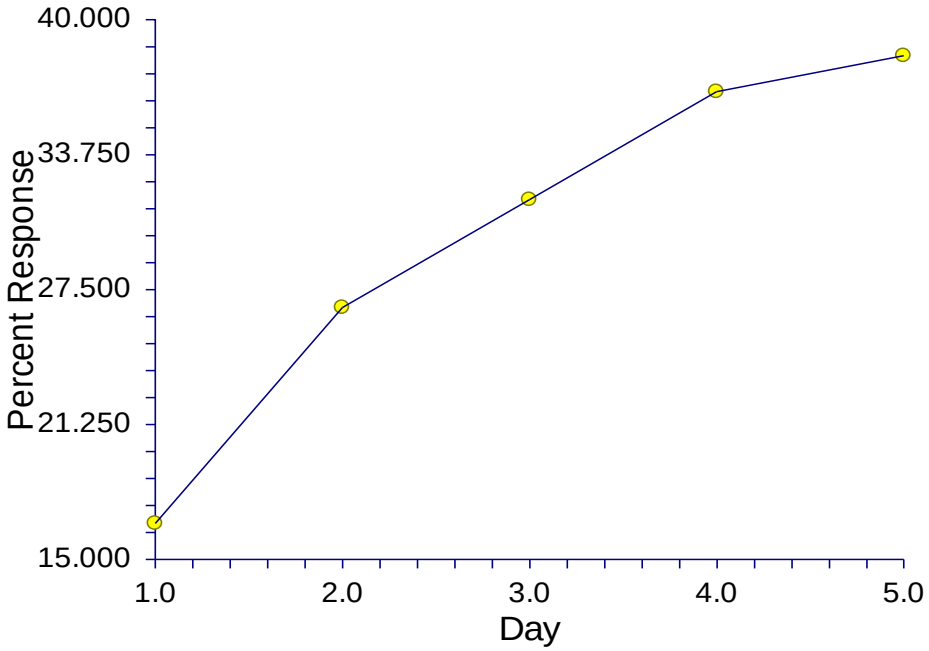

Fig. 3.22. Dose response plot showing the amount of days required to kill a certain percentage of *Eriosoma lanigerum* colonies using *Metarhizium brunneum* at a standard concentration of 1×10^7 conidia/ml over a period of 5 days under laboratory conditions.

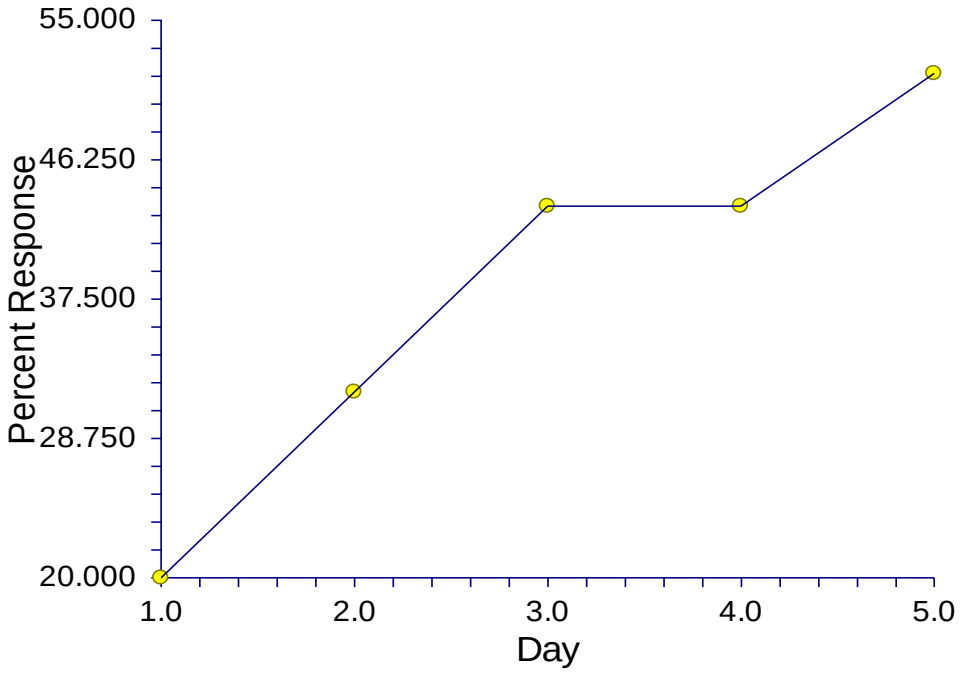

Fig. 3.13. Dose response plot showing the amount of days required to kill a certain percentage of *Eriosoma lanigerum* colonies using *Metarhizium pinghaense* at a standard concentration of 1×10^7 conidia/ml over a period of 5 days under laboratory conditions.

DISCUSSION

Entomopathogenic fungi secrete a wide range of biologically active secondary metabolites both *in vitro* and *in vivo*, of which some are restricted to specific genera, while others are commonly produced (Vey *et al.* 2001; Zimmermann 2007; Skrobek *et al.* 2008). The secreted metabolites display a wide array of biological activity, including insecticidal activity, and can thus be used in biological control of insect pests of agricultural crops across agroecosystems. Several studies have also reported the effective biological management of several hemipteran insects, including aphids and whiteflies, using EPF (Wraight *et al.* 1998). However, a limited number of studies have been conducted on assessing the effect of EPF in biological management of *E. lanigerum*. Screening the virulence of the six isolated fungi at a standard conidial concentration (1.0×10^7 conidia/ml), against the root colonies of WAA, showed that different species of EPF exhibit virulence against WAA. Bioassays were conducted to evaluate which of the locally isolated EPF had more potential for effective use in trials testing the effect of EPF on the root colonies of WAA. Evaluation of the virulence of the native strains of EPF not only allowed for the selection of the most suitable EPF for biological control, but it also provided information concerning the loss of virulence following the passage of the strains through artificial media, like SDA (Butt *et al.* 2006). The results obtained during the current

screening have shown that the root colonies of WAA on apple tree roots are susceptible to infection by the local isolates of the soil-dwelling EPF.

For the screening of the efficacy of the six EPF species against the WAA, the test arena used seemed to be extremely important. The 24-well bioassay plate method was used for screening in other studies, including the study of *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), the vine mealybug (Platt *et al.* 2018), and *Cydia pomonella* L. (Lepidoptera: Tortricidae), the codling moth (Odendaal *et al.* 2016). However, the method concerned proved not to be effective for testing the pathogenicity of the different EPF species against the WAA. In the present study, the majority of the female WAA used died of natural causes unrelated to the fungal treatments, 3 to 4 days following incubation when using the 24-well bioassay plate method. The above can be ascribed to the sensitivity in handling, and to damaging the insects' mouthparts when collecting the WAA from infested root samples. However, the currently used technique of dipping the WAA in conidial suspensions of the different EPF species, while they are still attached to the infested roots, with minimal disturbance, proved to be an effective method of testing the efficacy of the different EPF species against the WAA. The method was also employed by Stokwe (2016), when testing the efficacy of commercial EPF isolates against the root colonies of the WAA. In future research, the root-dipping technique, rather than the use of the 24-well bioassay plate method, when testing for the efficacy of EPF species against the WAA should be used, as the method has proved to be relatively effective.

In the current study, it was observed that exposure of WAA to the four local EPF species, *I. fumosorosea, M. pinghaense, M. robertsii*, and *M. brunneum*, resulted in mortality that was slightly higher than that of WAA insects exposed to *B. bassiana* and *P. lilacinum*. However, further analysis, undertaken by observing mycosis of dead WAA insects on SDA plates, and by using a microscope to verify whether the insects had died due to infection by the fungal pathogens, or due to natural causes, indicated that only two fungal isolates, *M. brunneum* and *M. pinghaense,* showed relatively high levels of virulence against WAA, when they were tested under laboratory conditions. The results obtained also indicated that the WAA was more susceptible to infection by these two species, which are members of the 'PARB' clade (Bischoff *et al.* 2009), when compared to other local fungal isolates. Similar observations were seen in a study conducted by Erler *et al.* (2014), when evaluating the effect of a *M. brunneum* strain in controlling pear psylla, *Cacopsylla pyri* L. (Hemiptera: Psyllidae), which is an insect pest of pears, that shows some level of resistance against insecticides. In other countries, *M. brunneum* has already been successfully commercialised against a variety of insects (Rehner & Kepler 2017). *Metarhizium pinghaense*, in a study conducted by Kirubakaran *et al.* (2018), was found to have some lethal effects when used against the rice leafroller, *Cnaphalocrocis medinalis* (Gunee) (Lepidoptera: Pyralidae), which is a destructive pest of rice crops. Stokwe (2016)

also showed the potential of two South African-registered commercial EPF products derived from *B. bassiana* and *M. anisopliae* to be effective in a field trial against the WAA in apple orchards.

