Biological control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr), using entomopathogenic nematodes and fungi

by

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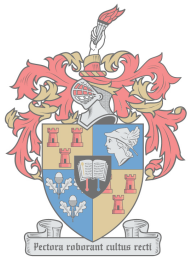

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Summary

The overall aim of this study was to establish guidelines, from research undertaken mainly in the laboratory, and from field application, for the use of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) to control the banded fruit weevil (BFW), *Phlyctinus callosus* (Schönherr), in vineyards and apple orchards in the Western Cape province. Discovering new species of entomopathogens is important, as more virulent, locally adapted species can be used to increase the potential of microbial control. The first objective of this study was to survey for EPNs and EPF strains/species in orchards and vineyards of the Western Cape, and to investigate their potential to control the BFW. The most virulent EPN species for the biological control of the BFW were selected in laboratory bioassays, and a field trial using *Steinernema yirgalemense* at different concentrations was conducted. In a further study, the best EPF strains for the biological control of the BFW were selected in laboratory bioassays. The most virulent EPN and EPF isolates were applied, in combination, to determine their interaction-effect on the mortality of BFW in the laboratory, as the final objective.

In total, 70 soil samples were collected from deciduous fruit orchards and vineyards in the Western Cape. The soil samples were baited with mealworms, *Tenebrio molitor* (Coleoptera: Tenebrionidae) to trap EPNs and EPF, which were characterised by using morphological and molecular techniques, and evaluated for their potential to control the BFW adults. EPNs were isolated from 17 % (12) of the samples, with *Heterorhabditis bacteriophora* Poinar and *Heterorhabditis safricana* as the only two EPN species isolated. *Heterorhabditis bacteriophora* (53 % mortality) resulted in significantly higher ($p < 0.05$) mortality of adult BFW, compared to *H. safricana* (37 % mortality), in laboratory trials. EPF were trapped from 37 % (26) of samples, consisting of a total of 14 *Beauveria bassiana* isolates and 12 isolates from the *Metarhizium anisopliae* complex. A *Metarhizium* isolate (79 % mortality) resulted in significantly higher ($p < 0.05$) mortality of BFW adults, compared to *B. bassiana* (63 % mortality) in laboratory trials. Results obtained from the study indicate that the soil samples from the deciduous fruit orchards and vineyards from the Western Cape contained both EPNs and EPF that can be used to control BFW.

Different EPN species were evaluated by laboratory screening for virulence against the different life stages of the BFW. A field trial to determine the performance of *Steinernema yirgalemense,* applied at different concentrations, followed. Results from a probit analysis showed *S. yirgalemense* to be six times more potent than *Heterorhabditis noenieputensis*, giving 95 %

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mortality of BFW larvae at a concentration of 400 infective juveniles (IJ) per insect, which was significantly higher ($p < 0.05$) compared to other concentrations. At a concentration of 100 IJs/insect, *Steinernema yirgalemense, H. noenieputensis*, and *Steinernema feltiae* resulted in significantly higher ($p < 0.05$) mortality of BFW larvae compared to the other EPNs, with no significant difference between each other. *Heterorhabditis indica* (70 % mortality) and *H. baujardi* (67 % mortality) resulted in significantly higher ($p < 0.05$) mortality of the BFW pupae, compared to *H. noenieputensis* (55 % mortality). In the case of adult BFW, *Heterorhabditis indica* (95 % mortality) and *S. yirgalemense* (94 % mortality) gave significantly ($p < 0.05$) higher mortality, compared to three other EPN species evaluated. In the field trials, *S. yirgalemense*, at 20 and 40 IJs/cm^2, gave 69 % and 78 % mortality of BFW larvae, respectively. The results showed that all EPNs screened controlled the juvenile and adults stages of BFW. *Steinernema yirgalemense* was also shown to be capable of controlling BFW under field conditions, even at low concentrations.

Different EPF isolates were tested at 1×10^6 conidia ml^{-1} for their virulence against the different life stages of the BFW. Results showed that Broadband® (*Beauveria bassiana* strain PPRI5339) (97 %) and Meta 69 (*Metarhizium anisopliae*) (93 %) gave significantly higher ($p < 0.05$) mortality of BFW larvae compared to Eco-Bb® (*B. bassiana*) (58 %), with no significant difference in mortality between Broadband® and Meta 69. In the case of pupae, Broadband® (92 %) gave significantly higher ($p < 0.05$) mortality, compared to Eco-Bb® (67.5 % mortality) and Meta 69 (65.8 % mortality). Broadband® (90 % mortality) gave significantly higher ($p < 0.05$) mortality of adult BFW compared to Eco-Bb® (69.2 % mortality) and Meta 69 (65 % mortality), of which neither differed from each other. When compared to a local EPF (*M. anisopliae* EA2), Broadband® (91.7 % mortality) gave significantly ($p < 0.05$) higher mortality of BFW adults, compared to Eco-Bb® (65 % mortality), Meta 69 (59.5 % mortality) and *M. anisopliae* isolate EA2 (64.2 % mortality), which did not differ significantly from one another. In a sand bioassay, Broadband® (85 % mortality) resulted in significantly ($p < 0.05$) higher mortality of BFW larvae compared to Eco-Bb® (55 % mortality) and Meta 69 (70 % mortality). The results indicated that all the EPF isolates tested were effective against juvenile and adults stages of the BFW, with Broadband® showing the most promise in controlling the BFW under laboratory conditions.

The last objective of the study was to evaluate the combined use of the EPF; Eco-Bb®, Broadband®, Meta 69 and a local isolate *M. anisopliae* EA2, with the EPN, *S. yirgalemense* against BFW larvae and adults. The EPF were either applied alone, or at the same time as *S. yirgalemense*, or *S. yirgalemense* was introduced 1 and 2 weeks after fungal application; the EPNs were also applied alone. Results showed that 100% larval and adult mortality was obtained when *S.*

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yirgalemense was applied 1 or 2 weeks after Eco-Bb® and BroadBand® application. Synergistic interactions were noted when Eco-Bb® and *S. yirgalemense*, BroadBand® and *S. yirgalemense*, Meta 69 and *S. yirgalemense*, and *M. anisopliae* isolate EA2 and *S. yirgalemense* when applied 1 or 2 weeks after application of the EPF. Additive interactions were observed when the EPF and *S. yirgalemense* were applied simultaneously.

Future research into the biological control of the BFW, using EPNs and EPF, should be focused on conducting large-scale field trials to demonstrate their potential use as biocontrol agents, within an integrated pest management programme. Both entomopathogens have shown outstanding potential to control the BFW when used alone, and in combination, could provide an economically viable control strategy against the BFW.

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Opsomming

Die doel van hierdie studie was om riglyne vas te stel, vanuit die resultate van navorsing in die laboratorium, asook veldproewe, vir die gebruik van entomopatogeniese nematodes (EPNs) en entomopatogeniese swamme (EPS) vir die beheer van die gebande vrugtekalander (GVK) in wingerde en appelboorde in die Wes-Kaap provinsie. Die ontdekking van nuwe spesies van entomopatogene is belangrik, omdat spesies wat plaaslik aangepas en effektief is vir gasheer spesies, gebruik kan word om die potensiaal van mikrobiese beheer te verhoog. Die eerste doel met hierdie studie was dus om 'n opname te doen van EPN en EPS in boorde en wingerde van die Wes-Kaap en om dan hul potensiaal om die GVK te beheer, te ondersoek. Biotoetse in die laboratorium het getoon dat *Steinernema yirgalemense* die effektiefste EPN spesie is vir die biologiese beheer van die GVK en is daarna gebruik vir veldproewe by verskillende konsentrasies. In 'n verdere studie is die beste EPS spesies vir die biologiese beheer van die GVK deur middel van biotoetse in die laboratorium geselekteer. As die laaste doel van die studie, is die effektiefste EPN en EPS isolate in kombinasie aangewend om die effek van hul interaksie te bepaal op die mortaliteit van die GVK in die laboratorium.

'n Totaal van sewentig grondmonsters is versamel vanuit sagtevrugte boorde en wingerde in die Wes-Kaap. Meelwurms, *Tenebrio molitor* (Coleoptera: Tenebrionidae), is gebruik as lok gashere en geplaas op die grondmonsters om EPN en EPS te isoleer. Hierdie spesies was dan gekarakteriseer deur gebruik te maak van morfologiese en molekulêre tegnieke en hul potensiaal vir die beheer van die volwassene GVK, geëvalueer. EPNs is geïsoleer vanuit 17 % (12) van die grondmonsters, met *Heterorhabditis bacteriophora* Poinar en *Heterorhabditis safricana Malan, Nguyen, De Waal & Tiedt* as die enigste twee EPN spesies wat gevind is. Gedurende proewe in die laboratorium, het *H. bacteriophora* (53 %) het aansienlik beter resultate gelewer ($p < 0.05$) in die mortaliteit van volwasse GVKs, in vergelyking met *H. safricana* (37 %). EPS is geïsoleer vanuit 37 % (26) van die grondmonsters en was geïdentifiseer as 14 *Beauveria bassiana* isolate en 12 isolate van die *Metarhizium anisopliae* kompleks. 'n *Metarhizium* isolaat (79 %) het aansienlik beter resultate getoon ($p < 0.05$) in mortaliteit van volwasse GVKs, in vergelyking met *B. bassiana* (63 %) in laboratorium proewe. Resultate van die studie toon dat die grondmonsters van die sagtevrugte boorde en wingerde in die Wes-Kaap beide EPNs en EPS bevat het wat gebruik kan word vir die beheer van grondlewende, asook nie-grondlewende fases van GVKs.

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Die effektiwiteit van verskillende EPN spesies is vergelyk deur hul vermoë om die verskillende lewensfases van die GVK te beheer, in die laboratorium te toets. Daarna is 'n veldproef uitgevoer om die sukses van *Steinernema yirgalemense* by verskillende konsentrasies te ondersoek. Resultate van 'n probitanalise het getoon dat *S. yirgalemense* ses keer meer effektief is as *Heterorhabditis noenieputensis*. *Steinernema yirgalemense* het 95% mortaliteit van GVK larwes getoon teen 'n konsentrasie van 400 infektiewe larwes (ILs)/insek, wat aansienlik hoër was ($p < 0.05$) as die vlak van beheer by ander konsentrasies. *Steinernema yirgalemense, H. noenieputensis*, en *Steinernema feltiae* het aansienlik beter gevaar ($p < 0.05$) in die mortaliteit van GVK larwes teen 'n konsentrasie van 100 ILs/insek wanneer hul vergelyk was met ander EPNs, met geen beduidende verskil tussen mekaar nie. *Heterorhabditis indica* (70 %) en *Heterorhabditis baujardi* (67 %) het aansienlik beter gevaar ($p < 0.05$) in die mortaliteit van GVK papies, in vergelyking met *H. noenieputensis* (55 %). *Heterorhabditis indica* (95 %) en *S. yirgalemense* (94 %) het aansienlik beter resultate getoon ($p < 0.05$) in die mortaliteit van volwassene GVKs, in vergelyking met die ander drie EPN spesies wat getoets was. In die veldproewe het *S. yirgalemense* 69% mortaliteit veroorsaak in GVK larwes teen 'n konsentrasie van 20 ILs/cm^2 en 78% mortaliteit teen 'n konsentrasie van 40 ILs/cm^2. Die resultate toon dat al die EPNs wat getoets was, die vermoë het om verskillende lewensfases van GVK te beheer. *Steinernema yirgalemense* het ook die vermoë getoon om die GVK te beheer in die veld, selfs teen lae konsentrasies.

Die vermoë van verskillende EPS isolate om die verskillende lewensfases van GVK te dood, was getoets teen 'n konsentrasie van 1×10^6 spore ml^{-1}. Resultate het getoon dat daar geen beduidende verskil was tussen die vermoë van Broadband® (*Beauveria bassiana* isolaat PPRI5339) (97 %) en Meta 69 (*Metarhizium anisopliae*) (93 %) om larwes van GVK te dood nie, maar dat die twee produkte wel aansienlik beter gevaar het ($p < 0.05$) as Eco-Bb® (*B. bassiana*) (58 %). Broadband® (92 %) het ook aansienlik beter gevaar ($p < 0.05$) as Eco-Bb® (67.5 %) en Meta 69 (65.8 %) in die mortaliteit van GVK papies. Daar was 'n beduidende verskil ($p < 0.05$) tussen Broadband® (90 %) se vermoë om volwasse GVKs te dood in vergelyking met Eco-Bb® (69.2 %) en Meta 69 (65 %), wat nie beduidend verskil het van mekaar nie. Toe die vermoë van die EPS om volwasse GVKs te dood vergelyk was met In plaaslike isolaat, was daar weereens 'n beduidende verskil ($p < 0.05$) tussen die resultate van Broadband® (91.7 %) in vergelyking met Eco-Bb® (65 %), Meta 69 (59.5 %) en *M. anisopliae* isolaat EA2 (64.2 %), wat nie beduidende verskille getoon het onder mekaar nie. In 'n sandbiotoets, was daar 'n beduidende verskil ($p < 0.05$) in die vermoë van Broadband® (85 %) om GVK larwes te dood, in vergelyking met die vermoëns van Eco-Bb® (55 %) en Meta 69 (70 %). Die resultate het getoon dat al die EPS isolate wat getoets

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was, effektief was teen die verskillende lewensfases van GVK, met Broadband® wat die meeste potensiaal getoon het vir mortaliteit van die GVK in die laboratorium.

Die laaste doel van die studie was om die gebruik van die Eco-Bb®, Broadband®, Meta 69 en *M. anisopliae* EA2, 'n plaaslike isolaat, gekombineer met *S. yirgalemense,* te toets teen die larwes en volwassenes van die GVK. Die EPS was of alleen, of saam met *S. yirgalemense* óf 1 en 2 weke na behandeling met swamme, aangewend. EPNs was ook op hul eie aangewend. Resultate het 100% mortaliteit van GVK larwes en volwassenes getoon in die behandelinge waar *S. yirgalemense* 1 of 2 weke na Eco-Bb® en BroadBand® aangewend is. Sinergistiese interaksies was opgemerk by behandelinge waar Eco-Bb® en EPN, BroadBand® en *S. yirgalemense*, Meta 69 en EPN, en *M. anisopliae* isolaat EA2 en *S. yirgalemense* aangewend is 1 of 2 weke na die aanwending van die *S. yirgalemense*. Bykomende interaksies is opgemerk toe die EPS en *S. yirgalemense* gesamentlik aangewend is.

Toekomstige navorsing op die biologiese beheer van die GVK deur die gebruik van EPNs en EPS, moet fokus op die uitvoer van grootskaalse veldproewe om hul potensiaal as biologiese beheermiddels te demonstreer, tesame met 'n geïntegreerde pes beheer program. Beide entomopatogene het uitmuntende potensiaal getoon om die GVK op hul eie te beheer en kan in kombinasie 'n ekonomies lewensvatbare beheerstrategie bied teen die GVK.

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Dedication

This dissertation is dedicated to my parents; Miriam Hlaleleni Ngwenya-Dlamini and Patrick Mgewu Dlamini who, despite all challenges, supported me all the way and encouraged me to give it all it takes to finish. To my wife, Nosisa T. Sibandze-Dlamini, and children, Ntsandvoyenkhosi S. Dlamini, Wenzokuhle P. Dlamini and Bongisisa U. Dlamini who were extremely affected in every way possible by this quest. Thank you. My love for you all can never be quantified.

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Biographical sketch	
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Education/Training

INSTITUTION	DEGREE	YEAR	FIELD OF STUDY
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Kasetsart University	Master of Science	2014	(Tropical Agriculture) in Entomology
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A. Research and Professional Experience

Professional Experience

- August 2013-Present: Lecturer, Agricultural Entomology, Crop Production Department, University of Swaziland
- July 2010-August 2014; Teaching Assistant, Crop Production Department, University of Swaziland
- April 2010-June 2010: Technician, Crop Production Department, University of Swaziland

B. Peer-reviewed Publications

1. DLAMINI, B.E., EARNSHAW, D.M, & MASARIRAMBI, M.T. 2012. Growth and Yield Response of Oyster Mushroom (*Pleurotus ostreatus*) Grown on Different Locally Available Substrates. Current Research. *Journal of Biological Sciences* **4** (5): 623–629.
2. EARNSHAW D M., DLAMINI, B.E. & MASARIRAMBI, M.T. 2012. Growth and Yield of Oyster Mushroom (*Pleurotus ostreatus*) Grown on Different Substrates Top Dressed with Varying Levels of Wheat Bran. *International Journal of Life Sciences* **1** (4): 111–117.
3. DLAMINI, B.E. & AMORNSAK, W. 2014. Effect of Host Age on Progeny Production of *Theocolax elegans* (Westwood) (Hymenoptera: Pteromalidae) Reared on *Sitophilus zeamais (*Motschulsky) (Coleoptera: Curculionidae). *Kasetsart Journal (Natural Science)* **48** (4): 1–11.
4. DLAMINI, B.E, MALAN, A.P. & ADDISON, P. 2018. Control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) using entomopathogenic nematodes. Austral Entomology (Submitted).

C. Research Projects and Funding

- Dlamini Bonginkhosi Edward

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- Completed: 2014
- Bio-pesticides produced, imported and exported by Swaziland
- This grant was supported by the United Nations Industrial Organization (UNIDO).

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Preface

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the *African Journal of Entomology*. Chapter three was submitted for publication in *Austral Entomology*.

Chapter 1	Literature review and project aims Potential biological control of the banded fruit weevil, *Phlyctinus callosus*, on deciduous fruit and grapevine, using entomopathogenic nematodes and fungi
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Chapter 1 Literature Review

Potential biological control of the banded fruit weevil, *Phlyctinus callosus*, on deciduous fruit and wine grapes, using entomopathogenic nematodes and fungi

1.1 INTRODUCTION

South Africa is among the leading producers and exporters of table grapes in the southern hemisphere, after Chile (De Villiers & Pringle 2007; Wettergreen *et al.* 2017). Major areas for table grape production include the Hex River Valley, the Berg River area and Olifants River area in the Western Cape, and the Lower Orange River area in the Northern Cape, Northern Provinces in Limpopo (SATI 2018). The above-mentioned areas include an estimated over 21 067hectares of table and wine grapes, which account for 34 % of the total area under deciduous fruit production in South Africa (SATI 2018). A total of 250 000 tons of table grapes were produced annually in South Africa, with total exports worth R4 billion per annum (Mogala 2014). The grape industry also employs up to 9 752 workers on a permanent basis and 49 501 seasonal workers, who have an estimated 49 433 dependants (SATI 2018).

Apples are important deciduous fruits that are grown in the Western Cape province (Pringle *et al*. 2015). Major areas for apple production include Ceres, Groenland, Villiersdorp (in the Western Cape), and Langkloof East (in the Eastern Cape) (Hortgro 2017). The total production area for apples is approximately 24 156 hectares (Hortgro 2017). South Africa was ranked 13th on the international competitiveness for apples in 2017, with Chile ranked the largest producer producing 31 % of its apples (Hortgro 2017). In terms of export, Chile and South Africa are the main exporters exporting 71 % collectively (Hortgro 2017). Apples were responsible for R2.9 billion of the total gross value of R8.37 billion value for deciduous fruits produced in South Africa (Hortgro 2017). The European Union and Russia were the largest export destination, responsible for buying 26 % of the fruits produced (Hortgro 2017). The apple industry also employs up to 27 033 workers, who have an estimated 108 131 dependants (Morokolo 2011). The apple industry is, therefore, important, when considering its potential to increase foreign exchange earnings, and to create employment and linkages with support institutions (Morokolo 2011).

A number of factors hamper the production of grapes and apples. The oversupply of fruits to established export markets, the increased inflation rate of labour, and the variability of currency are some major factors potentially limiting production (Morokolo 2011; Mogala 2014). The production of grapes and apples (at farm level) is negatively affected by climate, particularly in

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the Western Cape, where droughts are experienced, raising costs and irrigation water availability. In addition, hail, frost, and the presence of insect pests cause visual damage to the young grape bunches and apple fruits (De Villiers & Pringle 2007; Mogala 2014; Pringle *et al*. 2015). As a number of insect pests cause serious economic damage in the Western Cape, they are, therefore an economic challenge to farmers (Pringle *et al*. 2015). A number of important insect pests of grapes and apples are not only key pests in South Africa, but also cause economic losses in orchards and vineyards all over the world, including in Australia, California, Spain, Pakistan, and South America (Fisher & Learmonth 2003).

Insect damaged grapes and apples are usually unmarketable, because of the relatively low tolerance levels that the market have to cosmetic damage, particularly in the case of table grapes (Pringle *et al*. 2015; Allsopp *et al*. 2015). Many insect-infested fruit consignments are rejected prior to shipping, due to infestation and the presence of phytosanitary insect pests, or the visible symptoms that they cause (De Villiers & Pringle 2007). South Africa exports most of its fresh grapes to European and Asian markets, and relatively less to African markets (Mogala 2014). Most of the dried grapes are exported to several regions in the Americas. The presence of insect pests not only reduces the yield, but the depredations caused by phytosanitary insect pests also subject the country to risk from the loss of international markets (De Villiers & Pringle 2007). Although insect pests can be controlled by means of spraying with insecticide, doing so can present risks related to the residue levels permitted by the importing countries, the build-up of insect resistance to insecticides, and potential detriment to the environment (Chagnon *et al.* 2015).

Furthermore, demand patterns of the export market, particularly the European market, promote the use of environment-friendly pest management tactics (Chagnon *et al.* 2015). The country, therefore, risks losing its overseas markets, if the reliance on insecticides cannot be managed effectively. The need, therefore, exists to find alternative insect pest control tactics that will be effective in controlling the insect pests, while being environmentally friendly and resulting in low, or no, residues on grapes and apples.

The main aim of the current review was to consolidate the historical and currently available knowledge on *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), banded fruit weevil (BFW), and to provide information on the alternative control of BFW using bio-pesticides including entomopathogenic nematodes and fungi. The study emphasises the potential use of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF), and how the biologicals can be implemented in terms of an integrated pest management (IPM) system dedicated to the

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production of grapes and apples. Finally, the possibility of using such biologicals in combination, to obtain a synergistic effect for the control of BFW, is discussed.

1.2. BANDED FRUIT WEEVIL

1.2.1. Origin and distribution

The BFW, which is one of the major insect pests of grapes and apples, is indigenous to South Africa (Lounsbury 1896; Barnes 1989a;; Pringle *et al*. 2015; Allsopp *et al*. 2015). It is a phytosanitary pest, causing damage in many grape, apple and nectarine orchards, particularly in the Western Cape. The BFW was first reported as a major pest of grapes in New Zealand, from where it spread to all the other southern states in Australia in 1899, after it was recognised as a pest by Lounsbury (1896) in South Africa in 1896 (Kuschel 1972). The BFW is the only curculionid that is considered to be a major pest of grapevines and deciduous fruits in South Africa. Furthermore, the BFW has the potential to result in the causation of significant damage to several crops, as it has recently been found to be a serious pest of blueberries (Ferreira 2010; Pringle *et al*. 2015; Allsopp *et al*. 2015).

1.2.2. Description

BFW eggs are 0.9 mm long and oblong in shape; they turn black at the ends as they mature (Butcher, 1984). The larvae of BFW are c-shaped, soil-dwelling, and apodous, as well as having a brown head (Fig. 1.1A). The last-instar larvae, after finishing feeding, make smooth-sided earthen cells, in which the pre-pupal stage changes into a soft-bodied pupal stage (Fig. 1.1B). The BFW pupae are white, darkening as they mature; their eyes blacken, while the body becomes grey-brown, before adult emergence (Barnes 1989a). As the adult BFW have fused elytra, they are flightless. They are greyish-brown, with a bulbous abdomen, which has the characteristic pale-white V-stripe that is prominent at the end (Fig. 1.1C and D).

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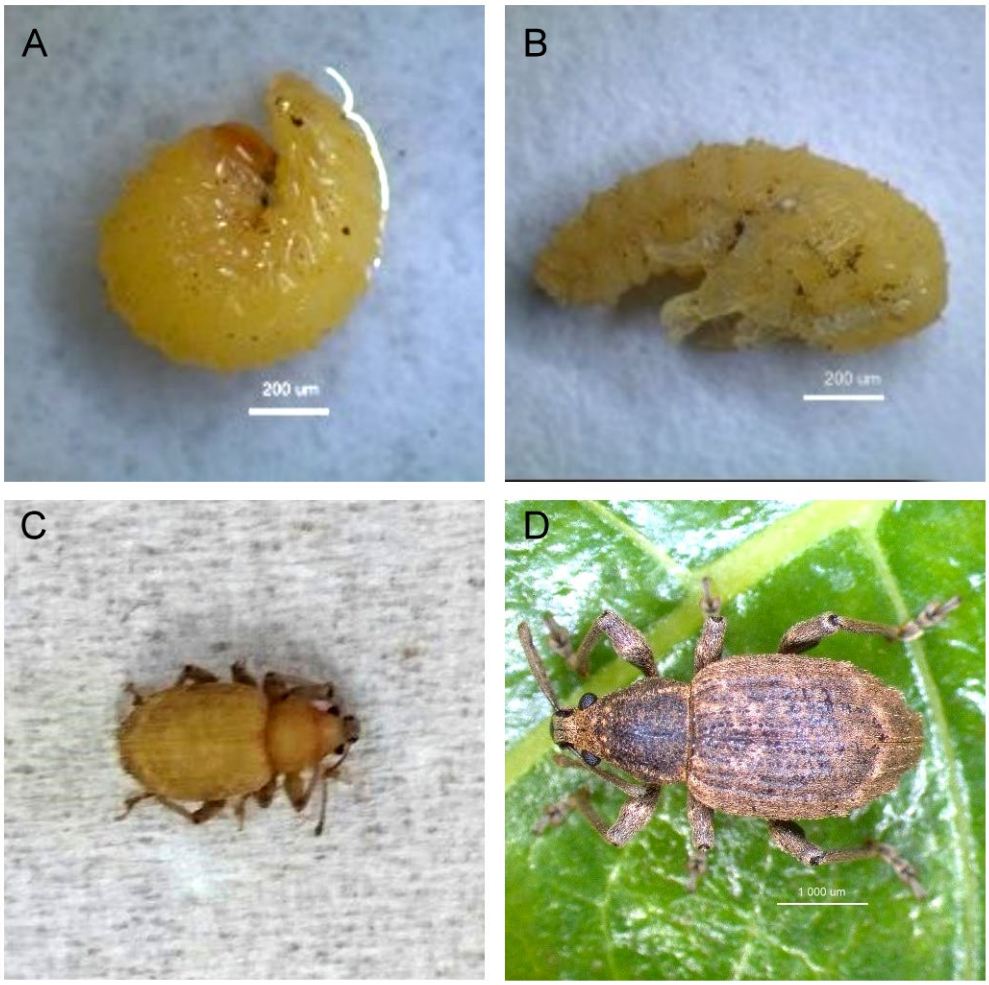

Fig. 1.1. Last-instar *Phlyctinus callosus* larvae (A), pupae (B), newly emerged adult (C), and adult, with the characteristic white band on the posterior part of the abdomen (D).

In vineyards and orchards, the adults reach the canopy by means of climbing on the tree trunks and supporting posts. The adults feed only at night and during the day, they shelter under the bark, as well as around the plant leaves and the main stem (Fisher & Learmonth 2003). The adults also hide under plant debris and in curled leaves, as well as in grape bunches and fruits. They tend to feign death (catalepsy), particularly when disturbed, and are likely to fall to the ground (Fisher & Learmonth 2003).

1.2.3. Life cycle

The BFW is indigenous to South Africa and adapted to the dry, hot summers and wet winters of the south-western parts of South Africa (Lounsbury 1896; Annecke & Moran 1982; Pringle *et al*. 2015; Allsopp *et al*. 2015). In South African apple orchards, the BFW can have one or two generations per annum, depending on the ground cover and the irrigation system used in orchards during the dry summer conditions (Barnes 1989a). However, in its natural habitat, away from the

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irrigated orchards or vineyards, the BFW tends to experience only one generation per year with the adult stages causing damage to a number of fruit crops (Barnes 1989a; Pringle *et al*. 2015).

