INTERACTIONS OF BIOPESTICIDES WITH SYNTHETIC PESTICIDES AND THEIR IMPLICATIONS FOR MANAGEMENT OF THE MACADAMIA SEED WEEVIL, *Kuschelorhynchus macadamiae* (COLEOPTERA: CURCULIONIDAE)

A Thesis submitted by

Kim Khuy Khun

For the award of

Doctor of Philosophy

April 2021

Abstract

In Australia, macadamias (*Macadamia integrifolia* and *M. tetraphylla*) are the only native nut trees to be commercially grown and generate annual revenue of more than AU$ 850 million. However, production has faced many challenges, including those caused by a number of insect pests. Macadamia seed weevil (MSW), *Kuschelorhynchus macadamiae*, is one of the key pests causing serious issues for growers. To control MSW the industry has been using broad-spectrum insecticides such as acephate and indoxacarb. Although chemical control is the current recommendation, the industry has a vision to produce macadamia nuts in a "clean and green" environment. To achieve this goal, the industry has committed to the development of an integrated pest and disease management (IPDM) program by reducing the use of broad-spectrum insecticides and integrating biological and cultural controls into the IPDM program. Entomopathogenic fungi (EPF) are recognised as among the important biological control agents for controlling many insect pests. Despite this, there is neither peer-reviewed information available on the use of EPF for controlling MSW nor on the integration of EPF with the IPDM program on macadamias. In this thesis, both the potential of EPF for controlling MSW and the possibilities to integrate EPF with the current IPDM program on macadamias are examined for the first time.

In the first study, six strains of *Beauveria* spp. and six strains of *Metarhizium* spp. were identified using molecular techniques. The DNA sequences of the 5' region of elongation factor-1 alpha (EFT1) and the B locus nuclear intergenic region (Bloc) of all strains confirmed that they belonged to the fungal species *Beauveria bassiana* and *Metarhizium anisopliae*. All twelve strains of the EPF and a commercial biopesticide (Velifer®) were used in laboratory assays on MSW and the results showed that *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 were the best strains in bioassays, as they induced the highest mortality to MSW and had the lowest median lethal time (LT_{50}) compared to other strains of their respective species. In the second study, these two strains were used to study horizontal transmission from fungus-infected adults and conidiated cadavers to healthy adults. The results showed that the mortality of healthy adults varied from < 50% to 100% depending on the ratios of

fungus-infected adults or conidiated cadavers with the healthy adults and the experimental conditions, i.e. a confined environment or the larger insect cages.

Insecticides (acephate, indoxacarb, trichlorfon, sulfoxaflor, spinetoram, beta-cyfluthrin, methidathion, diazinon) and fungicides (pyraclostrobin, carbendazim) are commonly used for controlling insect pests and plant diseases during the period when MSW is active. The *in vitro* study showed that acephate, indoxacarb and trichlorfon at their full field concentrations (FFCs) were compatible with both fungal species whereas sulfoxaflor and spinetoram at their FFCs were compatible to only *B. bassiana.* Beta-cyfluthrin, methidathion and diazinon at their FFCs were moderate to highly toxic to both fungal species whereas both fungicides were very toxic to the EPF even at 6.25% of their FFCs. The interactions of acephate and indoxacarb with EPF for controlling MSW were also investigated under laboratory and glasshouse conditions. Their synergistic and additive interactions were measured and they provided better control of MSW under both sets of experimental conditions than either insecticides or EPF alone.

In conclusion, this study demonstrates that EPF are potential biological control agents for managing MSW either directly or indirectly (via transmission). In addition, they are able to integrate with some insecticides, whereas fungicides cannot be integrated with EPF. Future studies, such as on attractants for MSW, the potential of applying compost/mulch with EPF to cover weevil infested nuts, and understanding the movement of MSW after emerging from the infested nuts may allow EPF to be used in more innovative ways.

Certification of Thesis

This Thesis is the work of **Kim Khuy Khun** except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Professor **Gavin J. Ash**

Associate Supervisor: Dr. **Bree A.L Wilson**

Associate Supervisor: Dr. **Ruth K. Huwer**

Associate Supervisor: Dr. **Mark M. Stevens**

Student and Supervisors signatures of endorsement are held at the university.

Statement of Contribution

The following detail is the agreed share of contribution by the candidate and co-authors in each publication/manuscript presented in this thesis.

Chapter 2 (Section 2.3)

Kim Khuy Khun, Bree A.L. Wilson, Mark M. Stevens, Ruth K. Huwer, Gavin J. Ash, 2020. Integration of entomopathogenic fungi into IPM programs: studies involving weevils (Coleoptera: Curculionoidea) affecting horticultural crops. *Insects* 11, Article ID 659. (Top 10% journal; Impact Factor: 2.14 and SNIP: 1.14). DOI: 10.3390/insects11100659

Kim Khuy Khun: designed the study, carried out literature search, analysed literature, synthesised concepts, drew the illustrations, drafted the manuscript and revised the final submission (70% contribution to the study). ***Bree A.L. Wilson***, ***Mark M. Stevens***, ***Ruth K. Huwer*** and ***Gavin J. Ash***: contributed to study design, edited the manuscript, provided comments and suggestions for revision of the manuscript (30% contribution to the study).

Chapter 3

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson, 2020. Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays. *Journal of Invertebrate Pathology* 174, Article ID 107437. (Top 10% journal; Impact Factor: 2.1 and SNIP: 0.88). DOI: 10.1016/j.jip.2020.107437

Kim Khuy Khun: designed the study, conducted all experiments, analysed the data, drafted the manuscript and revised the final submission (70% contribution to the study). ***Gavin J. Ash***, ***Mark M. Stevens***, ***Ruth K. Huwer*** and ***Bree A.L. Wilson***: contributed to study design, edited the manuscript, provided comments and suggestions for revision of the manuscript (30% contribution to the study).

Chapter 4

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson, 2021. Transmission of *Metarhizium anisopliae* and *Beauveria bassiana* to adults of

Kuschelorhynchus macadamiae (Coleoptera: Curculionidae) from infected adults and conidiated cadavers. *Scientific Reports* 11, Article ID 2188 (Top 10% journal; Impact Factor: 4.01 and SNIP: 1.37). DOI: 10.1038/s41598-021-81647-0

Kim Khuy Khun: designed the study, conducted all experiments, analysed the data, prepared all figures, drafted the manuscript and revised the final submission (70% contribution to the study). ***Gavin J. Ash***, ***Mark M. Stevens***, ***Ruth K. Huwer*** and ***Bree A.L. Wilson***: contributed to study design, edited the manuscript, provided comments and suggestions for revision of the manuscript (30% contribution to the study).

Chapter 5

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson, 2021. Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* to insecticides and fungicides used in macadamia production in Australia. *Pest Management Science* 77, 709-718 (Top 10% journal; Impact Factor: 3.26 and SNIP: 1.56). DOI: 10.1002/ps.6065

Kim Khuy Khun: designed the study, conducted all experiments, analysed the data, drafted the manuscript and revised the final submission (70% contribution to the study). ***Gavin J. Ash***, ***Mark M. Stevens***, ***Ruth K. Huwer*** and ***Bree A.L. Wilson***: contributed to study design, edited the manuscript, provided comments and suggestions for revision of the manuscript (30% contribution to the study).

Chapter 6

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson, 2021. Interactions of fungal entomopathogens with synthetic insecticides for the control of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae). *Journal of Applied Entomology* (Top 10% journal; Impact Factor: 1.83 and SNIP: 1.11). DOI: 10.1111/jen.12879

Kim Khuy Khun: designed the study, conducted all experiments, analysed the data, drafted the manuscript and revised the final submission (70% contribution to the study). ***Gavin J. Ash***, ***Mark M. Stevens***, ***Ruth K. Huwer*** and ***Bree A.L. Wilson***: contributed to study design, edited the manuscript, provided comments and suggestions for revision of the manuscript (30% contribution to the study).

Acknowledgement

Firstly, I would like to express my profound gratitude to my principal supervisor, Professor Dr. Gavin J. Ash for providing me with the opportunity to pursue this study. Professor Ash, I am so honoured to have you as my principal supervisor and to gain your expertise and trust. These have given me strength to work strategically to complete all tasks you have provided, and also to conduct high quality research in agriculture. Dr. Bree A.L. Wilson, my first associate supervisor, you are recognised for the significant contribution to my study. Dr. Wilson, thanks for being my great mentor in the research and providing me with generous support. Dr. Ruth K. Huwer, my second associate supervisor from Wollongbar Primary Industries Institute (WPII) of NSW DPI, you are also acknowledged as an important person to help with this research. Dr. Huwer, thanks for your benevolence and fieldwork support at Alstonville, NSW. Dr. Mark M. Stevens from NSW DPI, I am so thankful to have you as the last associate supervisor, but not the least to contribute to my study. Dr. Stevens, thanks for being my significant mentor in writing scientific papers. All my pre-submission manuscripts would not be refined without your attention to the details. Overall, I am fortunate enough to have four great supervisors and have once in a lifetime experience to learn almost everything I could from you all. The long journey of this study would not have been possible without you all.

I would like to express my sincere thanks to Dr. Sathya Khay from CARDI (Cambodia) for introducing me to Professor Ash and Mark Hickey, former director of WPII and leader of Northern Horticulture, NSW DPI. Thanks also to Mark for giving me the opportunity to meet and work with all the entomology staff at WPII. Thanks to Mark, Jeremy Bright (WPII) and their families for the warm welcome and accommodating me for a few nights at their beautiful places during my first field visit at Alstonville.

I am very grateful to the USQ international stipend and fees research scholarships I received, which supported my study and my stay in Australia from mid-2017 to the end of 2020. I really appreciate your generosity, USQ. I would also like to acknowledge Hort Innovation Australia with the Australian macadamia industry's research and development levy and contribution from the Australian government for partly funding my research project.

My sample collection in the field would not have been possible without the benevolent support of many people from northern NSW such as Jarrah Coates (a local consultant), Craig Maddox (WPII), Ian Purdue (WPII) and David Robertson (WPII). A big thank you also to Dr. Diana Leemon (QDAF) for providing me with several fungal entomopathogen strains to test.

My good, safe and successful time at USQ was also due to the contribution and support of many staff. Thanks goes to Dr. Susette Eberhand and her team for allowing me to get access and use the SEM, Dr. Joanna Turner for lending me her UV light meter, Associate Professor Anke Martin for lending me her data loggers, Lauren Huth and Katelynn Hadzi for all assistance in accessing facilities, Dr. Kerry Hancock for the safety guidance and regulation, Dr. Rachel King and Daniel Burrell for the useful statistical advice. Thanks to Prof. Levente Kiss, Liz Neary and Juanita Ryan for all their administrative support. Thanks to the CCH advisory group for the comments on my pre-submission manuscripts. My lab mate, Sudhan, and all mates in CCH are also thanked for being nice and friendly which made my time in the university enjoyable.

My acknowledgement is also extended to Royal University of Agriculture and Ministry of Agriculture, Forestry and Fisheries of Cambodia for all administrative support prior to my study in Australia.