The degree of clustering of the WAA on a piece of root, and the continuous production of the white waxy layer following dipping and exposure to the various fungal isolates, might also have influenced the level of mortality that was observed during the study. The WAA insects tended to cluster together during the time in which the experiments were conducted and they continued to produce the white waxy coating. Clustering of the WAA might have influenced the way in which some of the insects on the piece of root were exposed to the fungal pathogen, by preventing the conidial suspensions from reaching the insects and thus resulting in some level of protection to the exposure of the fungal pathogen. However, clustering of the WAA might also provide an opportunity to transmit the epizootics from infected individuals to healthy ones.

Testing the response of the WAA to the inoculation of *M. brunneum* and *M. pinghaense* at four different conidial concentrations showed that, with an increase in conidial concentrations, an increase in the number of infected WAA was observed for both fungal isolates. However, *M. pinghaense* showed better performance in terms of WAA infectivity (52 %) compared to that of *M. brunneum* (38 %), using the standard concentration. The exposure–time-response bioassays also showed that the median lethal time of exposure required to kill 50 % of the WAA population (LT_{50}), based on the laboratory trials was 5 days for *M. pinghaense*, which is shorter than the amount of time that was required for *M. brunneum* (9 days). The median lethal time required to kill 90 % of the WAA population (LT_{90}) for *M. pinghaense* (53 days) was less than that of *M. brunneum* (200 days).

In the current study, *E. lanigerum* was observed to have some form of association with soil-borne fungi, as the dead WAA insects in the control treatment, which had been treated with distilled water and 0.05 % Tween 20, showed fungal growth on the insect cadaver, when they were placed on an SDA growth medium. Similar observations were made by Damavandian (2000), where it was recorded that WAA had an association with at least six species of EPF, including *B. bassiana* and *P. lilacinus*, and saprophytic fungi. However, in the current study, the fungal growth that was observed to occur on some of the dead WAA in the control was a fast-growing saprophytic fungi. The growth of the saprophytic fungi on the insect cadavers in the control treatment could be the result of humid conditions, which is an environmental factor that is important for germination of fungal spores. However, in a previous study no EPF, but only saprophytic fungi, could be isolated directly from the female WAA (Chapter 2).

The results that were obtained in the present study provided useful information by giving insight into the potential of locally isolated EPF, which can be used in the integrated pest management of WAA, specifically *M. brunneum* and *M. pinghaense*. However, the results were obtained under optimum conditions, and at a relatively low concentration (1.0×10^7 conidia/ml). Further research

into the performance of the two fungal species against WAA, under both glasshouse and field conditions, and at relatively high conidial concentrations, as well as into their persistence under field conditions, should be undertaken.

Based on the results that were obtained through the concentration–dose- and exposure–time-response bioassays, *M. pinghaense* was shown to be the most effective local EPF species when used against the root colonies of the WAA. This local EPF isolate has shown that it requires a relatively short time, when compared to the other EPF species, to kill off WAA colonies. The results obtained therefore provide an opportunity of further testing *M. pinghaense* as a possible biological control agent that can be used for the management of this particular pest in apple orchards, and also for determining whether the WAA can be successfully managed when the isolate is used. The results from the study also broaden the range of possible biological control agents that can be used in WAA management, since various biotypes of the WAA are well-documented to have developed some level of resistance against a wide range of chemical insecticides, and currently only a few WAA management techniques are available, most of which have proven to be unsuccessful. From the current study, locally isolated EPF, specifically *M. pinghaense*, can be concluded as offering potential for effective use as biological control agents against both the root and the aerial colonies of WAA in apple orchards.

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CHAPTER 4

Persistence and infection of *Metarhizium pinghaense* (Hypocreales: Clavicipitaceae) and *Metarhizium brunneum* (Hypocreales: Clavicipitaceae) on apple bark

ABSTRACT

Eriosoma lanigerum (Hemiptera: Aphididae), or woolly apple aphid (WAA), is a serious insect pest affecting apple production across the world. The insect attacks the roots and above- ground parts of the apple tree. Root infestation results in the formation of hypertrophic galls that impede the flow of plant nutrients and water from the plant's root system to arboreal parts of the trees. WAA has developed some level of resistance against a variety of chemical insecticides that were previously used for its control. However, soilborne entomopathogenic fungi, *Metarhizium brunneum* and *M. pinghaense*, have shown the potential to be used as biological agents of WAA under laboratory conditions. The aim of the current study was to test the persistence of the two fungal species on apple bark over a period of three weeks, and to determine whether fungal conidia will attach to crawlers, or to the fourth-stage nymphs, as they move up tree trunks. The study was done by means of spraying apple bark with conidial suspensions of both fungi, at a standard infective conidial concentration of 1.0×10^7 conidia/ml. The persistence, or survival, of the fungal conidia on apple bark was measured using codling moth larva (*Cydia pomonella*) as an indicator. The results showed that the conidia of *M. pinghaense* showed better persistence on apple bark (63 ± 33.02 %) than did *M. brunneum* (11 ± 12.87 %) three weeks post application of the conidial suspension. Further analysis also showed that the fungal conidia of *M. pinghaense* are capable of inducing the mortality of WAA, four days after application to the apple bark. Based on the above-mentioned observations, *M. pinghaense* can be recommended as being the more effective fungal species to use, and should be further investigated as the microbial biological control agent of WAA under glasshouse and field conditions.

Key words: apples, nymphs, biological control, *Metarhizium brunneum*, *Metarhizium pinghaense*, conidia

INTRODUCTION

Woolly apple aphid (WAA), *Eriosoma lanigerum* L. (Hausmann) (Hemiptera: Aphididae: Pemphiginae), is an important insect pest that is native to North America. The aphid attacks apple trees across apple production areas both locally, and on a global scale (Klimstra & Rock 1985; Damavandian & Pringle 2002; Short 2003). The presence of WAA in South African apple orchards was initially recorded in 1895, with it having since spread throughout the apple- producing areas in the Western and Eastern Cape, which are the main regions of apple production in the country (Myburgh *et al.* 1973; Damavandian 2000; Heunis 2001). Infestations and feeding of the root colonies of *E. lanigerum* on an apple tree root system has a devastating effect on the plant's health, as they can result in damage to the root system through formation of hypertrophic galls on the inner bark of the host plant's root system (Sandanayaka & Hale 2003; Sandanayaka *et al*. 2003).

The formation and development of hypertrophic galls on the apple tree's root system restricts water and nutrient movement, and disrupts normal plant growth and development (Brown *et al.* 1991; Damavandian 2000; Short 2003; Damavandian & Pringle 2007; Dardeau *et al*. 2014). The root colonies of WAA are the main source of arboreal colonies, due to the continuously upward movement of the fourth-stage nymphs, known as crawlers, from the root colonies to the upper parts of apple trees (Nicholas *et al.* 2005; Damavandian & Pringle 2007; Berkvens *et al.* 2014; Lordan *et al.* 2015). The presence of WAA in apple orchards negatively affects productivity, as it affects the tree's vitality, which often leads to poor yields in terms of fruit quality and quantity (Asante & Cairns 1995; Brown *et al.* 1995; Dedryver *et al.* 2010; Costa *et al.* 2014). Increase in the WAA population outbreaks of arboreal colonies also has severe and prolonged effect on orchard yield, because the aphids reduce the number of fruit buds, weaken fruit-bearing wood, and stimulate the premature defoliation of plants (Short & Bergh 2004; Gresham 2013). Heavy infestations of WAA often result in crop losses in apple orchards (Pringle & Heunis 2008).