The eggs of the BFW are laid in batches of 20 or more on or near the soil surface in or on loose debris, foliage and organic litter (Barnes & Pringle 1989; Pringle *et al*. 2015). The BFW's growth is promoted by the maintenance of high ambient temperature (Barnes *et al.* 1994). Oviposition occurs throughout the year, usually peaks from August to October (spring), December to January (summer), and March to May (autumn) in apple orchards (Barnes 1989a). From 6 to 15 days after hatching, the first-instar larvae burrow into the soil and feed on the roots of the plants (Barnes 1989a). Most larvae of the BFW undergo from 5 to 8 instars, but up to 11 instars can occur, depending on the prevailing weather conditions (Barnes 1989b; Pringle *et al*. 2015). The highest larval population normally occurs from June to October (second-generation), and again from January to April (first-generation) (Barnes 1989a; Pringle *et al*. 2015).

The pre-pupal stage constructs a chamber in the upper 100 mm of the soil, with the pupal stage lasting from 1 to 3 weeks. In South Africa, the BFW start emerging from the soil from October to December, in the case of the first generation, and, where the second generation is present, the adults start emerging from March to May (Barnes 1989b). In Australia fruit damage by the adult weevil becomes visible from December onwards, extending to February (Fisher & Learmonth 2003). The adults can be controlled by means of trunk barriers, so as to prevent the weevils from reaching the fruit (Fisher & Learmonth 2003; Ferreira & Malan 2014; Pringle *et al*. 2015; Allsopp *et al*. 2015).

1.2.4. Damage

The feeding behaviour of the BFW was reported to result in up to R4 million damage annually 40 years ago (Barnes & Pringle 1989), with no recent estimates of this cost available. The damage to the fruit, which is caused by the adult weevils, are reported in relation to the grape berries and fruits of nectarines, apples, and other ornamental plants (Barnes 1989a, b; Barnes & Pringle 1989; Prinsloo & Uys 2015). The adults cause fruit damage by means of feeding on the skin and the underlying flesh, which results in the formation of shallow lesions on the damaged fruit (Fig. 1. 2C and D) (Barnes 1989a; Fisher & Learmonth 2003; Ferreira & Malan 2014; Pringle *et al*. 2015; Allsopp *et al*. 2015).

Other damage that is caused by the BFW adults includes foliage damage (Fig. 1. 2B and F), ringbarked stems (Fig. 1. 2A and E), due to feeding activity (Barnes & Giliomee 1992; Fisher &

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Learmonth 2003; Pringle *et al*. 2015; Allsopp *et al*. 2015). Allsopp *et al*. (2015) report that the adult weevil's damage to grapevines includes the shot-hole of leaves (Fig. 1. 2B and F) and leaf and stem notching, bud and shot-tip feeding, and scarring of the berry. The adults can feed on small green berries, causing them to drop prematurely (Allsopp *et al*. 2015).

Fig. 1.2. Adult damage to leaves and fruits; stem notching of grapes (A), shot-holing of apple leaves (B); the fruit scarring of (C) apples and (D) grapes; stem notching of apples (E) and shot-holing of grape leaves (F).

In apple orchards, the BFW larvae feed on the roots, but damage by the adults is of economic importance (Pringle *et al*. 2015; Allsopp *et al*. 2015). Just as with grape production, the adults feed on the leaves, stems, and fruits. Adult damage includes leaf and stem notching, buds and shoot-tip feeding, and scarring of the fruit (Pringle *et al*. 2015; Allsopp *et al*. 2015) (Fig. 2C and D). Large populations of BFW can completely defoliate young fruit trees (Barnes 1989a, b). The scarring of the fruit results in unmarketable fruits and the weevil may also be inadvertently packed in with export fruits (De Villiers & Pringle 2007; Pringle *et al*. 2015; Allsopp *et al*. 2015).

On grapevines, larvae of BFW, in contrast, feed on the roots, resulting in stunted growth, and can appear water-stressed particularly in the young vines (Barnes 1989a). Root feeding in mature vines is, however, not damaging to the vines. However, it is important to control the larvae involved, because the ease with which larvae are found in the soil is a good indication of

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subsequent adult abundance and consequently the severity of damage by adults later in the season. (Fisher & Learmonth 2003). The BFW was reported by Barnes (1989a) to be causing sporadic damage to other deciduous fruits, like pears, plums, and peaches.

1.2.5. Host plants

The BFW, which is a polyphagous pest, can feed on a number of plant groups, like grasses, weeds, herbs, and woody plants (Pringle *et al*. 2015; Allsopp *et al*. 2015). The type of host varies in the different countries in which the BFW has been recorded. In South Africa, the larvae can be found actively feeding on the roots of vines and on host weed species in the late winter / early spring (Pringle *et al*. 2015; Allsopp *et al*. 2015). The larvae feed on the roots of a number of plants, while a few feed on between rows, hence the presence or absence of weeds in an orchard or vineyard influences the distribution and abundance of the BFW larvae.

The adults can feed on the stems and leaves of a number of weeds and grasses (Barnes 1989a,b; Barnes & Pringle 1989). In South Africa, deciduous fruit and grapevine damage is caused on the leaves and fruit, including the fruit stalks and shoots. The adults also prefer to eat broad-leaved plants, particularly those with fleshy leaves (Pringle *et al*. 2015; Allsopp *et al*. 2015).

1.3. BFW MANAGEMENT STRATEGIES

The use of trunk barriers is effective in preventing the BFW from reaching the fruits, but such use is labour-intensive, particularly in large orchards and vineyards (Barnes *et al.* 1994; Fisher & Learmonth 2003). The use of synthetic chemicals like pyrethroids, in contrast, is no longer effective, because administering only low concentrations of bifenthrin (pyrethroid) allows some larvae to survive in certain parts of the plants (Cowles 2003). Such was reported in the case of the larval stages of the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), which is a close relative of the BFW. The BFW can develop resistance to pyrethroids, with the products being incompatible with the integrated pest management (IPM) programmes that are used in the orchards and vineyards in the Western Cape (Chagnon *et al.* 2015; Pringle *et al*. 2015; Allsopp *et al*. 2015). In Australia, Fisher and Learmonth (2003) report that the foliar application of esfenvalerate to control the BFW results in secondary pest outbreaks, such as those of the long-tailed mealybug, the grape leaf rust mite, and the grapevine scale.

The black vine weevil can be controlled by means of combining biocontrol agents (Ansari *et al.* 2008). Pringle *et al*. (2015) report that the curculionid has a number of natural enemies, including protozoans, entomopathogenic nematodes (EPNs) and fungi (EPF), parasitoids, and

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predators. The natural enemies recorded for BFW in the Western Cape are the mymarid egg parasitoid, *Cleruchus* sp. (Hymenoptera: Mymaridae); the braconid wasp, *Perilitus* sp. (Hymenoptera: Braconidae); nematode *Heterorhabditis* spp. (Rhabditida: Heterorhabditidae), and one *Steinernema* sp. (Rhabditida: Steinernematidae); mites, *Leptus* sp. (Trombidiforme: Erythraeidae); and guinea fowl (Fisher & Learmonth 2003; Barnes 2014; Ferreira & Malan 2014; Pringle *et al*. 2015).

The following three sections of the current paper synthesises details of entomopathogens as biological control agents, the focus of the current research.

1.4. ENTOMOPATHOGENIC NEMATODES

1.4.1. General taxonomy

EPNs, which are classified in the order Rhabditida, consist of two families, namely Steinernematidae and Heterorhabditidae (Hatting *et al.* 2018). The two genera, *Steinernema* and *Heterorhabditis*, are of paramount importance (Smart 1995). Feeding on bacteria and being the parasites of insects (Smart 1995; Hatting *et al.* 2018), EPNs are obligate parasites of insects that feed on, multiply, and kill their insect hosts with the help of an associated bacteria that is carried in the nematode's alimentary canal (Shapiro-Ilan & Gaugler 2002; Shapiro-Ilan *et al.* 2003a). The nematodes usually have a mutualistic relationship with the bacteria (Shapiro-Ilan & Gaugler 2002) found in steinernematids and heterorhabditids, which belong to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Kaya & Gaugler 1993; Shapiro-Ilan *et al.* 2003a; Hatting *et al.* 2018).

The bacteria serve as a source of nutrients for the nematodes, secreting antibiotics that inhibit any other competing microbes, and killing the insect host through the production of toxins, which leads to septicaemia (Akhurst & Boemare 1990; Shapiro-Ilan & Gaugler 2002). The most important role of nematodes in terms of the mutualistic relationship described is to act as the bacterial vector, but they also contribute to the host insect's death by suppressing its immune system, and by producing toxins (Akhurst & Boemare 1990). However, the bacteria concerned cannot survive in nature in the absence of the nematodes, and they are not pathogenic when ingested by the host insect (Akhurst & Boemare 1990; Shapiro-Ilan & Gaugler 2002).

1.4.2. Biology and life cycle

EPNs have three main stages: the egg stage, the four juvenile stages and an adult stage (Miles *et al.* 2012). The infective juvenile (IJ) is a special third larval stage, which is non-feeding, and

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which has arrested development. It is the only stage found outside of the insect hosts (Poinar 1990; Ciche 2007; Miles *et al.* 2012). The IJs enter the host through the latter's natural openings, including the mouth, anus and spiracles, and occasionally through the cuticle (Shapiro-Ilan *et al.* 2003a). EPNs usually complete up to three generations within the host, leading to the production of a new cohort of IJs that usually leaves the food-depleted host to seek new hosts (Poinar 1990; Smart 1995; Shapiro-Ilan *et al.* 2003a, b). The IJs, which range between 0.5 and 1.5 mm long, are free-living in the soil. They contain bacteria, and locate insect hosts (by means of ambushing or cruising), attack, and infect their insect hosts (Poinar 1990; Ciche 2007; Miles *et al.* 2012). Both the steinernematids and heterorhabditids usually take from between 6 to 14 days to complete their life cycle in an insect host, provided that the prevailing conditions are favourable (Miles *et al.* 2012). IJs usually emerge in from 6 to 11 days and from 12 to 14 days from the insect host's cadaver for the steinernematids and heterorhabditids, respectively, depending on the size of the insect concerned (Kaya & Koppenhöfer 1999; Miles *et al.* 2012).

1.4.3. Biotic and abiotic factors

Although IJs do not feed, they can live for weeks as active juveniles, and for months feeding on stored fat reserves and by means of entering a partially anhydrobiotic state (Smart 1995). Existence in either one of the two states is the most important survival strategy used by EPNs. The factors affecting the length of time that IJs can survive in the soil without the availability of a host are: temperature; humidity; natural enemies; and soil type.

However, EPNs, as biocontrol agents, can give poor results when they are not properly handled, transported, and stored (Shapiro-Ilan *et al.* 2002; Tofangsazi *et al.* 2015). Just like with any living organism, EPNs are affected by both biotic and abiotic factors that can be detrimental to the nematodes involved both before and during application (Tofangsazi *et al.* 2015). The nematodes highly favour sandy soils with a pH ranging from 4 to 8. Their activity is highly limited by freezing, high temperatures, exposure to ultraviolet (UV) light, and desiccation (Shapiro-Ilan *et al.* 2003a; Tofangsazi *et al.* 2015). EPNs, just like with any other living organisms, have natural enemies. The populations of EPNs in the environment are adversely reduced by the presence of nematophagous fungi, tardigrades, bacteria, predatory nematodes, and mites, to mention but a few of their natural predators (Kaya 1990; Smart 1995).

Further, EPNs can be applied to the soil using conventional spraying equipment, either through the irrigation system or above-ground (Smart 1995). Successful biological control with EPNs is mostly promoted by the nematode's possession of various beneficial traits (Gaugler 1987; Shapiro-

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Ilan *et al.* 2003b). The most crucial beneficial traits include virulence, reproductive potential, and environmental tolerance (Shapiro-Ilan *et al.* 2003b).

Furthermore, maximising the benefits to be gained from the use of nematodes can be achieved by following certain precautions. The safeguards include: matching the most suitable species to control the target insect pests; ensuring that the correct dosage of a viable nematode product is used; ensuring that the treated area is kept wet for at least 8 hours after application; and spraying the nematodes during early morning or evening hours, so as to reduce the amount of exposure to UV lights, and the effects of dry, unfavourable conditions (Tofangsazi *et al.* 2015). Inspecting EPNs to see whether they are viable can be done by means of observing the sinusoidal movement of the IJ stages concerned, using a 20× magnification hand lens or microscope (Tofangsazi *et al.* 2015).

1.4.4. Application

The use of EPNs has a number of advantages over the use of chemicals as an insect control strategy. The employment of EPNs is environmentally safe when compared to that of chemicals, which are pollutants. However, the application of non-indigenous species for masking local EPNs and the biological pollution of exotic organisms can be restricted (Georgis 1990; Smart 1995). In addition to application using conventional spraying equipment, EPNs can also be mixed with most of the pesticides that are available on the market to obtain enhanced results (Dutky 1974; Forschler *et al.* 1990; Rovesti & Deseo 1990, 1991). EPNs, can find their insect hosts even in cryptic habitats and in the soil, specifically targeting them (Georgis 1990; Smart 1995). However, as such nematodes are easily affected by desiccation and UV radiation, successfully applying them to the leaves (*i.e.* foliar application) is difficult. EPNs tend to reproduce in the cadaver of an insect host, resulting in the new IJs actively searching for additional insect hosts (Georgis 1990; Smart 1995).

EPNs have been reported to be efficient in controlling a number of economically important insect pests, including the larvae of a number of weevil species, like the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), and the larvae of the diaprepes citrus root weevil, *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae) (Table 1.1) (Shapiro-Ilan *et al.* 2002; Shapiro-Ilan *et al.* 2003b). A number of insect pests from different habitats, particularly soil-dwelling and insect pests in cryptic habitats, have been successfully controlled using EPNs. In such habitats, IJs tend to thrive easily, because they are protected from environmental factors (Table 1.1).

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Table 1.1 Entomopathogenic nematodes (EPNs) in the genus *Heterorhabditis* and *Steinernema* successfully used for the control of weevils (Shapiro-Ilan & Gaugler 2010; Ferreira & Malan 2014).

Crop(s)	Weevil	Effective EPNs	Reference
Berries, ornamentals	*Otiorhynchus sulcatus*	Hb, Hd, Hm, Hmeg, Sc, Sg	Shapiro-Ilan & Gaugler 2010
Citrus, ornamentals	*Pachnaeus* spp.	Sr, Hb	Shapiro-Ilan & Gaugler 2010
Citrus, ornamentals	*Diaprepes abbreviatus*	Hb, Sr	Shapiro-Ilan & Gaugler 2010
Forest plantings	*Hylobius albietis*	Hd, Sc	Shapiro-Ilan & Gaugler 2010
Nut and fruit trees	*Amyelois transitella*	Sc	Shapiro-Ilan & Gaugler 2010
Berries	*Otiorhynchus ovatus*	Hm	Shapiro-Ilan & Gaugler 2010
Sweet potato	*Cylas formicarius*	Hb, Sc, Sf	Shapiro-Ilan & Gaugler 2010
Laboratory assay	*Phlyctinus callosus*	Hz, Hb	Ferreira & Malan 2013

Hb = *Heterorhabditis bacteriophora*; Hm = *H. marelatus*; Hmeg = *H. megidis*; Hz = *H. zealandica*, Sc = *Steinernema carpocapsae*; Sf = *S. feltiae*; Sg = *S. glaseri*; Sr = *S. ribobravis*.

1.4.5. Previous studies on the use of EPNs to control the BFW

In a study by Ferreira and Malan (2013), EPNs were screened for their potential to control the BFW. Final larvae and adults were tested for their susceptibility against 400 IJs/per insect after 4 days in 24-well bioassay trays. All the EPNs used in the study were able to control the BFW final instar larvae and adults, with the final instar larvae found to be more susceptible compared to adult BFWs. *Heterorhabditis zealandica* was found to be the most effective isolate and was used to study the impact of vertical movement of the EPN in sand and sandy loam soil. Sandy loam soil gave a higher percentage mortality compared to sand soils. Ferreira and Malan (2013) concluded that BFW final instar larvae were not susceptible to EPNs as high concentration of up to 400 IJ/larva for 4 days were needed to give effective control.

1.5. ENTOMOPATHOGENIC FUNGI

EPF can infest an extensive range of economic insect pests, including lepidopterous larvae, aphids, beetles, and thrips (Shahid *et al.* 2012). Fungi, contrary to EPNs, are parasites that kill insect pests leading to disease symptoms in the host insects. EPF are important insect pathogens that significantly reduce the number of host insects and arthropod populations (Shahid *et al.* 2012). Although the absolute parasites live in association with the host insect, they benefit at its expense (Shahid *et al.* 2012). The causation of lethal infections by EPF serves as an ecosystem function, in terms of regulating the population of insects and mites by means of epizootics. In addition, EPF

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are host-specific, with the risk of attacking such non-target organisms as beneficial insects being low.

1.5.1. General taxonomy

Approximately 750 species of EPF from 85 different genera are currently known (Shahid *et al.* 2012). The effects of conventional chemicals on the residues of food crops, on groundwater contamination, on non-target organisms, and on insect resistance to synthetic chemicals, have resulted in the development of alternative control measures, of which some include EPF (Gillespie *et al.* 1988; Shahid *et al.* 2012). EPF are good biocontrol agents, because they act on contact, and they do not require ingestation, as well as having the ability to be easily mass-produced and formulated (Shahid *et al.* 2012).

EPF are grouped into the following divisions: Zygomycota; Ascomycota and Deuteromycota; and Chytridiomycota and Oomycota (Shahid *et al.* 2012). Deshpande (1999) reports that most of the fungal groups containing EPF include such genera as *Metarhizium*, *Beauveria*, *Verticillium*, *Nomuraea*, *Entomophthora*, and *Neozygites*. Notably, however, fungi can infect other soil arthropod species that are not the insect pests of cultivated crops (Zimmermann 1993; Shahid *et al.* 2012). EPF, which are reported to be infective to all the life stage of insect hosts, are found in almost all habitats.

A major EPF that is present in soils throughout the world is *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae). The fungus, which was first used as a biological control agent in the 1880s (Shahid *et al.* 2012), can infect and successfully control four insect types, namely beetles, termites, spittlebugs, and locusts (Zimmermann 1993; Shahid *et al.* 2012). A number of spores, or mycelia formulations, of *M. anisopliae* can be applied. After a fungal infection is achieved, the spores and vegetative cells produced from the infected insect tend to spread to other populations of insects (Zimmermann 1993; Shahid *et al.* 2012).

1.5.2. Fungal infection

The difference between EPF and other insect pathogens is that the former infect their insect hosts principally through their external cuticle (Inglis *et al.* 2001). When the EPF infect an insect host, they undergo four steps: adhesion; germination; differentiation; and, penetration (Shahid *et al.* 2012) (Fig. 1.3). Most taxa of EPF produce conidia that strongly adhere to insect cuticles. Such

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adherence occurs because of non-specific adhesion mechanisms that are brought about by the fact that the conidial cell wall is hydrophobic (Inglis *et al.* 2001; Shahid *et al.* 2012).

A wide range of biotic and abiotic factors influences each step of infection, and eventually determines the virulence and pathogenicity of the fungi involved. The successful infection of the host insect is achieved by means of the attachment or the adhesion of the spores (Shahid *et al.* 2012). The virulence of EPF is recognised first by the ability of the fungi to adhere, or to attach, to an insect body. The failure of a fungus to adhere to the epidermis of an insect host is considered when seeking out the features of virulent strains (Shahid *et al.* 2012).

The next factor to look for, after adhesion, when considering the virulence of EPF strains, is which enzymes serve to hydrolyse the epidermis of an insect host (Smith *et al.* 1981; Gillespie *et al.* 1988; Shahid *et al.* 2012). The enzymes that are usually secreted by EPF are lipases, proteases, and chitinases, which are produced sequentially and which reflect the order of the substrates that they encounter (Smith *et al.* 1981; Shahid *et al.* 2012).

Germination of the spores, is affected by a number of factors, including water, ions, fatty acids, and nutrients on the cuticle surface, as well as the physiological state of the insect host (Gillespie *et al.* 1988; Shahid *et al.* 2012). The germination of spores is usually successful when the utilisable nutrients can be assimilated, and when the spores can tolerate the presence of toxic compounds on the surface (Shahid *et al.* 2012). After germination, structures ranging from clavate or spherical, to slightly swollen terminal hyphae (appresoria), appear at the end of the short germ tubes (Gillespie *et al.* 1988) (Fig. 1.3).Penetration of the cuticle follows the germ tube, or an appressorium attaches to the cuticle, giving rise to a penetration peg (Shahid *et al.* 2012). Most EPF penetrate directly, but they rarely penetrate via wounds, sense organs, or spiracles (Gillespie *et al.* 1988; Shahid *et al.* 2012).

The EPF grow single or multicelled hyphal bodies once they reach the haemocoel (Inglis *et al.* 2001) (Fig. 3). Although the hyphal bodies lack a cell wall, their plasma membrane contains a thin fibrillar layer, and they are called blastospores. However, before the hyphal bodies can multiply in the haemocoel, the insect's defence response must be defeated. The above is achieved by means of producing toxins that weaken the efficacy of the insect defence response (Inglis *et al.* 2001). Insects usually respond to fungal infection by way of using humoral and/or cellular mechanisms (Bidochka *et al.* 1997; Boucias & Pendland 1998; Inglis *et al.* 2001).

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However, the insect defence response is unable to stop the hyphal bodies of a number of EPF species multiplying. *Nomuraea rileyi* (Farl.) Samson (Hypocreales: Clavicipitaceae) hyphal bodies, for example, are not phagocytosed by haemocytes (Boucias & Pendland 1998; Inglis *et al.* 2001; Sandhu *et al.* 2012). The above is because they cannot be recognised by humoral lectins, either due to them lacking specific surface residues, or because they copy the surface epitopes onto the insect haemocytes (Boucias & Pendland 1998; Inglis *et al.* 2001). The host insect dies because of a number of factors, including nutrient reduction, toxicosis, and the physical obstruction, or invasion, of the host insect organs (Inglis *et al.* 2001; Sandhu *et al.* 2012). Examples of toxic compounds produced by EPF include beauvericin, bassianolide, and oosporein produced by *Beauveria bassiana* (Balsamo.-Criv.) Vuillemin, and destruxins produced by *M. anisopliae* (Inglis *et al.* 2001). The toxins normally induce paralysis and suppress the immune system of the host insects concerned (Cerenius *et al.* 1990; Dumas *et al.* 1996; Inglis *et al.* 2001; Sandhu *et al.* 2012b).

The EPF continue growing on the insect host cadaver, releasing metabolites excluding competing microorganisms. The hyphae emerge from the cadaver by means of producing conidiogenous cells that sporulate on the host surface (Inglis *et al.* 2001). The EPF spread through hydrophobic conidia that are passively spread from the infected dead bodies (Shahid *et al.* 2012). Due to hydrostatic pressure, the conidia are actively discharged, and then carried on the wind, or by co-occurring insects (Hemmati *et al.* 2001; Roy *et al.* 2001; Shahid *et al.* 2012). If the primary conidia fail to find a suitable insect host on which to germinate, another form of higher-order conidia is discharged, or it results in the infective passively held secondary conidia with long stalks. In some EPF, the conidia are discharged while the host is still alive (Shahid *et al.* 2012).

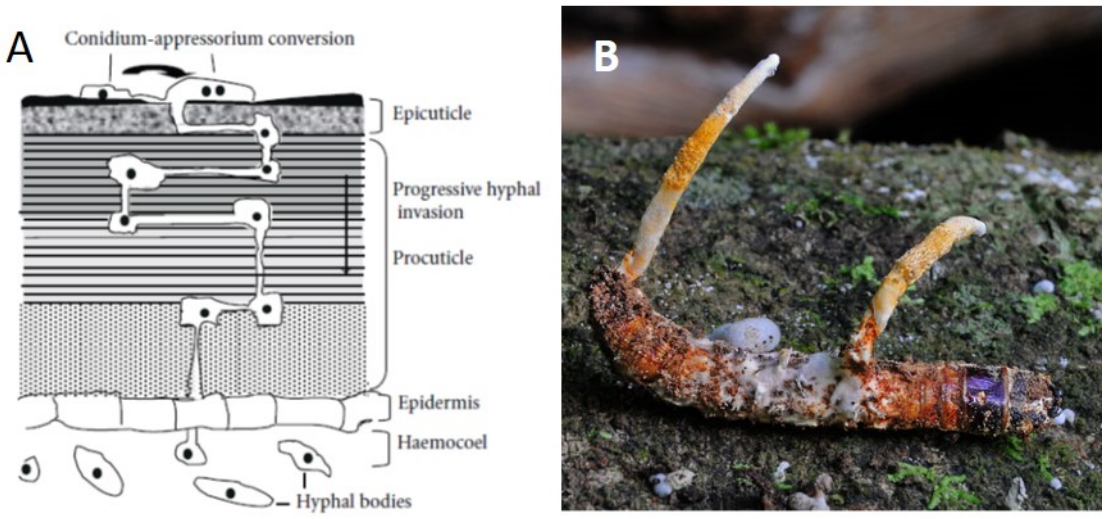

Fig. 1.3. (A) Schematic diagram showing the infection process of an EPF invading the host insect's cuticle, (B) the EPF fruiting body growing out of the thorax of an unidentified coleopteran larva. Photo credits: (A) Sandhu *et al.* 2012. (B) MycoImage.

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1.5.3. Biological control agents

A number of international companies produce biopesticides based on EPF, with the products concerned being used in a number of crops, including coffee, maize, bean, potato, cabbage, and tomato (Table 1.2) (Sandhu *et al.* 2012). In addition, EPF are successful in controlling the pests that are of medical and veterinary importance, including such haematophagous insect pests as mosquitoes and flies (Florez 2002; Sandhu *et al.* 2012). Biopesticides are not only environmentally friendly, but they also have a number of benefits, compared to chemical pesticides. One benefit is that pest resistance to biopesticides is unlikely to develop, as such pesticides have a complex mode of action (Sandhu *et al.* 2012). However, the integration of natural enemies with other control strategies can result in the long-term control of insect pests. The most important EPF biocontrol agents are discussed below.

1.5.3.1 *Beauveria bassiana*

The *Beauveria* sp. are filamentous EPF belonging to the class Deuteromycete (Shahid *et al.* 2012). *Beauveria bassiana* occurs naturally throughout the world in soils, with it controlling the population of a number of insect species by means of causing white muscardine disease (Sandhu & Vikrant 2004; Thakur *et al.* 2005; Shahid *et al.* 2012). *Beauveria bassiana* is an asexually reproducing form (*i.e.* an anamorph) of *Cordyceps bassiana*. The sexually reproducing form (*i.e.* the teleomorph) has only, so far, been collected in Asia (Li *et al.* 2001). The EPF has a high level of persistence in the environment, which can provide the long-term suppression of the insect host population, particularly if an epizootic is caused (Hamlen 1979; Shahid *et al.* 2012).

Several *Beauveria* strains are highly host-specific, with a number of them having been isolated, using a number of insect pests (Shahid *et al.* 2012). The fungi are genetically different variants that are associated with a geographical location and host. The spores of *B. bassiana* are sprayed on the affected crops in the form of a wettable powder, or an emulsified suspension (Shahid *et al.* 2012).

Unlike most EPF, the white muscardine disease, which is a parasite of a number of arthropod hosts, is non-selective (Thakur & Sandhu 2010; Shahid *et al.* 2012). The EPF can control insect pests of combined medicinal, agricultural, and forest importance. The insect hosts of medicinal importance include, but are not limited to, the vectors of tropical infectious diseases, such as tsetse fly, *Glossina morsitans* (Diptera: Glossinidae), and sand fly *Phlebotomus* spp. (Diptera: Psychodidae). Agricultural hosts include, but are not limited to: the codling moth, *Cydia*

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pomonella (Lepidoptera: Tortricidae); the false codling moth (Lepidoptera: *Thaumatotibia leucotreta*); the Colorado potato beetle (Coleoptera: Chrysomelidae); and the African bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Thakur & Sandhu 2010).

Of the 18 known *Beauveria* species, only four occur in South Africa, including *B. bassiana*, *B. brongniartii*, *B. caledonica*, and *B. pseudobassiana* (Rong & Grobbelaar 1998; Morar-Bhana *et al.* 2011; Goble *et al.* 2012; Abaajeh 2014; De Beer *et al.* 2017; Hatting *et al.* 2018). A total of five *B. bassiana* containing products are registered in South Africa. The five consist of: BB Plus WP®, which is registered to control aphids and spider mites; BB Weevil EC®, which is registered to control curculionids; Beauvitech® WP, which is registered against whitefly; Broadband®, which is registered against red spider mite, diamond-back moth, potato tuber moth, whitefly, thrips, stinkbug, false codling moth, and red scale; and Eco-Bb®, which is registered against red spider mite, whitefly, fall army, worm, false codling moth, and tomato leaf miner (Zimmermann 2007b; Hatting *et al.* 2018).