My profound gratitude to my parents for their endless love and support throughout my entire life. My parents, Sim Veng and Sothea Mong, taught me to understand and appreciate the value of knowledge. When I was small, they taught me many things and said many words of wisdom. Among those, they said “ship leaves, harbour stays”, this translates as “human will die in some day, but your achievement and invention (or findings) are staying with the mankind”. They were unfortunate to not get high education during their time, as Cambodia had come across many civil wars. I am extremely fortunate to get to school with their wisdom and I have now reached the highest degree in a modern country. Even you both are unlikely to be able to read or understand my findings, mum and dad, I produce this one for you both! The Khun team, Kim Eng, Kim Ang, Siv Lang and Siv Luy, you all are thanked for being the good brothers and sisters, bringing joy during the video conferences and taking care of our parents during my absence from Cambodia.

Lastly, I would like to thank my fiancée and future wife, Satya Ouk, for her love, patience and encouragement throughout my study while we are thousands of miles apart. Our future plans have given me strength to complete this study.

Table of Contents

List of Figures

Abbreviations

AMS	Australian Macadamia Society
ANIC	Australian Nut Industry Council
APVMA	Australian Pesticides and Veterinary Medicines Authority
BOM	Bureau of Meteorology
EPF	Entomopathogenic fungi
HVP	Hidden Valley Plantations
INDFC	International Nut and Dried Fruit Council
NIS	Nut-In-Shell
NZ TCA	New Zealand Tree Crops Association
pers. comm.	Personal Communication
QDAF	Queensland Department of Agriculture and Fisheries
US EPA	United States Environmental Protection Agency

Chapter 1: Introduction

1.1. Introduction to macadamias

Macadamias are evergreen nut trees in the family Proteaceae, and are endemic to the east coast of Australia (Hely *et al.*, 1982; Wilkinson, 2005). Two main edible macadamia species have been commercially grown, *Macadamia integrifolia* Maiden and Betche (smooth shell nut) and *Macadamia tetraphylla* L. Johnson (rough shell nut) (Rosengarten, 2004). Several characteristics are useful to separate these two species, such as the number of leaves at each node (*M. integrifolia* has three leaves with smooth edges at each node while *M. tetraphylla* has four spiny leaves at each node) and the colour of the new flush of growth and flowers (*M. integrifolia* has a green flush and creamy white flowers whereas *M. tetraphylla* has a pink flush and flowers) (Vock, 1999; Orwa *et al.*, 2009a, 2009b). The macadamia is a follicle and consists of a green to brown husk, a hard brown shell and the kernel or macadamia nut (Figure 1), which contains up to 82% mono-unsaturated fat (Maguire *et al.*, 2004) and 3 – 5% sugar when dry (Fourie and Basson, 1990; Wall and Gentry, 2007). The green husk normally splits open along a suture as the nut matures. A mature macadamia tree may reach a height of 18 m or more and a canopy width of around 12 m (Figure 2) and generally produces the first nuts in the sixth to seventh year after seeding, or in the fifth year after grafting (Hamilton *et al.*, 1983; Rosengarten, 2004). Interestingly, some specifically bred varieties, for examples A4, A29, A38, A104, A217 and A268 start producing their first nuts in the third year (HVP, 1999; NZ TCA, 2017). Varieties A4, A16, A38, 246 (Keauhou), 344 (Kau), 660 (Keaau), 741 (Mauka), 814, 816, 842 and 849 are popular and widely planted in Australia (Quinlan and Wilk, 2005; AgriFutures Australia, 2017). Typically, the maximum yield is reached between the twelfth and fifteenth year with an average of 12 – 13 kg of nut-in-shell (NIS) per tree or around 3.5 – 4.0 t/ha in Australia with the industrial density of 313 trees/ha (O'Hare *et al.*, 2004; AMS, 2017). Macadamia trees can continue to produce nuts for 60 years or more (Hamilton *et al.*, 1983; Rosengarten, 2004).

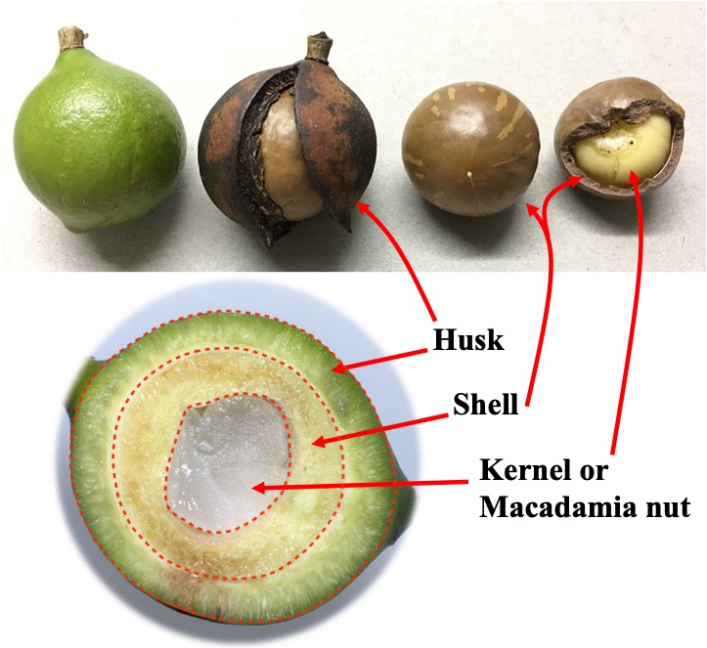


Figure 1: Immature macadamia nut cross-section and terminology for mature macadamia nuts

Figure 2: Mature macadamia trees at the Centre for Tropical Horticulture (CTH) at Alstonville, New South Wales

1.2. Macadamia industry in Australia

Macadamias are the only Australian native nut to be grown commercially (ANIC, 2019). In Australia, macadamias are the second largest nut crop grown (ANIC, 2019) with 28,000 ha in plantations, a total farm-gate value of AU$ 267 million and retail value of more than AU$ 850 million (AMS, 2019). In 2018, Australian macadamias were produced in three states; more than 57% of the total production was in Queensland, 40% in New South Wales (NSW) and less than 3% in Western Australia (Figure 3) (AMS, 2019). In total, Australia produced yield of NIS of 52,900t (ANIC, 2019) and the main part of the crop was exported (49,300t) (INDFC, 2019). The top markets for Australian macadamia nuts include the European Union (27% of total export), China (20%), Japan (19%) and the USA (15%) (INDFC, 2020). At least 25 countries are producing macadamia nut in commercial quantities (Figure 4). Australia and South Africa are the largest producers (Figure 5) and together, they are responsible for around 48% of world supplies (INDFC, 2019) or around 51% of world production (INDFC, 2020).

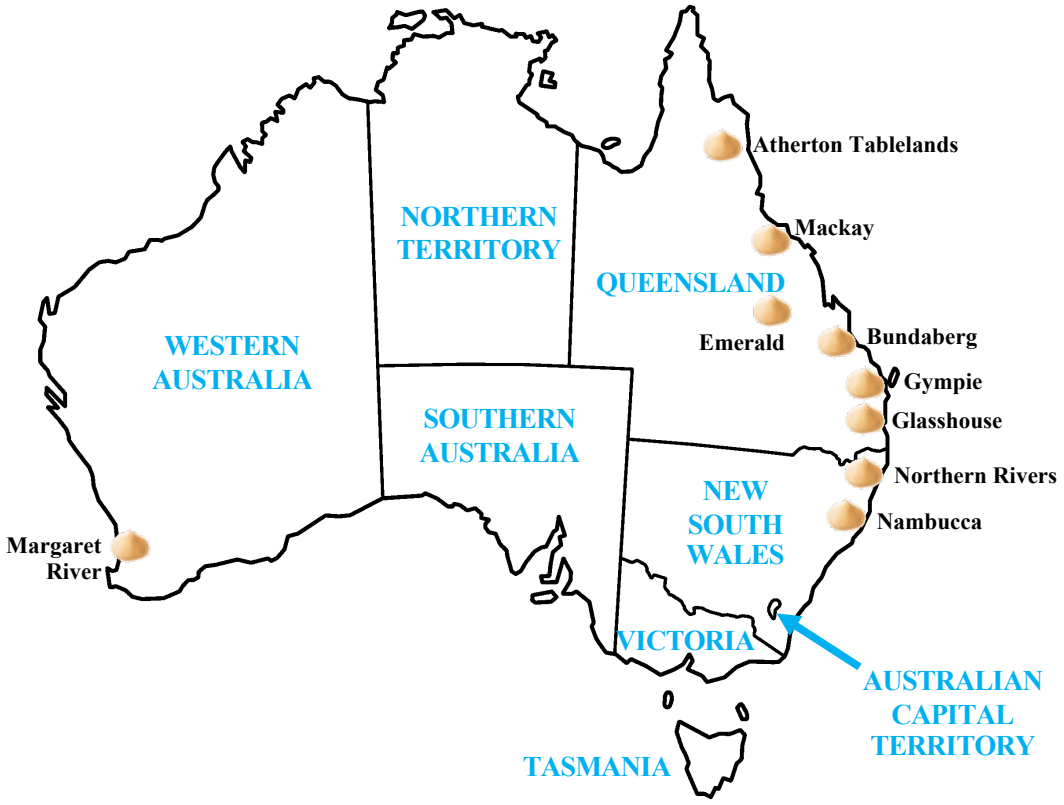


Figure 3: Macadamia producing regions in Australia (Redrawn from AMS, 2019)

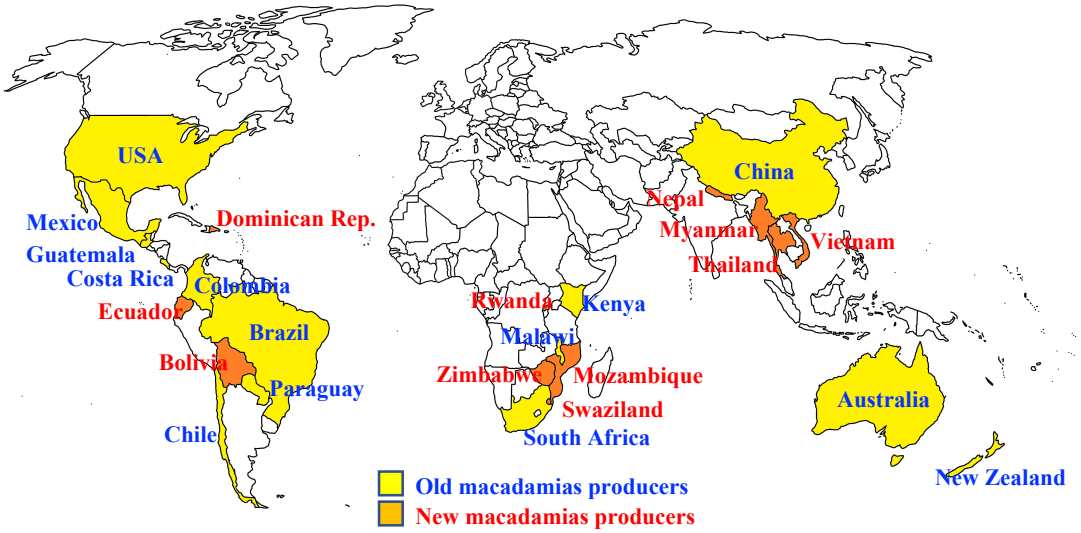


Figure 4: World macadamia producers (Redrawn from Nichols, 2017)