Eriosoma lanigerum has also developed some level of resistance against a wide array of chemical insecticides that have been used in the past for its control (Christians 2003). Such chemicals include chlorpyrifos and endosulfan, which have been used extensively in apple orchards, where the strains of *E. lanigerum* are now resistant to the application of Vamidothion (Pringle *et al.* 1994; Nicholas *et al.* 2003). The development of resistance to chemical insecticides by pests, and concerns over the deleterious effects of chemicals on the environment, have provided a strong drive for the development of microbial agents for the control of insect pests (Inglis *et al.* 2001).

The soil environment is an important reservoir of a large diversity of fungal species, with various ecological functions (Shah & Pell 2003; Meyling & Eilenberg 2007). Entomopathogenic fungal pathogens, which play an important role in pest insect population regulation, are generally considered as very promising biological control agents of insect pests (Charnley & St Leger 1991;

St Leger *et al.* 1996; Gilbert & Gill 2010). The entomopathogens are among the natural enemies that cause epizootics in arthropod species in agroecosystems, by means of inducing lethal infection to their host insects, which helps with the regulation of pest populations (Leger *et al.* 1992; Roy *et al.* 2006; Meyling & Eilenberg 2007; Vega *et al.* 2009; Shahid *et al.* 2012). Apple orchards provide long-term stable habitats for populations of various soilborne entomopathogenic fungi (EPF), and thus EPF populations are more likely to be high in apple orchard soils (Cross *et al.* 1999).

Important EPF species with biocontrol potential are found mainly across two fungal divisions: Ascomycota and Entomophthoromycota. The fungal division Ascomycota, contains Hypocreales fungal species including the *Metarhizium anisopliae* Metch. Sorokin (Hypocreales: Clavicipitaceae) complex and *Beauveria* species, of which some are commercially produced and used globally for biological control against a variety of agricultural pests in agroecosystems (De Faria & Wraight 2007; Quesada-Moraga *et al.* 2007; Hatting *et al*. 2018). *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and *M. anisopliae*, which are among the most well-studied fungal species within agroecosystems, have been used to control a number of insect pests in some countries (Chase *et al.* 1986; Leger *et al.* 1992; Wraight *et al.* 2000; Meyling & Eilenberg 2007).

The use of EPF as mycoinsecticides for insect pests is also considered as an environmentally acceptable alternative to chemical pesticides, as they pose less harm to the environment (St Leger *et al.* 1996; Shah & Pell 2003). They have long been used as biological control agents for controlling many pest species, with various levels of success (Dedryver *et al.* 2010). The use of local strains of EPF against crop-damaging insects across agricultural ecosystems is considered preferable, because the fungi are already adapted to the local climatic conditions (Abaajeh & Nchu 2015). EPF cause mycosis in many different taxa of arthropods, and in almost every order of insects, and they infect all life stages of insects (Dedryver *et al.* 2010; Shahid *et al.* 2012).

Metarhizium brunneum (Petch) and *Metarhizium pinghaense* Chen & Guo (Ascomycota: Hypocreales: Clavicipitaceae) have both been tested and used successfully in controlling various insect pest within agroecosystems. In a study conducted by Kirubakaran *et al.* (2018), *M. pinghaense* was shown to have relatively great potential for control against the rice leaf folder *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), which is a destructive insect pest that attacks rice crops. Another study, conducted by Cossentine *et al.* (2010), assessing the susceptibility of apple clearwing moth, *Synanthedon myopaeformis* (Lepidoptera: Sesiidae), which is an invasive European moth species that lays its eggs on the damaged bark near graft unions and pruning cuts, to *B. bassiana* and *M. brunneum*, found that the insect pest was susceptible to infection by both of the EPF species concerned.

The persistence of an entomopathogen in the habitat of the target host insect, mainly the ability of the pathogen to remain active on the surface in contact with the host insect, is one of the main

factors affecting the effectiveness of a pathogen as a biological control agent (Jaques 1983). Therefore, the persistence of local EPF, within their targeted ecosystems, is one of the most important factors that should be taken into consideration, if the fungal isolate is to be used as a biological control agent of insect pests. The current study addressed the question: can the fungal conidia of *M. brunneum* and *M. pinghaense* persist for relatively long periods on apple tree bark, and still cause epizootics in WAA colonies post-application? The EPF conidia were predicted to persist for extended periods on apple tree bark following application, with fungal conidia still capable of causing epizootics in WAA colonies after application to the tree bark. The aim of the current study was to test the persistence of fungal conidia on apple bark, by determining whether fungal conidia would be able to infect the WAA. The apple bark was, therefore, sprayed with conidial suspensions and the persistence of the fungal conidia was determined. The natural infections and the mortality of WAA, following exposure to the apple tree bark treated with conidial suspensions over time were also determined.

MATERIALS AND METHODS

Source of insects

The codling moth (CM), *Cydia pomonella* L. (Lepidoptera: Tortricidae), last instar larvae used in the current study were obtained from stored cultures provided by Entomon Technologies (Pty) Ltd, an insect-rearing facility located at Welgevallen Experimental Farm in Stellenbosch, Western Cape. The larvae were reared from eggs in an artificial diet in a growth chamber under diapause conditions, with controlled temperatures of 25 ºC ± 2 ºC and 60 % relative humidity. Once having reached the last instar larvae, the cultures were stored at temperatures of ± 6°C, until their use in the experimental trials.The codling moth last instar larvae were used as indicator species for conidial persistence on the apple bark due to their easy susceptibility to infection by EPF. The WAA adult females were collected from roots collected in an apple orchard in Grabouw and kept in moist containers in the laboratory, and used as needed (Chapter 2).

Source of entomopathogenic fungi

Metarhizium pinghaense isolate collected during the current study (5HEID) in apple orchards in Grabouw, and an isolate collected from an apple orchard on Baldric Farms, Kylemore, Western Cape province (DO1) (-33.929668; 18.958976) were used in the present study.

Preparation of conidial suspensions

Fungal conidia were harvested from 2-3 week-old surface cultures by scraping from SDAY (Saboraud dextrose with yeast) plates (Coombes 2012). The collected fungal spores were

suspended in 20 ml of autoclaved distilled water supplemented with 0.05 % Tween 20, in 28-ml McCartney wide-mouth glass bottles. The bottles containing the fungal conidia were sealed and vortex-mixed for 2 min to produce a homogenous conidial suspension, which was then poured through organza fabric into a sterilised 100-ml glass beaker, so as to remove the fungal hyphae and mycelium. Following the above procedure, the fungal concentrate in the beaker was poured back into the 28-ml McCartney bottles and vortex-mixed for 2 min, with the concentrate then being used as the conidial stock for the serial dilutions concerned.

Conidial concentration dilutions

For the concentration dilution process, 9 ml of sterile distilled water in 28-ml McCartney bottles was used. One ml of the 20 ml conidial suspension for each fungal strain was pipetted in 9 ml sterile distilled water, following which the conidial suspensions were vortex-mixed for 2 min. To determine the conidial concentrations, a Neubauer haemocytometer was used, and the conidial suspensions were used within 3 h of enumeration (Coombes *et al.* 2013).

The conidial suspension (200 µl) was loaded onto the haemocytometer. The number of spores on both ends of the haemocytometer were counted under a compound microscope to determine the conidial concentration of the suspension. Following the above, a formula was used to determine the conidial concentrations, with the total number of spores counted on each side of the haemocytometer being added, and the average of the two calculated. The average number obtained from the conidial concentration was then multiplied by 5 x 10^4 to give a concentration of 1 x 10^7 conidia/ml (Inglis *et al.* 2012).

Following calculation of the conidial concentration of the fungal suspensions, the volume of the conidial suspension and that of the sterile distilled water were calculated to make a conidial suspension of 10 ml, with a conidial concentration of 1 x 10^7 conidia/ml. The following formula was used:

$C_1V_1 = C_2V_2$; $V_1 = \frac{C_2V_2}{C_1}$ where: C_1 = initial concentration; V_1 = required volume of conidial suspension stock to be added to the distilled water, or the required volume for dilution of the original aliquot; C_2 = desired concentration; V_2 = desired final volume of the inoculum.