1.5.3.2. *Lecanicillium lecanii*

Lecanicillium lecanii is distributed in the tropical and subtropical regions, in the warm and humid environments of the world, where it causes epizootic diseases (Table 2) (Nunez *et al.* 2008; Shahid *et al.* 2012). The EPF attacks nymphs and adults of the greenhouse whitefly, *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae), and several aphid species, by means of adhering to the underside of leaves, using its filamentous mycelia (Nunez *et al.* 2008; Shahid *et al.* 2012).

1.5.3.3. *Metarhizium* complex

Metarhizium anisopliae, which is a major EPF that is present in soils throughout the world (Hatting *et al.* 2018), was first used as a biological control agent in the 1880s (Shahid *et al.* 2012). Although some isolates of the fungi have restricted host ranges, the group is mainly known for its ability to kill a wide range of insects, from seven different insect Orders. The sign of insect death that is caused by *Metarhizium* is the green muscardine that appears on the insect cadaver. *Metarhizium anisopliae* can infect and successfully control beetles, termites, spittlebugs, and locusts (Zimmermann 1993; Shahid *et al.* 2012). Several spore, or mycelia, formulations of *M. anisopliae* are available. After a fungal infection is achieved, the spores and vegetative cells produced from the infected insect spread to the other populations of the insects (Zimmermann 1993; Shahid *et al.* 2012).

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In South Africa, the fungus, *Metarhizium acridum* strain IMI 330189, was registered as part of the LUBILOSA (Lutte Biologique contre les Locustes et Sauteriaux) project (Lomer *et al.* 2001) against locusts and grasshoppers (Bateman *et al.* 1994; Price *et al.* 1997), leading to the registration of Green Muscle® in 1998 (Müller 2000; Hatting *et al.* 2018). A number of species are categorised under the *M. anisopliae sensu lato* species complex, but members of the 'PARB' clade, including *Metarhizium pingshaense*, *M. anisopliae*, *M. robertsii*, and *M. brunneum*, are important as EPF (Bischoff *et al.* 2009; Rehner & Kepler 2017; Hatting *et al.* 2018). *Metarhizium anisopliae* and *M. brunneum* have successfully been commercialised in a number of countries, with *M. anisopliae*, isolate ICIPE69, being registered against thrips, whiteflies, and snout beetles in South Africa (De Faria & Wraight 2007; Zimmermann 2007a; Ekesi *et al.* 2011; Rehner & Kepler 2017; Hatting *et al.* 2018).

Metarhizium rileyi, previously known as *Nomuraea rileyi* (Kepler *et al.* 2014), is a dimorphic hyphomycete that causes deaths in a number of insects, particularly in those from the Orders Lepidoptera and (some) Coleoptera (Ignoffo 1981). *Metarhizium rileyi* has not yet been commercialised in South Africa, but local research into *M. rileyi* has shown that it is an important EPF of noctuid pests (Hatting 2012; Fronza *et al.* 2017). As the EPF concerned is host-specific and environment-friendly, its use as an EPF is encouraged (Rajak *et al.* 1991; Mathew et *al.* 1998). The fungi have been known to control a number of insect pests, including cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae); corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae); *Plathypena scabra* (Lepidoptera: Noctuidae); and velvetbean caterpillar moth, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (Mathew *et al.* 1998).

1.5.3.4. *Paecilomyces* sp.

As *Paecilomyces* is a genus of fungi that parasites harmful nematodes, they are known as being nematophagous. When applied to soils, they can act as a bionematicide for the control of nematodes (Shahid *et al.* 2012). That root-knot and cyst nematodes can be controlled by means of *Paecilomyces lilacinus*. The fungus can also control *Bemisia* and *Trialeurodes* spp. in the greenhouse and field conditions (Nunez *et al.* 2008). Yellow muscardine is caused by the presence of *Paecilomyces fumosoroseus* (Wize) on whiteflies. As the EPF tends to grow rapidly on the leaf surface under humid conditions, it can easily spread and control whitefly populations (Wraight *et al.* 2000; De Faria & Wraight 2001). The EPF, however, cannot be relied upon for control, because only specific species can cause high mortality, with the natural epizootics depend on the prevalence of particular environmental conditions and crop production practices (Shahid *et al.* 2012).

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Table 1.2. Bioactive products based on entomopathogenic fungi (EPF) used for commercial field application.

Product	EPF	Biological action
Verelac	*Lecanicillium lecanii*	Fungal pesticide
Mycotal	*L. lecanii*	Fungal pesticide
Pfr21	*Paecilomyces fumosoroseus*	Sucking pests
Beevicide	*Beauveria bassiana*	Borer-type pests
Grubkill	Selected fungus and bacteria	Borers and sucking pests
Pelicide	*Paecilomyces lilacinus*	Nematodes
Biologic Bio 1020	*Metarhizium anisopliae*	Mycelium granules
Bioter	*L. lecanii*	Termites
Brocaril	*B. bassiana*	Wettable powder
Ostrinil	*B. bassiana*	Microgranules of mycelium used as pesticide
Boverol	*B. bassiana*	Dry pellets as pesticide
Naturalis	*B. bassiana*	Liquid formulation
Mycontrol-WP	*B. bassiana*	Wettable powder as pesticide
Betel	*Beauveria brongniartii*	Microgranules of mycelium used as pesticide
Engerlingspilz	*B. brongniartii*	Barley kernels colonised with fungus used as pesticide
Biopath	*M. anisopliae*	Conidia on a medium used as pesticides
Biomite	*L. lecanii*	Effective against mites
Biogreen	*M. anisopliae*	Conidia produced on grain used as pesticide
Naturalis-O and BotaniGard	*B. bassiana*	Effective against whiteflies
Trypae Mix	*Trichoderma* and *Paecilomyces*	Effective against fungal pathogens and nematodes in soil

Source: Sandhu *et al.* 2012.

1.6. COMBINED USE OF ENTOMOPATHOGENS

Apart from the evidence that entomopathogens are effective in controlling weevils, the combined use of both microbial control agents has additional advantages to their use alone. Such benefits include their potential to infect the insects in cryptic habitats, to reduce the pesticide residues on food, to facilitate mass production, and not endanger the safety of humans and a number of non-target organisms (Emelianoff *et al.* 2008; Malan *et al.* 2011; Shahid *et al.* 2012). Shahid *et al.* (2012) report that entomopathogens manage the population of insects and mites in nature by means of causing lethal infections, which tend to result in temporary outbreaks of disease. The improved efficacy of the combined use of biocontrol agents comes from the

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synergetic and additive effects of the biocontrol agents involved (Koppenhöfer & Kaya 1997; Koppenhöfer *et al.* 1999; Ansari *et al.* 2008).

Shapiro-Ilan *et al.* (2003a) report that both *Heterorhabditis* and *Steinernema* spp. nematodes and fungi (*B. bassiana* and *M. anisopliae*) are pathogenic to a number of insect groups, including coleopteran pests. Ansari *et al.* (2008) also report that the black vine weevil can be easily controlled by a combination of EPNs and EPFs. The heterorhabditid and steinernematid EPNs, when combined with *M. anisopliae*, have been shown to control the black vine weevil efficiently.

1.7. CONCLUSION

EPNs are highly virulent, causing high mortality to insect pests within 48 hours. With Ferreira and Malan (2014) having already reported that EPNs can be used as biological control agents against BFW, the current study was carried out to evaluate the potential of EPNs to control its immature soil, and adult, stages. EPF are reported to control the black vine weevil, *Otiorhynchus salcatus*, effectively within 21 days (Grewal *et al.* 1997; Ansari *et al.* 2008). Furthermore, the combination of both the EPNs and EPF may results in the high mortality of the BFW larvae, because the combined use of microbial control agents has both synergetic and additive effects (Koppenhöfer & Kaya 1997; Koppenhöfer *et al.* 1999; Ansari *et al.* 2008). The current study evaluated the potential of using both EPNs and EPF in combination to control the BFW. EPNs can be stored for several months in a refrigerated water bubble tank, and EPF can be stored as conidia, blastospores and mycelia. However, the production costs, and the maintenance of the viability of both EPNs and EPF, are higher when using the two in combination. Both EPNs and EPF are usually formulated into solid, or semi-liquid, substrates post production. Such formulations are currently available throughout, and are highly accepted by, the different markets on a global scale.

1.8. MAIN AIM OF THE STUDY

To establish guidelines, from research done in the laboratory and from field applications, for the use of EPNs and EPF against the BFW in vineyards and apple orchards.

Objectives of the study

The main aim of the current study was to investigate the biological control potential of EPNs and EPF to control the BFW in grapevines and apple orchards, in an integrated pest management system. The study was divided into the following objectives:

1. To survey for the presence of EPNs and EPF strains/species in the orchards and vineyards of the Western Cape province, and to investigate their potential to control the BFW.

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2. To select the best EPNs for the biological control of the BFW in laboratory bioassays.
3. To select the best EPF strains for the biological control of the BFW in laboratory bioassays.
4. To determine the combined mortality of EPNs and EPF on BFW in the laboratory.

As the dissertation appears in the form of individual journal publications, some repetition might occur between the different chapters. The format of the journal of *African Entomology* was followed.

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Chapter 2

Entomopathogens from agricultural soil and their potential to control the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)

2.1. ABSTRACT

The banded fruit weevil (BFW) is a major indigenous pest of deciduous fruits and grapevine in the Western Cape province of South Africa and difficult to control without the use of chemicals. Entomopathogens are effective in controlling some soil-dwelling and above-ground stages of insect pests. Soil samples were collected from selected deciduous fruit orchards and vineyards in the Western Cape, baited with mealworms, *Tenebrio molitor* (Coleoptera: Tenebrionidae) to isolate entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF), identified using molecular techniques, and evaluated for their potential as the biocontrol agents of BFW. A correlation analysis was performed to determine the effect of organic matter, magnesium, potassium and phosphorus on the presence of the entomopathogens. EPNs were trapped in 12 (17.1 %) samples, while *Heterorhabditis bacteriophora* and *Heterorhabditis safricana* were the only two species isolated. *Heterorhabditis bacteriophora* (53 % mortality) gave significantly higher ($p < 0.05$) mortality of adult BFW than did *H. safricana* (37 % mortality). EPF were isolated from 26 (37 %) samples baited, with 14 fungi isolates being identified as *Beauveria bassiana* and 12 from the *Metarhizium anisopliae* complex. The *M. anisopliae* complex (79 % mortality), compared to *B. bassiana* (63 % mortality), gave significantly higher ($p < 0.05$) mortality of BFW adults. The presence of organic matter, magnesium and phosphorus had a negative relationship on EPN occurrence, while organic matter and potassium had a negative relationship on EPF occurrence. The results indicate that soils from the deciduous fruit orchards and vineyards of the Western Cape contain both EPNs and EPF that can potentially be used in an integrated pest management system against soil-dwelling and above-ground stages of BFW.

Keywords: biocontrol, deciduous fruit, entomopathogenic fungi, entomopathogenic nematodes, soil-dwelling stages

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2.2. INTRODUCTION

Coleopteran insects are among the most destructive insect pests of economically important crops (Wakil *et al*. 2017). The banded fruit weevil (BFW), *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), is indigenous to South Africa, first being reported as a major insect pest in 1896 (Lounsbury 1896). Primarily a pest of grapes and apples, it is a phytosanitary pest causing damage in many grape, apple and nectarine orchards, particularly in the Western Cape (Barnes & Capatos 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). The BFW was first reported as a major pest of grapes in New Zealand in 1899, from where it spread to all the southern states in Australia (Kuschel 1972). The BFW has the potential to result in significant damage to several crops, as it has recently been found to be a serious pest of blueberries (Ferreira 2010; Pringle *et al*. 2015).

This polyphagous pest can feed on grasses, weeds, herbs and woody plants (Prinsloo & Uys 2015). Larvae feed on the roots of a number of host plants in South Africa (Pringle *et al*. 2015; Allsopp *et al*. 2015). In Australia, Fisher and Learmonth (2003) reported that a number of weeds growing in the drip zone can feed a large number of BFW larvae, while a few were found feeding on weeds in the mid-row, hence their deduction that the presence of weeds influences the distribution and abundance of the BFW larvae. The adults can feed on the stems and leaves of a number of weeds and grasses (Barnes 1989a, b; Barnes & Pringle 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). In South Africa, deciduous fruit and grapevine damage is caused on the leaves, fruit, fruit stalks, and shoots. The adults also prefer to consume broad-leaved weeds, particularly those with fleshy leaves (Pringle *et al*. 2015; Allsopp *et al*. 2015).

Although trunk barriers are widely used in the Western Cape to prevent the BFW from reaching fruits, an alternative control strategy is required (Barnes 1991). Trunk barriers are labour-intensive, particularly in large orchards and vineyards (Barnes 1991; Barnes *et al*. 1994; Fisher & Learmonth 2003). Fisher and Learmonth (2003) report that the foliar application of esfenvalerate (synthetic pyrethroid) to control the BFW results in secondary pest outbreaks, such as those of the long-tailed mealybug, the grape leaf rust mite and the grapevine scale. It has been shown that many curculionids can be controlled by combining relevant biocontrol agents (Ansari *et al*. 2008; Ferreira & Malan 2014). Biological agents reported for the BFW in the Western Cape include: the mymarid egg parasitoid, *Cleruchus* sp. (Hymenoptera: Mymaridae); the braconid wasp, *Perilitus* sp. (Hymenoptera: Braconidae); the EPNs *Heterorhabditis* spp. (Rhabditida: Heterorhabditidae), and a *Steinernema* spp. (Rhabditida: Steinernematidae); the EPF *Beauveria* spp. and *Metarhizium* spp.; a mite, *Leptus* sp. (Trombidiforme: Erythraeidae); and guinea fowl (Fisher & Learmonth 2003; Barnes 2014; Pringle *et al*. 2015).

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Using biocontrol agents, in particular entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF), is safe for humans and leads to the development of an epizootic, and repeated, infection cycles of insect pests in the micro-environment, thereby increasing the longevity and the persistence of the biocontrol agents involved (Bateman *et al*. 1993; Thomas *et al*. 1995; Wood & Thomas 1996; Hidalgo *et al*. 1997; Wakil *et al*. 2014). Both EPNs and EPF that help control population flushes of insects in nature are found in soils worldwide. Discovering new species of entomopathogens is important, as species that are more virulent can be used to increase the potential of microbial control (Tanada & Kaya 1993; Shapiro-Ilan *et al*. 2002; Malan *et al*. 2006, 2008, 2011). Malan *et al*. (2011) was able to isolated EPNs in 17 % of the tested soil samples from citrus orchards in South Africa. In a survey of EPNs conducted in the south-western parts of South Africa, the *Heterorhabditis* spp. were found to be the most dominant EPNs in the Western Cape, whereas the *Steinernema* spp. were found to be the least present (Malan *et al*. 2006). During a survey in Kwazulu-Natal, *Steinernema* species were more common, as compared to the *Heterorhabditis* species (Spaull 1990, 1991).

The main objective of the current study was to survey both EPNs and EPF in deciduous fruit orchards and vineyards in the Western Cape province. Soil samples from different agricultural habitats were collected and the entomopathogens isolated by using a live 'bait', and identified. A correlation analysis was performed to establish the effect of land use practices and soil types on the presence of entomopathogens. Laboratory bioassays to establish the virulence of the entomopathogens identified during the survey against *P. callosus* were conducted.

2.3. MATERIALS AND METHODS

2.3.1 Source of insects used

Adult BFW that were collected from vineyards and orchards in the Western Cape from October to December and were mass reared at the Stellenbosch University Insectary (IPM Initiative), Stellenbosch University, South Africa. Corrugated cardboard bands were used to collect the weevils by tying them onto apple and vine trunks (Fig 2.1A, B). The adult weevils, ages ranged from 2 to 4 weeks old, were kept in a ventilated Perspex boxes measuring 15 × 20 cm. Weevils were reared on carrots (personal communication, D. Stenekamp) (Fig. C, D).

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Fig. 2.1. A corrugated cardboard band tied around a vine trunk (A); an adult *Phlyctinus callosus* on a corrugated cardboard band (B); larvae being reared on carrots and sweet potatoes on river sand (C); and adults feeding on *Caprosma* sp. branches and leaves (D).

2.3.2. Soil sample survey

A total of 70 soil samples samples were collected during the 2016/2017 growing season from different orchards and vineyards throughout the Western Cape province. Soil samples of approximately 1.5 kg were collected 10 m apart at a soil depth ranging from 0 to 20 cm, using either a shovel, a spade or a pick, depending on the soil type. A total of five samples were collected from each vineyard and orchard (n = 14) and combined to increase the probability of obtaining different nematode and fungal species. The samples were placed in plastic ziplock bags, which were placed away from direct sunlight and any other source of heat, and transported to the laboratory in cool boxes, where they were stored at 20° C, and baited with *T. molitor* larvae within a month. A global positioning system (GPS) was used to record the locations of the orchards and vineyards sampled. The soil samples were thoroughly mixed and sieved (to remove stones), using a 4-mm-aperture metal sieve upon reaching the laboratory. Soil samples of 200 ml were transferred to 400 ml plastic containers to which 10 *T. molitor* larvae were added. Dry soil samples were moistened, using distilled water to keep them humid during the baiting process, and the plastic containers were each closed with a lid and incubated at 22 °C. The samples were checked after 7 days, with the dead larvae being removed from the plastic containers and replaced with fresh larvae, for a total of 14 days.

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2.3.3. Entomopathogen isolation

Signs of EPN infection in *T. molitor* larvae were recognised by noting the change in colour of the cadavers, their softness, and their lack of fungal growth. The dead insects, showing signs of EPN infection, were placed on a White trap (White 1927), and the emerging IJs were collected into a culture flask containing 150 ml distilled water, in a final concentration of ± 2000 IJs mL^{-1}. The IJs were stored in 50 ml distilled water in culture flasks at 14 °C. To isolate the EPF, a selective medium, as described by Meyling (2007), was used. The selective media were prepared by means of suspending 65 g of Sabouraud Dextrose Agar (SDA) in 1 L of distilled water in a schott bottle. A fungicide-inhibiting (1 ml Dodine) opportunistic external saprophytic fungal growth was added and mixed with the medium. The bottle used was marked with an autoclave tape (indicating correct sterilization) and autoclaved for 20 min at 120 °C. The medium was cooled to approximately 60 °C and 50 mg/L of chloramphenicol (antibiotic bacteria inhibitor) and 50 mg/L Rifampicin (antibiotic bacteria inhibitor) was added. The medium was poured into 9 mm diameter petri dishes and used for isolation and culture of fungi at 25 °C in the dark.

2.3.4. Source of entomopathogens

The IJ rearing and harvesting procedures were carried out according to the methods described by Kaya and Stock (1997), using *T. molitor* larvae at room temperature (± 25°C). The IJs, which were harvested from the White trap within 1 week of emergence, were stored horizontally using 500-ml vented culture flasks. The flasks, containing 150 ml of distilled water, were kept at 14 °C, with the collected IJs being used within one month after harvesting. The culture flasks were shaken on a weekly basis to increase the aeration and survival of the IJs in storage. The nematode concentrations used for different experiments were calculated using the equation developed by Navon and Ascher (2000).

EPF cultures (2–3 weeks old) were used to harvest the conidia by means of scraping the surface cultures using a glass rod. The conidia were suspended in 20 ml sterilised distilled water, augmented with 0.05 % Triton X-80 in sterile McCartney bottles. The bottles were sealed and vortexed for 2 min, resulting in a homogenous suspension.

The germination response of EPF was studied by using a SDA medium in polystyrene Petri dishes (60 × 15 mm). A 100 µl of spore suspension (1×10^6 conidia ml^{-1}) was spread onto four SDA plates, with each plate containing one of the four EPF. After a cover slip was placed on each plate, the plates were incubated at 25 °C in a growth chamber. The percentage of germination was determined by counting 100 spores from each isolate, at 40 × magnification after 24 h (Ekesi *et al*. 2002).

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2.3.5. Identification of entomopathogens

In the case of the EPNs, the required DNA was extracted by means of using a single first-generation young female. For each isolate, the female was cut into small pieces in a 0.5-ml microcentrifuge tube with 30 μl lysis buffer (500 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatine, and 3 μl of proteinase K [600 µg/ml]), on the side of a 0.5-µl microcentrifuge tube (Nguyen 2007), using a syringe needle. The tubes were frozen for 1 h at -80 °C, and incubated in a thermocycler at 65 °C for 1 h, and at 95 °C for 10 min. The tubes were centrifuged at 12 000 rpm for 2 min, after which the supernatant (top 20 µl) was removed, and added to new 0.5-ml tubes. The DNA extractions obtained were kept at -20 °C until use.

Cultures of each EPF isolate were sent to the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI, Pretoria, South Africa) for morphological identification. Molecular identification was carried out, using a separate fungal culture of each isolate, to confirm the morphological identification thereof. Fungal cells from 50 to 100 mg (wet weight) were suspended in up to 200 µl of water or isotonic buffer (*e.g.* PBS), or in up to 10-200 mg of tissue, in a ZR BashingBead™ lysis tube. The tube was secured in a bead beater fitted with a 2.0-ml tube holder assembly (Scientific Industries' Disruptor Genie™, Cat. No. S6001-2 from Zymo Research Corp.), which was processed, at maximum speed, for 5 min. The ZR BashingBead™ lysis tube was centrifuged in a microcentrifuge at ≥10 000 × g for 1 minute. The technique supplied by the DNA extraction kit was used.

The polymerase chain reaction (PCR) was used to amplify the ITS (internal transcribed spacer (ITS) region with the primers for nematodes suggested by Hominick *et al*. (1997), a forward primer TW81 (5-TTTCCGTAGGTGAACCTGC-3) and a reverse primer AB28 (5-ATATGCTTAAGTTCAGCGGGT-3). The primers for EPF fungi amplification were ITS4-TCCGTAGGTGAACCTGCGG and ITS1-TCCTCCGCTTATTGATATGC. To distinguish between the different species in the *Metarhizium* complex, the elongation factor (EF), with the primers EF1 (F) -TGCGGTGGTATCGACAAGCGT and EF2 (R) -AGCATGTTGTCGCCGTTGAAG, were amplified. Nguyen's (2007) technique was followed for the purpose of polymerase chain reaction (PCR) amplification. Each PCR reaction contained 5 µl of 10 × PCR buffer, 1.25 µl of 10 pM forward primer, 1.25 µl of 10 pM reverse primer, 12.5 µl of KAPPA, 5 µl of distilled water, and 5 µl of the DNA suspension from the DNA extraction, which served as the template DNA in a final reaction volume of 25 µl. A thermocycler (GeneAmp 2720) was used to perform the PCR reaction, in accordance with the cycling profile suggested by Nguyen *et al*. (2004): 1 cycle of 95 °C for 3 min, followed by 3

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cycles of 95 °C for 20 s, 48 °C for 20 s, and 72 °C for 30 s. Finally, the last cycle was performed at 72 °C for 5 min.

The products from the PCR were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Santa Clarita, CA, USA). The purified DNA was sequenced in both directions, using the aforementioned primers on an automated sequencer (ABI 3100), at the Department of Genetics, Stellenbosch University, South Africa, using Big Dye 3.1 chemistry (PE Applied Biosystems). The CLC Main Workbench version was used for sequence editing, for verifying the base calls, and for obtaining a consensus sequence (using both the forward and the reverse sequence, if possible). The final sequence obtained was aligned with sequences in GenBank, using a BLAST comparison for species verification (Nguyen 2007). All consensus sequences were deposited in GenBank.

2.3.6. Effect of soil parameters on the presence of entomopathogens in the soil

The soil samples collected from the commercial apple orchards and vineyards in the Western Cape were sent to Bemlab (Strand, Cape Town) for pH determination and analysis; organic matter (OM); nitrogen (N); calcium (Ca); potassium (K); magnesium (Mg); manganese (Mn); phosphorus (P) and zinc (Zn) available in the soil samples. A correlation analysis was performed to determine the effect of the above-mentioned elements on the occurrence of biologicals in the different soil samples collected.

2.3.7. Bioassay protocol

Virulence experiments were conducted in 24-well bioassay trays (flat-bottom, Nunce, Cat. No.144530, Thermo Fisher Scientific (Pty) Ltd, Johannesburg, Gauteng, South Africa). Alternate wells were lined with a circular (13-mm-diam.) filter paper, to obtain an even distribution throughout the wells. Each piece of filter paper was inoculated with a concentration of 200 IJs/50 µl filtered tap water for the adults, and with a concentration of 100 IJs/50 µL for the final instar larvae. The control treatment comprised 50 µl distilled water only. After one insect was added to each well, it was closed with a lid, and placed in a closed plastic container lined with moistened tissue paper, and placed in a growth chamber, where it was kept at 25° C for two days. Mortality was determined by confirming the infection of the insect concerned by dissection with the aid of a stereomicroscope. The bioassays were repeated for all treatments on different test dates, and with freshly prepared nematode inoculum.

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2.3.8. Susceptibility of BFW adults against nematodes

Using the 24-well bioassay protocol, the two EPNs isolated from the, at 200 IJs/insect, were used to test their ability to control adult BFWs. Five bioassay plates, each containing 12 adults, were used for each nematode species, with the controls receiving water only. The mortality caused by each EPN. and the number of IJs that penetrated the host, was determined for each EPN species studied. The procedure was repeated on different dates, and with freshly prepared inoculum.

2.3.9. Susceptibility of BFW adults against fungi

Five replicates of 12 BFWs were treated with the fungal suspensions, or with distilled water as the control. The adult BFW were individually immersed in a 100 ml conidial suspension of the different EPF for 60 seconds, with the control being individually immersed in a 100 ml aqueous solution of 0.01% Tween-80 (without fungal spores) (Merck, KGaA, Darmstadt, Germany). The adult BFWs treated with the EPF and control were then transferred to 24-well bioassay trays, in which they were kept at 25° C in a growth chamber, for a period of 21 days. The 24-well bioassay trays were checked daily to record the mortality that had occurred. The experiment was repeated on a different test date with a fresh culture of fungal spores.

2.3.10. Data analysis

Correlation analysis (SAS Institute 1985) was used to determine the relationships between the soil parameters measured and the pathogen occurrence (number of positive sites). A one-way ANOVA was performed to analyse the mortality of the entomopathogens obtained from the surveys, and the germination response of the selected EPF isolates. If the F value was significant ($p \leq 0.05$), the means were differentiated by means of LSD MEANS (SAS Institute 1985). The mortality data were corrected for the corresponding control mortality using the formula: CM (%) = $\{(T-C)/(100-C)\} \times 100$, with CM being the corrected mortality, T being the percent mortality in the treated insects, and C being the percent mortality in the untreated insects (Abbott, 1925). If no significant interaction was found to have occurred between the main effect of the test dates and the treatments concerned, the data obtained from the two test dates were pooled and analysed, using a one-way ANOVA.

2.3. RESULTS

2.3.1 Entomopathogens identified

The EPNs were isolated from 12 of 70 (17.1 %) soil sampling sites representing all the sampled habitats; seven out of 40 sites (17.5 %) and five out of 30 sites (16.7 %) from the commercial and

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organic orchards, respectively (Table 2.1). All the EPNs isolated from the survey belonged to the genus *Heterorhabditis*, with the different species identified being *Heterorhabditis bacteriophora* Poinar and *Heterorhabditis safricana* Malan, Nguyen, De Waal & Tiedt (Table 2.1). Of the isolates, seven (58.3 %) were *H. bacteriophora* and five (41.7 %) were *H. safricana*. Both EPN species were isolated from commercial orchards and vineyards, and from organic orchards. Of the seven *H. bacteriophora* isolates recovered, four were from the commercial apple orchards and vineyards, and three from the organic orchard under quince fruit. Of the four *H. safricana* isolates recovered, two were isolated from a commercial apple orchard, and one from an organic plum orchard and a commercial peach orchard.

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Table 2.1. *Heterorhabditis* species and isolates identified from a survey of orchards and vineyards in the Western Cape province, South Africa. Unless stated otherwise, all habitats were conventional, commercial plantings.