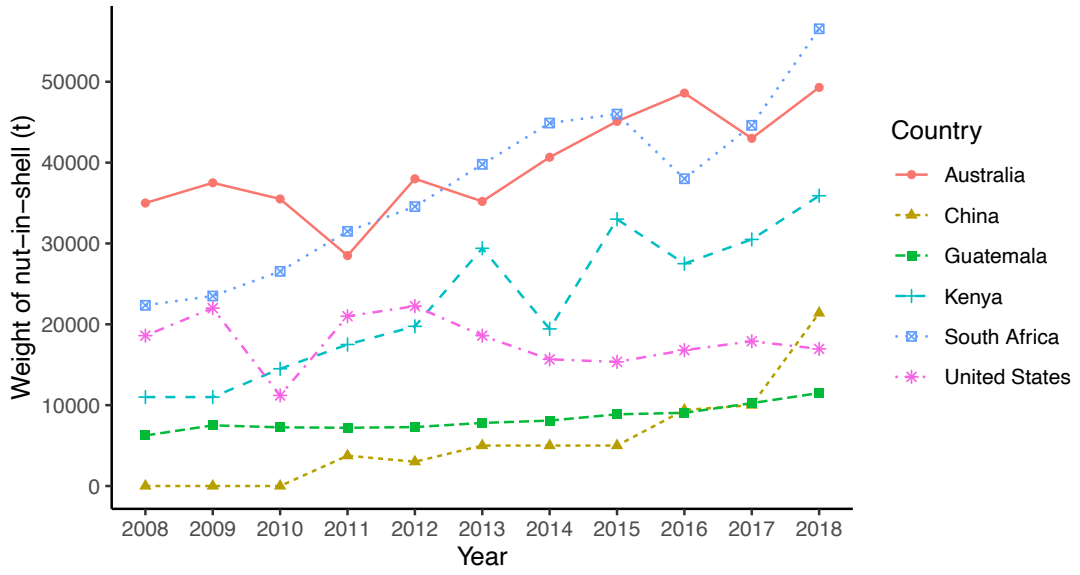


Figure 5: The six major macadamia nut-in-shell suppliers in the world between 2008 and 2018 (Compiled data from INDFC, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019)

1.3. Insects problems on Australian macadamias

According to the Queensland Department of Agriculture and Fisheries (2019), unfavourable weather such as storm/hail, hot/dry weather were considered as the first major factor affecting macadamia production. Insects pests were recognised as the second major factor affecting macadamia production, with management costs increasing from less than AU$ 300/ha between 2003 – 2006 (QDAF, 2016) to more

than AU$ 500/ha in 2018 (QDAF, 2019). Insects were also the number one problem causing the rejection of nuts by processors between 2009 – 2018 (QDAF, 2019). At least 150 insect species have been recorded on macadamia in Australia (Kawate and Tarutani, 2004) of which 41 species were considered common pests, with only 26 of these species prevalent on flowers and nuts (Vock, 2003). Among the insects pests the fruitspotting bug (*Amblypelta nitida* Stål, Hemiptera: Coreidae) and macadamia seed weevil (*Kuschelorhynchus macadamiae* Jennings and Oberprieler, Coleoptera: Curculionidae) are considered the most important on macadamia in Australia (QDAF, 2019).

Chemical control is the current industry standard for management of various pests and plant diseases in macadamia (Bright, 2019). For example, in order to control macadamia lace bug (*Ulonemia* spp., Hemiptera: Tingidae), *A. nitida* and *K. macadamiae*, trichlorfon, beta-cyfluthrin, methidathion, sulfoxaflor, diazinon, acephate, indoxacarb are recommended (Bright, 2019). For control of husk spot disease caused by *Pseudocercospora macadamiae* Beiharz, Mayers and Pascoe (Capnodiales: Mycosphaerellaceae), carbendazim and pyraclostrobin are recommended as rotational fungicides (Akinsanmi *et al.*, 2008; Bright, 2019). Although these agrochemicals are widely used, the Australian macadamia industry is committed to the development of an integrated pest and disease management program. This is aimed at reducing the use of broad-spectrum chemicals and integrating biological and cultural controls into pest management practices in order to conserve beneficial insects and protect the environment in the macadamia agro-ecosystem (AMS, 2019).

Entomopathogenic fungi (EPF), *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) are common biological control agents or biopesticides and have shown potential for controlling many economically important insect pests (Dolinski and Lacey, 2007; Lacey and Shapiro-Ilan, 2008; Lacey *et al.*, 2015). However, no peer-reviewed studies have investigated the potential of EPF to control insect pests on macadamia, especially those of economic importance like *K. macadamiae*.

1.4. Aim and Objectives

The overall aim of this study was to select potential biopesticides (EPF such as *B. bassiana*, *M. anisopliae*) and integrate them with registered synthetic pesticides in order to maximise the success of weevil management and minimise the unintentional impact of synthetic pesticides on biopesticide performance. In order to achieve this overall aim, four objectives were established, requiring a series of experiments in the laboratory and glasshouse. The key research objectives were:

- To identify, evaluate and compare the pathogenicity of *M. anisopliae* and *B. bassiana* strains to *K. macadamiae*
- To evaluate the potential of conidia transmission from fungus-infected adults and conidiated cadavers to fungus-free/healthy adults
- To evaluate the compatibility of registered synthetic pesticides used in macadamia production with *M. anisopliae* and *B. bassiana*
- To evaluate the synergistic interactions of the registered insecticides and EPF on *K. macadamiae*

1.5. Rationale and Outline

Chapter 1

This chapter provides general information on macadamia trees, the Australian macadamia industry, pest problems on macadamia, pest management practices, and research gaps and opportunities. It details the individual aim and objectives of the study.

Chapter 2

This chapter reviews the literature on *K. macadamiae,* in particular its classification, morphology, distribution, biology, symptoms of crop damage, economic impacts and its management. As *K. macadamiae* is restricted to Australia and is only known from macadamia, there are no peer-reviewed studies examining the control of *K. macadamiae* with EPF. To provide a better perspective of how to control *K. macadamiae* with EPF, various studies on *M. anisopliae* and *B. bassiana* on weevils affecting horticultural crops, which share similar habitats to *K. macadamiae* were compiled and synthesised, and a model on how to integrate EPF with other IPM

programs was designed. The compilation and synthesis for the integration of EPF with IPM programs is presented as a published paper in the journal *Insects.*

Chapter 3

This chapter presents the results of various experiments, which are the foundation of this study. The first experiment characterises the EPF (*B. bassiana* and *M. anisopliae*) using molecular methods and screens various strains of EPF, comparing them to a commercialised biopesticide based on *B. bassiana*. The most promising strains of both fungal species were selected for further tests on *K. macadamiae.* This study is presented as a published paper in *Journal of Invertebrate Pathology.*

Chapter 4

The sustainable philosophies related to the use of EPF for managing pests suggested that EPF efficacy in the field should not rely only on the success of controlling pests immediately after application, but should also focus on the ability of the EPF to remain active and continue the on-going suppression of pest populations over time. This means the fungus-infected insects should ideally transfer conidia or infective propagules to others in the first cycle, and cadavers of weevils killed by EPF should conidiate under suitable conditions and become a further source of fungal inoculum. Consequently, they could trigger more mortality to other insects via horizontal infection in the second cycle. Chapter 3 showed that EPF were potentially able to control *K. macadamiae* and were also able to conidiate well on the cadavers, and this chapter describes the potential for conidia transmission to adults of *K. macadamiae* from fungus-infected adults and conidiated cadavers. This study is presented as a published paper in the journal *Scientific Reports.*

Chapter 5

The results from Chapters 3 and 4 clearly show that EPF are potentially able to control *K. macadamiae.* Weather conditions in the field and the dense canopy of mature macadamia trees seem to provide a suitable habitat and shade for the EPF to persist in their activity and suppress the pests. However, we did not know whether the other agricultural practices related to pest and disease management during the active season of *K. macadamiae* may have any effects on the efficacy and persistence of EPF. This chapter investigates the side effects of commonly used pesticides on EPF. The results

of this study are presented as a published paper in the journal *Pest Management Science.*

Chapter 6

In Chapters 3 and 4 the most promising strains of *B. bassiana* and *M. anisopliae* were identified, and Chapter 5 demonstrated the compatibility of the insecticides acephate and indoxacarb (the registered insecticides for controlling *K. macadamiae*) with both fungal species *in vitro.* This chapter examined the compatibility of EPF with acephate and indoxacarb when applied to the weevils as combination treatments. Their synergistic interactions for the management of *K. macadamiae* under laboratory and glasshouse conditions were also explored. This study is presented as a published paper in the *Journal of Applied Entomology*.

Chapter 7

This chapter discusses the implications of this research project, provides a summary of outcomes, and identifies opportunities for further research. The conclusion of this thesis is also included.

Chapter 2: Literature review

2.1. Macadamia seed weevil, *Kuschelorhynchus macadamiae*

2.1.1. Classification

The Coleoptera is the largest order of insects with a total of more than 386,700 described species in 29,595 genera (Bouchard *et al.*, 2017). Macadamia seed weevil, *Kuschelorhynchus macadamiae* is classified under the order Coleoptera (Jennings and Oberprieler, 2018) and belongs to the family Curculionidae, which is the largest family of the seven families in the superfamily Curculionoidea with a total of 51,000 described species and makes up 82% of all weevil species or around 13% of all Coleoptera (Oberprieler *et al.*, 2007; Bouchard *et al.*, 2017). Macadamia seed weevil is classified under the subfamily Curculioninae and belongs to the genus *Kuschelorhynchus,* which is a new genus of the tribe Cryptoplini (Curculionidae: Curculioninae) and is only associated with macadamia (Oberprieler *et al.*, 2007). Only six genera, *Cryptoplus, Haplonyx, Menechirus, Sigastus, Zeopus* and *Kuschelorhynchus,* are in the Cryptoplini Tribe. They are confined to Australia except for one species, *Menechirus oculatus* Hartmann (Coleoptera: Curculionidae), which occurs in New Guinea (Jennings and Oberprieler, 2018). *Kuschelorhynchus* was previously misidentified as *Sigastus* (Fay *et al.*, 2001) but a recent study confirmed that they can be differentiated by their pronotal and elytral sculpture and their male and female genitalia (Jennings and Oberprieler, 2018). In addition, larvae of *Kuschelorhynchus* develop inside young macadamia nuts or nutlets (Proteaceae), but not in those of Myrtaceae where "true" *Sigastus* is found (Jennings and Oberprieler, 2018). A detailed key to all genera of Cryptoplini is available in Jennings and Oberprieler (2018).

2.1.2. Morphology

Egg: round to oval, pale yellow and 1 – 2 mm long (Figure 6).

Larvae: legless, plump and creamy white. The mature larvae are up to 15 mm long with segmented bodies, reddish-brown head capsules and strong black chewing jaws (Figure 6).

Pupae: creamy to grey in colour and about the size of the adult (Figure 6).