Assessment of conidia viability germination

The viability of conidia was determined by means of spread-plating 100 µl of the diluted conidial suspension, with the concentration of 1.0 × 10^7 conidia/ml, on three SDAY plates per isolate. Each plate was sealed with a Parafilm, and then incubated in a growth chamber at an ambient temperature of 25 °C. The percentage of conidia germination was then examined after 24 h from 100-spore

counts on each plate. The above was done under a compound microscope at 40 x magnification, with the spores that had developed a germ tube twice the length of the spore being counted as viable, or living, spores and the spores without a germ tube being counted as dead, or non-viable, spores (Ekesi *et al.* 2002; Inglis *et al.* 2012). The average number of both the dead and the living spores was calculated for the three plates, so as to give the viability of the fungal spores for the fungal isolate being tested. The viability of the fungal conidia was checked to determine the number of viable spores per unit volume of the conidial suspension, as this might influence the effectivity of the fungal strain in killing the WAA insects. Fungal cultures with a viability of >80 % were used in bioassays.

Persistence of conidial spores

Pieces of apple bark, harvested from apple trees, were used in the current study. The bark was first autoclaved, and then dried, followed by spraying 30 pieces with a conidial concentration of 1 x 10^7 conidia/ml (standard concentration) of *M. pinghaense* and *M. brunneum*, respectively, suspended in sterile distilled water and 0.05 % Tween 20. The sprayed pieces of bark were air dried and divided into three groups, consisting of 10 pieces of bark per group for each fungal isolate (Fig. 4.1A). Each piece of bark from the three groups was transferred and sealed in Petri dishes (90-mm-diameter), and incubated at 25 ºC (Fig. 4.1B).

Weekly, for three weeks, 10 of the treated bark pieces from each fungal isolate were removed from the incubator and washed using 5 ml sterile distilled water to collect the fungal conidia off from the bark. The above was done by placing each piece of bark in a 50-ml plastic Falcon centrifuge tube containing 5 ml sterile distilled water, where it was vortex-mixed for 2 min (Fig. 4.1C). Following the above, the apple bark was removed from the tube and the suspension was centrifuged for a period of 15 min. Following centrifugation, the top 1 ml of supernatant was removed and the remaining 4 ml was mixed with the centrifuged pellet.

Fig. 4.3. Persistence of fungal conidia on apple bark. (A) Collected apple bark sprayed with fungal conidia; (B) treated apple bark a week post treatment; (C) apple bark in 5 ml of sterile distilled water.

The persistence and virulence of the spores was determined using 10 CM larvae per piece of apple bark. Each of the CM larvae was dipped in the 4 ml conidial suspension and placed in a 9-cm Petri dish fitted with filter paper, moistened using distilled water. The Petri dishes were then sealed, and incubated in a growth chamber at a controlled temperature of 25 ± 2 ºC. The process was repeated weekly, and every day for a period of 10 days dead insects were removed from the Petri dishes, surface-sterilised using 70 % ethanol, and placed on a water agar medium to check for mortality and mycosis.

Natural infections

The apple bark pieces were prepared as above, with a double conidial concentration of 2 x 10^7 conidia/ml of *M. pinghaense*. Here, 10 ml conidial suspensions of the normal concentration (1 x 10^7 conidia/ml) were transferred to centrifuge tubes, and centrifuged for 20 min, following which the top 5 ml of water was removed, and the remaining 5 ml water was vortex-mixed to mix the pellet at the bottom of the centrifuge tube. The conidial suspension was then applied to the apple tree bark, and the bark was left to dry overnight. After every 24 h, 20 pieces of the bark were lightly misted with sterile water.

The same procedure was followed for persistence, except in this case the codling moth larvae, or the adult WAA, were not dipped in the collected conidia, but were enclosed in a Petri dish with moistened pieces of dried bark (Fig 4.2). The Petri dishes were then placed within 2- L plastic

containers, fitted with moist paper towels, and incubated in a growth chamber at 25 ± 2 ºC. The same procedure was followed for the evaluation of infection.

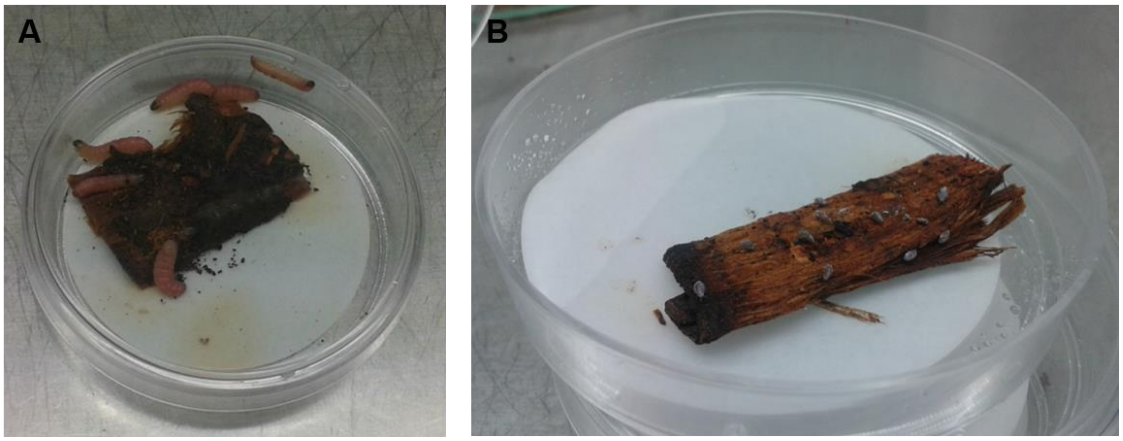

Fig. 4.4. (A) Codling moth larvae (*Cydia pomonella*) and (B) *Eriosoma lanigerum* (WAA) adult females on a piece of apple tree bark treated with *Metarhizium pinghaense* fungal conidia.

Data analysis

Analysis of collected data was done using the statistical software, STATISTICA, version 13.3 (TIBCO Soft Inc. 2016). Data was analysed using one-way ANOVA (analysis of variance), comparison of means using LSD tests (least significant difference), and post-hoc tests (the Games-Howell post-hoc test), at 95 % confidence intervals.

RESULTS

Persistence of conidial spores

The average percentage of conidial persistence, measured by washing and concentrating the EPF spores, and by using the infection of codling moth larvae as an indicator, for both *M. pinghaense* and *M. brunneum* on apple bark, showed a decline over time (Fig. 4.3). For *M. pinghaense*, the results showed a significant difference in conidial persistence between the 3 weeks of the trial ($F_{(2,27)}$ = 9.74, ρ < 0.01). The first week after application showed the highest level of infection (63 ± 33.02 %), which declined from the second week (37 ± 29.83 %) to the third week, with the lowest percentage in conidial persistence (8 ± 18.74 %). For *M. brunneum*, no significant difference in the average percentage of conidial persistence was observed between the 3 weeks after application ($F_{(2,27)}$ = 6.32, ρ > 0.05). A decline in persistence was observed from the first week (11 ± 12.87 %), through the second week (1 ± 3.162 %), with zero persistence in the third week.