EPN species	Isolate number	Number of samples	GenBank number ITS	Nearest town	GPS reading	Habitat*
H. safricana	272	10	MH333118	Ceres	33°13'45.3"S; 19°13'19.6"E	Apple
H. safricana	273	10	MH333122	Ceres	33°13'45.3"S; 19°13'19.6"E	Apple
H. safricana	288	2	MH333121	Ceres	33°13'45.3"S; 19°13'19.6"E	Apple
H. bacteriophora	283	10	MH333232	Grabouw	34°08'16.1"S; 19°01'15.6"E	Apple
H. bacteriophora	285	2	MH333233	Grabouw	34°08'18.4"S; 19°01'15.5"E	Apple
H. bacteriophora	287	3	MH333235	Grabouw	34°08'18.4"S; 19°01'15.5"E	Apple
H. bacteriophora	301	10	MH333236	Grabouw	34°08'23.4"S; 19°01'16.9"E	Wine grape
H. bacteriophora	280	5	MH333230	Robertson	33°43'22.5"S; 19°47'21.5"E	Quince (organic)
H. bacteriophora	281	2	MH333231	Robertson	33°43'36.5"S; 19°47'27.0"E	Quince (organic)
H. bacteriophora	286	5	MH333234	Robertson	33°43'36.5"S; 19°47'27.0"E	Quince (organic)
H. safricana	282	10	MH333120	Robertson	33°43'32.1"S; 19°47'26.2"E	Plum (organic)
H. safricana	302	10	MH333122	Robertson	33°43'32.1"S; 19°47'26.2"E	Peach (organic)

*Apple (*Malus domestica* B.); plum (*Prunus salicina* L.); grapevine (*Vitis vinifera* L.); quince (*Cydonia oblonga* M.); peach (*Prunus persica*).

The EPF were found in 26 (37.1 %) of the 70 soil samples representing all the sampled habitats (Table 2.2). The different species morphologically identified from the different habitats were the *Beauveria bassiana* and the *Metarhizium anisopliae* complex (Fig. 2.2, Table 2.2). *Beauveria bassiana* was found in 14 soil samples; 12 soil samples were collected from the organic deciduous fruit orchards, and two soil samples were obtained from the commercial deciduous fruit orchards and vineyards. *Metarhizium anisopliae* was found in 12 soil samples in total, with all the soil samples having been collected from the commercial orchards and vineyards involved.

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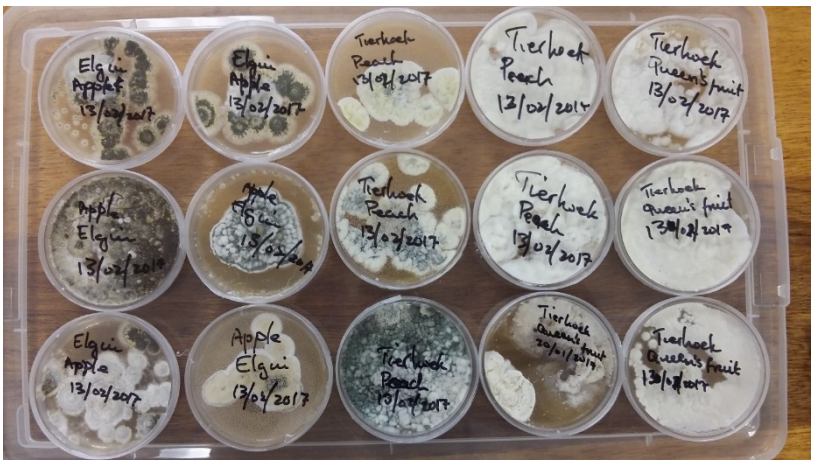

Fig. 2.2. Culture morphology of entomopathogenic fungi isolates obtained from the soil sample collected in the Western Cape province, South Africa.

Table 2.2. *Beauvaria* and *Metarhizium* species identified from orchards and vineyards in the Western Cape province, South Africa. Unless stated otherwise, all habitats were conventional, commercial plantings.

Isolate name	Morphological ID	Molecular ID	GenBank Number	Positive samples	Nearest town	GPS reading	Habitat
CA 5	*B. bassiana*	*B. bassiana*	Pending	2	Ceres	33°13'45.3"S; 19°13'19.6"E	Apple
CA 1	Pending	*M. anisopliae*	Pending	4	Ceres	33°13'45.3"S; 19°13'19.6"E	Apple
EA2	*M. anisopliae*	*M. anisopliae*	Pending	3	Grabouw	34°08'16.1"S 19°01'15.6"E	Apple
EP 3	*M. anisopliae*	Pending	Pending	3	Grabouw	34°08'18.4"S; 19°01'15.5"E	Pear
EG 4	*M. anisopliae*	Pending	Pending	2	Grabouw	34°08'23.4"S; 19°01'16.9"E	Wine grapes
TQ1 3	*B. bassiana*	*B. bassiana*	Pending	5	Robertson	33°43'22.5"S; 19°47'21.5"E	Quince(organic)
TQ2 5	*B. bassiana*	*B. bassiana*	Pending	5	Robertson	33°43'36.5"S; 19°47'27.0"E	Quince (organic)
TP1/ES1	*B. bassiana*	*B. bassiana*	Pending	2	Robertson	33°43'32.1"S; 19°47'26.2"E	Peach (organic)

*Apple (*Malus domestica* B.); pear (*Pyrus* communis L.); plum (*Prunus salicina* L.); grapevine (*Vitis vinifera* L.); quince (*Cydonia oblonga* M.).

2.3.2. Effect of soil parameters on the presence of entomopathogens

2.3.2.1. Entomopathogenic nematodes

The organic matter, magnesium and phosphorus levels (Table 2.3) in the soil had a negative relationship with EPN occurrence (r = -0.092, P = 0.05; r =-0.092, P = 0.05; and r = -0.075, P = 0.05 for the organic matter (OM), magnesium (Mg) and phosphorus (P) levels, respectively) (Table 2.3). The levels of calcium, manganese, potassium, zinc, and pH were each positively correlated with EPN occurrence (r = 0.036, P = 0.05; r = 0.069, P = 0.05; r = 0.012, P = 0.05; r = 0.014, P = 0.05; and r = 0.083, P = 0.05 respectively).

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Table 2.3. Soil parameters for commercial and organic deciduous fruit and grapevine surveyed for nematodes. Unless stated otherwise, all habitats were conventional, commercial plantings.

Location	Orchard	Soil type	pH	OM	Ca	K	Mg	Mn	P	Zn
Ceres	Apple	Loam	5.5	1.38	3.28	38	1.55	23.8	154	11.8
Grabouw	Apple	Loam	4.7	2.39	3.30	88	1.42	34.2	34	6.5
Grabouw	Pears	Loam	4.4	2.13	2.77	54	1.21	20.4	28	3.4
Grabouw	Grapes	Loam	5.0	1.86	4.27	65	1.38	14.8	24	3.6
Robertson(organic)	Quince fruit 1	Clay	5.5	2.78	7.08	65	3.55	362.1	44	15.3
Robertson(organic)	Quince fruit 2	Loam	6.2	3.19	9.13	71	4.28	391.1	45	16.7
Robertson(organic)	Plum	Sand	6.7	3.44	10.95	0.59	4.66	229.6	337	50.5
Robertson(organic)	Peach	Loam	6.4	2.99	10.42	0.28	3.57	355.5	143	33.8

[a]OM (organic matter) are percentages; K, Mn, P, & Zn are in mg/kg; Ca & Mg are in cmol(+)/kg.

2.3.2.2. Entomopathogenic fungi

The soil characteristics from each orchard, and the occurrence, in terms of the EPF correlation analysis, indicated the existence of several significant relationships. A negative relationship was detected between the EPF (*B. bassiana* and *M. anisopliae* combined) occurrence and the organic matter (OM) levels in the soil ($r = -0.069$; $P = 0.05$). Another negative relationship between the EPF occurrence and the K levels in the soil was detected ($r = -0.303$; $P = 0.05$). The levels of Ca, Mg, Mn, P, Zn and pH were each positively correlated with the EPF occurrence ($r = 0.312$, $P = 0.05$; $r = 0.350$, $P = 0.05$; $r = 0.440$, $P = 0.05$; $r = 0.217$, $P = 0.05$; $r = 0.216$, $P = 0.05$; and $r = 0.544$, $P = 0.05$ respectively).

2.3.3. Susceptibility of BFW adults to nematode infection

The BFW adults were found to be susceptible to the two EPN species isolated from the commercial apple orchards in the Western Cape. The mortality of *H. safricana* was 37 % and for *H. bacteriophora* 53 %, after they were exposed to 200 IJs/insect over a period of 3 days (Fig. 2.3A). No significant interactions were found between the test dates and the treatments. A significant effect ($F_{(2, 27)} = 31.9005$; $P = 0$) of the treatment was detected on the percentage adult mortality. The *H. bacteriophora* isolated from a commercial apple orchard in Grabouw gave significantly higher ($p < 0.05$)mortality of the adult BFWs on both test dates than did the *H. safricana* isolated from an apple orchard in Ceres.

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The mean number of IJ penetration ranged from 5.8 for *H. safricana* and 9.5 nematodes per BFW larva (Fig. 2.3B) for *H. bacteriophora*. No significant interactions were found between the test dates and the treatments concerned. A significant effect ($F_{(2, 27)} = 195.9$; $p = 0.001$) of the treatment on the adult BFW penetration rate was observed (Fig. 2.3B). The *H. bacteriophora* isolated from a commercial apple orchard in Grabouw gave a greater mean number of nematodes that penetrated the adult BFWs on both test dates involved (9.5) than did the *H. safricana* isolated from an apple orchard in Ceres ($p < 0.05$) (Fig. 2.3B).

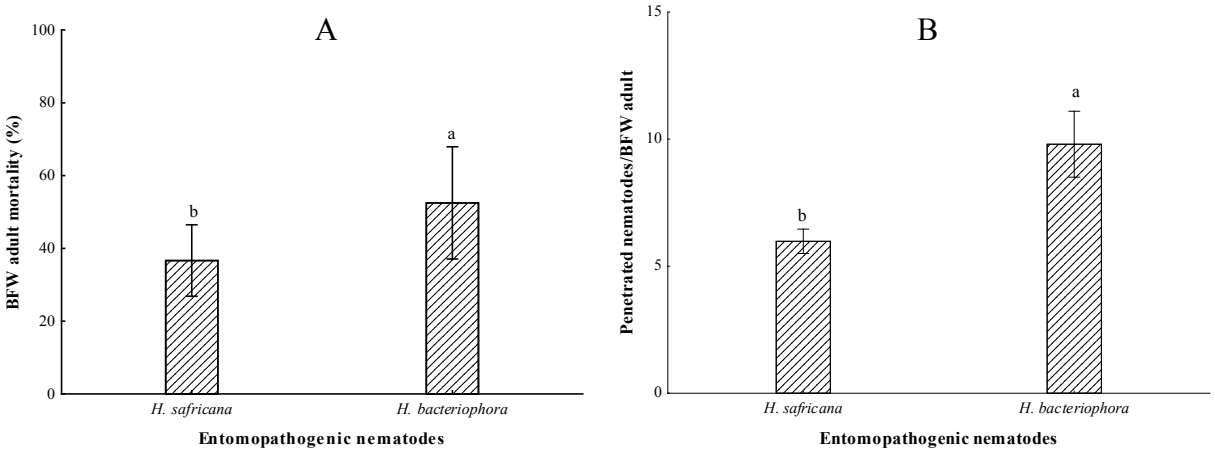

Fig. 2.3. Mean percentage mortality (A) and mean number of nematodes that penetrated (B) (± 95% confidence level) *Phlyctinus callosus* adults inoculated with 200 IJs/insect of *Heterorhabditis safricana* and *H. bacteriophora*. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

2.3.4. Susceptibility of BFW adults to fungi

The mean mortality of the two EPF species tested ranged between 63 % and 79 % after inoculation with 1×10^6 conidia ml^{-1} over a period of 21 days (Fig. 2.4). A significant effect ($F_{(1,8)} = 7$, $p = 0$) of the treatment on the percentage mortality was observed. *Metarhizium anisopliae* gave significantly higher ($p < 0.05$) mortality of the BFW adults, compared to that given by the *Beauveria bassiana* (Fig. 2.4).

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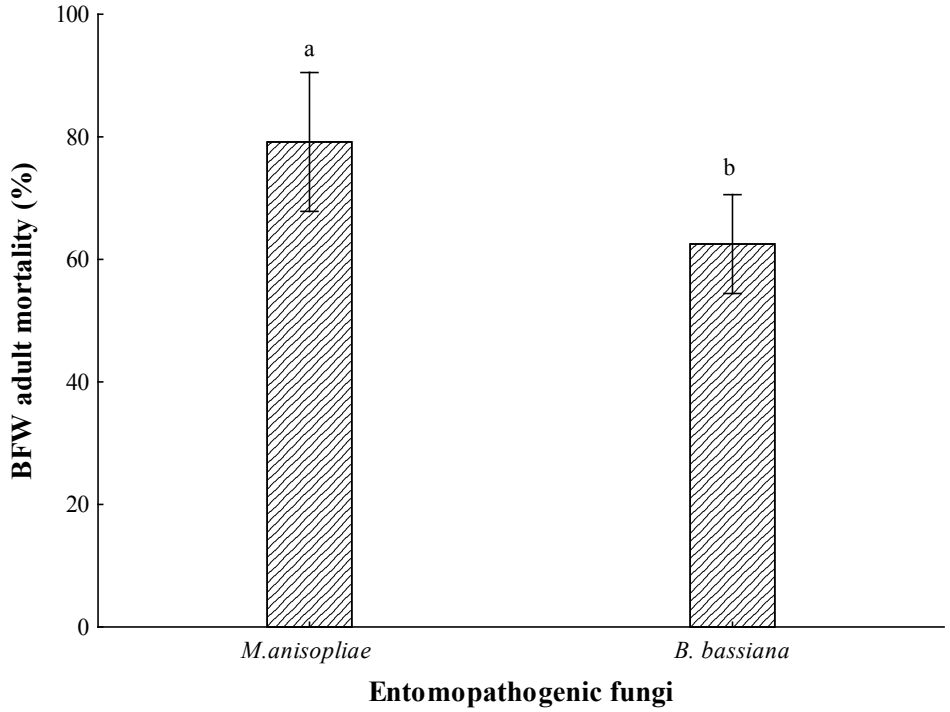

Fig. 2.4. Mean percentage mortality (±95% confidence level) of *Phlyctinus callosus* adults inoculated with 1×10^6 conidia ml^{-1} of *Metarhizium anisopliae* and *Beauveria bassiana* (one-way ANOVA: $F_{(1, 18)} = 7$, $p = 0$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

2.4. DISCUSSION

The 17.1 % occurrence of EPNs (as a percentage of positive soil samples) obtained from the study is within the ranges reported in the surveys conducted elsewhere. The relevant examples include the surveys conducted by Shapiro-Ilan *et al*. (2003) in the south-eastern United States, and by Mráček *et al*. (1999) in Czechoslovakia, who reported 20 % and 28.5 % EPNs, respectively. In another study conducted by Malan *et al*. (2011) to identify EPNs from citrus orchards in South Africa, 17 % of the tested soil samples showed positive for the presence of EPNs. The results obtained in the current study are within the ranges reported in the survey by Malan *et al*. (2011).

In a survey of EPNs conducted in the south-western parts of South Africa, the *Heterorhabditi*s spp. were found to be the most dominant EPNs in the Western Cape, whereas the *Steinernema* spp. were found to be the least present (Malan *et al*. 2006). During a survey in Kwazulu-Natal, *Steinernema* species were more common, as compared to the *Heterorhabditis* species (Spaull 1990, 1991). In the current survey, *Heterorhabditis* was the only genus isolated, with *H. bacteriophora* being the more common species found, while the *Steinernema* species was not isolated. *Heterorhabditis safricana* is originally described from South Africa, being isolated from a peach orchard in the Western Cape (Malan *et al*. 2008). Since then, the presence of this EPN has neither been reported from outside the Western Cape, nor from any other country.

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Significantly higher incidence of the EPNs was noted on the commercial farms compared to the organic deciduous fruit orchard. Ignoffo (1992) reports several factors as affecting the persistence of EPNs (*e.g.* light, water, chemicals), with sunlight being the most destructive environmental factor concerned. The previous author, however, stresses that sunlight alone cannot affect the persistence of EPNs, whereas the presence of commercial pesticides can. The results from the current survey do not concur with the fact that the use of commercial pesticides has an adverse effect on the persistence of EPNs. However, such a finding might have been made because only one organic farm was used, while more commercial farms were used, giving a potentially biased result. The effect of pesticides on EPN and EPF persistence should still be tested on the local species.

As soil parameters are important for both the surface and the below-ground applications of nematodes, knowing the soil type and the structure where the nematodes are to be applied is important (Kaya 1990; Kung *et al*. 1990; Shapiro-Ilan *et al*. 2012). Kaya (1990) and Barbercheck (1992) report that the movement and survival of nematodes in the soil is affected by the soil texture, with light soils with a high content of clay reducing the amount of movement of nematodes. The availability of oxygen levels are reduced, as clay soils are poorly aerated, which reduces the efficacy and survival of the nematodes concerned (Georgis & Poinar 1983; Georgis & Gaugler 1991; Shapiro-Ilan *et al*. 2000). The soil pH, in contrast, affects the distribution of EPNs in nature, with soil pH from 10 upwards being detrimental to the application of EPNs, while a range of soil pH from 4 to 8 has no effect on the application of EPNs (Kung *et al*. 1991; Kanga *et al*. 2012). The current study found that the pH is positively correlated with EPN occurrence.

A much higher incidence of the entomopathogens (EPNs and EPF) was noted in the organic deciduous fruit orchard compared to the incidence that was found on the commercial farms investigated. The above was due to the commercial farms, from which the soil samples were collected, using several different chemical pesticides to reduce the populations of a number of insect pests previously encountered there. The results from the current experiment also support that the use of commercial pesticides adversely affects the persistence of entomopathogens (EPNs and EPF).

In the current study, many soil samples from which only *B. bassiana* was isolated came from the organic orchards involved, while the *M. anisopliae* was associated with soil samples collected from both the commercial orchards and the vineyards surveyed. The findings agree with those made by Quesada-Moraga *et al*. (2007), who suggest a strong association between *M. anisopliae* and the soils collected from cultivated commercial habitats. The high occurrence of *M. anisopliae* in commercially cultivated habitats is reported to be due to *M. anisopliae* conidia persisting for

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fairly long periods without infecting the hosts, while the lack of susceptible hosts in commercial heavily cultivated orchards minimises the persistence of *B. bassiana* in the soils (Fargues & Robert 1985; Vänninen 1996). *Metarhizium anisopliae* is more tolerant to synthetic pesticides than is *B. bassiana*, leading to reducing the competitiveness of *B. bassiana* in commercial orchards (Bidochka *et al*. 1998).

A higher persistence of EPF was recorded in the organic deciduous fruit orchard compared to the level of persistence that was encountered on the commercial farms. The findings also concur with those of Klingen *et al*. (2002), who showed that EPF are more commonly isolated from the soil samples collected from organically managed fields than they are from conventionally managed, arable fields. The above is due to the use of synthetic pesticides considerably reducing the number of hosts, which affects the persistence of the EPF in the soil, leading to the inference being made that, in organic orchards, the probability of conidia infecting and successfully killing an insect is higher than that which is experienced in commercial orchards. The use of pesticides not only reduces the number of insect hosts available, but it also greatly impacts on the presence of EPF, by way of deleteriously affecting the EPF in the soil (Mietkiewski *et al*. 1997).

The soil nutrients found to be positively related to the EPF occurrence included the macronutrients (calcium, magnesium and phosphorus), the micronutrients (manganese and zinc), and the soil pH. An increase in the occurrence of such nutrients in the soil tends to increase the persistence of the EPF in the soil, hence helping to control host insects in the field. Other key soil parameters affecting the occurrence of EPF include the presence of nitrogen content and organic matter (Lingg & Donaldson 1981; Studdert *et al*. 1990; Rosin *et al*. 1996; Shapiro-Ilan *et al*. 2003). In the current study, organic matter was found to affect the persistence of EPF in the soil negatively, with an increase in the amount of organic matter present reducing the degree of persistence of EPF in the soil, as well as the degree of control of host insects in the field. Koppenhöfer & Fuzy (2006) evaluated the effect of soil type on performance and persistence on for different EPN species and concluded that the EPN performance and persistence in different soil types differed according to the nematode species concerned. The effect of the presence of such nutrients and pH must be considered if the use of EPF is to be integrated into the IPM strategies that are currently employed in the Western Cape. The findings made concerning the relationship between the soil nutrients and the EPF have implications for BFW management practices, in terms of promoting the persistence of natural enemies in both orchards and vineyards.

Ferreira and Malan (2014), in reporting that EPNs have the potential to control BFW, state that the percentage mortality of the BFW larvae found ranged from 41 to 73 %, whereas that of adult BFWs ranged from 13 to 45 % after they were inoculated with 400 IJs/insect over a period of 4

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days. In the current study, the adult percentage mortality was found to range from 36.7 % and 52.5 % for the EPNs surveyed, after they were inoculated with 200 IJs/insect over a period of 3 days. The study findings, therefore, show that BFW adults can be suppressed by means of EPNs used in commercial orchards, as the EPN species isolated from the commercial orchards surveyed were able to control the BFWs involved. The persistence of the EPNs in the commercial orchards indicates that they have the potential for incorporation with other management strategies to control the BFWs, both in the Western Cape and elsewhere.

The major pest status of the BFW justifies the conducting of field trials to find the efficiency of EPNs in the field, which is of paramount importance for incorporating the use of the pathogens in an IPM strategy. Since the BFW has two generations in a year, with the soil stages being found throughout the year, good persistence of EPNs in the soil should prove possible, since the host is likely always to be available.

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Chapter 3

DLAMINI, B.E, MALAN, A.P. & ADDISON, P. 2018. Control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) using entomopathogenic nematodes. *Austral Entomology* (Submitted).

Potential of entomopathogenic nematodes to control the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)

3.1. ABSTRACT

Phlyctinus callosus, the banded fruit weevil (BFW), is a key pest of deciduous fruit and grapevine in the Western Cape province of South Africa. Entomopathogenic nematodes (EPNs), as a biocontrol agent, are found to be effective in controlling both soil-borne and above-ground insect pests. Different EPN species were screened for virulence against different life stages of the BFW, which was followed by a field trial, in which *Steinernema yirgalemense* was applied at different concentrations. The results indicated *S. yirgalemense* to be six times more potent than *Heterorhabditis noenieputensis,* at 400 infective juveniles (IJs)/insect (95 % mortality) with significantly higher ($p < 0.05$) control of BFW larvae. *Steinernema yirgalemense, H. noenieputensis*, and the exotic *Steinernema feltiae* gave good control of BFW larvae at 100 IJs/insect, but with no significant difference between treatments. In the case of BFW pupae, *Heterorhabditis indica* (70 % mortality) and *H. baujardi* (67 % mortality) differed significantly ($p < 0.05$) in their control, compared to *H. noenieputensis* (55 % mortality). *Heterorhabditis indica* (95 % mortality) and *S. yirgalemense* (94 % mortality) gave significantly higher ($p < 0.05$) control of BFW adults, compared to three other nematode species tested. In the field trials, *S. yirgalemense*, at 20 and 40 IJs/cm^2, gave 69 % and 78 % mortality of BFW larvae, respectively. The results indicated that all EPNs tested were effective against the immature and adult stages of the BFW, showing clear differences among the EPN species tested. *Steinernema yirgalemense*, at low concentration, can effectively control BFW under field conditions, the conducting of large-scale field trials being recommended to demonstrate further the potential use of this biocontrol agent within integrated pest management programmes.

Keywords: Biocontrol, pome fruit, integrated pest management, deciduous fruit, grapevine

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3.2. INTRODUCTION

The banded fruit weevil (BFW), *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), which is a major insect pest of grapes and apples, is indigenous to the Western Cape province of South Africa (Lounsbury 1896; Barnes & Capatos 1989; Prinsloo & Uys 2015). The phytosanitary pest causes significant damage to deciduous fruit orchards and vineyards in the Western Cape (Pringle *et al*. 2015; Allsopp *et al*. 2015). The weevil has two generations, with eggs laid in batches of 20 or more on or near the soil surface, or on loose debris, foliage and organic litter (Barnes 1989a,b; Barnes & Pringle 1989; Fisher & Learmonth 2003; Pringle *et al*. 2015; Allsopp *et al*. 2015). Eggs hatch after 6 to 15 days, with the first-instar larvae feeding on the roots in the soil (Fisher & Learmonth 2003). The larvae undergo 5-8 instars, and sometimes up to 11, depending on the prevailing weather conditions (Pringle *et al*. 2015; Allsopp *et al*. 2015). Damage to fruits by the adults starts in December and extends to February (Fisher & Learmonth 2003). As the adults cannot fly, they need to crawl up the tree from the soil, seeking shelter during the day, and feeding at night.

The control of BFW in the Western Cape includes the use of trunk barriers to prevent weevils reaching the fruit (Fisher & Learmonth 2003; Pringle *et al*. 2015). In Australia, the combination of pyrethroids and trunk exclusion barriers is used to minimise the extent of damage caused by the BFW (Fisher & Learmonth 2003). However, the use of synthetic insecticides has potentially detrimental effects for the environment, with such use having the ability to leave residues on the fruit if they are applied as broad spectrum sprays. An alternative to chemical control is biological control, which uses pathogens like bacteria, fungi, nematodes, protozoa, and viruses (Lacey *et al.* 2001). Microbials are efficient and non-toxic to humans and other non-target organisms, reduce the amount of pesticide residues, and can be applied using standard pesticide equipment. They also encourage the conservation of biodiversity by heightening the extent of preservation in ecosystems (Lacey *et al.* 2001; Shapiro-Ilan *et al.* 2006).

Entomopathogenic nematodes (EPNs) are responsible, in nature, for regulating insect populations. They are non-segmented, soft-bodied roundworms, which are naturally occurring obligate parasites of insects (Kaya & Gaugler 1993; Stuart *et al.* 1997; Campos-Herrera 2015). The genera *Heterorhabditis* and *Steinernema*, which kill insect hosts with the help of a mutualistic bacterium, have been successfully used as biological insecticides throughout the world (Shapiro-Ilan *et al.* 2003; Grewal *et al.* 2005). They can effectively control several insect pests, including numerous weevil species (Gaugler & Kaya 1990; Kaya & Gaugler 1993; Ferreira & Malan 2013).

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EPNs locate host insects in response to chemical cues, vibration and carbon dioxide released by the insect (Kaya & Gaugler 1993). The infective juveniles (IJs) carry the bacteria in their intestinal tracts (Khan & Brooks 1977; Endo & Nickle 1991; Bowan 1995). The EPNs and symbiotic bacteria work together to kill, and to utilise, a number of economic insect pests (Bleakley & Nealson 1988; Boemare & Akhurst 1988; Akhurst & Boemare 1990; Forst & Nealson 1996). IJs do not feed, but are actively searching for an insect host in the soil (Bowan 1995; Bowen & Ensign 1998). IJs enter the insect through natural openings, eventually making their way into the haemocoel, where the bacteria are released (Poinar 1990; Forst & Nealson 1996). The bacteria release toxins that play an important role in the killing of the host insect. The nematodes, on their own, are unable to kill the host insect, but, together with their symbiotic bacteria, are highly virulent (Lu *et al.* 2017).

The use of EPNs to control the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), has been successful in terms of glasshouse crops and potted plants (Lola-Luz & Downes 2007; Susurluk & Ehlers 2008; Haukeland & Lola-Luz 2010). EPNs have been found to be able to reduce the number of *O. sulcatus* on strawberry, after the EPNs were applied to the soil, or straw, concerned (Wilson *et al.* 1999). Lola-Luz *et al.* (2005) also reported up to 93.4 % and 51.3 % mortality with a single application of *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987 and *Heterorhabditis downesi* Stock, Griffin & Burnell, 2002, respectively, against black vine weevil larvae. In South Africa, Ferreira & Malan (2013) reported that locally isolated EPNs (*Heterorhabditis bacteriophora* (SF134), *H. zealandica* (SF41), and *Steinernema khoisanae* (106-C)) can be used as biological control agents against the BFW larvae and adults. However, their study did not consider the susceptibility of BFW pupae to EPNs, and no field trials were conducted.

In the current study, the main objective was to establish the potential of EPNs to control the larvae, pupae, and adults of BFW under laboratory conditions. Further bioassays were conducted to establish optimum nematode concentrations; to compare the virulence levels between two local EPN species; and to establish the ability of EPNs to detect, and infect, the BFW in a sand column bioassay. Finally, field trials were conducted to determine the control of the BFW larvae, after the application of EPNs at different concentrations, in a small-scale orchard environment.