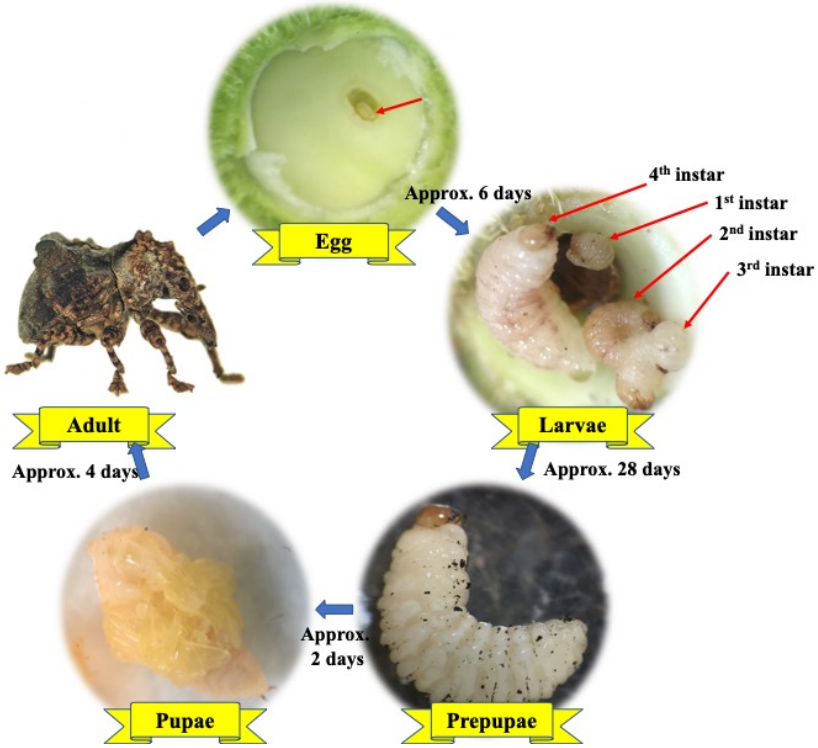


Figure 6: Life cycle of *K. macadamiae* at 25˚C

Adult: a detailed description is available in Jennings and Oberprieler (2018). Briefly, adults have a short body, which is subhexagonal in shape. Adults are typically 7.1 – 9.5 mm in length in both sexes (holotype 8.4 mm) (Jennings and Oberprieler, 2018), although individuals can be smaller due to variation in the size and nutritional quality of the nutlet in which they develop (Fay *et al.*, 2001; Vock, 2003). The width of adults is approximately 75% of their length and they bear large tubercles on their pronotum and elytra (Figure 7a, b, i). Adults are densely covered with a mixture of silvery white, pale and dark brown scales; the head has a complex pattern of dark brown and pale brown/whitish scales (Figure 7h); pronotum is greenish-grey except for centre and dorsal anterior margin around the tubercles with reddish-brown and reddish-brown elongate lateral patches (Figure 7b). *Rostrum*. Short (about 80% as long as pronotum in both sexes), very robust, straight, dorsoventrally slightly flattened, apically broadened in dorsal view (Figure 7g). *Antennae*. Inserted in apical third of rostrum; scapes reaching to below anterior margin of eye in repose; funicles with segment 1 around 200% longer than segment 2, segments 3 to 7 progressively shorter towards club; clubs shortly elongate, 250% longer than broad in dorsal view (the narrow side), finely pubescent (Figure 7c). *Legs*. Femora with two unequal ventral teeth, anterior tooth smaller (very small on metafemora) (Figure 8); tibiae with

premucro prominent but smaller than uncus (very small on metatibiae) (Figure 8); tarsi with claws slightly divergent (Figure 7d-f) (Jennings and Oberprieler, 2018).

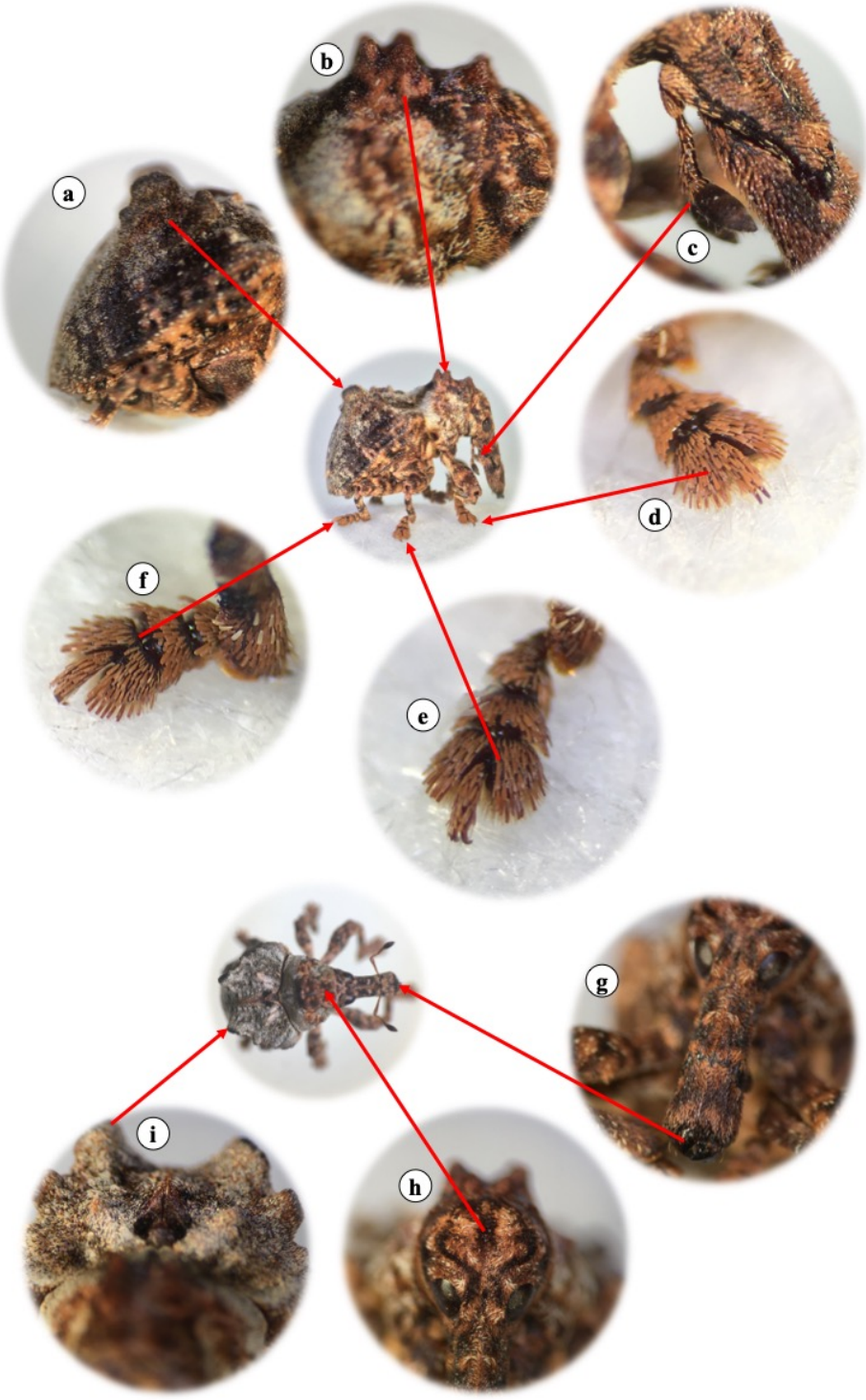


Figure 7: Adults of *K. macadamiae* in lateral view: (a) tubercles on elytra, (b) tubercles on pronotum, (c) antennae, (d-f) tarsi; in dorsal view: (g) rostrum, (h) head, (i) tubercles on elytra

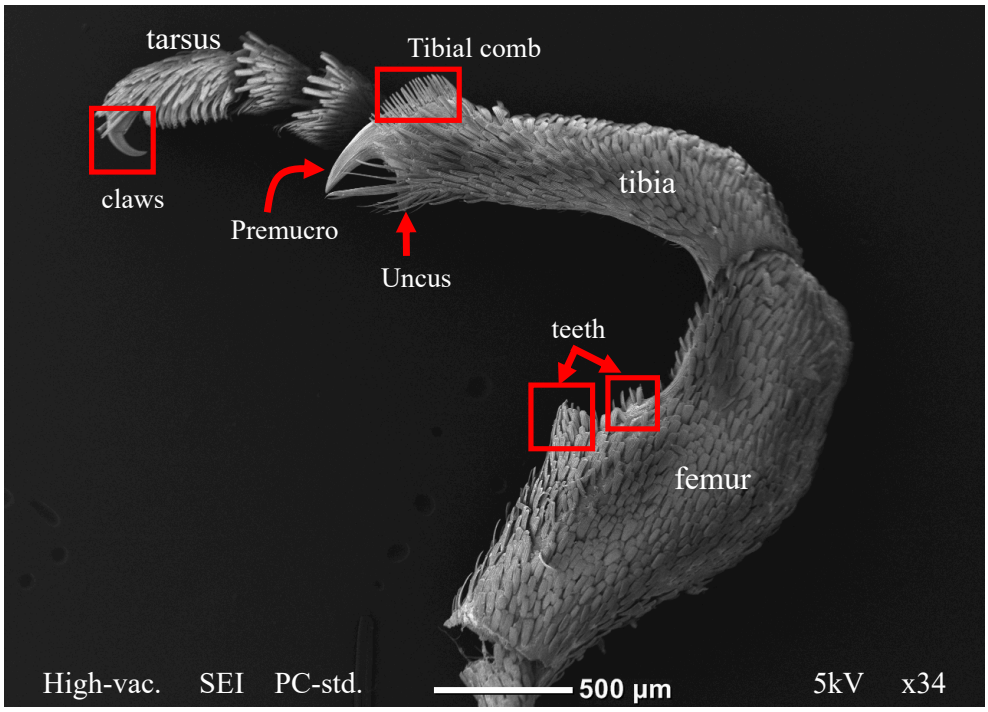


Figure 8: Scanning electron micrograph of the foreleg of *K. macadamiae* showing claws on the tarsus, premucro, uncus comb on the tibia and ventral teeth on the femur. Methods for specimen preparation and imaging are described in Chapter 4.

2.1.3. Distribution

Kuschelorhynchus macadamiae was first found on macadamia trees on the Atherton Tablelands, Queensland in 1994 (Fay *et al.*, 2001) and later in the Clunes/Eureeka area (Northern Rivers, NSW) in 2010 (Lee, 2014). It is not clear why this weevil was only found in two macadamia producing regions which are about 1,500 km apart. This could be the result of movement of seeds or seedlings infested by *K. macadamiae* from the Atherton Tablelands south to Clunes/Eureeka (J. Coates, pers. comm., October 31, 2017) (Figure 9). In 2014, *K. macadamiae* was found in many orchards in the Northern Rivers. Its expanded distribution was believed to be caused by the strong winds during a major storm event in 2013 (Lee, 2014). By 2015 the weevil's distribution was reported to have extended to 22 km from Clunes/Eureeka (Maddox *et al.*, 2015) and an isolated detection of *K. macadamiae* in the Gympie area was reported, but it remains absent from Glasshouse, Central Queensland (Mackay, Emerald, Bundaberg) and the Mid North Coast region (Nambucca) of NSW (Bright, 2019). In 2020, *K. macadamiae* remains confined to the Northern Rivers of NSW and Mareeba districts in far north Queensland (Bright, 2020).