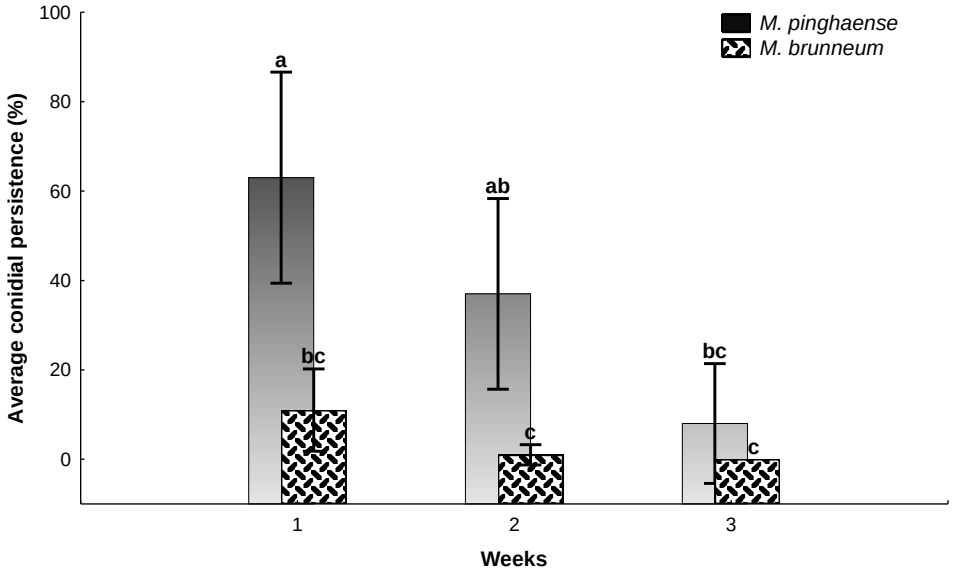

Fig. 4.3. A comparison of conidial persistence (95 % confidence) of *Metarhizium pinghaense* and *M. brunneum* on apple bark, sprayed with a conidial concentration of 1 x 10^7 conidia/ml over 3 weeks. The dried conidia were washed off from the bark and concentrated, with codling moth being used as the indicator of conidial persistence. Different letters indicate a significant difference between the fungal treatments ($\rho < 0.05$).

The infection of codling moth larvae with *M. pinghaense* was observed by the hardening of the insect cadaver and by the growth of hyphae, with the formation of mycosis and green conidia on the cadaver, following incubation on the water agar medium (Fig. 4.4).

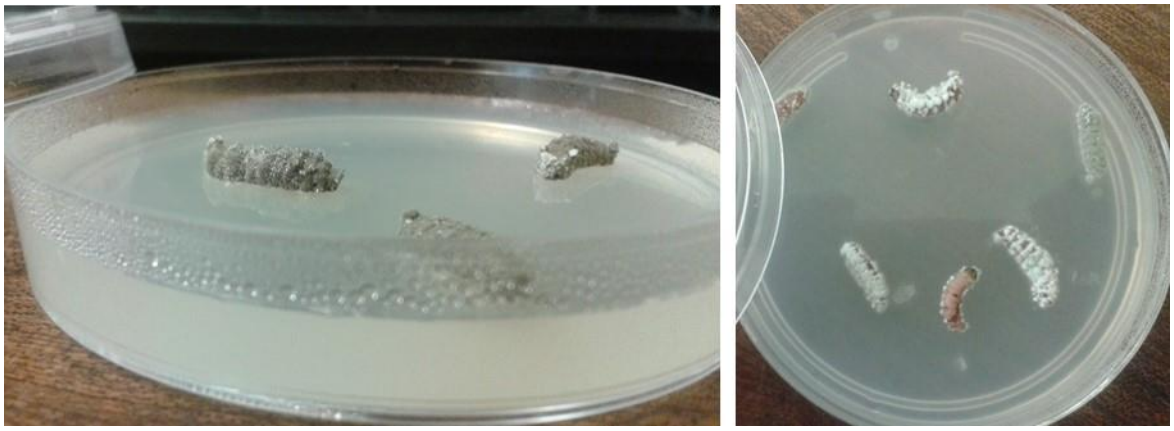

Fig. 4.4. The growth of *Metarhizium pinghaense* on codling moth larvae (*Cydia pomonella*), following exposure to apple tree bark exposed to dried *Metarhizium pinghaense* conidia, washed, and concentrated after 7 days.

Natural infection of codling moth

The persistence of *M. pinghaense* conidia on apple bark following application after 7 days is depicted in Fig. 4.5, using codling moth as the indicator. A significant difference in conidial persistence was observed across the 7 days of the trial ($F_{(6,63)}$ = 17.052, ρ < 0.01). No significant difference was found in the persistence of the infective fungal conidia of *M. pinghaense* between the first (68 ± 13.17 %) and second day (67 ± 23.59 %), following application to the tree bark. A significant difference was observed between the first 2 days and days 3 to 6, as well as day 7 with a low infection rate of 3 ± 6.75 %. The results showed a sharp decline in *M. pinghaense* conidial persistence dried on the bark, over the 7 days, with low infection on day 7.

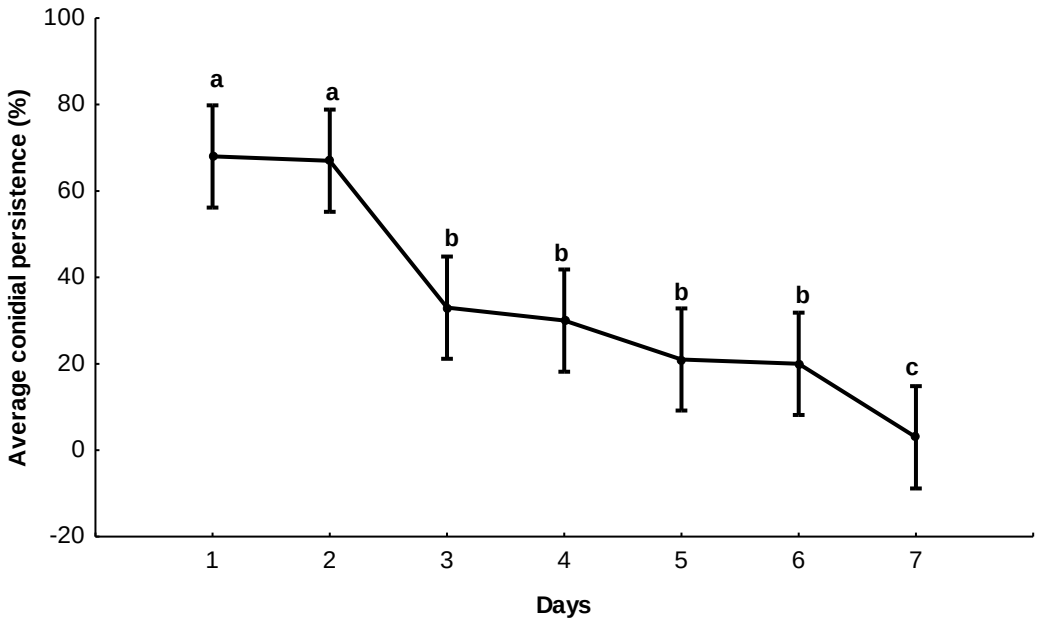

Fig. 4.5. Percentage of *Metarhizium pinghaense* persistence (mycosis of codling moth larvae) (95 % confidence interval), after being dried on apple bark over a period of 7 days (one-way ANOVA; $F_{(6, 63)}$ = 17.052, ρ < 0.01). Different letters indicate a significant difference between the weeks (ρ < 0.05).

Natural infection of the woolly apple aphid

Experiment 1 (pilot study)

The percentage of WAA mortality, due to fungal infection, following exposure to apple bark treated with double the normal concentration of dried *M. pinghaense* (2 x 10^7 conidia/ml), for a period of 4 days, is indicated in Fig. 4.6. No significant difference in WAA percentage mortality was observed across the 4-day trial period ($F_{(3,36)}$ = 1.659, ρ = 0.15). Exposure of the WAA to *M. pinghaense*-treated apple bark a day after application showed 73 ± 17.67 % in percentage mortality, with the

exposure of WAA to the treated bark after 2 days resulting in an average mortality of 69 ± 24.24 %. After 3 days 58 ± 19.89 %, and after 4 days 56 ± 18.97 % average mortality was observed. The results obtained showed that the persistence and efficacy of *M. pinghaense* conidia on the tree bark declined over time, after application. However, decline in coniudial persistence on the apple bark over the four day trial was not significant (ρ > 0.05).