3.3. MATERIALS AND METHODS

3.3.1. Source of insects

Adult BFW that were collected from vineyards and orchards in the Western Cape from October to December and were mass reared at the Stellenbosch University Insectary (IPM Initiative),

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Stellenbosch University, South Africa. Corrugated cardboard bands were used to collect the weevils by tying them onto apple and vine trunks. The adult weevils, ages ranged from 2 to 4 weeks old, were kept in a ventilated Perspex boxes measuring 15 × 20 cm. Weevils were reared on carrots (personal communication, D. Stenekamp).

3.3.2. Source of nematodes

The IJ rearing and harvesting procedures were carried out according to the methods presented by Kaya & Stock (1997), using mealworm larvae kept at room temperature (±25°C). The IJs were harvested from the White trap during the first week of emergence, stored horizontally using 500-ml vented culture flasks, and used within one month after harvesting. The culture flasks were shaken on a weekly basis, to increase the amount of aeration, and the survival of the IJs, during storage. The endemic EPN species that had been collected in previous local surveys were used because they were readily available, with them being currently maintained in Stellenbosch University's nematode collection. The origin of the different species used in the study is indicated in Table 3.1 below.

Table 3.1. Different *Steinernema* and *Heterorhabditis* isolates, with their associated host plants, origin and GenBank accession number.

Species	Isolate	Associated host plant	Origin	GenBank number
S. jeffreyense	J194	Guava tree	Jeffrey's Bay, Eastern Cape	KC897093
S. yirgalemense	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295
S. feltiae	-	-	e-nema, Germany	-
S. sacchari	SB10	Sugarcane	KwaZulu-Natal	KC633095
H. bacteriophora	SF351	Grapevine	Wellington, Western Cape	-
H. baujardi	MT19	Natural vegetation	KwaZulu-Natal	MF535520
H. noenieputensis	SF669	Fig tree	Noenieput, Northern Cape	JN620538
H. zealandica	SF41	Natural vegetation	Patensie, Eastern Cape	EU699436
H. indica	SGS	Grapevine	Bonnievale, Western Cape	GQ377411

3.3.3. Bioassay protocol

Virulence experiments were conducted in 24-well bioassay plates (flat-bottom, Nunce, Cat. No.144530, Thermo Fisher Scientific [Pty] Ltd, Gauteng, Johannesburg, South Africa). Alternate wells were lined with a circular piece of 13-mm-diameter filter paper, to secure an even distribution throughout the well. Each piece of filter paper was inoculated with a predetermined concentration (Navon & Ascher 2000) of IJs in filtered tap water. A control treatment comprised

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of 50 µl filtered tap water. One insect was added to each alternate well, and the trays were closed with the lid, placed in a closed plastic container lined with moistened tissue paper, and left in a growth chamber at 25°C for 48 hours. For each treatment, five trays with 12 wells were used (n = 60). Mortality was determined by means of confirming infection by dissection, and by way of visually observing the presence of nematodes, using a stereomicroscope. All bioassays were repeated for all the treatments on different test dates, and with a freshly prepared nematode inoculum.

3.3.4. Screening

The 24-well bioassay protocol was used to test the potential of *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams 2004, *Steinernema jeffreyense* Malan, Knoetze & Tiedt and *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt 2014 to infect the BFW larvae under optimal laboratory conditions. Five trays were used for each nematode species, with another five trays being used for the control. Each alternate bioassay well with a BFW larva (n = 60), was inoculated with 100 IJs / 50 µl water, while the control received distilled water only. Mortality by nematode infection was confirmed after 48 h, by dissection. The procedure was repeated on a separate date, with a different batch of nematodes.

3.3.5. Nematode concentrations

To determine the effect of different concentrations of the EPNs on the mortality rate of the BFW larvae, the 24-well bioassay protocol was used. The BFW larvae were exposed to different concentrations (400, 200, 100, 50, 20, and 0 IJs/insect) with *S. yirgalemense* and *H. noenieputensis*. For each concentration tested, five bioassay plates with 12 larvae (n = 60) were used, while the control treatments received distilled water only. The mortality caused by the nematode infection was confirmed by means of dissection after 48 h. Probit analysis was performed to obtain the LD_{50} and LD_{90} for *S. yirgalemense* and *H. noenieputensis*.

3.3.6. Susceptibility of different life stages

Using the 24-well bioassay protocol, available *Steinernema feltiae* Wouts, Mráček, Gerdin & Bedding 1982, *H. noenieputensis*, *Heterorhabditis indica* Poinar, Karunakar & David, *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens, S. *yirgalemense Steinernema sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan and *Heterorhabditis zealandica* Poinar, at 100 IJs/insect, were used to test the susceptibility of the BFW larvae and pupae, while 200 IJs/insect

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were used to test the susceptibility of the BFW adults. Fresh inoculum of the nematode species and insect stage tested, depended on the availability of both at the same time. Five bioassay plates (n = 60) were used for each nematode isolate, with the control plates receiving only water. Mortality were determined after 48 h, whereupon the insects were removed from the plates, and washed to remove the surface nematodes. The number of IJs penetrated was then determined by means of dissection with the aid of a dissection microscope. The procedure was repeated on a separate date, with a different batch of nematodes.

3.3.7. Sand bioassay

The use of a sand bioassay is regarded a more natural way of screening for EPN. Sand was autoclaved, dried and sieved through a mesh screen to result in 500-µm particle size. Centrifuge tubes measuring 1.5 cm in length 15 cm in diameter were filled moist river sand (10:100 v/v, river sand to water) that was frozen overnight (Yu *et al.* 2008). The BFW larvae were placed in Eppendorf tubes with 0.2-ml thin walls, used mostly for polymerase chain reactions (PCRs), so as to simplify retrieval. The Eppendorf tubes were pierced, using a heated needle to make 10 holes (Kehres *et al.* 2001). The tubes containing a BFW larva were placed at the bottom of each of the 30 sand-filled centrifuge tubes. *Heterorhabditis baujardi*, *H. indica* and *S. yirgalemense* at 100 IJs/insect, for each treatment, were added by means of pipetting onto a 13-mm-diameter bioassay disk, turned upside down, and placed on top of the sand in each tube to prevent the nematodes from being washed into the soil. The control treatments received water only. All the columns were covered to prevent excess water loss. The mortality caused by infection of the BFW larvae was confirmed by means of dissection after 48 h. The experiment was conducted on two separate test dates, and with freshly prepared inoculum.

3.3.8. Field trial

Eight apple trees in four rows, which received treatments in a Completely Randomised Design, were exposed to *S. yirgalemense* at 0, 10, 20, and 40 IJs/cm^2 over a period of 7 days. The first and last plants, and the first and the last rows, were not treated, so as to avoid edge effects. The four treatments were applied at the rate of 10 BFW larvae per plant. The 10 perforated 0.2-ml Eppendorf tubes were tied together using a piece of cotton thread with BFW larvae in each tube, after which the lids were closed (Le Vieux & Malan 2013). The Eppendorf tubes were buried close to the treatment plant, and at a depth of 15 cm, with the thread left extending above the soil, for ease of detection and retrieval.

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The desired concentration of the pathogens was prepared in 200 ml water, with an area of 0.8 × 1 m being measured around each treatment plant. After burying the BFW tubes and four iButton temperature loggers (Wdsen Electronic Technology Co. Ltd, Philippines), the EPN suspensions were sprayed onto the soil, using a handheld spray bottle. Each treatment plant was drip irrigated only if no natural rainfall had occurred between treatments. The BFW tubes were removed from the soil after 7 days, rinsed with water, placed on moist filter paper in Petri dishes, and sealed using Parafilm®. The Petri dishes were placed in a 25°C growth chamber for 24 h, with the mortality rate being recorded. The mortality caused by the EPNs was confirmed by means of dissecting the BFW larvae and observing the presence of IJs.

3.3.9. Data analysis

Virulence assays were analysed using analysis of variance (ANOVA); if the F value was significant ($p < 0.05$), the means were differentiated by LSD MEANS (SAS Institute 1985). The mortality data were corrected for the corresponding control mortality, using the formula: CM (%) = {(T–C)/(100–C)}×100, where CM is the corrected mortality, T is the percent mortality in treated insects, and C is the percent mortality in untreated insects (Abbott 1925). If no significant interactions were found to occur between the main effect of the test dates and the treatments concerned, the data obtained from the two test dates were pooled and analysed, using a one-way ANOVA.

3.4. RESULTS

3.4.1. Screening

A significant effect ($F_{(1, 58)} = 4.1$, $p < 0.05$) of the nematode treatments (100 IJs/insect) was found on the BFW larval mortality, after a period of 48 h. Mortality of 70.8 % and 54.4 % was recorded for *S. yirgalemense* and *H. noenieputensis*, respectively, with the mortality rate concerned differing significantly ($p < 0.05$) between the two (Fig. 3.1).

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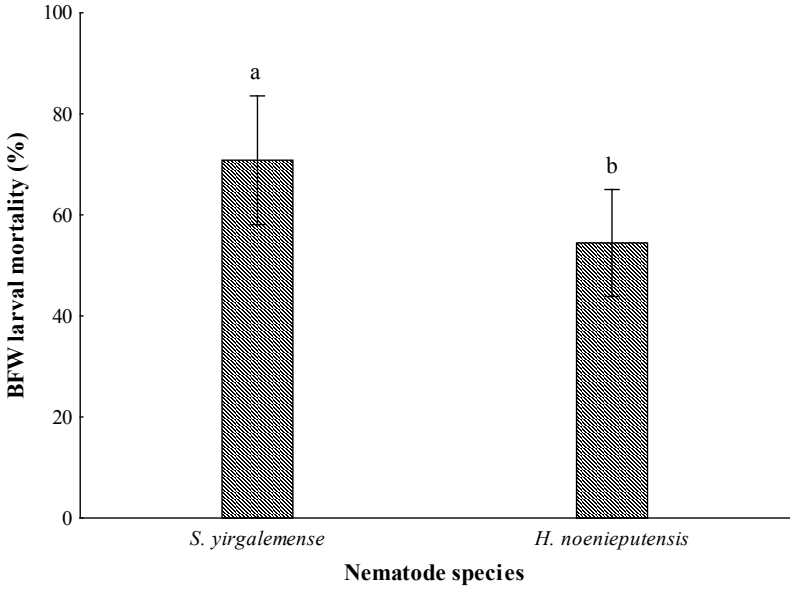

Fig. 3.1. Mean percentage mortality (95 % confidence level) of *Phlyctinus callosus* (BFW) larvae, after exposure to *Steinernema yirgalemense* and *Heterorhabditis noenieputensis* at 100 IJs/insect for 48 h (one-way ANOVA: $F_{(1, 58)} = 4.1$, $p < 0.05$). Bars with the same letter do not differ significantly ($p < 0.05$).

3.4.2. Nematode concentrations

The mean mortality ranged from 53.3 % to 95.0 % after the BFW larvae were inoculated with *S. yirgalemense* and *H. noenieputensis* during the different nematode treatments over a period of 48 h. A significant effect ($F_{(5, 108)} = 8$, $p < 0.001$) of the concentration levels on mortality was found. All nematode treatments differed significantly ($p < 0.05$) from the control treatment. *Steinernema yirgalemense* at 400 IJs/insect (95.0 % mortality) showed no significant difference in BFW larval mortality, in comparison with *H. noenieputensis* (89.2 %) at 400 IJs/insect, and of *S. yirgalemense* at 200 IJs/insect (93.3 % mortality) and 100 IJs/insect (85.0 % mortality) (Fig. 3.2). *Steinernema yirgalemense* at 25 IJs/insect (67.5 % mortality) differed significantly ($p < 0.05$) from the two higher concentrations (200 IJs/insect and 400 IJs/insect), with no significant difference, compared to *S. yirgalemense*, at 50 IJs/insect (80.3 % mortality) (Fig. 3.2). In the case of *H. noenieputensis*, the only significant difference were found in the highest nematode treatment of 400 IJs/insect compared to the other nematode treatments (Fig. 3.2).

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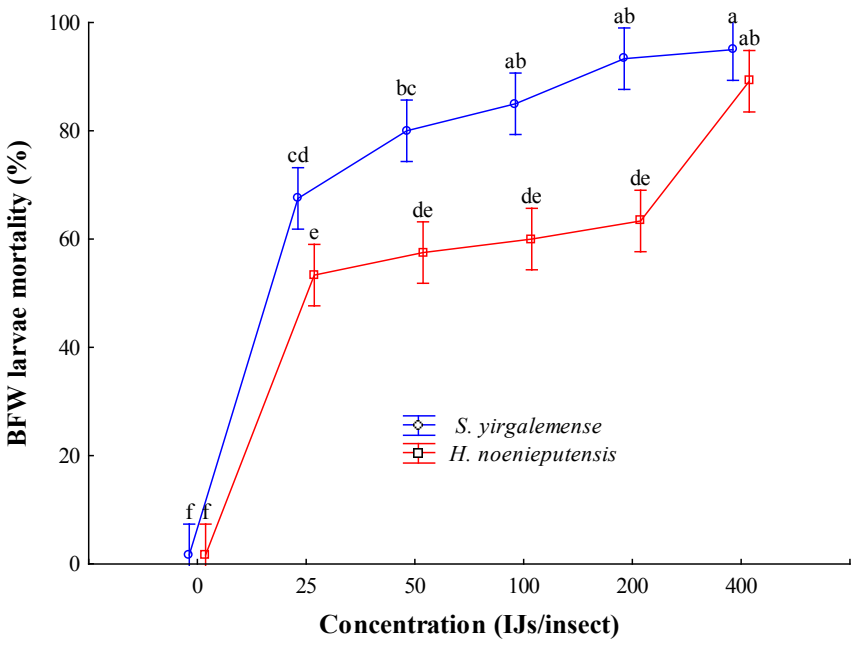

Fig. 3.2 Mean percentage mortality (95 % confidence level) of *Phlyctinus callosus* (BFW) larvae after exposure to five different concentrations of *Heterorhabditis noenieputensis* and *Steinernema yirgalemense* IJs for 48 h (one-way ANOVA: $F_{(5, 108)} = 8$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

Results of a probit analysis showed that the probit regression lines for *S. yirgalemense* and *H. noenieputensis* differed. The probit regression line for *S. yirgalemense* was y = 0.828 x + 4.42, while the probit regression line for *H. noenieputensis* was y = 0.828 x + 3.74, with *S. yirgalemense* being found to be over six times (6.76) more potent than *H. noenieputensis* (Fig. 3.3). The data fitted the model employed well, showing a positive relationship between nematode concentration and percentage mortality. *Steinernema yirgalemense* had the lowest LD_{50} of 4.94 IJs per larva (90 % fiducial limits: 0.84-11.90), and an LD_{90} of 177.18 IJs/larva (90 % fiducial limits: 101.44-407.31), whereas *H. noenieputensis* had an LD_{50} of 33.38 IJs/larva (90 % fiducial limits: 14.72-54.87), and an LD_{90} of 1198.2 (90 % fiducial limits: 535.09-6172.9).

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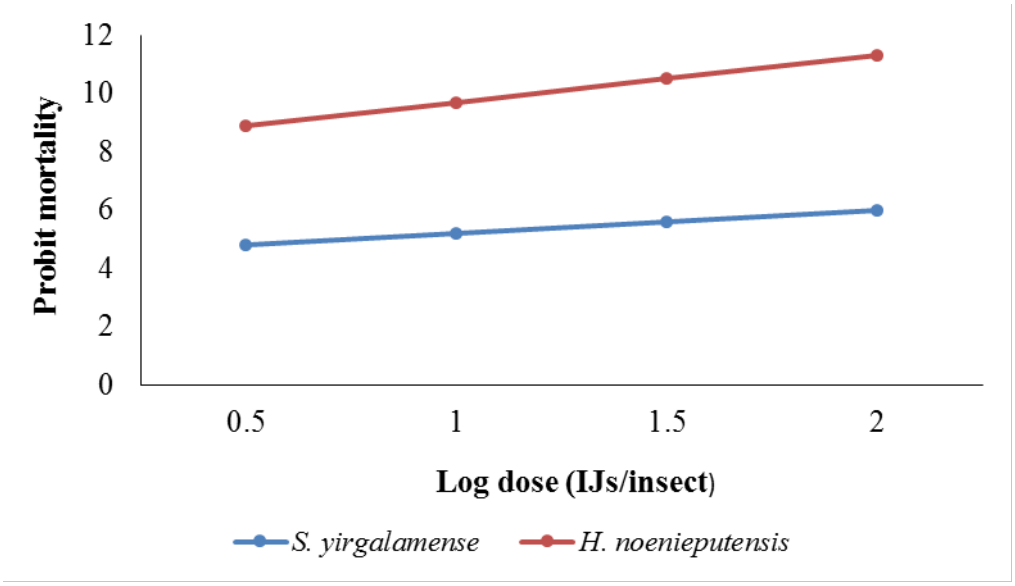

Fig. 3.3. Probit mortality of fourth-instar *Phlyctinus callosus* larvae at different concentrations (log dose) of *Steinernema yirgalemense* and *Heterorhabditis noenieputensis*; 0, 25, 50, 100, 200, and 400 IJs/larva.

3.4.3. Susceptibility of different life stages

3.4.3.1. Larvae

The mean mortality of the three EPN species inoculated with 100 IJs/insect ranged between 43.3 % and 65.8 % over a period of 48 h (Fig. 3.4A). Analysis using a one-way ANOVA showed no significant effect ($F_{(3, 36)} = 26.34$, $p < 0.001$) of the treatment on percentage mortality. *Steinernema yirgalemense, H. noenieputensis* and *S. feltiae* provided mortality (65.8 %, 47.7 %, and 43.3 % mortality, respectively) not significantly different from one another (Fig. 3.4A).

Penetration ranged from 13 to 44 nematodes per BFW larva (Fig. 3.4B), with a significant difference ($F_{(3, 36)} = 24.69$, $p < 0.001$) in penetration rate. *Steinernema feltiae* (44.0) had a significantly higher ($p < 0.05$) number of nematodes that penetrated the BFW larva, in comparison with *H. noenieputensis* (13.0), but not different from *S. yirgalemense* (28.5). The number of nematodes that penetrated the BFW larvae in the case of *S. yirgalemense* and *H. noenieputensis* (28.5 and 13.0, respectively) did not differ significantly from each other (Fig. 3.4B).

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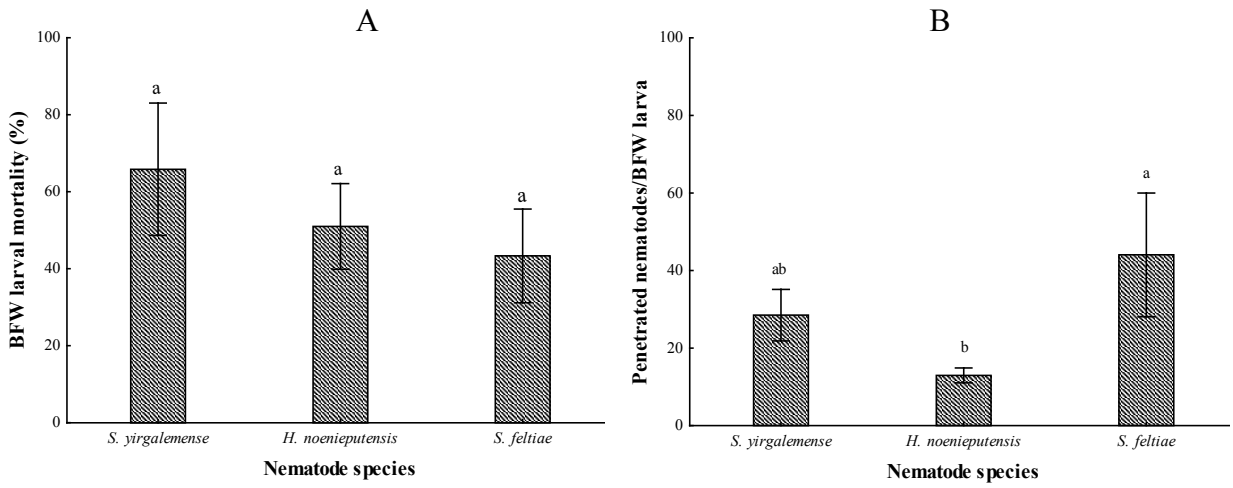

Fig. 3.4. Mean percentage mortality (A) and number of nematodes penetrated (B) (95 % confidence level) *Phlyctinus callosus* (BFW) larvae inoculated with 100 IJs/insect of *Steinernema yirgalemense*, *Heterorhabditis noenieputensis* and *S. feltiae* after 48 h. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.4.3.2. Pupae

The mean mortality of the pupae ranged from 55.2 % to 69.8 % after inoculation with 100 IJs/insect over a period of 2 days (Fig. 3.5A). A significant effect ($F_{(3, 28)} = 65.12$, $p < 0.001$) of the treatment was seen in the percentage mortality. Although *H. indica* (69.8 % mortality) and *H. baujardi* (66.7 % mortality) gave significantly higher ($p < 0.05$) mortality of BFW pupae, the mortality did not differ significantly from each other. However, *H. noenieputensis* (55.2 % mortality) gave significantly lower ($p < 0.05$) control of the BFW pupae (Fig. 3.5A).

The penetration by IJs ranged from 9.5 to 12.4 nematodes per BFW pupa (Fig. 3.5B). No significant difference ($F_{(3, 28)} = 37.51$, $p < 0.001$) was found between the different treatments on the penetration rate of the BFW pupae. *Heterorhabditis indica*, *H. baujardi* and *H. noenieputensis* gave 10, 9.5, and 12.4 nematodes that penetrated the BFW pupae, respectively (Fig. 3.5B).

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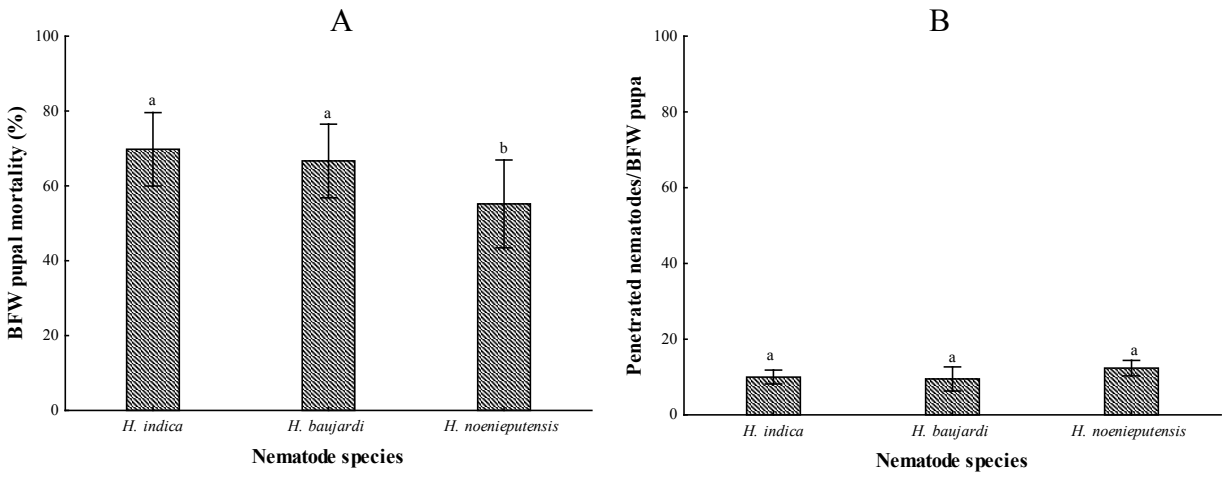

Fig. 3.5. Mean percentage mortality (A) and number of nematodes penetrated (B) (95 % confidence level) of *Phlyctinus callosus* (BFW) pupae inoculated with 100 IJs/insect of *Heterorhabditis indica*, *H. baujardi* and *H. noenieputensis*. Different letters above the vertical bars indicate significant differences.

3.4.3.3 Adults

Adult mortality ranged between 24.2 % and 95.0 % after inoculation with 200 IJs/insect over a period of 2 days. The treatment had a significant effect ($F_{(5, 54)} = 77.21$; $p < 0.001$) on the percentage of adult mortality. *Heterorhabditis indica* (95 % mortality) and *S. yirgalemense* (94.2 % mortality) rendered significantly higher ($p < 0.05$) mortality of the adult BFW, with the mortality of the two not being significantly different from each other. *Heterorhabditis bacteriophora* (35 % mortality), *H. zealandica* (24 % mortality), and *S. jeffreyense* (28 % mortality) gave significantly lower ($p < 0.001$) mortality of the adult BFW, with their mortality not significantly different from that of one another (Fig. 3.6A).

The penetration by IJs ranged from 2.2 to 107.5 nematodes per BFW adult, with a significant difference ($F_{(4, 45)} = 576.57$, $p < 0.001$) being found between the penetration rate of the BFW adults. *Heterorhabditis indica* (107.5) gave a significantly higher ($p < 0.05$) penetration rate than did the other nematodes species. *Steinernema jeffreyense* (3.0), *S. yirgalemense* (5.2), *H. bacteriophora* (5.2), and *H. zealandica* (2.2) gave a significantly lower ($p < 0.05$) number of penetrated nematodes, with the number concerned not significantly different from one another (Fig. 3.6B).

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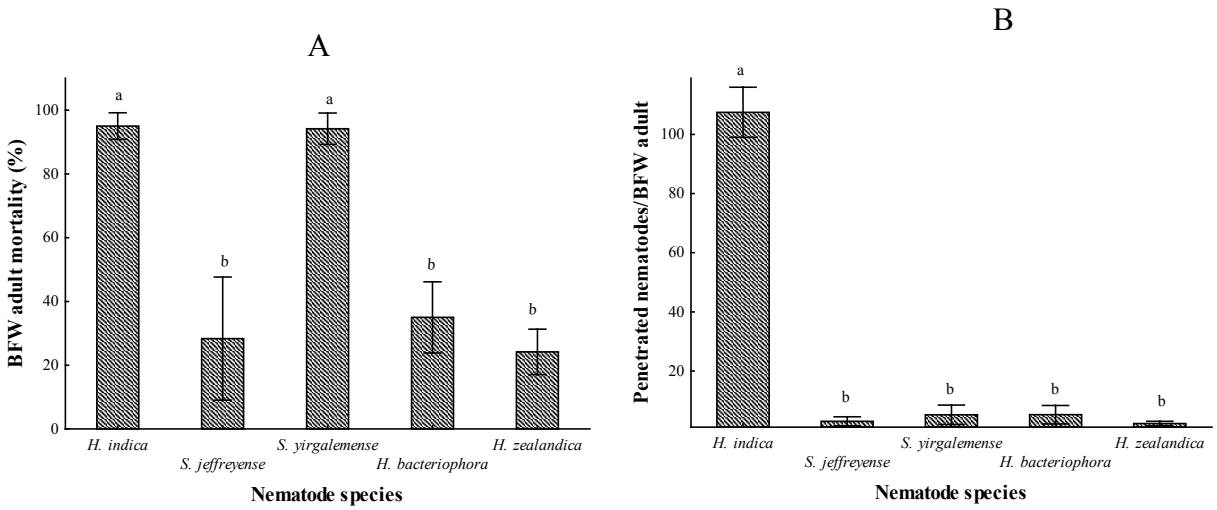

Fig. 3.6. Mean percentage mortality (A) and number of nematodes (B) penetrated (95 % confidence level) adult *Phlyctinus callosus* (BFW) inoculated with 200 IJs/insect of *Heterorhabditis indica*, *Steinernema yirgalemense*, *H. bacteriophora, H. zealandica*, and *S. jeffreyense*. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

Heterorhabditis indica was further evaluated for virulence, and for its ability to suppress the BFW adults, compared to other, smaller, EPNs (*H. noenieputensis, H. baujardi*, and *S. sacchari*). The mortality ranged from 4.2 % to 92.5 % after inoculation with 200 IJs/insect over a period of 48 h. A significant effect ($F_{(4, 45)} = 140.26$; $p < 0.001$) of the treatment was found on the percentage of adult mortality. *Heterorhabditis baujardi* (93 % mortality) and *H. indica* (84.2 % mortality) rendered significantly higher ($p < 0.05$) mortality of the adult BFW, with the differences concerned not being significant. *Heterorhabditis noenieputensis* (29.2 % mortality) and *S. sacchari* (4.2 % mortality) gave significantly lower ($p < 0.05$) mortality of the adult BFW, with the two not differ significantly from each other (Fig. 3.7A).