Figure 9: Distribution and the year of the first report of *K. macadamiae* in two eastern states of Australia

2.1.4. Biology

Kuschelorhynchus macadamiae has a life cycle with complete metamorphosis (Figure 6) with adult, egg, larval and pupal stages. The adults are very active and can be easily found on leaves and nutlets, although they are well camouflaged when on the macadamia branches (personal observation). An adult female lays up to 280 eggs per generation (Bright, 2017a), but only a few eggs are laid each day (Fay *et al.*, 2001). Eggs are laid singly inside each nutlet when they are about 10 mm in diameter, in the tissue between the shell and the husk (Fay *et al.*, 2001). The larvae hatch from the egg in about 6 days and develop inside the nutlet by feeding on the developing shell tissue and kernel. The larval stage lasts around 4 weeks; passing through 4 larval instars. The adult weevil emerges after a prepupal period of about 2 days and pupal period of about 4 days (Bright, 2017a). The life cycle from egg-laying to adult emergence takes about 40 days at 25˚C (Bright, 2017a). The weevil passes through 3 generations in a year, with the first and second generations in November and December and the third and overwintering generation from March to October (Bright, 2017c).

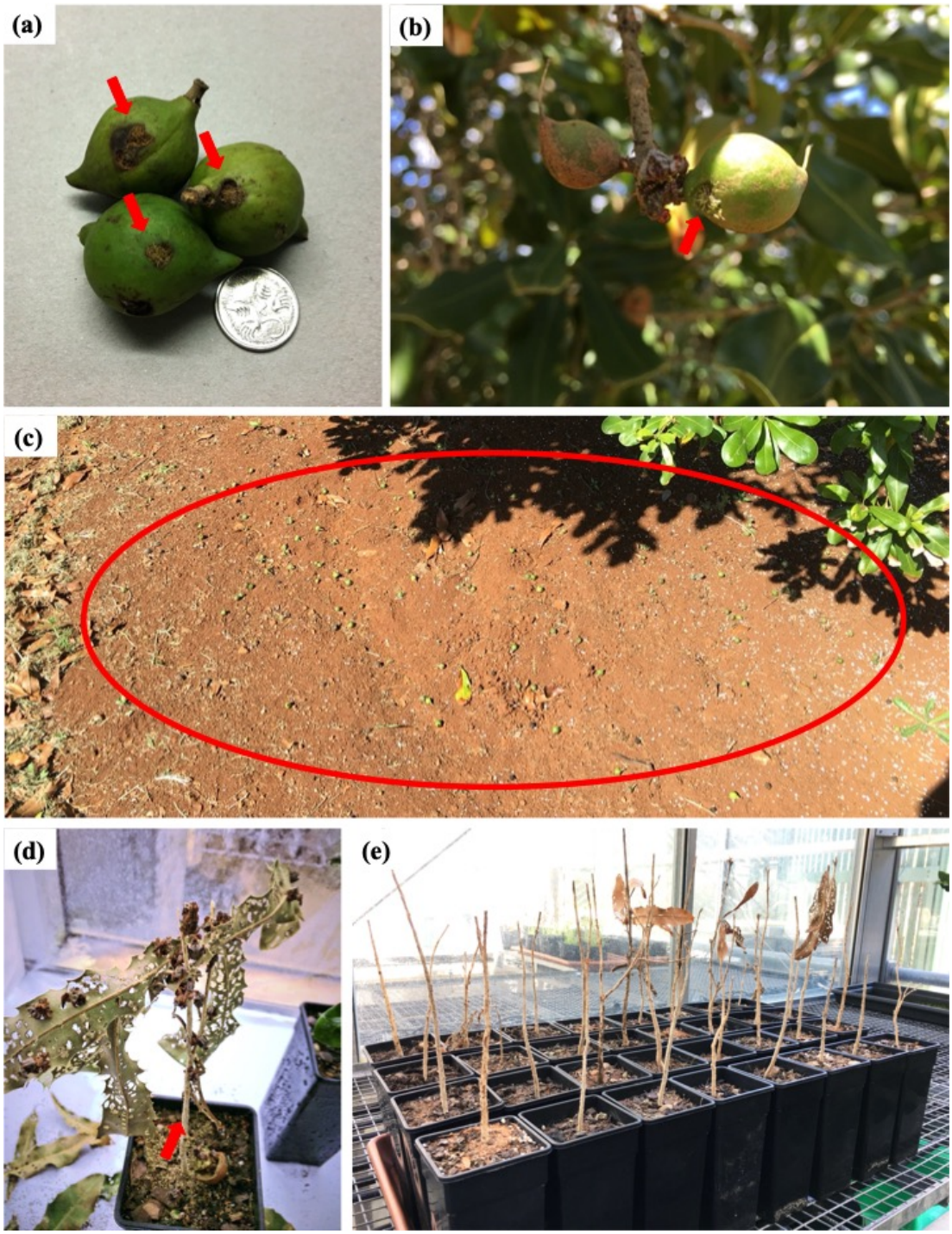


Figure 10: The damage caused by adults of *K. macadamiae*, (a) oviposition site showing the triangle shaped scarification mark, (b) the action of the female chewing the petiole of the nutlet to stop nut development and induce macadamia drop, (c) the abundance of dropped nutlets and (d & e) dead seedlings with bark removal and defoliation caused by adults

2.1.5. Plant damage and symptoms

The primary host damage caused by *K. macadamiae* involves nutlets drop, which is induced by adult females (Figure 10c). After laying one or a few eggs inside a nutlet, the female chews about half way through the petiole to stop nut development and induce macadamia drop (Figure 10b) (Fay *et al.*, 2001). The number of fallen young nuts increases rapidly through September and early October, then slows down before declining further in early December/January. When the shell hardens (about mid-December) the macadamia nuts are no longer suitable for oviposition (Fay *et al.*, 2001). Adult weevils also feed on young leaves and the green surface of the husk (Vock, 2003), and are also able to completely remove the bark of the macadamia seedlings (Figure 10d-e). The symptoms of damage can be easily identified, with a scarified area on the surface of each macadamia nut (Figure 10a) (Fay *et al.*, 2001). The scarified area is triangular in shape with blackened husk attached on top. The adults chew the macadamia husk to the depth of the shell and deposit their eggs then they put back the husk tissue to cover the oviposition site and protect the eggs (personal observation). Normally, the egg or larva can be found just under the scarified area.

2.1.6. Economic importance

Fay *et al.* (2001) found that *K. macadamiae* could cause significant damage to macadamia orchards, estimating a yield reduction of up to 30% in unsprayed farms, especially on susceptible varieties such as 344 and 741. Observations and sample collection in this study showed that the wild macadamia in the germplasm block (28°50'49.2"S 153°27'23.1"E) and other commercial varieties (246, 660, 814 etc.) were also severely infested when insecticides were not applied. A new study has estimated that *K. macadamiae* reduces the overall yield of macadamia by around 15% annually. This equates to approximately AU$ 15 million worth of lost production (Huwer, 2016).

2.1.7. Status of the management program

Over the past 20 years the management of *K. macadamiae* has relied on chemical control. In the study by Fay *et al.* (2001), three potential insecticides were identified to control the weevil, beta-cyfluthrin, carbaryl and methidathion. Later Maddox *et al.* (2015) compared these insecticides with new chemicals and they found that acephate, carboxamide, beta-cyfluthrin, bifenthrin, cyantraniliprole, diazinon,

endosulfan, flupyradifurone, methidathion, methomyl, spinetoram, sulfoxaflor and trichlorfon were more effective than carbaryl. By the end of 2015, acephate was the only registered insecticide for controlling *K. macadamiae* (APVMA, 2015). Recently, the management on this weevil has moved away from multiple applications of acephate (registered until 31 January, 2021) (APVMA, 2015) to a maximum of two applications of indoxacarb (registered until 30 September, 2021) per season (APVMA, 2018) combined with the collection and destruction of the fallen nuts that contain developing larvae (Bright, 2020). The recommended schedule for fallen nut collection is monthly for 5 months, from September to January (depending on macadamia varieties) (Bright, 2017b).

2.2. Entomopathogenic fungi – biopesticides

2.2.1. Overview

The European Union gave the definition of biopesticides as "*a form of pesticides based on micro-organisms and natural products*" (European Commission, 2008) whereas the United States Environmental Protection Agency defined biopesticides as "*a certain type of pesticides derived from animals, plants, micro-organisms and certain minerals*" (US EPA, 2016). Three classes of biopesticides have been identified; microbial biopesticides (e.g. bacteria, fungi, virus, protozoa, nematodes, etc.), biochemical pesticides (e.g. plant extracts, insect sex pheromones) and plant-incorporated-protectants or genetically modified plants that induced gene expression and production of metabolites effective against pests and diseases (US EPA, 2016).

Entomopathogenic fungi (EPF) were the first microbial biopesticides to be developed and used to control insect pests (Olson, 2015). In 2007, around 171 biopesticide products based on EPF were in commercial production. Of these, around 119 biopesticides utilised two specific fungal species, *B. bassiana* and *M. anisopliae*, as their main EPF (de Faria and Wraight, 2007). *Beauveria bassiana* and *M. anisopliae* are from the order Hypocreales and have a cosmopolitan distribution (Roberts and St. Leger, 2004; Rehner and Buckley, 2005). At least 700 insect species in 15 insect orders including the economically important orders Coleoptera, Lepidoptera, Diptera, Hemiptera, Orthoptera and Thysanoptera have been reported to be infected either naturally or artificially in agricultural and forest systems (Zimmermann, 2007a, 2007b).

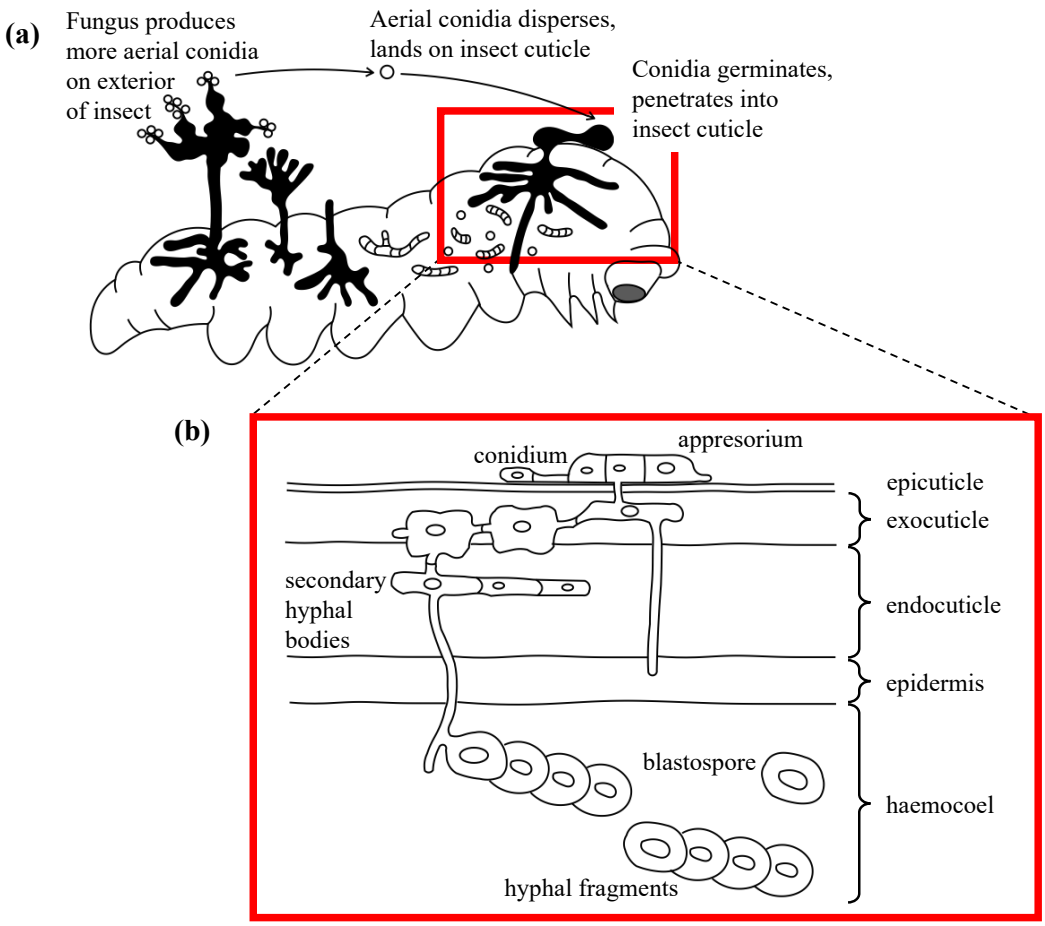


Figure 11: (a) Cycle of EPF infection on insect; (b) infection process of EPF on insect cuticle (Redrawn and modified from Hajek and St. Leger, 1994; Jaronski, 2014)