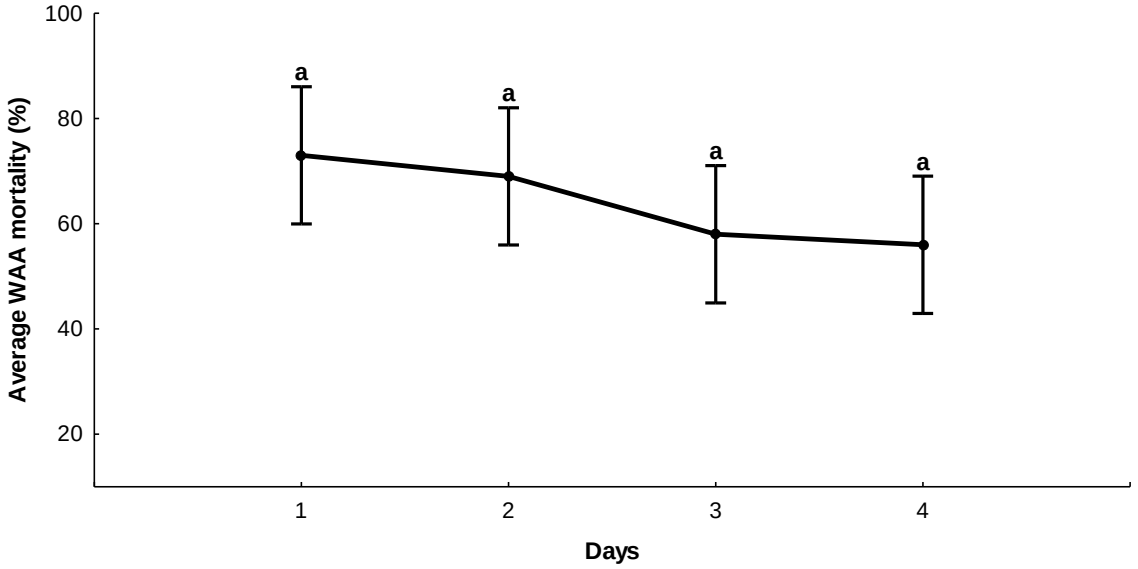

Fig. 4.6. Average percentage (95 % confidence interval) of *Eriosoma lanigerum* (WAA) mortality due to infection following exposure to apple bark exposed to *Metarhizium pinghaense* (5HEID) (one-way ANOVA; $F_{(3, 36)} = 1.659$, ρ = 0.15). Different letters indicate a significant difference between the weeks (ρ < 0.05).

The growth of *M. pinghaense* on codling moth larvae (Fig. 4.7A) and on WAA females (Fig. 4.7B) following exposure to apple bark treated with a double concentration (2 x 10^7 conidia/ml) of *M. pinghaense*.

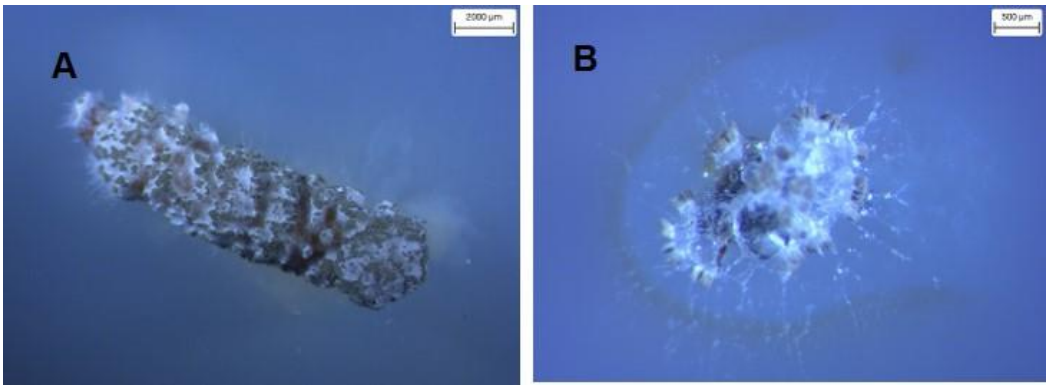

Fig. 4.7. The growth of *Metarhizium pinghaense* on codling moth larvae (*Cydia pomonella*) (A) and on adult female WAA (B), following exposure to apple tree bark exposed to *M. pinghaense* fungal conidia.

Experiment 2

Analysis of the results of date and treatment with regard to the percentage mortality of WAA, due to infection by *M. pinghaense* (two-way ANOVA; $F_{(4,90)} = 1.35$, $\rho = 0.26$) showed no significant difference. Therefore, the data from both trials were pooled and analysed using one-way ANOVA. The results showed a significant difference in WAA mortality over time ($F_{(4,95)} = 30.33$, $\rho < 0.01$). The mortality of WAA following exposure to apple bark treated with dried *M. pinghaense* declined over time, with the highest percentage being obtained for day 2 (82 % ± 15.76 %), followed by that which was obtained for day 4 (74 ± 16.67 %), day 6 (64 ± 13.53 %), day 8 (46 ± 15 %), and day 10 (39 ± 11.91 %). No significant difference was observed for mortality between days 2 and 4, and between days 8 and 10 ($\rho > 0.05$), whereas day 6 differed significantly in mortality from all the other days ($\rho < 0.05$) (Fig. 4.8).

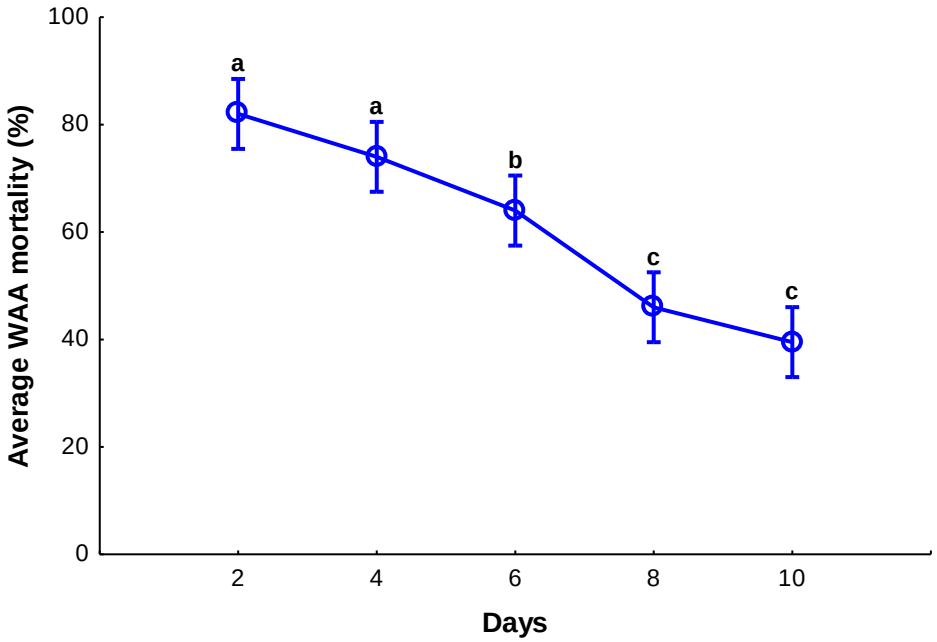

Fig. 4.8. Average percentage (95 % confidence interval) of *Eriosoma lanigerum (*WAA) mortality, due to infection following exposure to apple bark exposed to dried *Metarhizium pinghaense* (DO1) (one-way ANOVA; $F_{(4,95)} = 30.33$, $\rho < 0.01$). Different letters indicate a significant difference between the weeks ($\rho < 0.05$).

DISCUSSION

The success of EPF in managing pest insects, and in its use in crop protection, is partially dependent on the persistence of the fungal inoculum, following field application (Inyang *et al.* 2000). The persistence of EPF refers to the ability of the fungal isolate to remain viable in the field for longer periods post-application. EPF isolates that exhibit relatively long field persistence, tend to have an increased probability of coming into contact with a sufficient number of target pest insects, and with them being capable of causing epizootics in populations of the pest insects. Fungal isolates that are capable of persisting for relatively long periods are comparatively suitable as a biological control agent (Inglis *et al.* 2001; Coombes *et al.* 2013).