The penetration by IJs ranged from 2.9 to 102.7 nematodes per BFW adult, with a significant difference ($F_{(3, 36)} = 154.28$, $p < 0.001$) being detected in the penetration rates of the BFW adults. *Heterorhabditis indica* (102.7) gave a significantly higher ($p < 0.05$) number of penetrated nematodes than did the other nematode species. *Heterorhabditis baujardi* (58.3) gave a significantly lower ($p < 0.05$) number of penetrated nematodes than did *H. indica,* but higher than *H. noenieputensis* (2.9) and *Steinernema sacchari*. *Heterorhabditis noenieputensis* and *S. sacchari* (6.5) gave a significantly lower ($p < 0.05$) number of penetrated nematodes, with them not being significantly different from each other (Fig. 3.7B).

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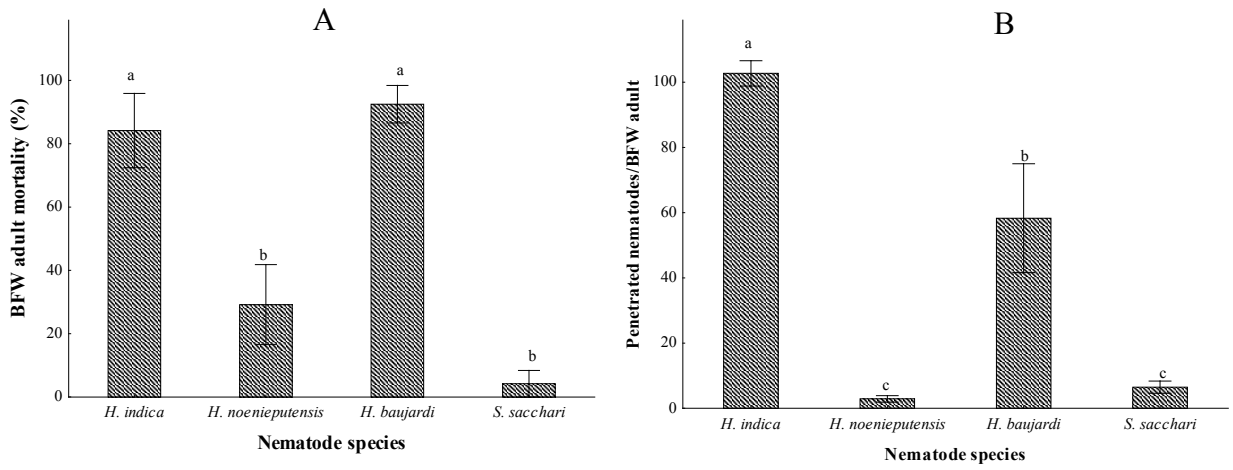

Fig. 3.7. Mean percentage mortality (A) and number of nematodes penetrated (B) (95 % confidence level) of the adult *Phlyctinus callosus* (BFW), inoculated with 200 IJs/insect of *Heterorhabditis indica*, *H. noenieputensis, H. baujardi*, and *Steinernema sacchari*. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.4.4 Sand bioassay

The mortality of BFW larvae in sand ranged from 58.3 % to 66.7 % after inoculation with 100 IJs/insect over a period of 48 h. No significant effect ($F_{(3, 20)} = 40.54$, $p < 0.001$) of the treatment was detected in terms of the percentage mortality. Although the inoculation of *H. baujardi* (58.3 % mortality) resulted in lowering of the mortality, it did not differ significantly from that of *H. indica* (66.7 % mortality) and *S. yirgalemense* (61.7 % mortality) (Fig. 3.8A).

The penetration rate of *H. indica*, *H. baujardi*, and *S. yirgalemense* ranged from 5.5 to 31.6 nematodes per BFW larva in the sand, which had a significant effect ($F_{(3, 20)} = 42.61$, $p < 0.001$) on the larvae that had been penetrated in the sand. *Steinernema yirgalemense* and *H. indica* (31.6 and 16.0, respectively) gave a significantly higher ($p < 0.05$) number of nematodes that penetrated the BFW larvae in the sand, with no significant difference between the two. *Heterorhabditis baujardi* gave significantly lower ($p < 0.05$) number of nematodes that penetrated BFW larvae in the sand (5.5), although the number of such did not significantly differ from that of *H. indica* (Fig. 3.8B).

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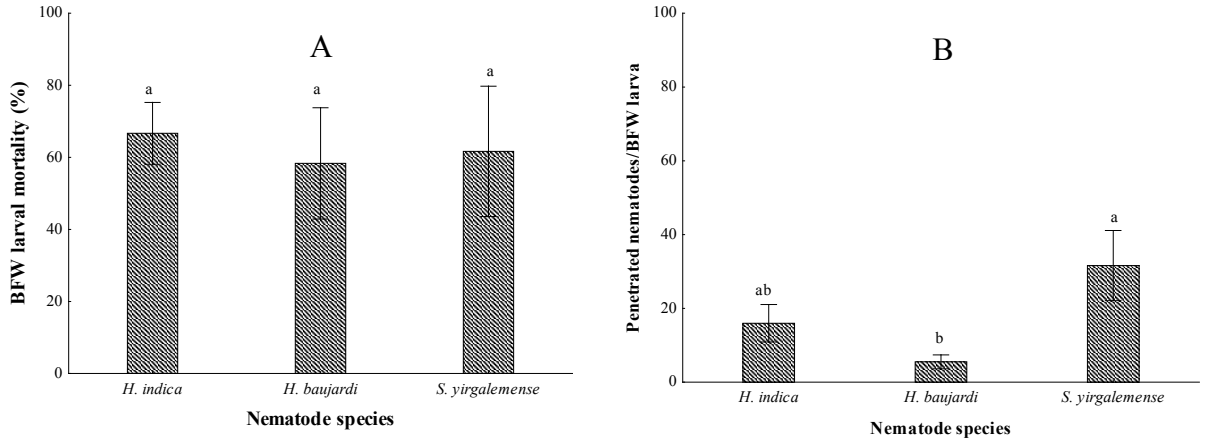

Fig. 3.8. Mean percentage mortality (A) and number of nematodes (B) penetrated (95 % confidence level) of *Phlyctinus callosus* (BFW) larvae inoculated with 100 IJs/insect of *Heterorhabditis indica*, *H. baujardi* and *Steinernema yirgalemense*. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.4.5 Field trial

The soil temperatures during the exposure period ranged from between 8°C in the morning to 30°C in the afternoon. The mortality of BFW larvae in the field trial ranged from 20 % to 77.5 % after inoculation with 0, 10, 20, and 40 IJs/cm^2 over a period of 7 days, with significant effect ($F_{(3, 20)} = 40.54$, $p < 0.001$) of the treatment on the percentage mortality (Fig. 3.9). *Steinernema yirgalemense*, at 40 IJs/cm^2 (77.5 % mortality), gave significantly greater ($p < 0.05$) mortality of the BFW larvae, which did not differ significantly from that obtained with 20 IJs/cm^2 (68.8 % mortality). *Steinernema yirgalemense*, at 20 IJs/cm^2, gave mortality of BFW larvae not significantly different from 10 IJs/cm^2 (43.8 % mortality). *Steinernema yirgalemense* at 10 IJs/cm^2 (43.8 % mortality) gave significantly lower ($p < 0.05$) control of BFW larvae, with no significant difference from that which was obtained with 0 IJs/cm^2 (20 % mortality) (Fig. 3.9).

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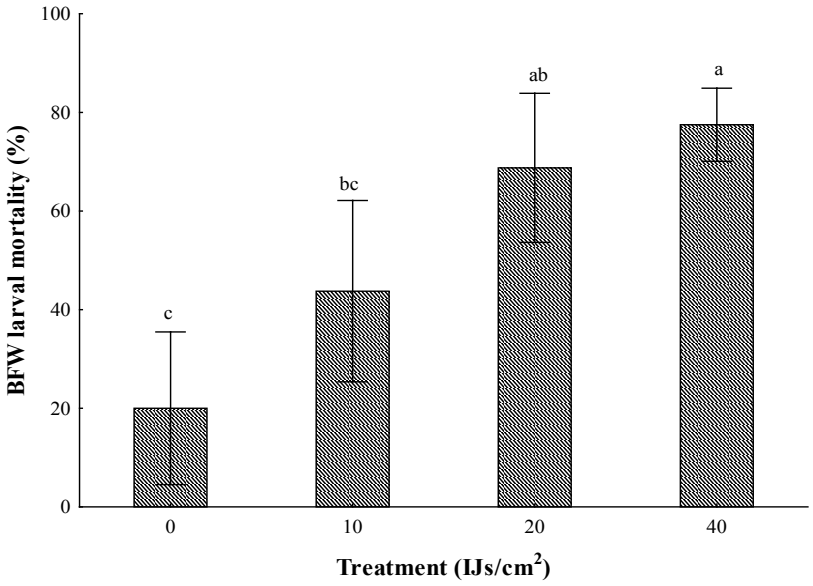

Fig. 3.9. Mean percentage mortality (95 % confidence level) of *Phlyctinus callosus* (BFW) larvae inoculated with 0, 10, 20, and 40 IJs/cm^2 of *Steinernema yirgalemense* (one-way ANOVA: $F_{(3, 28)} = 17.50$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.5. DISCUSSION

In an initial screening, *S. yirgalemense* and *H. noenieputensis* were used for the first time against laboratory-cultured larvae of the BFW, with successful control being obtained for both species. When using *S. yirgalemense*, *H. noenieputensis,* and *S. feltiae*, no significant differences were found between the three EPN species. This indicates that the exotic species *S. feltiae* did not obtain significantly better control than did the local species. In both screenings, *S. yirgalemense* was the best performer against the BFW larvae. Ferreira & Malan (2013) obtained good results with the control of BFW larvae in the laboratory, using the local isolates of *H. zealandica* and *H. bacteriophora*, with *H. zealandica* being the best performing nematode. However, the concentration and exposure time of the nematodes to *P. callosus* larvae was double the concentration, in comparison to the present study. Previously, *Heterorhabditis* spp. were thought to be the superior performer in the control of the black vine weevil (Lola-Luz *et al.* 2005; Lola-Lux & Downes, 2007). However, Shapiro & McCoy (2000) showed in their study of nine different species of EPNs, that specific species of *Steinernema* provided enhanced control of BFW larvae over that obtained with the *Heterorhabditis* species. The above concurs with the results obtained in the present study, as *S. yirgalemense* gave superior results in terms of the control of BFW larvae in comparison with that obtained with the *Heterorhabditis* species.

Using different nematode concentrations, a positive relationship was found between the number of IJs and the mortality of BFW larvae. At lower concentrations, significant differences were found

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between *S. yirgalemense* and *H. noenieputensis*, but, at the higher concentration (400 IJs/insect), no significant differences were found, with the mortality being close to 100 %. The lethal doses were recorded as an LD_{90} of 177 and 1198 IJs/larva for *S. yirgalemense* and *H. noenieputensis*, respectively. Ferreira & Malan (2013) also found a positive relationship between the IJ concentration and the mortality of BFW larvae after exposure to *H. zealandica* for 4 days, with the LD_{90} value recorded as 278 IJs/50 ml after 4 days.

The current research is the first laboratory study to have been undertaken on the potential use of EPN to control the pupal stages of the BFW. Mortality was shown to range from 55 % to 70 % with *H. indica*, *H. baujardi*, and *H. noenieputensis* when administering 100 IJs/insect. In general, the pupal stages seem to be at least half as sensitive as are the larval stages, requiring increased concentrations, with the lengthening of exposure times to the nematodes, showing some resistance to nematode infection. De Waal (2008) showed that, in the case of *Cydia pomonella* (Lepidoptera: Tortricidae) codling moth, the pupa is not as susceptible to nematode infection as is the larval stage. The same finding was earlier made by Malan *et al.* (2011) in the case of false codling moth.

The adult BFW mortality was found to range between 24 % and 95 %, using double concentrations (200 IJ/insect) of *H. indica*, *S. yirgalemense*, *H. bacteriophora, H. zealandica*, and *S. jeffreyense*. In all cases, mortality was determined after 2 days of exposure to nematodes in the laboratory. Ferreira & Malan (2013) found that the mortality of BFW adults ranged from 13 % to 45 %, using double the concentration of IJs/insect for *H. zealandica*, *H. bacteriophora,* and *S. khoisanae* after 4 days. Usually, nematodes are not as effective against adult BFWs, because of the short period during emergence from the soil that the insects concerned are susceptible, as well as due to their grooming behaviour. However, as the BFW is flightless, they have to crawl up the tree, which opens up new possibilities of contact with nematodes, and of subsequent control through the use of targeted application methods.

The IJ penetration of BFW larvae was found to range from 13 to 37 nematodes for *S. yirgalemense*, *H. noenieputensis*, and *S. feltiae*. Pupal penetration by IJs ranged from 10 to 12 nematodes with *H. indica*, *H. baujardi* and *H. noenieputensis*. In both cases, 100 IJs/insect and exposure to the nematode of 48 h were applied. The adult BFW penetration ranged from 2 to 108 nematodes per BFW adult. *Heterorhabditis indica* obtained the highest number of nematode penetration for adult weevils, which coincided with high mortality. In the follow-up trial, using four heterorhabditid species, *H. baujardi* performed better than *H. indica* concerning adult mortality, while also showing the second-highest penetration of the four heterorhabditids tested.

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The results reflected that, in general, the lowest penetration was found for the pupae, followed by that which was obtained for the larvae. Surprisingly high IJ penetration, with correspondingly high mortality of adults, was found in the case of *H. indica* and *H. baujardi*, even though the same concentrations and exposure times were used.

EPNs tend to penetrate insect hosts that have already been occupied by EPNs of the same species, leading to overcrowding (Lewis *et al.* 1996; Griffin 2012). Glazer (1997) states that the time since the first occupation has taken place is, however, limiting. *Steinernema* spp. were limited from entering the insect host that had been exposed to IJs of the same species 6 to 9 hours before the experiment. To further support the above, Christen *et al.* (2007) report that the penetration by IJs of *Steinernema riobrave* Cabanillas, Poinar & Raulston declined over time, with the IJs penetrating the host for 72 hours after first infection. Such behaviour did not correspond adequately with the fact that overcrowding leads to lower reproduction rate of the EPNs involved (Koppenhöfer & Kaya 1995; Ryder & Griffin 2002). To explain such behaviour, Christen *et al.* (2007) state that in the case where there are no possible hosts available, or when only low-quality hosts are available, the IJs prefer penetrating the already penetrated host than to reject it and die of starvation. In the current study, however, overcrowding was not observed.

The control of BFW larvae in the sand bioassay ranged between 58 % and 67 % having been inoculated with 100 IJs/insect of *H. indica*, *H. baujardi*, and *S. yirgalemense* after 48 h. However, Le Vieux & Malan (2013) report a much higher mean percentage mortality, ranging between 82 % and 95 % in sand bioassays, when *H. zealandica* and *S. yirgalemense* were used to control the vine mealybug, *Planococcus ficus* (Signoret). In another study by Ferreira & Malan (2013), they reported a percentage mortality ranging between 71 % and 80 %, when *P. callosus* larvae were buried at four different depths and inoculated with *H. zealandica* at a concentration of 400 IJs/100ml for 7 days. The current results showed that EPNs are able to locate and infest BFW adults and immature stages in the soil, making EPNs viable biocontrol agents to manage the soil borne stages of BFW.

Laboratory conditions are not necessarily representative of the field performance of nematodes. Different local EPN species, of which the freshly harvested IJs coincided with the availability of a specific life stage of BFW, were tested for susceptibility under optimum conditions in field trials. During the trial, *S. yirgalemense* at 20 and 40 IJs/cm^2 gave >60 % control of BFW larvae. This is the first field application of EPNs that has been performed for the control of BFW under South African conditions, with it indicating that BFW larvae could be controlled by the use of EPNs in

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the field. In another field trial in Australia, Curran & Patel (1988) recorded that *H. bacteriophora* could successfully reduce the number of vine weevil, *Otiorhynchus sulcatus* (Fabricius), and *P. callosus* larvae and pupae by up to 59 % and 25 %, respectively, when *H. bacteriophora* was applied using a trickle irrigation system in strawberry plants. In New Zealand, Prestidge & Willoughby (1990) reported 90 % and 100 % control of BFW larvae and pupae after 62 hours, when 10 and 15 IJs/larva of *H. bacteriophora* were topically applied. They also found 55 %, 70 %, and 80 % control of BFW larvae and pupae in pots after 28 days when 5×10^4, 10×10^4, and 250×10^4 IJs/pot were inundatively applied. In the citrus orchards in Florida, the generally recommended rate for the application of *Steinernema riobrave* Cabanillas, Poinar & Raulston, 1994 is 25 IJs/cm^2 against citrus root weevil, *Diaprepes abbreviates* (Linnaeus 1758), to ensure control has been found to be comparable to that of chemical application (Georgis & Hague 1991; Gaugler *et al*. 2000).

The results from the current study indicated that the local EPN species were effective against all stages of BFW in both the laboratory bioassays and the field trials, with clear differences among the species tested. *Heterorhabditis indica* and *S. yirgalemense* were found to be highly virulent against the pupae and adults of BFW, respectively. *Steinernema yirgalemense*, at 20 and 40 IJs/cm^2, showed potential to control the soil stages of BFW in the field. However, further research into the biological control of BFW is still required, including the screening of entomopathogenic fungi (EPF), and the combination of the two biological control agents, to assess possible synergistic and additive effects (Koppenhöfer & Kaya 1997; Koppenhöfer *et al.* 1999; Ansari *et al.* 2008). The present study showed BFW adults to be susceptible at 200 IJs/insect, opening the possibility of controlling the adults using *H. indica* and *S. yirgalemense*. *Steinernema yirgalemense* was also found to be highly virulent against the adult female vine mealybug, *Planococcus ficus* (Signoret) (Le Vieux & Malan 2013), which is also a major pest of grapevine in South Africa, making the biological control option concerned relatively financially viable.

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Chapter 4

Potential of entomopathogenic fungi to control the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae)

4.1. ABSTRACT

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae), banded fruit weevil (BFW) is a sporadic pest, causing damage to fruits, leaves and roots of deciduous fruits and grapevines in the Western Cape province. Using trunk barriers to prevent the flightless weevils from reaching the leaves and fruits is labour-intensive. Entomopathogenic fungi (EPF) are effective biocontrol agents for the control of soilborne and above-ground insect pests. Different EPF isolates were screened at 1×10^6 conidia ml^{-1} for virulence against different life stages (larvae, pupae and adults) of the BFW. The results indicated that Broadband® (*Beauveria bassiana*) and Meta 69® (*Metarhizium anisopliae*) resulted in significantly higher BFW larval mortality (96.7% and 92.5% mortality, respectively) than Eco-Bb® (*B. bassiana*) (58.3 %). In the case of pupae, Broadband® (91.7 %) resulted in significantly higher mortality than Eco-Bb® (67.5 %) and Meta 69® (65.8 %). In the case of adult BFW mortality, Broadband® caused 90 % mortality, which differed significantly from that obtained with for Eco-Bb® (69.2 %) and Meta 69® (65 %), whose percentage of control did not differ from each other. In terms of comparing the adult mortality attained with that which was achieved with a local EPF isolate, Broadband® (91.7 %) again gave significantly higher mortality of BFW adults compared to what was attained with Eco-Bb® (65 % mortality), Meta 69® (59.5 % mortality) and *M. anisopliae* isolate EA2 (64.2 % mortality). In a sand bioassay using final instar BFW larvae, Broadband® (85 %) resulted in higher mortality than either Eco-Bb® (55 % mortality) or Meta 69® (70 % mortality). The results indicated that all the EPF isolates tested were effective against the immature and adult stages of the BFW, but with clear differences between the EPF isolates. Broadband® consistently cave the best results BFW under laboratory conditions. Large-scale field trials are recommended to further demonstrate the potential use of this biocontrol agent in integrated pest management programmes.

Keywords: *Beauveria bassiana*, biocontrol, entomopathogenic fungi, *Metarhizium anisopliae*, trunk barriers

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4.2 INTRODUCTION

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae), or banded fruit weevil (BFW), is one of the major insect pests of grape and apple varieties, that and are indigenous to the Western Cape province (Lounsbury 1896; Barnes & Capatos 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). Lounsbury (1896) reported the weevil as being a major pest of grapes in South Africa, even before it spread to New Zealand and the southern states of Australia in 1899 (Lounsbury 1896; Kuschel 1972). The weevil has the potential to cause considerable damage to several crops, with it recently having been found to be a serious pest of blueberries (Ferreira 2010; Pringle *et al*. 2015; Allsopp *et al*. 2015).

Annual losses due to BFW damage were reported to be up to R4 million by Barnes and Pringle (1989). Damage is reportedly caused by adult BFW on the fruits, whereas the larvae feed on the roots of various plants (Barnes 1989a, b; Barnes & Pringle 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). Adults feed on the skin and the underlying flesh of apples, resulting in the formation of shallow lesions on the damaged fruits (Barnes 1989a; Fisher & Learmonth 2003; Pringle *et al*. 2015). Other damage caused by adults to apples includes foliage damage, resulting from feeding (Barnes & Giliomee 1992; Fisher & Learmonth 2003; Pringle *et al*. 2015).Adult damage on grapevines includes the shot-holing of leaves, as well as leaf and stem, notching, bud and shot-tip feeding, and berry scarring. The adults feed on small green berries, causing the latter to drop (Pringle *et al*. 2015; Allsopp *et al*. 2015). Damage in orchards due to BFW larvae on the roots is less crucial than is the adult damage caused on the fruits, leaves and stems (Pringle *et al*. 2015; Allsopp *et al*. 2015). Fruit scarring results in unmarketable fruits, and the weevils that are unintentionally packed together with export fruit consignments results in the fruits being rejected (De Villiers & Pringle 2007; Pringle *et al*. 2015; Allsopp *et al*. 2015).

Although the use of trunk barriers is effective in preventing the BFW from reaching the fruits, the need exists for an alternative control strategy, because applying trunk barriers is labour-intensive, particularly for large orchards and vineyards (Barnes 1991; Barnes *et al*. 1994; Fisher & Learmonth 2003). The use of pyrethroids, in contrast, has a number of disadvantages, and they are not compatible with the integrated pest management programmes that are used in the Western Cape (Pringle *et al*. 2015; Allsopp *et al*. 2015). Therefore, the need exists for new and environmentally-friendly BFW control strategies.

Ansari *et al*. (2008) report that curculionids can be controlled by means of entomopathogenic fungi (EPF). The fungi, which act on contact, do not require ingestion, can easily be mass-produced, and are host-specific (Shahid *et al*. 2012). EPF are found in the divisions Ascomycota,

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Zygomycota, Deuteromycota, Chytridiomycota, and Oomycota (Shahid *et al*. 2012). A group of more than 750 species from 85 genera are found in the different classes of fungi that can parasitise almost all insect groups (Shahid *et al*. 2012). Deshpande (1999) reports that most groups containing EPF include such genera as *Metarhizium*, *Beauveria*, *Verticillium*, *Nomuraea*, *Entomophthora*, and *Neozygites*. EPF can, however, infect other arthropods that are not the insect pests of cultivated crops. They are also infective to all the life stages of insect hosts, and are found in almost all habitats (Zimmermann 1993; Shahid *et al*. 2012). The use of EPF as a biocontrol agent against the BFW has, to our knowledge, not previously been reported in South Africa.

In the current study, the main objective was to establish the potential of three commercial EPF to control the larvae, pupae and adults of BFW under laboratory conditions. Bioassays were conducted to compare the virulence to adults of one local EPF and with that of three commercial EPF isolates for adults, and to establish the ability of EPF to infect the BFW, using a sand column bioassay.

4.3 MATERIALS AND METHODS

4.3.1 Source of insects

Adult BFW that were collected from vineyards and orchards in the Western Cape from October to December and were mass reared at the Stellenbosch University Insectary (IPM Initiative), Stellenbosch University, South Africa. Corrugated cardboard bands were used to collect the weevils by tying them onto apple and vine trunks. The adult weevils, ages ranged from 2 to 4 weeks old, were kept in a ventilated Perspex boxes measuring 15 × 20 cm. Weevils were reared on carrots (personal communication, D. Stenekamp).

4.3.2. Fungal cultures

Fungi used in this study included the commercially available fungal formulations, Eco-Bb® (*Beauveria bassiana*), Broadband® (*B. bassiana* strain PPRI5339), Meta 69 (*Metarhizium anisopliae*), and a local isolate, *M. anisopliae* (EA2, from an apple orchard in Grabouw). Conidia for the assays were obtained by culturing the EPF on Sabouraud Dextrose Agar (SDA) medium (supplemented with 1 ml Dodine, 50 mg/L chloramphenicol, and 50 mg/L Ampicillin, or 50 mg/L Rifampicin) for 14 days at 25 ± 1 °C (Goettel & Inglis 1997). The EPF cultures were used immediately, and stored at 25 °C for 3 to 4 weeks.

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4.3.3 Conidial concentrations and viability

EPF cultures (2–3 weeks old) were used to harvest conidia by means of scraping the surface of Petri dish cultures, using a glass rod. The conidia was suspended in 20 ml sterilised distilled water (H_2O) augmented with Tween® 20 in sterile McCartney bottles. The bottles were sealed and vortexed for 2 min, resulting in a homogenous suspension.

The concentration of suspensions of conidia was determined using a haemocytometer. Before the chamber was used, it was rinsed with 70 % ethanol. The conidia in the middle square, or five squares highlighted in red within the middle square, were used to calculate the number of conidia (Fig. 4.1). If only conidia in the middle square were counted, the number of conidia was multiplied by 10^4 to give the number of conidia/ml. Otherwise, if the five squares highlighted within the middle square were counted, the number of conidia obtained was multiplied by 5×10^4 to give the number of conidia/ml.

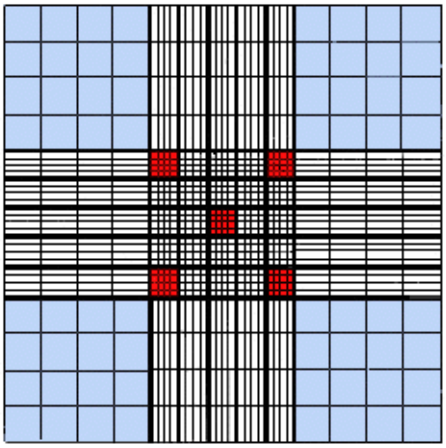

Fig. 4.1. Haemocytometer, indication the squares for counting conidia in red, as seen under 400-fold magnification of a research microscope.

The germination response of the four EPF was tested by using an SDA medium in Petri dishes (60 × 15 mm), after which 100 µl of spore suspension (1×10^6 conidia ml^{-1}) was spread onto four SDA plates, with each plate containing one of the four EPF. A cover slip was placed on each plate, with each being incubated at 25 °C. The percentage of germination was determined after 24 h by means of counting 100 spores from each isolate, at 40 × magnification (Ekesi *et al*. 2002).

4.3.4. Susceptibility of different life stages of BFW

Final instar larvae, pupae and adults of BFW were individually immersed in a 100 ml conidial suspension of Eco-Bb®; BroadBand®, Meta 69 and *M. anisopliae* (EA2), a local isolate isolated in an apple orchard in Grabouw (Chapter 2) (only used for adults), each with a concentration of 1 ×

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10^6 conidia ml^{-1} with 0.01% Tween® 20 (Merck, KGaA, Darmstadt, Germany) for 60 sec. The controls were immersed in a 100 ml solution of distilled water and 0.01% Tween® 20. The treated BFW were placed in 24-well bioassay trays, kept at 25 °C in a growth chamber for a period of 21 days. Five replicates of 12 were treated to with the fungal suspensions, used as the controls (n = 60). The 24-well bioassay trays were checked weekly for mortality, and the entire bioassay was repeated on a different test date with newly prepared conidial suspension. The BFW were considered mycosed if fungal sporulation of the isolate occurred (Fig. 4.2). The mycosed insects were placed on the SDA plates after 14 days.