2.2.2. Mode of action

The mode of EPF infection is unlike other entomopathogens, including bacteria, viruses, protozoa and nematodes, which penetrate the host insect through only the midgut (therefore requiring ingestion) (Samson *et al.*, 1988) or anus (entomopathogenic nematodes only) (Boucias and Pendland, 1998). Entomopathogenic fungi infect insect hosts through contact (Mohammadbeigi and Port, 2013; Klieber and Reineke, 2016), especially at joints between body segments and the mouth (Zimmermann, 2007a, 2007b), parts of dorsal thoracic and abdominal sclerites (Quesada-Moraga *et al.*, 2008) and via the spiracles (respiratory openings) (Boucias and Pendland, 1998). Whenever conidia land on the cuticle of a suitable host, cuticle components like free amino acids or peptides may trigger the attachment, germination and production of an appressorium for penetration into the insect host (Zimmermann, 2007a, 2007b). After penetration into the insect's cuticle, EPF may

produce fungal toxins, obstruct hemolymph circulation, deplete nutrients and invade the internal organs (Inglis *et al.*, 2012) in order to overcome the host response and immune system, followed by proliferating within the host and formation of hyphal bodies or blastospores (Figure 11b) (Zimmermann, 2007a, 2007b). This development causes the death of the host (Samson *et al.*, 1988) in several days to a few weeks (Zimmermann, 2007a, 2007b). After host death and under humid conditions, the white muscardine fungus (*B. bassiana,* Figure 12a-c) (Zimmermann, 2007a) or the green muscardine fungus (*M. anisopliae,* Figure 12d-f) (Zimmermann, 2007b) may start its saprophytic growth out of the host body and produce aerial conidia (Figure 11a), which may allow horizontal infection into the insect population (Charnley and Collins, 2007).

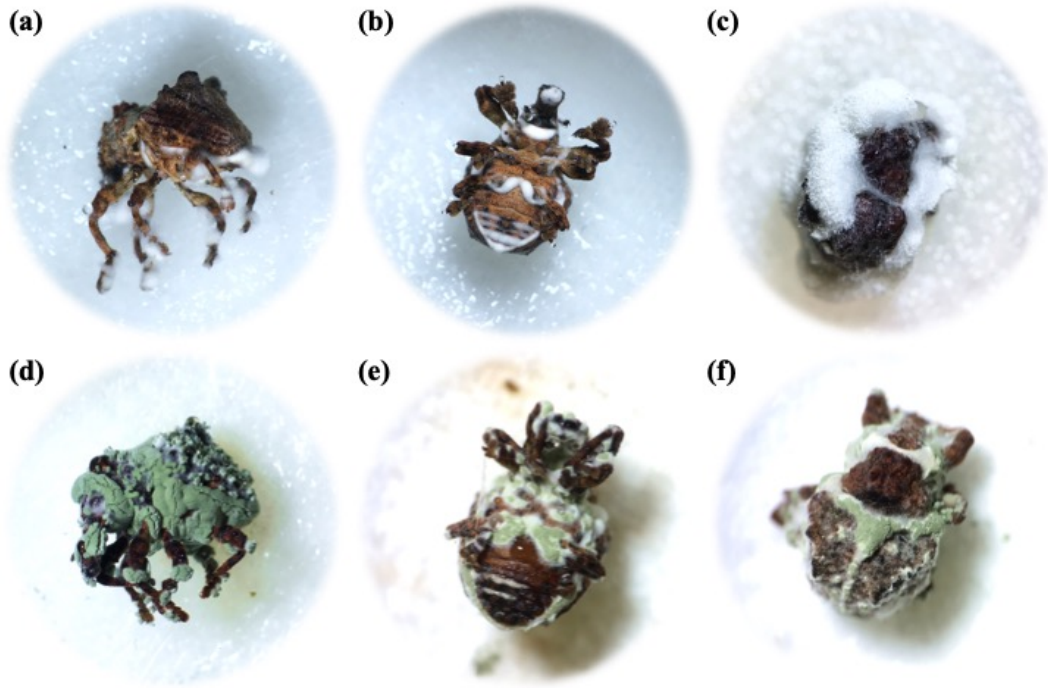


Figure 12: The white (a-c) and green (d-f) muscardine fungi conidiated on cadavers of *K. macadamiae*

In some cases, EPF are not able to attach, germinate and infect the insect host due to the secretion of antimicrobial compounds on the epicuticle that has evolved for the purpose of preventing or reducing the activities of microbes (Boucias *et al.*, 2018). These antimicrobial compounds on the insect's cuticle include quinones, formic acid, aldehydes, alkaloids and norharmane (beta-carboline), and are limited to some species of beetles, ants, bugs and termites (Gross *et al.*, 2002; Dossey, 2010; da Silva *et al.*, 2015; Pedrini *et al.*, 2015; Boucias *et al.*, 2018).

Over the past few decades, genuine oral infection by EPF inside the insect gut has remained unproven, although some studies have demonstrated the ability of EPF to kill insects after ingestion of conidia. As an example, the oral ingestion of *M. anisopliae* conidia led to significant mortality of *Hylobius pales* Herbst (Coleoptera: Curculionidae) (Schabel, 1976). Several studies that have examined insects histologically could only confirm that fungal conidia failed to attach, germinate and penetrate the gut wall, and could not cause inflammation of the midgut epithelium. Consequently, the conidia were removed from the digestive system in the form of faecal pellets (Chouvenc *et al.*, 2009; Butt *et al.*, 2013). Through whole genome sequencing and RNA-seq transcriptomics, Xiao *et al.* (2012) demonstrated that a number of toxins produced by *B. bassiana* could be found inside the gut of chickpea pod borer, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) after infection with a conidial suspension (e.g. heat-labile bacteria-like enterotoxins, vegetative insecticidal toxin, Cry-like delta enterotoxins, bacteria-like zeta toxins) which shared similarities to bacterial toxins and might have the ability to cause mortality to insects. Even so, the EPF failed to cause mortality to insects by ingestion in many studies. This could be due to the ability of the insects to detoxify the bacteria-like toxins (Biswas *et al.*, 2018), the unfavourable conditions in the gut (such as pH and digestive enzymes) for the fungi to be active (Mannino *et al.*, 2019) and the diverse microbiota in the insect gut which are potentially antagonistic to the fungi (Zhang *et al.*, 2013; Kabaluk *et al.*, 2017). In general, the microbiota in the insect gut is far more diverse than microbiota on the insect cuticle (Boucias *et al.*, 2018) and the insect gut microbiota, particularly bacteria, are antagonistic to foreign micro-organisms (Dillon and Charnley, 1986; Zhang *et al.*, 2018). Some examples of this antagonism include the effect of *Pseudomonas reactans* (Pseudomonadales: Pseudomonadaceae), a bacteria isolated from the gut of the German cockroach (*Blattella germanica* L., Blattodea: Ectobiidae), on the infection ability of *B. bassiana* (Zhang *et al.*, 2013) and the antagonistic effect of four bacteria species, *Pantoea agglomerans* (Enterobacterales: Erwiniaceae), *Pandoraea pnomenusa* (Burkholderiales: Burkholderiaceae), *Nocardia pseudovaccinii* (Corynebacteriales: Nocardiaceae) and *Mycobacterium frederiksbergense* (Corynebacteriales: Mycobacteriaceae) in the gut of wireworms (*Agriotes obscurus* L., *A. lineatus* L., Coleoptera: Elateridae) on the infection capacity of *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) (Kabaluk *et al.*, 2017). In some cases, however, the microbiota of the gut has a synergistic interaction

with EPF. For example in mosquitoes, the microorganisms can interact synergistically to enhance the effectiveness of the EPF (Wei *et al.*, 2017). The results of these studies demonstrate that mortality of insects caused by oral EPF ingestion is not consistent and it is effective only when insects are not able to detoxify the bacteria-like toxins, or when the microbiota of the gut has a synergistic interaction with the EPF.

2.2.3. Effect of environmental factors (humidity, temperature and solar radiation)

Humidity: Humidity is the most important environmental factor affecting the viability, efficacy and virulence of EPF on host insects and in the environment. Generally speaking, on growth media conidia require 98% relative humidity (RH) for germination (Milner *et al.*, 1997) or water activity (a_w) of at least 98 (Hallsworth and Magan, 1999; Lazzarini *et al.*, 2006); whereas water activity of 93 or 94% RH or lower was found to slow or inhibit germination (Milner *et al.*, 1997; Lazzarini *et al.*, 2006). *Metarhizium anisopliae* conidia have been shown to be more sensitive to low humidity compared to *B. bassiana*. A study found that the conidia of 10 strains of *B. bassiana* germinated faster and at higher rates than 10 strains of *M. anisopliae* when the moisture conditions were unfavourable (≤ 0.93 a_w), whereas when the water activity was high (≥ 0.99 a_w), the conidia of most strains of *M. anisopliae* germinated faster and at a higher rate than strains of *B. bassiana* (Lazzarini *et al.*, 2006). In contrast to germination on media, conidia germinate and infect insect hosts when the humidity is around 70% RH or more (Haraprasad *et al.*, 2001; Shipp *et al.*, 2003; Mishra *et al.*, 2015) although some exceptional infections have been reported at low RH (e.g. the mortality of larvae and pupae of false codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) at 12% RH was as high as that obtained at 98% RH when infected by *M. anisopliae* and *B. bassiana* (Acheampong *et al.*, 2020)) or low soil humidity (e.g. the mortality of adult Mexican fruit fly (*Anastrepha ludens* Loew, Diptera: Tephritidae) in response to *B. bassiana* at soil humidity of 6% was higher than at soil humidity of 21% (Wilson *et al.*, 2017)). When the humidity is lower than 70%, the efficacy of EPF on insects is reduced significantly, for example, < 30% mortality of adult housefly (*Musca domestica* L., Diptera: Muscidae) at 50% RH compared to > 70% mortality at 75% RH caused by *B. bassiana* (Mishra *et al.*, 2015) or 69% mortality of adult coffee berry borer (*Hypothenemus hampei* Ferrari, Coleoptera: Curculionidae) at 50% RH, compared to 87% mortality at 70% RH, also in response to *B. bassiana* (Haraprasad *et al.*, 2001). Formulations containing oil

reduce the desiccation stress on conidia (Hong *et al.*, 2005), consequently improving the ability of EPF to cause mortality (Brito *et al.*, 2008; Kaiser *et al.*, 2020).