In the current study, the persistence of two EPF isolates, *M. brunneum* and *M. pinghaense*, following application to apple bark, at a standard conidial concentration (1.0×10^7 conidia/ml) under laboratory conditions, was investigated. The results obtained, after washing and concentrating the spores, showed that *M. pinghaense* had higher levels of conidial persistence on the apple bark over 3 weeks, relative to *M. brunneum*. For both EPF isolates, the persistence of conidia on the apple bark (indicated by codling moth larval infection) was also observed to decrease with an increase in time after application. These results are similar to those that were obtained through a study conducted by Coombes (2012), assessing the persistence of fungal isolates derived from *M.*

anisopliae and *B. bassiana* species, under field conditions. They found that, over time the persistence of fungal conidia under field conditions also declined. However, the results from the current study conflict with those of Coombes (2012), who showed the extended persistence of infective EPF conidia for a minimum of 6 months in the soil, under field conditions. However, such persistence can be attributed to the high conidial concentrations used for their study, relative to the current study, where only a relatively low standard concentration of 1.0×10^7 conidia/ml was used to test for EPF conidial persistence. Coombes (2012) also highlights that *M. anisopliae*-derived isolates persist better under field conditions than do *B. bassiana* isolates. Such persistence is not out of the ordinary, as the isolates derived from the *M. anisopliae* EPF species have been shown to be well adapted to surviving under fluctuating environmental conditions (Bidochka *et al.* 1998).

Application of *M. pinghaense* to apple bark has shown that the isolate might be capable of infecting the crawlers, or the fourth-stage nymphs of WAA, as they continue to move up the apple tree trunk to arboreal parts of the apple trees in apple orchards. The fungal conidia of the species were able to persist when they were exposed on the bark at a level of >60 % for 7 days post-treatment. Although a decline in the percentage of conidial persistence was observed from 63 % to 37 % from week 1 to week 2, the *M. pinghaense* isolate offered a better chance for the effective control of WAA relative to *M. brunneum*, which did not show enhanced performance in conidial persistence, following application. Therefore, further evaluation of the conidial persistence of *M. pinghaense*, when applied under field conditions, should be conducted at a higher conidial concentration of $>1 \times 10^7$ conidia/ml against the WAA in apple orchards. The application of *M. pinghaense* at a conidial concentration slightly higher than 1×10^7 conidia/ml under field conditions might ensure a good probability that the WAA insects will come into contact with a sufficient number of fungal conidia, as the insects move up the apple tree trunks in apple orchards.

Natural infection of the WAA females, following exposure to apple tree bark treated with a double concentration of *M. pinghaense* and dried over a period of 4 days, was tested in a pilot trial, as this had not been previously investigated. The results showed that the dried conidia of *M. pinghaense* persisted on the tree bark, and that the conidia were capable of causing natural infections and mortality of WAA after 4 days. The average percentage mortality of WAA was 73 % on day 1, and 56 % on day 4, with no significant difference being encountered after 4 days. The results show that the success of *M. pinghaense* in effectively managing colonies of WAA in apple orchards depends on the persistence of the fungal conidia over time. Follow-up research of conidia persistence over 10 days, using *M. pinghaense* (DO1), showed a decline in the persistence of the fungal species on apple bark over time, with the WAA mortality induced ranging between 82 % on day 1 and 39 % on day 10. The results are evident that conidial persistence declines over time, and that with increased conidial concentrations, the higher the chances of EPF persistence and of it coming into contact with

a sufficient number of the target insect pest. Using relatively high concentrations should also ensure the success of the *M. pinghaense* isolate in causing epizootics in WAA populations.

Taking into consideration that the above-mentioned results were obtained under controlled environmental conditions in the laboratory, the results obtained might vary under field conditions. In the field, a variety of abiotic environmental factors, like rainfall, solar radiation, environmental temperature, wind, water availability and relative humidity, might drastically affect the efficacy of EPF against target pest insects, and they could affect the persistence of the fungus over time (Inglis *et al.* 2001; Goble 2009). Under field conditions, the EPF are highly susceptible to damage by solar radiation when exposed to sunlight, more particularly when exposed to the UV-B portion (258-315 nm) of the solar spectrum, which could result in the inactivation of the fungal conidia (Inglis *et al.* 2001; Goble 2009). In a study conducted by Inglis *et al.* (1993), investigating the persistence of the EPF, *B. bassiana*, on the Phylloplanes of crested wheatgrass (*Agropyron cristatum*) and alfalfa (*Medicago sativa*), the fungal conidia persistence was observed to decline when the conidia were exposed to solar radiation, under field conditions.

Environmental temperatures will also have a significant effect on the efficacy and success of *M. pinghaense* against WAA, under field conditions. Ambient temperatures are well documented as affecting the rate of infection and the time of death of insect pests that have been treated with hyphomycetous EPF, with optimum ambient temperature requirements of between 20 ºC and 25 ºC. Temperatures of above 30 ºC are likely to inhibit the vegetative growth of the EPF on infected insects (Inglis *et al.* 2001). Relative humidity might also affect the effectiveness of *M. pinghaense* conidia under field conditions, as high moisture levels are required for conidiogenesis to occur on the surfaces of the insect cuticle (Inglis *et al.* 2001). Lower levels of relative humidity also lowers the production of conidia, and thus decrease the chances of transmission of the infective conidia from the infected insects to healthy individuals (Inglis *et al.* 2001; Goble 2009).

The results obtained through the current study provide insights into the potential persistence of fungal isolates on the bark of apple trees. Depending on the success of *M. pinghaense* under field conditions, the above provides an opportunity to use the fungal isolate in the integrated pest management of the WAA, which has developed some level of resistance against a variety of chemical insecticides. Under laboratory conditions, the fungal isolate, *M. pinghaense*, has shown potential to be successfully integrated into WAA management, as was observed in Chapter 3 of the present study, where it was noted that the isolate's pathogenicity against the insect pest outperformed the other five locally isolated EPF. Similar observations were also made in a previous study conducted by Stokwe (2016), who showed that despite different fungal species being pathogenic to the adult WAA females, the degree of pathogenicity of the different EPF isolates was found to vary. Stokwe (2016) also found that two commercial isolates, Meta 69®, derived from *M. anisopliae*, and Eco-Bb® strain R444, derived from *B. bassiana*, offered better chances for the

successful control of adult WAA under laboratory conditions, at a control rate of >70 %. The two commercial isolates also performed better than the local EPF isolates collected during the study described. However, the current study has shown that some of the local isolates also have the potential for successful use for WAA management, especially in terms of the local isolate of *M. pinghaense*.

The current study concludes that EPF offer a good chance of managing WAA in apple orchards. The study has indicated that *M. pinghaense* is, currently, the best local fungal candidate for the integrated management of WAA. Future studies should test *M. pinghaense* against the WAA under glasshouse, or field conditions, with the fungus either being applied directly to the root system of apple trees to control the root colonies, or applied to the arboreal parts (e.g. the tree trunks) of the apple trees concerned. Another option requiring investigation is the use of conidia-infected barriers, to target WAA crawlers as they move up the tree trunk to the arboreal parts of the tree.

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CHAPTER 5

GENERAL DISCUSSION

Eriosoma lanigerum L. (Hausmann) (Hemiptera: Aphididae), or woolly apple aphid (WAA), is a serious pest of apple trees, *Malus domestica* (Borkhausen), in all apple-producing regions globally. Its presence in South African apple orchards was first recorded in 1895, and the aphid has since spread throughout the apple-producing areas in the Western and Eastern Cape, which are the main apple production regions in the country. Edaphic populations of WAA pose a major threat to apple production in South Africa, as they cause damage to the root system, before the effects of arboreal colonies are detected. An increase in WAA population outbreaks of arboreal colonies also has severe and prolonged effects on orchard yield, as this aphid reduces the number of fruit buds, weakens the fruit-bearing wood, and stimulates the premature defoliation of plants. The management of WAA using chemical insecticides such as Vamidothion and Imadacloprid has proven to be unsuccessful, as the WAA has developed some level of resistance against a wide array of chemical insecticides. However, the use of entomopathogenic fungi (EPF) has provided an opportunity to explore the microbes for possible use in biological control against insect pests of crops that have developed resistance against chemical insecticides such as the WAA.