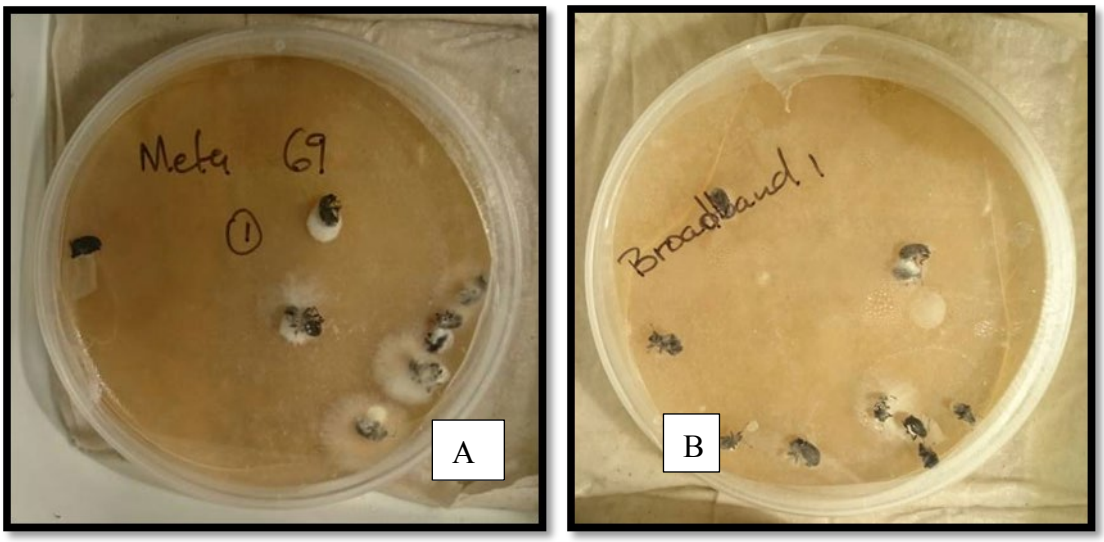

Fig. 4.2. *Phlyctinus callosus* (BFW) adults showing signs of fungal mycosis after incubation on SDA plates; (A) Adults showing signs of mycosis after treatment with Meta 69; (B) Adults showing signs of mycosis after treatment with Broadband®.

4.3.5. Sand bioassay

River sand was autoclaved, dried and sieved through a mesh screen to result in 500-µm particle size. The 50 g sterilised moist sand (10:100 v/v, sand to water) was transferred into a 90-mm-diam Petri dish, and 5 ml of each of the three EPF stock suspension was titrated to the test concentration of 1×10^6 conidia ml^{-1}. After being inoculated onto 50 g of sand in each Petri dish, the conidial suspension was mixed. The sand of the controls were treated with distilled water, and Tween® 20. Ten BFW larvae were introduced into each Petri dish, which constituted a replicate. Five replicates with 12 insects each, were used per fungal isolate (n = 60). The Petri dishes were incubated in a 25 °C growth chamber for 7 days. The test insects were surface sterilised in 70 % ethanol, transferred onto Sabouraud Dextrose Agar (SDA) plates, and maintained at the same conditions as above. The criteria for scoring mycoses was larval death, followed by fungal sporulation.

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4.3.6. Data analysis

Virulence assays were analysed using analysis of variance (ANOVA); if the F value was significant ($p < 0.05$), the means were differentiated by means of LSD MEANS (SAS Institute 1985). The mortality data were corrected for the corresponding control mortality, using the formula: CM (%) = {(T–C)/(100–C)} × 100, where CM is the corrected mortality, T is the percentage mortality in treated insects, and C is the percentage mortality in untreated insects (Abbott 1925). If no significant interactions were found to have occurred between the main effect of the test dates and the treatments concerned, the data obtained from the two test dates were pooled and analysed, using a one-way ANOVA.

4.4. RESULTS

4.4.1 Susceptibility of insect life stages

4.4.1.1 Final instar larvae

The mean mortality of BFW larvae inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 ranged between 58.3 % ± 3.51 % and 96.7 % ± 1.84 % over a period of 21 days (Fig. 4.3). Analysis using a one-way ANOVA showed a significant effect ($F_{(2, 27)} = 58.6997$, $p < 0.001$) of the treatment on percentage mortality. The application of Broadband® (96.7 % ± 1.84 % mortality) and Meta 69 (92.5 % ± 2.12 % mortality) resulted in mortality that was not significantly different from each other, but which was significantly higher ($p < 0.05$) than Eco-Bb® (58.3 % ± 3.51 % mortality) (Fig. 4.3). The mortality for the control caused by natural death after a period of 21 days was 3.3 %.

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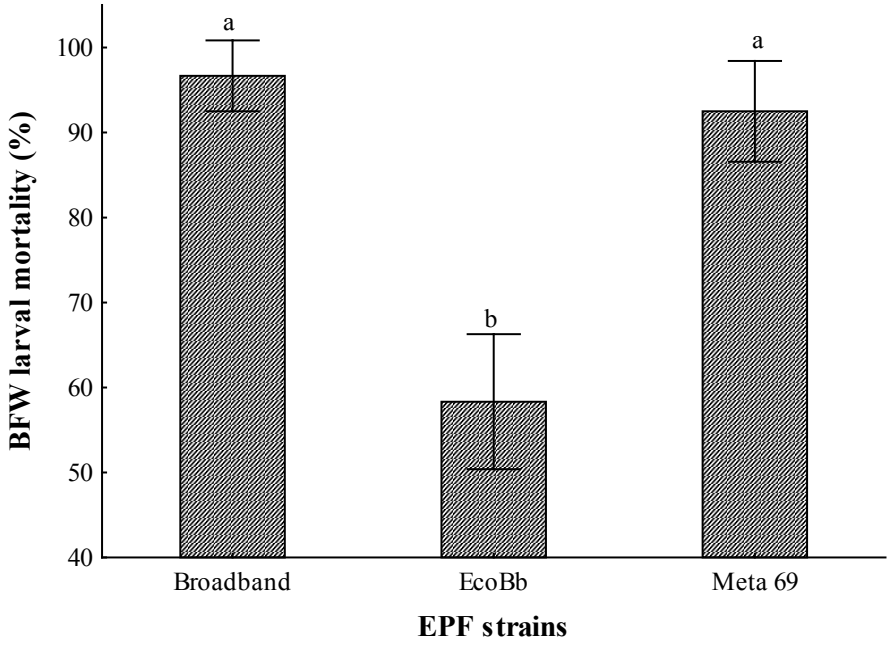

Fig. 4.3. Mean percentage mortality (95 % confidence level) after 21 days of *Phlyctinus callosus* (BFW) larvae inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 (one-way ANOVA: $F_{(2, 27)} = 58.6997$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

4.4.1.2. Pupae

The mean mortality of BFW pupae inoculated with 1×10^6 conidia ml^{-1} was highest for Broadband® at 91.7 % ± 3.04 %, followed by that for Eco-Bb® at 67.5% ± 2.90 %, with Meta 69 having the lowest mean mortality at 65.8 % ± 3.39 % over a period of 21 days (Fig. 4.4). Analysis using a one-way ANOVA showed that a significant effect ($F_{(2, 27)} = 21.5$, $p < 0.001$) of the treatment on percentage mortality. The application of Broadband® resulted in a mortality that was significantly higher ($p < 0.05$) than was that which was attained for Eco-Bb and Meta 69. The mortality of BFW pupae, treated with Eco-Bb® and Meta 69, did not differ significantly from each other (Fig. 4.4). The mortality for the control caused by natural death after a period of 21 days was 4.1 %.

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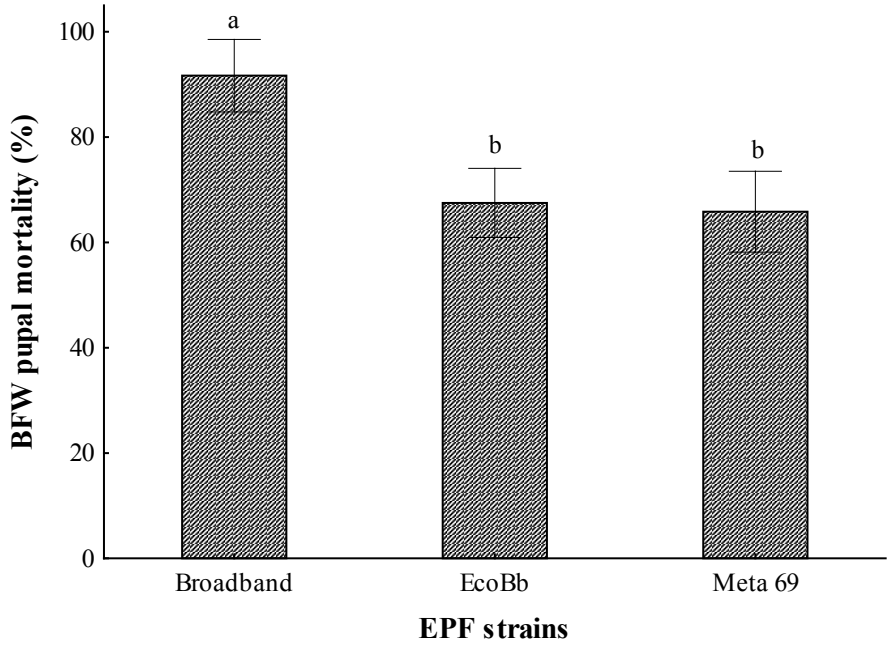

Fig. 4.4. Mean percentage mortality (95 % confidence level) after 21 days of *Phlyctinus callosus* (BFW) pupae inoculated with 1 × 10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 (one-way ANOVA: $F_{(2, 27)} = 21.5$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

4.4.1.3 Adults

The mean mortality of BFW adults inoculated with 1 × 10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 ranged between 65 % ± 2.99 % and 90 % ± 3.47 % over a period of 21 days. Analysis using a one-way ANOVA showed that the treatment had a significant effect ($F_{(2, 27)} = 17.7481$, $p < 0.001$) on the percentage mortality. The application of Broadband® (90 % ± 3.47 % mortality) resulted in mortality that was significantly higher ($p < 0.05$) than that which was attained with Eco-Bb® and Meta 69 (69.2 % ± 3.05 and 65 % ± 2.99 % mortality, respectively). Mortality, with Eco-Bb® and Meta 69, did not differ significantly from each other (Fig. 4.5). The mortality for the control caused by natural death after a period of 21 days was 3.3 %.

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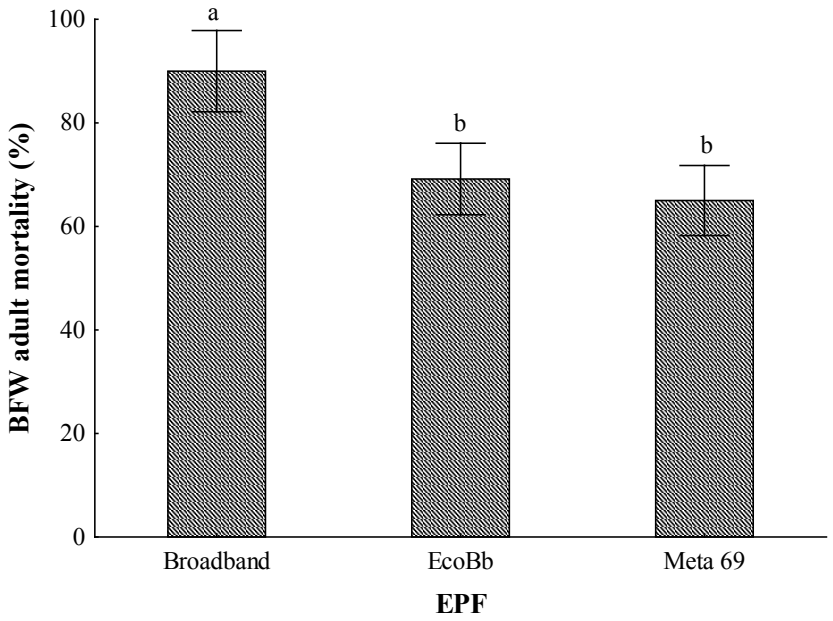

Fig. 4.5. Mean percentage mortality (95 % confidence level) after 21 days of *Phlyctinus callosus* (BFW) adults inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 (one-way ANOVA: $F_{(2, 27)} = 17.7481$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

Broadband®, Eco-Bb® and Meta 69 were compared to *M. anisopliae* (EA2), a local isolate isolated in an apple orchard in Grabouw (Chapter 2), for virulence to the BFW adults. The mean mortality of BFW adults inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb®, Meta 69 and *M. anisopliae* (EA2) ranged between 59.5 % ± 4.23 % and 91.7 % ± 3.51 % over a period of 21 days. Analysis using a one-way ANOVA showed the significant effect ($F_{(3, 36)} = 18.7171$, $p < 0.001$) of the treatment on the percentage mortality. The application of Broadband® (91.7 % ± 3.51 % mortality) resulted in mortality significantly higher ($p < 0.05$) than Eco-Bb®, Meta 69 and *M. anisopliae* isolate EA2 (65 % ± 2.72%, 59.5 % ± 4.23 % and 64.2 % ± 2.79 % mortality, respectively). Mortality, after treatment with Eco-Bb®, Meta 69 and *M. anisopliae* (EA2), did not significantly differ from one another (Fig. 4.5). The mortality for the control caused by natural death after a period of 21 days was 1.6 %.

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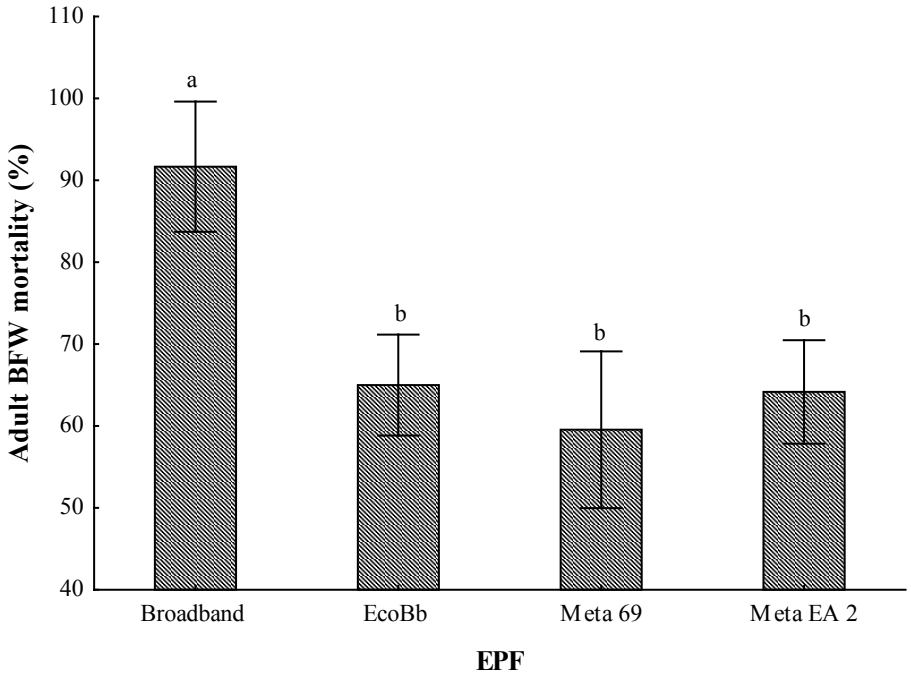

Fig. 4.6. Mean percentage mortality (95 % confidence level) after 21 days of *Phlyctinus callosus* (BFW) adults inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb®, Meta 69, and *M. anisopliae* (EA2) (one-way ANOVA: $F_{(3, 36)} = 18.7171$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

4.4.2. Sand bioassay

The mean mortality of BFW larvae inoculated with 1×10^6 conidia ml^{-1} of Broadband®, Eco-Bb® and Meta 69 in sand ranged between 55 % ± 2.22 % and 85 % ± 2.72 % after a period of 21 days (Fig. 4.7). Analysis using a one-way ANOVA showed the significant effect ($F_{(2, 27)} = 14.4834$, $p < 0.001$) of the treatment on the percentage mortality. Broadband® (85 %± 2.72 % mortality) resulted in mortality that was significantly higher ($p < 0.05$) than it was for Eco-Bb® and Meta 69 (55 % ± 2.22 and 70 % ± 5.85 % mortality, respectively). The mortality after exposure to Meta 69 was significantly higher ($p < 0.05$) than that for Eco-Bb®, which was lower than for all the other treatments. The mortality for the control caused by natural death after a period of 21 days was 3.3 %.

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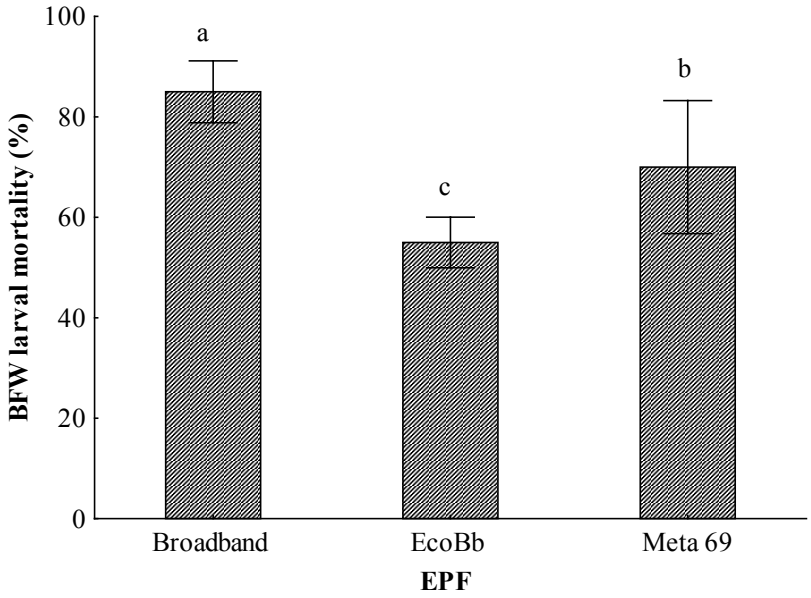

Fig. 4.7. Mean percentage mortality (95 % confidence level) after 21 days of *Phlyctinus callosus* (BFW) larvae inoculated with 1 × 10^6 conidia $ml^{-1}$of Broadband®, Eco-Bb® and Meta (one-way ANOVA: $F_{(2, 27)} = 14.4834$, $p < 0.001$). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

4.5 DISCUSSION

The use of EPF as a biocontrol agent against the BFW has, to my knowledge, not previously been reported in South Africa. EPF such as *M. anisopliae* and *B. bassiana* are known to be pathogenic to other weevils (Ansari *et al*. 2008). In the current study, the pathogenicity of the commercially available strains of *M. anisopliae* and *B. bassiana* in South Africa was tested against the different developmental stages of BFW.

The results of the study support that the application of Eco-Bb®, BroadBand®, Meta 69, *M. anisopliae* (EA2) can effectively control all stages of BFW, as exceptionally high mortality of up to 96.7 % was obtained when both BroadBand® and Meta 69 were used. Gindin *et al*. (2006) recorded from 80 % to 100 % mortality of larvae and adult weevils after 4 to 5 weeks, when *M. anisopliae* and *B. bassiana* spore suspensions were used to control the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) under laboratory conditions. Such results concur with those that were obtained in the present study, in spite of the different screening methods used.

Although all the EPF strains used in the present study were, to some extent, effective in controlling BFW, Broadband® proved throughout to be the most virulent isolate for controlling all developmental stages of the BFW. The isolates that tend to be most virulent towards a host insect are generally those that have been isolated from the same host species. Yeo *et al*. (2003) state that the most effective EPF for controlling a target host has to be isolated from a host species related

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to the target host. The use of Broadband®, which is an African strain that is isolated from the tortoise beetle, *Charidotella sexpunctata* (Coleoptera: Chrysomelidae), might actively contribute to the high virulence levels displayed towards BFW. The climatic conditions where the host naturally occurs are another factor affecting the ability of an EPF to control a target host. Such conditions contribute to the virulence of the EPF to the target host insect concerned. In the current study, constant temperature of approximately 25 °C was maintained, with the EPF more effective from 15 to 30 °C (Moorhouse *et al*. 1994).

Moorhouse *et al*. (1994) studied the susceptibility of the larvae of the black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), to *Metarhizium* isolates at 20 °C. They reported that most isolates of *M. anisopliae* are tropical isolates, whereas the BFW involved is from a Mediterranean climate, which might have contributed to the reduced mortality recorded with Meta 69 in terms of the control of BFW. However, there was no evidence to show that the EPF from the *Metarhizium* spp. can control insect related to the original host, as two other extremely virulent strains were originally sourced from other coleopteran hosts, with one being from a lepidopteran larva (Moorhouse *et al*. 1994).

The method of immersing BFW in a conidial suspension used in our laboratory assays to infect BFW differs from contact with EPF spores under field conditions. The usefulness of EPF under field conditions to achieve the efficient control of BFW is open to doubt, despite the susceptibility of all the developmental stages of the BFW to EPF under laboratory conditions. Many factors affect the field efficacy of EPF, but, in most cases, the behaviour of the insect host in its natural habitat is important. As the natural habitat of EPF is the soil, and the BFW larvae and pupae, as well as, occasionally, the adults, inhabit the soil, their infection with EPF spores is more likely to be attained by treating the soil concerned. Since BFW pupation occurs inside smooth-sided earthen cells, and not within a spun cocoon, and young adults remain in the earthen cells for a few days before they emerge, implementing fungal control in the soil is relatively feasible. This concurs with the results obtained in the present study, because up to 85 % larval mortality was recorded from the sand bioassay, with all the EPF involved showing promising control of the BFW in the soil.

In the current study, Broadband® proved to have the highest level of virulence toward the BFW in respect of killing, and in terms of eventually showing signs of mycosis on the cadaver. Godonoua *et al*. (2009) report that the main benefit to be gained from using EPF in managing insect pests is that they tend to have the potential to kill their insect host, and to spread disease quickly. Our results showed that the various life stages of BFW (except for that of the eggs, which

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were not tested) were infected by the different EPF, while the adult stage was the only one that with the potential to disseminate EPF to the non-infested BFW. Considering adults as the target in any BFW control strategy involving EPF should prove to be important, because the EPF can finish its life cycle in the adult BFW, and the BFW that are infected with EPF can spread the entomopathogen to other BFW populations, as well as to succeeding generations.

The present study showed BFWs to be susceptible to all the EPF tested, but Broadband®, at a concentration of 1×10^6, proved that it should be considered as a biological control agent against BFW. The adult BFW normally reach their target fruit by means of climbing up the tree trunk (Barnes *et al*. 1994), which is the site of the trapping strategy that is currently used against the weevil in the Western Cape. Corrugated cardboard trunk barriers can be treated with a high concentration of Broadband spores prior to use on tree trunks, which may be able to infect the adults moving over the barrier or shelter underneath it, and thereby spread the EPF within their own population, which can lead to the occurrence of an epizoic event.

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Chapter 5

Effect of combining entomopathogenic fungi and *Steinernema yirgalemense* against the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), in the laboratory

5.1. ABSTRACT

Phlyctinus callosus (Schönherr) (Coleoptera: Curculionidae), or banded fruit weevil (BFW), is one of the coleopteran insect pests causing damage to the fruits, leaves and roots of deciduous fruits and grapevines in the Western Cape province. The weevils are prevented from reaching leaves and fruits by the trunk barriers, which is a labour-intensive physical control method. The control of the BFW can be improved by means of combining entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPNs). Following positive results earlier with EPNs and EPFs evaluated separately against BFW, in this study we evaluate the combination of the EPF, Eco-Bb® (*Beauveria bassiana*), Broadband® (*B. bassiana* strain PPRI5339), Meta 69 (*Metarhizium anisopliae*), and a local isolate *M. anisopliae* EA2, with the EPN, *Steinernema yirgalemense* (Rhabditida: Steinernematidae) against BFW larvae and adults in the laboratory. Four treatment regimens were used: BFW were exposed to the EPFs and the EPN separately, and to a combination of EPFs and *S. yirgalemense* either 0, 1 or 2 weeks apart. The combined application of the different EPF isolates with *S. yirgalemense* resulted in higher mortality of the BFW in the laboratory. When *S. yirgalemense* was applied 1 or 2 weeks after application of Eco-Bb® and BroadBand®, 100% larval and adult mortality was obtained. Synergy was noted in Eco-Bb® + *S. yirgalemense*, BroadBand® + *S. yirgalemense*, Meta 69 + *S. yirgalemense* and *M. anisopliae* isolate EA2 + *S. yirgalemense*, when applied 1 or 2 weeks after application of the EPF. Interactions observed showed that the EPF and *S. yirgalemense* have an additive effect when applied simultaneously, and a synergistic effect when applied 1 or 2 weeks after the application of the different EPF. The results indicate that the combination of EPFs and an EPN shows potential as economically viable control strategy for BFW in the field.

Keywords: additive, biocontrol, entomopathogenic fungi, entomopathogenic nematode, synergistic

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5.2. INTRODUCTION

Coleopteran insect pests are important insect pests of a number of economically important agricultural crops (Wakil *et al*. 2017). *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), or banded fruit weevil (BFW), is an indigenous insect pest causing sporadic damage on grapes, apples and nectarines, mostly in the southern portions of the Western Cape province of South Africa (Lounsbury 1896; Barnes & Capatos 1989; Barnes & Pringle 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). In New Zealand, the BFW was first reported as a major pest of grapes in 1899, with it later spreading to all the other southern states in Australia (Kuschel 1972). The BFW, which is also a phytosanitary pest, is difficult to control in the Western Cape province (Barnes 1991; Barnes *et al*. 1994; Pringle *et al*. 2015; Allsopp *et al*. 2015).

The curculionid has also become a serious pest of blueberries (Ferreira 2010; Pringle *et al*. 2015). Damage of up to R4 million annually by the adult weevils on grape berries, nectarines, apples and other ornamental plants was reported (Barnes 1989a, b; Barnes & Pringle 1989; Pringle *et al*. 2015; Allsopp *et al*. 2015). The scarring of the fruit results in unmarketable fruits, with the weevils often accidentally being packed with export fruits, resulting in quarantine issues (De Villiers & Pringle 2007; Pringle *et al*. 2015; Allsopp *et al*. 2015).

The recommended control strategy that is currently available to farmers in South Africa is trunk barriers (physical and chemical), whereas tree trunks are drenched with chemical insecticides in Australia to prevent the adults from reaching the fruits (Barnes 1991; Barnes *et al*. 1994; Fisher & Learmonth 2003). Trunk barriers have, so far, proved to be effective in preventing the BFW from reaching the fruits, but they are labour-intensive, particularly in large orchards and vineyards (Barnes *et al*. 1994; Fisher & Learmonth 2003). Due to problems associated with the use of chemical insecticides, research aimed at developing alternative control strategies is warranted. The BFW, as shown for other curculionids, can be controlled by combining various biocontrol agents (Ansari *et al*. 2008; Wakil *et al*. 2017).

Using laboratory-cultured stages of larvae, pupae and adults of the BFW (Chapter 3), showed that a range of South African EPNs are effective against the BFW, with clear differences among the EPN species tested. *Steinernema yirgalemense* was shown to be effective in controlling BFW larvae under field conditions. In a follow-up study (Chapter 4,), different commercially available entomopathogenic fungi (EPF) (Hatting *et al*. 2018) isolates were screened for virulence against the different life stages of the BFW. The results indicated that all the EPF isolates tested were effective against the immature and adult stages of the BFW, with clear differences being found

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among the EPF isolates tested. Broadband® (*Beauveria bassiana* strain PPRI5339) was shown to be an effective biocontrol agent against BFW under laboratory conditions (Chapter 3).

Entomopathogenic nematodes (EPNs) and EPF, which suppress insect populations in nature, are both potential alternatives for the control of BFW. However, they can also potentially be combined in a management strategy, as has been shown by previous research. The combination of EPNs and EPF to control insect pests can have additive, synergistic or antagonistic effects on mortality. Additive effects were observed when *Heterorhabditis indica* Poinar, Karunakar & David, 1994 was combined with *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Cordycipitaceae), for the control of *Curculio caryae* (Coleoptera: Curculionidae) (Shapiro-Ilan *et al*. 2003). However, when *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982 was combined with *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) for the control of *C. caryae*, it resulted in an antagonistic interaction (Shapiro-Ilan *et al*. 2003). Synergistic interactions were observed when controlling *Cyclocephala* spp. with EPNs and *Paenibacillus popilliae* (Dutky), or with *Bacillus thuringiensis* subspecies *japonensis* Berliner (Thurston *et al*. 1993, 1994; Koppenhöfer & Kaya 1997; Koppenhöfer *et al*. 1999). Additive and synergistic interactions were reported when *H. bacteriophora* was combined with *B. bassiana* or *M. anisopliae*, with more frequent synergism interactions in *H. bacteriophora* and *B. bassiana* combinations, than there were in *H. bacteriophora* and *M. anisopliae* combinations. The above occurred mostly with early instars, than with the older instars, of red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) (Wakil *et al*. 2017).