Temperature: Temperature affects the germination, growth, infection and conidiation of EPF. Most EPF strains from different geographical locations grow effectively (germination and mycelial growth) on media at 25 – 30°C (Ekesi *et al.*, 1999; Dimbi *et al.*, 2004; Devi *et al.*, 2005; Acheampong *et al.*, 2020), although there are some exceptional cases that show that they can be active at temperatures as low as 3°C (McCammon and Rath, 1994; Rath *et al.*, 1995) and as high as 45°C (Rangel *et al.*, 2005; de Oliveira *et al.*, 2018). On insects, EPF are most effective at 25 – 30°C whereas at lower or higher temperatures (≤ 20°C and ≥ 35°C) their efficacy is reduced (Vandenberg *et al.*, 1998; Milner *et al.*, 2003; Mishra *et al.*, 2015; de Oliveira *et al.*, 2018; Tumuhaise *et al.*, 2018; Thaochan *et al.*, 2020). Strains that tolerate and grow at low temperature (18°C) could be very useful for targeting insects that occupy colder habitats, for example black vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) (Klingen *et al.*, 2015). To avoid heat shock as the result of exposure to high temperature, studies have investigated heat protectant formulations and have found that oil can improve viability and infectivity of EPF in comparison to oil-free formulations (Mola and Afkari, 2012; de Oliveira *et al.*, 2018). It is also important to note that high temperatures do not only effect the germination, mycelial growth and infectivity of the EPFs, but also conidiation on the dead cadavers (Arthurs and Thomas, 2001). Again, 25 – 30°C is the optimum temperature for EPF to conidiate on insect cadavers, whereas temperatures of ≥ 35°C or ≤ 20°C have been shown to significantly reduce conidiation (Arthurs and Thomas, 2001).

Solar radiation: Sunlight, which consists of UV-B (wavelength of 280 – 320 nm) and UV-A (wavelength of 320 – 400 nm) (Parisi and Wong, 1997), is very detrimental to EPF (Fernandes *et al.*, 2007; Yao *et al.*, 2010; Fernández-Bravo *et al.*, 2017). Several studies have shown that the germination of *M. anisopliae* strains is reduced significantly after conidia are exposed to sunlight for 4 h (Braga *et al.*, 2001a; 2001b). Similarly, Fargues *et al.* (1996) found that exposing conidia of 65 strains of *B. bassiana* and 23 strains of *M. anisopliae* from six continents to sunlight for 2 h reduced their viability significantly (germination of < 60% for 62 *B. bassiana* strains and < 42% for all tested *M. anisopliae* strains) and almost eliminated the viability of all isolates when they were exposed for 8 h (germination of < 2% for both fungal species). To provide protection, Alves *et al.* (1998) examined the role of various oils

as protectants in formulations and demonstrated that the germination of *M. anisopliae* in a peanut oil formulation was around 69% after exposure to solar radiation for 6 h, whereas the germination of *M. anisopliae* without peanut oil was only 10% after exposure to solar radiation for the same period. Other studies used sunscreen and other formulation additives (such as lignin, liposoluble photoprotectants and humic acid) as solar radiation protectants for EPF (Leland and Behle, 2005; Hedimbi *et al.*, 2008; Mochi *et al.*, 2017; Kaiser *et al.*, 2019). Although oil and other additives could provide a degree of UV protection, there is no way to avoid the detrimental effects of solar radiation completely. A study by Moore *et al.* (1993) showed that although conidia were protected by the formulation additives to some extent, they were still damaged by solar radiation and as a consequence the conidia may have diverted energy to repair damaged cells, delaying the germination process. Interestingly, this delay did not have any significant impact on fungal virulence to adult mosquitoes (*Aedes aegypti* L., Diptera: Culicidae) (Moore *et al.*, 1993; Falvo *et al.*, 2016). However, without any formulation additives for solar radiation protection, virulence was reduced significantly, for example 30% versus 60% mortality of medfly (*Ceratitis capitata* Wiedemann, Diptera: Tephritidae) after being challenged with *M. brunneum* previously exposed to UV-B for 48 h, compared to the same formulation not pre-exposed to sunlight (Fernández-Bravo *et al.*, 2017).

2.3. Integration of entomopathogenic fungi into IPM programs: studies involving weevils (Coleoptera: Curculionoidea) affecting horticultural crops

Kim Khuy Khun, Bree A.L. Wilson, Mark M. Stevens, Ruth K. Huwer, Gavin J. Ash

Insects 11, 659.

https://doi.org/10.3390/insects11100659

Note 1: "*Synergy*" in this review paper is defined as "*the joint action of two agents resulting in a greater effect than the sum of the activities of the agents acting alone*" (Koppenhofer, 2007, p. 658)

Note 2: supplementary materials associated to this paper are included in the appendix A.

insects

MDPI

Review

Integration of Entomopathogenic Fungi into IPM Programs: Studies Involving Weevils (Coleoptera: Curculionoidea) Affecting Horticultural Crops

Kim Khuy Khun [1,2,*], Bree A. L. Wilson [2], Mark M. Stevens [3,4], Ruth K. Huwer [5] and Gavin J. Ash [2]

1 Faculty of Agronomy, Royal University of Agriculture, P.O. Box 2696, Dangkor District, Phnom Penh, Cambodia
2 Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, Toowoomba, Queensland 4350, Australia; bree.wilson@usq.edu.au (B.A.L.W.); gavin.ash@usq.edu.au (G.J.A.)
3 NSW Department of Primary Industries, Yanco Agricultural Institute, Yanco, New South Wales 2703, Australia; mark.stevens@dpi.nsw.gov.au
4 Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga, New South Wales 2650, Australia
5 NSW Department of Primary Industries, Wollongbar Primary Industries Institute, Wollongbar, New South Wales 2477, Australia; ruth.huwer@dpi.nsw.gov.au
* Correspondence: kimkhuy.khun@usq.edu.au or khun.kimkhuy@rua.edu.kh; Tel.: +61-46-9731208

Received: 7 September 2020; Accepted: 21 September 2020; Published: 25 September 2020

Simple Summary: Horticultural crops are vulnerable to attack by many different weevil species. Fungal entomopathogens provide an attractive alternative to synthetic insecticides for weevil control because they pose a lesser risk to human health and the environment. This review summarises the available data on the performance of these entomopathogens when used against weevils in horticultural crops. We integrate these data with information on weevil biology, grouping species based on how their developmental stages utilise habitats in or on their hostplants, or in the soil. These patterns of habitat usage can help identify the stages during which pest species are at their most vulnerable, and also help to determine the most effective ways to deploy entomopathogens for their control.

Abstract: Weevils are significant pests of horticultural crops and are largely managed with insecticides. In response to concerns about negative impacts of synthetic insecticides on humans and the environment, entomopathogenic fungi (EPF) have been developed as an alternative method of control, and as such appear to be "ready-made" components of integrated pest management (IPM) programs. As the success of pest control requires a thorough knowledge of the biology of the pests, this review summarises our current knowledge of weevil biology on nut trees, fruit crops, plant storage roots, and palm trees. In addition, three groups of life cycles are defined based on weevil developmental habitats, and together with information from studies of EPF activity on these groups, we discuss the tactics for integrating EPF into IPM programs. Finally, we highlight the gaps in the research required to optimise the performance of EPF and provide recommendations for the improvement of EPF efficacy for the management of key weevils of horticultural crops.

Keywords: attract-and-kill; *Bacillus thuringiensis*; *Beauveria*; endophyte; entomopathogenic nematode; *Metarhizium*; repellent volatile; sterile male; transmission; weevil

1. Introduction

Insect pests are one of the main constraints to global crop production and reduce crop yields by 30–40%, equating to US$300 to 470 billion worth of production losses each year [1,2]. To manage them, synthetic insecticides are routinely used by commercial growers, with at least US$16 to 20 billion being spent on insecticides annually [1,3]. However, a sole reliance on insecticides is not considered sustainable as they are often harmful to endemic natural enemies within crops and may induce insecticide resistance in the target pest [4]. The same insecticides may also increase the frequency of primary and secondary pest outbreaks [5]. For example, pecan scab (*Venturia effusa*), pecan weevil (*Curculio caryae*) and pentatomid stink bugs (*Nezara viridula* and *Euschistus* sp.) are the key problems in pecan (*Carya illinoinensis*) plantations and to minimise their impact, preventive applications of broad-spectrum pesticides are used. Pyrethroids and carbaryl are used for control of late-season pecan weevil and kernel-feeding hemipterans; however, these insecticides also destroy aphidophagous insects and repel or kill predatory mites. Consequently, aphid and phytophagous mite resurgences are often observed [6]. Fungicides can also contribute to pest outbreaks and resurgence as a consequence of their impact on entomopathogenic fungi (EPF) [6,7]. The fungicides applied to control pecan scab are reported to kill the EPF that provide control of pecan aphids [8] and as a consequence, secondary outbreaks of aphids require additional application of insecticides [6].

Microbial biopesticides have been recognised as alternatives to synthetic insecticides, since they can have minimal impacts on non-target organisms, prevent pesticide resistance, and are less toxic to both humans and the environment [9,10]. On a global scale, microbial biopesticides account for approximately US$3.3 billion or around 8% of all pesticides sold [11], but they have long-term potential for increased usage over the next few decades [12]. Among the microbial biopesticides, EPF are the second-highest selling, accounting for around 9% of all microbial biopesticides sold globally [11]. Their popularity stems from their potential to control a wide range of insect pests [13,14] and their suitability for organic and sustainable crop production [15]. In addition to their direct impact on insect pests, EPF have also been reported to act as endophytes within host plants [16], can be integrated with attractants for attract-and-kill pest management approaches [17,18], and can be combined with sterile males for integration with the Sterile Insect Technique (SIT) [19–21]. Entomopathogenic fungi may also have synergistic interactions with some beneficial arthropods (predators, parasitoids, pollinators) [22–24], other entomopathogens (bacteria and nematodes) [25,26] and synthetic insecticides [27,28] that could be exploited within IPM programs on various crops.

Weevils are amongst the most important pests of horticultural crops. They often have behaviours and habitats that can make some insecticides difficult to deploy. For example, pecan weevil is a major pest of pecans in the southern United States [29] where the damage caused by larvae and adults can reduce yields by up to 80% [30]. This weevil has a lengthy and complicated life cycle (90% of the population complete their life cycle in 2 years while 10% take up to 3 years) with the larvae and pupae occurring inside nuts and in the soil, respectively [29]. This makes the opportunities to control this weevil using contact insecticides limited to only the adults.

Another important weevil in horticulture is the coffee berry borer (*Hypothenemus hampei*). It is a major pest of coffee (*Coffea arabica* and *C. canephora*) worldwide [31], with the damage caused by the larvae and adults estimated to cost the industry around US$500 million annually [32]. This borer is also difficult to control as the larval and pupal stages only occur inside the coffee berries [31]. There are many other weevil species in which one or more developmental stages live within the host plant and/or the soil and cause significant damage to horticultural crops (Table 1).