The overall aim of the current study was to conduct a survey of EPF in the local apple orchards of the Western Cape, and to screen them for their potential to control WAA. The first objective of the study involved a survey of EPF found in local apple orchards, which was undertaken by means of collecting soil and WAA-infested root samples. The collected soil samples were baited for the presence of EPF using susceptible host insects. The collected EPF isolates were tested for their pathogenicity against insects under laboratory conditions, and isolates that were entomopathogenic were identified, both morphologically and molecularly, to species level. From the survey, six EPF species were successfully isolated, including *Beauvaria bassiana, Metarhizium brunneum*, *Metarhizium pinghaense*, *Metarhizium robertsii*, *Purpureocillium lilacinum* and *Isaria fumosorosea* (Chapter 2). Some of the collected EPF isolates were commonly found in apple orchards, whereas other isolates were unique to certain orchards. The results indicate the diversity of EPF isolates in the local apple orchards of the Western Cape with potential as biological control agents against insect pests. Future research should focus on conducting a comprehensive survey of EPF across the various fruit orchards of the Western Cape, so as to isolate other potential EPF species. Conducting a comprehensive survey in the various fruit orchards might also result in the discovery of other EPF isolates that are capable of inducing infections in WAA populations that could not be

isolated in the apple orchards. Future research should also be extended to undertaking a survey of EPF in areas with natural vegetation.

The second objective of the study involved screening the pathogenicity of collected EPF isolates against the root colonies of the WAA. Only two EPF isolates, *M. pinghaense* and *M. brunneum*, were found to offer higher level of pathogenicity against the insect pest in comparison to the other four EPF species (*M. robertsii*, *P. lilacinum*, *B. bassiana* and *I. fumosorosea*). The above was observed through higher levels of mortality and mycosis that occurred on the insect cadavers following their death (Chapter 3). The results indicate that different EPF isolates have different levels of efficacy, when they are used against various insect pests. It is well documented that different insect species at certain life stages (usually immature life stages), are more susceptible to infection by EPF. However, in the current study only the adult WAA females were used to test the efficacy of the fungal isolates, which might have affected the results obtained, as the adult stage is usually less susceptible than the earlier stages are to infection for most insect pest species, as the insect's cuticle is usually fully formed at this stage.

Further study objectives involved the analysis of the pathogenicity and efficacy of *M. brunneum* and *M. pinghaense* against the WAA, in a concentration dose–response bioassay. Four different conidial concentrations, at lower levels than the standard concentration of 1×10^7 conidia/ml were used. The results obtained from this process showed that, with an increase in conidial concentration, the number of adult WAA insects that died because of infection due to exposure to the EPF isolates also increased. The above indicated that, with an increase in conidial concentrations, there was an enhanced opportunity to cause epizootics in WAA colonies. The study also showed that *M. pinghaense* was more successful in causing the mortality of WAA colonies, relative to *M. brunneum*. Therefore, *M. pinghaense* can be considered as the most effective fungal isolate candidate with a potential for development for the biological control of the WAA in apple-producing regions.

However, the studies with EPF were conducted under controlled environmental conditions in the laboratory, which differ extensively from field conditions. The conidial concentrations used in the laboratory were regarded as low, and therefore under field conditions it would be advisable to use concentration levels of above 1×10^7 conidia/ml. Doing so should ensure an enhanced chance of the insects coming into direct contact with the EPF conidia, and causing epizootics to the WAA populations under field conditions. The exposure time–response bioassays showed that the exposure of the WAA colonies to conidial concentrations of 1×10^7 conidia/ml for *M. pinghaense* was able to kill off 50% of the WAA colonies. The procedure involved took 5 days (LD_{50}), whereas to kill off 90% of the insect colonies required 53 days (LD_{90}), which was less time than that which was required to kill off 50% (LD_{50} = 9 days) and 90% (LD_{90} = 200 days) of the WAA population, using *M. brunneum*. The results obtained showed that *M. pinghaense* requires less time to kill off colonies

of WAA than does *M. brunneum*. In future studies, it would be advisable to test the exposure time required to kill the WAA colonies at a conidial concentration of above 1 x 10^7 conidia/ml for both isolates, so as to be able to determine whether less time will be required to kill off the insect pest when using relatively high EPF conidial concentrations.

The final objective of the study was to determine the persistence and the natural contact infection of EPF conidia on apple bark. The conidia of both *M. pinghaense* and *M. brunneum* were applied at a conidial concentration of 1 × 10^7 conidia/ml to apple tree bark, and then completely dried, with the EPF persistence being determined over a period of 3 weeks. The above was done to simulate field conditions, to predict the length of time of the conidial persistence on the apple bark, and to test whether the fungal conidia will have the potential to infect the WAA crawlers, as they migrate from the root systems to the arboreal parts of the trees. *Metarhizium pinghaense* was observed to show higher levels of persistence on the apple tree bark than did *M. brunneum*. The finding confirms that different EPF isolates have different levels of persistence over time, when applied to cultivated crops for the management of insect pests across agricultural fields.

To evaluate the conidial persistence of *M. pinghaense*, and whether it would be able to induce the natural infection of codling moth after application of the conidial suspensions, a double concentration of *M. pinghaense* (2.0 × 10^7 conidia/ml) was applied to apple tree bark, and completely dried overnight. The persistence of the fungal conidia was checked daily for 7 days, by means of adding codling moth larvae to 10 pieces of the dried bark pieces, with the persistence of fungal conidia being observed to decline over time. The results that were obtained from a pilot trial, using WAA females, showed high mortality (73% - 56%) due to infection, with no significant decline in persistence over 4 days. A follow-up replicated trial, which was conducted for a period of 10 days, showed a similar trend compared to the pilot trial, where the mortality of the WAA due to infection by *M. pinghaense* showed significant decline after 6 days, with ≈ 40% mortality on day 10. The results obtained give the expectation that, after application of the *M. pinghaense* to apple trees in the field, the fungus is likely to be able to induce mortality in WAA crawlers, as they move up the tree trunk to the aerial parts of the tree. These results are regarded as novel, as the persistence of completely dried conidia on apple bark has not been investigated before.

The current study has provided insight into the benefits of integrating the use of locally isolated EPF in biological management of insect pests, especially those, like the WAA, that have developed some level of resistance against chemical insecticides. The results obtained through the study provide guidelines as to determining which of the fungal isolates can be used in managing population outbreaks of WAA in apple orchards. Previous studies have also shown that the WAA is susceptible to infection, when it is exposed to some of the already commercialised EPF products developed from *B. bassiana* and *M. anisopliae* strains, such as Eco-Bb R444 and Meta 69. Therefore, the present

study also broadens the range of fungal isolates that can be made available to apple producers for the successful management of this particular pest species. The study is also important to farmers who are particularly interested in integrated pest management (IPM) as a way of managing insect pests on their cultivated crops, with it being even more so important for apple producers.

WAA biotypes have been well documented as having developed some level of resistance against a wide array of chemical insecticides, including Imidacloprid and Vamidothion, used for managing WAA across apple orchards. Therefore, apple farmers would be likely to find it highly beneficial to invest in the use of EPF for the management of the WAA in their farms as part of an IPM system, as the use of chemical control has proven to be unsuccessful. Applying local strains of EPF in apple orchards would also help reduce the use of chemical insecticides, as EPF offers an environmentally-friendly way of managing the insect pest.

The timing of applications of EPF in apple orchards is also crucial, depending on the seasons involved, because although the EPF isolates are well adapted to the local climatic conditions, their success as biological control agents is affected by a variety of environmental factors such as environmental temperature, solar radiation and relative humidity. The application of EPF isolates during the hot and dry seasons might affect the efficacy of the isolates concerned. For EPF isolates to be successful as biological control agents, it is well recorded that high levels of humidity and temperatures of approximately 25 ºC are required within their targeted environments. Relatively low levels of humidity due to the absence of rainfall and to the presence of high temperatures, will likely affect both the efficacy and the persistence of the fungal isolates under field conditions.

Future research, prior to the development of a biological control product using *M. pinghaense*, should focus on testing the efficacy of *M. pinghaense* isolates in apple orchards. The fungus can be applied directly to apple trees, either by means of conventional spraying equipment or by means of applying the conidial suspension to bands placed around the trees. The latter should serve to ensure the persistence of the fungus on the trees, and ensure the possible infection of the insect as it moves up the trees. Using such application methods might help to reduce the amount of damage that the insect not only causes to the root system of the apple trees, but also the amount of damage that is caused to the aerial parts of the trees. Doing so should also reduce contamination of the apple fruit, which is a major issue with the infestation caused by the WAA in apple orchards, as it results in fruit contamination problems during the export and import of the apple fruit.