In the current study, the effect of combining entomopathogenic fungi and *S. yirgalemense* on mortality of larvae and adult BFW was investigated in the laboratory. Synergy, additivity and antagonism between the different microbial agents were tested in turn.

5.3 MATERIALS AND METHODS

5.3.1. Source of insects

Adult BFW that were collected from vineyards and orchards in the Western Cape from October to December and were mass reared at the Stellenbosch University Insectary (IPM Initiative), Stellenbosch University, South Africa. Corrugated cardboard bands were used to collect the weevils by tying them onto apple and vine trunks. The adult weevils, ages ranged from 2 to 4 weeks old, were kept in a ventilated Perspex boxes measuring 15 × 20 cm. Weevils were reared on carrots (personal communication, D. Stenekamp).

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5.3.2. Source of nematodes

Steinernema yirgalemense infective juvenile (IJ) rearing and harvesting procedures were carried out according to the methods presented by Kaya & Stock (1997), using mealworm larvae kept at room temperature (± 25 °C). The IJs were harvested from the White traps, during the first week of emergence, stored horizontally using 500-ml vented culture flasks, and used within one month after harvesting. The culture flasks were shaken on a weekly basis, so as to increase the amount of aeration, and the survival of the IJs during storage.

5.3.3. Source of fungal cultures

Fungal isolates used in this study included the commercially available fungal formulations, Eco-Bb® (*B. bassiana*), Broadband®, and Meta 69 (*M. anisopliae*), as well as the local isolate, *M. anisopliae* (EA2), from an apple orchard in Grabouw. To obtain the conidia, the different strains were cultured on Sabouraud Dextrose Agar (SDA) medium (supplemented with 1 ml Dodine, 50 mg/L chloramphenicol and 50 mg/L Ampicillin or 50 mg/L Rifampicin) for 14 days at 25 ± 1 °C (Goettel & Inglis 1997). The EPF cultures were either used immediately after sporulation, or stored at 25 °C for 3 to 4 weeks.

5.3.4. Conidial concentrations and viability

SDA medium in Petri dishes (60 × 15 mm) was used to test the germination response of the four EPF. A 100 µl of spore suspension (1×10^6 conidia ml^{-1}) was spread onto four SDA plates, with each plate containing one of the four different EPF isolates. A cover slip was placed on each SDA plate, and incubated at 25 °C growth chamber. The percentage germination attained was determined by means of counting 100 spores from each isolate at 40 × magnification after 24 h (Ekesi *et al*. 2002).

5.3.4. Bioassay protocol

Steinernema yirgalemense inoculum was prepared at a concentration of 100 IJs / 50 µl water for BFW larvae and 200 IJs / 50 µl water for adults, with it being pipetted into the 24-well bioassay trays lined with Whatman filter paper. Twelve final instar larvae for each treatment were used, in each well, and each treatment was replicated five times ($n = 60$).The conidia of two-to-three-week-old EPF cultures were harvested by means of scraping the surface of the Petri dish cultures, using a glass rod. The sterilised distilled water (H_2O) (20 ml) with the suspended conidia was augmented with Tween 80 in sterile McCartney bottles. The bottles carrying the conidial suspension were

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sealed and vortexed for 2 min. A haemocytometer was used to determine the concentration of the conidial suspensions (Chapter 4). After being dipped in the predetermined conidial concentration (1×10^6 conidia ml^{-1}), the larvae, or adults, were added to the 24 wells of the bioassay plates. The control treatment received 50 µl of distilled water only. All 24-well bioassay trays were kept at 25 °C in a growth chamber. Mortality was determined after 48 h for the EPNs, and after 21 days for the EPF. All treatments were repeated on a different test date. The technique used was adapted from those of Ansari et al. (2008) and Wakil et al. (2017).

5.3.5. Susceptibility of BFW to the combination of EPF and EPN

Whereas the four different EPF strains were each tested in combination with *S. yirgalemense*, the EPF in combination with each other were not tested. The combined treatments comprised EPF and EPN applied at different time intervals, or simultaneously, according to the following combinations: 1) Each of the four different EPF strains, plus the *S. yirgalemense*, were applied simultaneously (week 0). After immersion in EPF suspensions (1×10^6 conidia ml^{-1}), the BFW larvae or adults were transferred to 24-well bioassay trays that were preinoculated with *S. yirgalemense* (100 IJs/well), as described in the bioassay protocol above. 2) The BFW larvae or adults were first dipped in each of the four different EPF strains, maintained at 25 ± 2 °C and 65 ± 5% RH for one week, and then transferred to 24-well bioassay trays pre-inoculated with *S. yirgalemense* IJs. 3) After first being dipped in each of the four different EPF strains, the BFW larvae were maintained at 25 ± 2 °C and 65 ± 5% RH for 2 weeks, after which they were transferred to 24-well bioassay trays pre-treated with *S. yirgalemense* and 4) For the control, BFW were immersed in aqueous solution with 0.01% Tween 80 and maintained in 24-well bioassay trays, lined with moistened filter paper. All bioassay plates were kept in a 25 °C growth chamber. The BFW mortality was recorded 3 weeks post application; if the larvae failed to respond on slight prodding with a blunt needle, they were considered to be dead.

5.3.6. Data analysis

Virulence assays were analysed using analysis of variance (ANOVA); if the F value was significant ($p < 0.05$), the means were differentiated by LSD MEANS (SAS Institute 1985). The mortality data were corrected for the corresponding control mortality, using the formula: CM (%) = $\{(T–C)/(100–C)\}\times100$, where CM is the corrected mortality, T is the percentage mortality found in the treated insects, and C is the percentage mortality found in the untreated insects (Abbott 1925). To determine whether the EPF–EPN interactions were additive, antagonistic or synergistic, the comparison of observed versus expected values of insect mortality was applied (Finney 1964;

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McVay *et al*. 1977; Ansari *et al*. 2008; Wakil *et al*. 2017). A procedure, first described by Finney (1964) and later modified by McVay *et al*. (1977), was used to determine the type of interaction occurring between the *S. yirgalemense* and the EPF isolates.

The mortality of the BFW was determined by means of subtracting the number of surviving BFWs from the number used in each replicate. To determine the expected additive proportional mortality ME for the EPN–EPF combinations, the formula $M_E = M_{EPN} + M_{EPF} (1 – M_{EPN})$ was used. M_{EPN} and M_{EPF} were the observed proportional mortalities induced by the EPN and the EPF alone, respectively. A χ^2 test was used to obtain the observed and expected results, $\chi^2 = (M_{EPNEPF} – M_E)^2/M_E$, where M_{EPNEPF} refers to the observed mortality for the EPN–EPF combination, which was compared to 3.83, which is the χ^2 table value for 1 degree of freedom. The interaction was labelled synergistic/antagonistic when the calculated χ^2-values exceeded the table value, and additive when the calculated χ^2-values did not exceed the table value (Finney 1964). The interaction was considered to be synergistic if the differences $M_{EPNEPF} – ME = D$ indicated a positive value, and to be antagonistic if D had a negative value (Finney 1964; Ansari *et al*. 2008).

5.3 RESULTS

5.3.1 Susceptibility of BFW larvae to the combination of EPF and EPN

To determine the individual mortality potential against BFW larvae for the different fungal strains, the mortality concerned at a concentration of 1×10^6 conidia ml^{-1} was determined after 21 days. The mortality for the fungal isolates ranged from between 54.4 % ± 1.9 % and 89.4 % ± 3.7% (Table 5.1). Analysis using a one-way ANOVA showed a significant effect ($F_{(4.45)} = 149.0224$, $p < 0.001$) of the treatment on the percentage mortality. Broadband® (89.4 % ± 3.7% mortality) resulted in significantly ($p < 0.05$) higher mortality of BFW larvae, in comparison with the other entomopathogens, which included Eco-Bb® (63.3% ± 2.5% mortality), Meta 69 (54.4 % ± 1.9 % mortality) and *M. anisopliae* (EA2) (57.5 % ± 2.3 % mortality). The mortality of *S. yirgalemense* (66.5 % ± 1.9 % mortality) at a concentration of 100 IJs/insect was determined after a period of 48 h.

Additive and synergistic interactions were observed between *S. yirgalemense* and EPF treatments employed against the BFW larvae. The interactions were additive when *S. yirgalemense* was applied simultaneously with the different EPF species, and synergistic when *S. yirgalemense* was applied 1 or 2 weeks after the application of the EPF. The degree of synergy increased when *S. yirgalemense* was applied 1 week after the application of *M. anisopliae* isolate EA2 ($\chi^2 = 21$,

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d.f. = 1, $p < 0.001$) (Table 5.1). The average control mortality for Eco-Bb®, BroadBand®, Meta 69, and *M. anisopliae* EA2 was 1.66, 3.32, 4.98 and 3.32 %, respectively.

Table 5.1. Mean mortality (% ± SE) of the last instar larvae of *Phlyctinus callosus* treated with Eco-Bb®, BroadBand®, Meta 69, *Metarhizium anisopliae* (EA2) and *Steinernema yirgalemense* (EPN). The entomopathogenic fungi were used at 1×10^6 conidia ml^{-1} concentration, and the *S. yirgalemense* was applied at 100 IJs/insect.

Treatments	Intervals[1] (weeks)	Observed[2] mortality* (%)	Expected[3] mortality	Chi-sq. χ^2	Interaction[4]
Eco-Bb®	–	63.3 ± 2.5b	–	–	–
BroadBand®	–	89.4 ± 3.7a	–	–	–
Meta 69	–	54.4 ± 1.9b	–	–	–
M. anisopliae (EA2)	–	57.5 ± 2.3b	–	–	–
EPN	–	66.5 ± 1.9b	–	–	–
Eco-Bb® + EPN	0	74.4	87.7	2.00	Additive
BroadBand® + EPN	0	98.5	96.4	0. 04	Additive
Meta 69 + EPN	0	77.5	84.7	0. 60	Additive
EA2 + EPN	0	80.5	85.7	0. 30	Additive
Eco-Bb® + EPN	1	100.0	70.1	12.00	Synergistic
BroadBand® + EPN	1	100.0	77.9	6.00	Synergistic
Meta 69 + EPN	1	84.2	53.7	17.00	Synergistic
EA2 + EPN	1	88.6	54.6	21.00	Synergistic
Eco-Bb® + EPN	2	100.0	74.0	9.10	Synergistic
BroadBand® + EPN	2	100.0	78.0	6.20	Synergistic
Meta 69 + EPN	2	92.5	68.0	8.8	Synergistic
EA2 + EPN	2	96.0	70.0	9.7	Synergistic

*Mortality means followed by the same letter are not significantly different ($p > 0.05$)

[1]EPNs were applied in the combination treatments 0, 1 and 2 weeks after being dipped into EPF conidial suspension

[2]Mean percentage calculated from five replicates, each infested with 12 BFW larvae ($n = 60$)

[3]Expected mortality ME = $M_{EPN} + M_{EPF} (1 - M_{EPN})$, where M_{EPN} and M_{EPF} are the observed proportional mortalities caused by *S. yirgalemense* and EPF alone

[4]Interactions based on the χ^2 ratio of expected: observed mortality

5.3.2 Susceptibility of BFW adults to the combination of EPF and EPN

To determine the individual mortality potential against BFW adults for the different fungal strains, the mortality was determined after 21 days at a concentration of 1×10^6 conidia ml^{-1}. The mean mortality of the BFW adults inoculated with 1×10^6 conidia ml^{-1} and 200 IJs/insect ranged

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between 51.7 % ± 1.0 % and 79.2 % ± 3.1% (Table 5. 2). Analysis using a one-way ANOVA showed the significant effect ($F_{(3, 36)} = 37.46$, $p = 0.001$) of the treatment on percentage mortality. Broadband® (79.2 % ± 3.1% mortality) resulted in significantly higher ($p < 0.05$) mortality of the BFW adults, in comparison with the other fungal isolates. Eco-Bb® (56.7 % ± 1.7 % mortality), Meta 69 (51.7 % ± 1.0 % mortality) and *M. anisopliae* (EA2) (54.2 % ± 1.9 % mortality) resulted in mortality not significantly different from one another. *Steinernema yirgalemense* (68.5 % ± 1.8 % mortality), at a concentration of 200 IJs/insect, resulted in mortality significantly different from that which was caused by any one of the EPF after a period of 48 h (Table 5. 2).

Additive and synergistic interactions were observed between the *S. yirgalemense* and EPF treatments against the BFW adults (Table 5.2). The interactions were additive when *S. yirgalemense* was applied simultaneously with the different EPF species, and when *S. yirgalemense* was applied 1 or 2 weeks after application of the EPF. A synergistic interaction was only observed when *S. yirgalemense* was applied 2 weeks after the application of *M. anisopliae* isolate EA2. The degree of synergy attained increased when *S. yirgalemense* was applied 2 weeks after the application of *M. anisopliae* isolate EA2 ($\chi^2 = 3.95$, d.f. = 1, $p < 0.001$). The average control mortality for Eco-Bb®, BroadBand®, Meta 69, and *M. anisopliae* isolate EA2 was 3.32, 3.32, 4.98 and 3.32 %, respectively.

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Table 5.2. Mean mortality (% ± SE) of *Phlyctinus callosus* adults treated with Eco-Bb®, BroadBand®, Meta 69, *Metarhizium anisopliae* (EA2) (EPF), and *Steinernema yirgalemense* (EPN). The fungi were each used at a concentration of 1×10^6 conidia ml^{-1}, and the *S. yirgalemense* was applied at 100 IJs ml^{-1}.

Treatments	Intervals[1]	Observed mortality* (%)[2]	Expected mortality	Chi-sq. χ^2	Type of interaction
Eco-Bb®	-	56.7 ± 1.7c	-	-	-
BroadBand®	-	79.2 ± 3.1a	-	-	-
Meta 69	-	51.7 ± 1c	-	-	-
EA2	-	54.2 ± 1.9c	-	-	-
EPN	-	68.5 ± 1.8b	-	-	-
Eco-Bb® + EPN	0	87.6	86.4	0.01	Additive
BroadBand® + EPN	0	100	93.4	0.50	Additive
Meta 69 + EPN	0	87.8	84.8	0.10	Additive
EA2 + EPN	0	89.7	85.6	0.20	Additive
Eco-Bb® + EPN	1	90.5	80.1	1.35	Additive
BroadBand® + EPN	1	100	97.9	0.04	Additive
Meta 69 + EPN	1	94.2	80.7	2.26	Additive
EA2 + EPN	1	96.6	81.6	2.75	Additive
Eco-Bb® + EPN	2	96.8	84.1	1.91	Additive
BroadBand® + EPN	2	100	98.9	0.01	Additive
Meta 69 + EPN	2	98.5	81.7	3.45	Additive
EA2 + EPN	2	100	82.0	3.95	Synergistic

*Mortality means followed by the same letter are not significantly different ($p > 0.05$)

[1]EPNs were applied in the combination treatments 0, 1 and 2 weeks after being dipped into EPF conidial suspension

[2]Mean percentage calculated from five replicates, each infested with 12 BFW adults ($n = 60$)

[3]Expected mortality $ME = M_{EPN} + M_{EPF} (1 - M_{EPN})$, where M_{EPN} and M_{EPF} are the observed proportional mortalities caused by *S. yirgalemense* and EPF alone

[4]Interactions based on the χ^2 ratio of expected: observed mortality

5.4. DISCUSSION

The combined use of EPF and EPN to control the BFW has not previously been reported. In the current study, the use of commercially available EPF and a local isolate *M. anisopliae* EA2, in combination with *S. yirgalemense*, was evaluated for their combined insecticidal properties against BFW larvae and adults, and resulted to both additive and synergistic interactions. The results concurred with those by Ansari *et al*. (2008) who reported that EPF such as *M. anisopliae* or *B. bassiana*, combined with EPNs such as *Steinernema feltiae* Bovien, *Steinernema kraussei*

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(Steiner) Travassos and *Heterorhabditis bacteriophora* Poinar (Heterorhabditidae) are known to be pathogenic to other weevils, and to result in additive and synergistic interactions .

In earlier evaluations of BFW larvae, the EPF strains Eco-Bb®, BroadBand®, Meta 69, *M. anisopliae* EA2 combined with *S. yirgalemense* used separately against the laboratory-cultured larvae of the BFW, good control was obtained for all biologicals (Chapters 3 and 4). In evaluations reported here, when *S. yirgalemense* was applied 1 or 2 weeks after the application of Eco-Bb® and BroadBand®, 100% larval mortality was obtained. In the current study, increased larval and adult mortality in an additive or a synergistic way was reported. Stronger synergistic effects were noted with the combinations Eco-Bb® + *S. yirgalemense*, BroadBand® + *S. yirgalemense*, Meta 69 + *S. yirgalemense* and *M. anisopliae* isolate EA2 + *S. yirgalemense* when they were applied 1 or 2 weeks after application of the EPF. The findings from the current study are similar to those of Ansari *et al*. (2008), who found that the combined application of EPNs (*H. bacteriophora*, *S. feltiae*, and *S. kraussei*) with *M. anisopliae* resulted in increased control of black vine weevil.

In the same study by Ansari *et al*. (2008), the combination of EPNs and *M. anisopliae* resulted in 100% larval mortality when the EPNs were applied 1 or 2 weeks after the application of the fungus. Similar results were also reported by Ansari *et al*. (2004) when *M. anisopliae* CLO 53 and EPNs like *Heterorhabditis megidis* and *Steinernema glaseri* were applied against third-instar *Hoplia philanthus* in laboratory and greenhouse experiments. Combined application of *M. anisopliae* with the EPNs increased larval mortality either in an additive or a synergistic way. In the current study, strong synergistic interactions were only observed when the BFW larvae were exposed to the EPF for 1 or 2 weeks before the addition of the EPN. The findings were different from those reported by Ansari *et al*. (2004) who found stronger synergistic effects when larvae were exposed to *M. anisopliae* for at least 3 or 4 weeks before the addition of nematodes.

In earlier evaluations with BFW adults, the EPF strains Eco-Bb®, BroadBand®, Meta 69, *M. anisopliae* EA2 combined with *S. yirgalemense* against BFW adults showed successful control for all strains. When *S. yirgalemense* was applied simultaneously, 1 or 2 weeks after application of BroadBand® and *M. anisopliae* isolate EA2, 100% larval mortality was obtained. Additive interactions were mostly noted, with a synergistic interaction only being noted in the case of *M. anisopliae* isolate EA2 + *S. yirgalemense*, when it was applied 2 weeks after application of the EPF. The findings from the current study differed from those of Shapiro-Ilan *et al*. (2003) on the pecan weevil, *Curculio caryae* Horn, where additivity or synergy was reported between the EPF or between the bacterium and the EPNs only, when the entomopathogens were applied simultaneously. In the same study, most of the combinations resulted in antagonistic interactions,

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with an exception being the combination of *H. indica* with *M. anisopliae*, where additive effects were observed.

The synergistic interactions shown between the different EPF and *S. yirgalemense* promote the control of the BFW by means of reducing the cost of control, while maximising the efficiency of the control strategy. In another study, undertaken by Wakil *et al*. (2017), to investigate the insecticidal properties of *B. bassiana* or *M. anisopliae* in combination with *H. bacteriophora* for the control of red palm weevil, *Rhynchophorus ferrugineus* (Olivier), both additive and synergistic interactions were reported. However, the synergism was observed mostly in the *H. bacteriophora* and *B. bassiana* combinations compared to the *H. bacteriophora* and *M. anisopliae* combinations. Ansari *et al*. (2008) report that combining biocontrol agents has additional benefits, including extended control of the host insect in the field. The reason is that EPF can persist for more than 18 months longer than can the EPNs in growing medium (Coombes *et al*. 2013, 2015). The fungal conidia and the infective juveniles (IJs) can spread to the surviving populations, thus providing long-term protection (Ansari *et al*. 2008).

The current study showed that the EPF isolates, in combination with *S. yirgalemense* under laboratory conditions, resulted in high mortality against both the larvae and the adults of the BFW. The incorporation of Eco-Bb®, BroadBand®, Meta 69, and *M. anisopliae* EA2 in combination with *S. yirgalemense* for the control of BFW can be more effective than are single- application treatments. However, further research is required under field conditions to confirm the degree of success that can be attained with using combinations of entomopathogens in apple orchards and vineyards.

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Chapter 6

Conclusion

The current study is the first to report on the use of EPNs and EPF as potential biological control agents against the BFW in the Western Cape. The study aimed to investigate the biological control potential of both EPNs and EPF to control the BFW in grapevines and apple orchards, in an integrated pest management system. Scant research has yet been conducted on the BFW, compared to other weevil pests such as the black vine weevil, *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae). One reason is that the BFW is a sporadic insect pest, which is found in patches in orchards and vineyards, and evaluating the different control options is, therefore, difficult. The study, in addition to being of importance to the field of IPM, has also sought to provide a potential alternative control mechanism for the BFW in orchards and vineyards. The study successfully isolated local pathogens, and excellent control was obtained with most EPNs and EPF tested in laboratory and small scale field trials against larvae, pupae and adults.

Two EPN species and a number of EPF isolates were obtained from Western Cape orchards and vineyards, proving that entomopathogens can survive in these areas, and are a potential option for controlling the BFW. However, the success attained in trapping EPNs from vineyards and orchards was surprisingly low. Upon screening the *Heterorhabditis bacteriophora* Poinar and *Heterorhabditis safricana* Malan, Nguyen, De Waal & Tiedt for their pathogenicity against BFW adults under optimum laboratory conditions, the two were found to differ significantly from each other and from the control, with *H. safricana* causing lower mortality compared to *H. bacteriophora*. After screening the two EPF species for their pathogenicity against BFW adults under optimum laboratory conditions, both EPF species gave mortality > 50%, with the two species, *Beauvaria bassiana* and a *Metarhizium* sp. differing significantly from each other (Chapter 2). The findings were promising, as half the EPN concentration (200 IJs/insect) gave good results, compared to those achieved in a previous study (Ferreira 2010). The possibility of seeding orchards and vineyards with the suitable EPN species, which may then persist, and to provide a long-term ecological service, is a viable proposition to consider for future control of the BFW.

A number of EPN species have been reported as having been successful in controlling a number of economic insect pests worldwide. In the current study, a total of nine EPN species, consisting of eight local EPN isolates and *Steinernema feltiae* from e-nema, Schwentinental, Germany, were screened for their pathogenicity against BFW under optimum laboratory conditions. The five local

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Heterorhabditis species used were *H. bacteriophora*, *H. baujardi*, *H. noenieputensis*, *H. zealandica*, and *H. indica*, while the three local *Steinernema* species used were *S. jeffreyense*, *S. yirgalemense*, and *S. khoisanae*. All the EPN species screened in the current study provided good mortality of BFW, with the local EPNs providing better mortality of the BFW compared to the imported *S. feltiae*, in laboratory bioassays (Chapter 3). Such success can be attributed to the adaptability of the indigenous EPN species to the warm climatic conditions in South Africa, as compared to *S. feltiae*, which are adapted to cold conditions in Europe. From the laboratory trials, two local EPN species, *S. yirgalemense* and *H. noenieputensis*, were selected, so as to determine their lethal dosages against the fourth instar larvae of BFW, which indicated *S. yirgalemense* to be six times more potent than was *H. noenieputensis*, with better mortality of BFW larvae. Field trials are important for determining the potential of the EPNs in natural conditions, such as apple orchards and vineyards, where the EPN efficacy can be affected by both biotic and abiotic interactions. In a small-scale field trial local *S. yirgalemense* used at a low concentration (20 and 40 IJs/cm^2) resulted in mortality of more than 69 %, further demonstrating the potential of using the biocontrol agent within an IPM programme to control the BFW.

After selecting one promising EPN species, it was important to select an EPF isolate that would be effective in controlling the BFW under laboratory conditions. Commercially available fungi can provide a viable option for the control of BFW, since they are already registered, and they do not need to undergo the process of mass culture and formulation. A total of four EPF isolates, including two *Beauveria bassiana* isolates: Broadband® and Eco-Bb®, and two *Metarhizium anisopliae* isolates: Meta 69 and *M. anisopliae* isolate EA2, were screened at 1×10^6 conidia ml^{-1} for their virulence against the different larvae and adults BFW. All the EPF isolates were able to control the BFW, with Broadband® providing the best results compared to the other two commercial isolates and the indigenous isolate tested in the study (Chapter 4). High mortality of both the immature and adult stages of BFW was reported.

Combinations of biocontrol agents can improve the control of any insect pest, due to the synergistic or additive effects (Ansari *et al*. 2004). Some biocontrol specialists have postulated that synergistic interactions work by having one of the biocontrol agents stressing or changing the behaviour (in terms of reduced feeding or lack of movement) of the insect host, while becoming more susceptible to the other biocontrol agent (Ansari *et al*. 2008). However, the underlying processes of synergistic interactions remain unclear. In this study, four EPF isolates, including two *Beauveria bassiana* isolates: Broadband® and Eco Bb®, and two *Metarhizium anisopliae* isolates: Meta 69 and *M. anisopliae* isolate EA2, were applied either alone, or in simultaneous combination

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with *S. yirgalemense*, or 1 and 2 weeks after EPF application, to evaluate their insecticidal properties against BFW larvae and adults. The combined application of the different EPF isolates with *S. yirgalemense* resulted in mortality of the BFW of up to 100 % (Chapter 5). Additive interactions were mostly recorded when the biocontrol agents were applied simultaneously, while synergistic interactions were recorded when the biocontrol agents were applied 1 or 2 weeks after the application of the different EPF. The results strongly suggest that combining these biocontrol agents can result in good levels of BFW pest control in the field. However, in some cases, higher levels of insect pest control, compared to what can be achieved with either biocontrol agent when used alone, have been recorded, even when the interactions between the EPNs and the fungi were neither additive, nor synergistic (Choo *et al*. 2002; Acevedo *et al*. 2007; Ansari *et al*. 2008). This was not, however, the case in the present study, because both additive and synergistic interactions were recorded in the study. Both entomopathogens have shown great potential for the control the BFW in the field when they are used alone, as well as when they are used in combination, and may provide an economically viable control strategy for the BFW.

The findings from this study suggest that both biocontrol agents can play an important role in the control of the BFW. The locally isolated EPN and EPF species, together with *S. yirgalemense* and *H. indica*, and Broadband®, can effectively control the BFW under optimum conditions. In addition to improving the control of BFW in orchards and vineyards, other synergies may arise, such as a possible reduction in production costs for growers, because each biocontrol agent used would be applied at a lower dose than when used alone (Koppenhöfer & Kaya 1997; Koppenhöfer *et al*. 1999). The integrated use of EPF and EPNs into the currently used management strategies of the BFW, so as to optimise the strategies concerned, is recommended. The biocontrol agents can be applied using conventional spraying equipment, and they can be mixed with most of the pesticides available on the market (Smart 1995), making them a practical solution for BFW control. The timing of the application (spring) of the biocontrol agents is key to reducing the population of BFW that might otherwise make its way to the fruits and aerial parts of the plants concerned.

6.1. FUTURE RESEARCH

Research into BFW is problematic, because the insect is not easy to mass culture. The study was only made possible because the different life stages of the BFW were cultured in the laboratory, albeit with low numbers and a low survival rate. Future research should involve finding effective techniques to culture the BFW with a higher survival rate. Discovering new species of

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entomopathogens is important, as species that are comparatively virulent can be used to increase the potential of microbial control, requiring the conducting of more surveys to find new species of biocontrol agents. Additional research to determine the effect of organic matter on the persistence of the biocontrol agents in the orchards and vineyards is recommended. The above is because organic matter was found to affect the persistence of EPF in the soil negatively (Chapter 2), with an increase in the amount of organic matter present reducing the degree of persistence of EPF in the medium, as well as the degree of control of host insects in the field. Future research into the biological control of the BFW with the help of EPNs and EPF should continue on more small scale field trials with a later focus on conducting large-scale field trials to demonstrate the potential use of EPF and EPNs within an IPM programme. Since *S. yirgalemense* and Broadband® were found to be the most effective in controlling the BFW, future research should focus on their compatibility with the different control strategies currently used, including agrochemicals and herbicides. Additional research should be conducted on the formulation of effective biocontrol agents, so as to allow for ease of application of the biocontrol agents to the BFW in the field, and so as to increase the shelf life and persistence in the environment following on application. Since the adult stage of the BFW can easily transmit the biocontrol agents to the non-infested BFW, future research should focus on finding a method for the application of such biocontrol agents as spores and infective juveniles, on the trunk barriers that the adults must cross to access the canopy of the vine/tree concerned.

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