2. Methodology

This review describes the potential use of EPF (particularly *Beauveria* spp. and *Metarhizium* spp.) alone or in combination with other management techniques to control weevils in horticultural crops. Studies using *Metarhizium* spp. or *Beauveria* spp. for managing weevils affecting horticultural crops were identified using databases including Web of Science (http://www.webofknowledge.com),

SCOPUS (https://www.scopus.com), CAB Abstracts (https://www.cabdirect.org) and Google Scholar (https://scholar.google.com). A total of 1666 articles (Figure S1) were identified from searches using the terms "*Metarhizium*", "*Beauveria*", "Weevil", and "Curculionidae". After removing duplicates, 566 article titles were screened and 391 were excluded from this review because they fell outside the scope of this review due to the host crop involved, or other factors. The final 175 full-text articles, which show a strong bias towards perrenial crops, are included in this review. Fourty-four weevil species were identified as having a major impact on horticultural crops (Table 1). Studies published between 1973 and 2020 that dealt with the use of EPF for weevil control involved 26 of these species, and of these, data on life cycle duration was available for 21 (Table 2, Figure S2). The 26 weevil species used in experiments involving EPF could be grouped according to their patterns of habitat utilisation throughout their life cycles (Figure 1, Table 3). Successful pest management requires a thorough knowledge of pest biology [33], and in this review we combine published data with these patterns of habitat utilisation to identify the optimal approaches for integrating EPF into weevil IPM programs, targeting the most vulnerable developmental stages for each weevil group.

In this review, weevils are defined as the superfamily Curculionoidea, following the taxonomy of Oberprieler et al. [34] where the Curculioninae, Cyclominae, Dryophthorinae, Entiminae, Molytinae, and Scolytinae have subfamily status within the Curculionidae, and the Brentinae are a subfamily within the Brentidae.

Table 1. Important weevil species of horticultural crops, including crops impacted, geographical distribution and economic impact.

Weevil Species	Common Name	Family: Subfamily [1]	Distribution [2]	Crops	Damaging Stages [3]	Economic Impact [4]	Ref.
Aclees sp. cf. *foveatus* (Voss)	Fig weevil	Cur: Mol	IT	Fig	A & L	n/a	[35]
Aegorhinus superciliosus (Guérin)	Raspberry weevil	Cur: Cyc	AR & CL	Blueberries, raspberries, strawberry	A & L	n/a	[36,37]
Anthonomus musculus (Say)	Cranberry weevil	Cur: Cur	North-Eastern US & CA	Blueberries, cranberries	A & L	n/a	[38]
Anthonomus piri (Kollar)	Apple bud weevil	Cur: Cur	EUR & GB	Apple, pears	A & L	n/a	[39]
Anthonomus pomorum (L.)	Apple blossom weevil	Cur: Cur	EUR	Apple, pears	A & L	n/a	[39]
Anthonomus rubi (Herbst)	Strawberry blossom weevil	Cur: Cur	EUR & GB	Strawberry, blackberry, raspberry	A & L	MCL between 36–90%	[40,41]
Anthonomus signatus (Say)	Strawberry bud weevil	Cur: Cur	US & CA	Strawberry	A & L	MCL up to 100% in New York & 70% in Quebec	[42]
Blosyrus asellus (Olivier)	Rough sweetpotato weevil	Cur: Ent	US	Sweetpotato	A & L	n/a	[43]
Conotrachelus nenuphar (Herbst)	Plum curculio	Cur: Mol	Eastern & central NAM (US, CA)	Pome & stone fruits	A & L	MCL up to 85% in unsprayed orchard	[44,45]
Conotrachelus psidii (Marshall)	Guava weevil	Cur: Mol	BO, BR, CO, MX, PY & VE	Guava	L	MCL up to 100% in Rio de Janeiro, Brazil	[46,47]
Cosmopolites sordidus (Germar)	Banana weevil	Cur: Dry	Tropical regions worldwide	Banana & plantain	A & L	MCL up to 50%	[31,48]
Curculio caryae (Horn)	Pecan weevil	Cur: Cur	Southern US	Pecan	A & L	MCL between 30–80%	[29,30]
Curculio caryatrypes (Boheman)	Larger chestnut weevil	Cur: Cur	Central-eastern US	Chestnut	A & L	n/a	[49]
Curculio elephas (Gyllenhal)	Chestnut weevil	Cur: Cur	Central & Southern EUR, North AFR	Chestnut	A & L	MCL up to 90% in Italy	[39,50]
Curculio nucum (L.)	Hazelnut weevil	Cur: Cur	PAL, also present in North AFR	Hazelnut	A & L	MCL up to 80% in the unprotected orchards in Spain	[51,52]
Curculio sayi (Gyllenhal)	Lesser chestnut weevil	Cur: Cur	Central-eastern US	Chestnut	A & L	n/a	[49]
Curculio sikkimensis (Heller)	Chestnut weevil	Cur: Cur	CN, IN, JP & KR	Chestnut	A & L	n/a	[53,54]
Cylas formicarius (F.)	Sweetpotato weevil	Bre: Bre	Tropical regions worldwide	Sweetpotato	A & L	MCL up to 100%	[31,55]

Table 1. *Cont.*

Weevil Species	Common Name	Family: Subfamily [1]	Distribution [2]	Crops	Damaging Stages [3]	Economic Impact [4]	Ref.
Cylas puncticollis (Boheman), *C. brunneus* (F.)	African sweetpotato weevil	Bre: Bre	AFR (sub-Saharan)	Sweetpotato	A & L	MCL up to 97%	[55,56]
Diaprepes abbreviatus (L.)	Citrus root weevil	Cur: Ent	US & several CAR	Citrus, sugarcane	L	n/a	[57]
Heilipus lauri (Boheman)	Avocado seed weevil	Cur: Mol	CO & MX	Avocado	A & L	MCL between 60–70% in Mexico	[58,59]
Hypothenemus hampei (Ferrari)	Coffee berry borer	Cur: Sco	AFR, ASI, OCE, SCA & US	Coffee	A & L	MCL between 40 - 90%. EAL around US$215–358 million in Brazil or around US$500 million worldwide	[31,32,60]
Kuschelorhynchus macadamiae (Jennings & Oberprieler)	Macadamia seed weevil	Cur: Cur	Eastern AU	Macadamia	A & L	MCL up to 15%	[61,62]
Odoiporus longicollis (Olivier)	Banana stem weevil	Cur: Dry	Tropical ASI	Banana & plantain	A & L	MCL between 10–90%	[63]
Otiorhynchus clavipes (Bonsdorff)	Red-legged weevil	Cur: Ent	Western EUR	Plum, apple, berry crops, grapevine	A & L	n/a	[39]
Otiorhynchus ovatus (L.)	Strawberry weevil	Cur: Ent	EUR & NAM	Strawberry, berry crops	A & L	MCL up to 100% in Saxony, Germany	[39]
Otiorhynchus rugifrons (Gyllenhal)	Strawberry root weevil	Cur: Ent	EUR	Strawberry	A & L	n/a	[39]
Otiorhynchus rugosostriatus (Goeze)	Rough strawberry root weevil	Cur: Ent	EUR, NAM & MED	Strawberry, berry crops	A & L	n/a	[39]
Otiorhynchus singularis (L.)	Clay-coloured weevil	Cur: Ent	EUR & NAM	Apple, pear, berry crops, grapevine	A & L	n/a	[39]
Otiorhynchus sulcatus (F.)	Black vine weevil	Cur: Ent	EUR, NAM & AUA	Grapevines, berry crops	A & L	n/a	[39,64]
Pantorhytes plutus (Oberthür)	Cacao weevil	Cur: Ent	PG	Cacao	L	n/a	[65,66]
Phlyctinus callosus (Schönherr)	Banded fruit weevil	Cur: Ent	AU, NZ & ZA	Grapevines, pome fruit, stone fruits	A & L	MCL up to 40%	[67,68]
Pityophthorus juglandis (Blackman)	Walnut twig beetle	Cur: Sco	south-western US & MX	Walnut	A & L	n/a	[69]
Rhynchophorus bilineatus (Montrouzier)	Black palm weevil	Cur: Dry	ID, PG & SB	Palm	L	n/a	[70]
Rhynchophorus cruentatus (F.)	Palmetto weevil	Cur: Dry	Florida & south-eastern US	Palm	L	n/a	[70]
Rhynchophorus ferrugineus (Olivier)	Red palm weevil	Cur: Dry	ASI, AU & MED	Palm	L	EAL around US$5–26 million in the Middle East	[71,72]

Table 1. *Cont.*

Weevil Species	Common Name	Family: Subfamily [1]	Distribution [2]	Crops	Damaging Stages [3]	Economic Impact [4]	Ref.
Rhynchophorus palmarum (L.)	American palm weevil	Cur: Dry	MX & SCA	Palm	L	MCL up to 15%	[70,73]
Rhynchophorus phoenicis (F.)	African palm weevil	Cur: Dry	AFR	Palm	L	n/a	[70]
Rhynchophorus quadrangulus (Queden)	n/a	Cur: Dry	AFR	Palm	L	n/a	[70]
Scolytus amygdali (Guérin-Méneville)	Almond bark beetle	Cur: Sco	MED	Almond, apricot, peach	A & L	n/a	[39]
Scolytus mali (Bechstein & Scharfenberg)	Large fruit bark beetle	Cur: Sco	EUR & PAL	Apple, plum, pear	A & L	n/a	[39]
Scolytus rugulosus (Müller)	Fruit bark beetle	Cur: Sco	EUR	Apple, pear, plum	A & L	n/a	[39]
Xyleborus affinis (Eichhoff)	Ambrosia beetle	Cur: Sco	MX & US	Avocado, mango, macadamia, walnut	A & L	n/a	[74–76]

[1] Bre: Bre = Brentidae: Brentinae, Cur: Cur = Curculionidae: Curculioninae, Cur: Cyc = Curculionidae: Cyclominae, Cur: Dry = Curculionidae: Dryophthorinae, Cur: Ent = Curculionidae: Entiminae, Cur: Mol = Curculionidae: Molytinae, Cur: Sco = Curculionidae: Scolytinae. [2] AFR = Africa, AR = Argentina, ASI = Asia, AU = Australia, AUA = Australasia, BO = Bolivia, BR = Brazil, CA = Canada, CAR = Caribbean nations, CL = Chile, CN = China, CO = Colombia, EUR = Europe, GB = United Kingdom, ID = Indonesia, IN = India, IT = Italy, JP = Japan, KR = Korea, MED = Mediterranean area, MX = Mexico, NAM = North America, NZ = New Zealand, OCE = Oceania, PAL = Palaearctic, PG = Papua New Guinea, PY = Paraguay, SCA = South and Central America, SB = Solomon Islands, US = United States, VE = Venezuela, ZA = South Africa (code follows https://www.iso.org/). [3] A = adults, L = Larvae. [4] n/a = Specific data not available, MCL = may cause yield or crop loss, EAL = estimated annual loss. Ref. = References.

Table 2. Weevil life stage durations (where known) for important horticultural pest species used in experiments with EPF.

Weevil Species	Egg (Days)	Larvae (Days)	Pupae (Days)	Adult (Days)	Generation	Ref.
Aclees sp. cf. *foveatus*	10–20	n/a	n/a	n/a	2 generations/year	[35]
Anthonomus signatus	6–14	21–28	5–8	n/a	32–64 days/generation, 1 generation/year	[42,77]
Conotrachelus nenuphar	2–12	14–21	30	n/a	57 days/generation	[78]
Conotrachelus psidii	2–6	8–27	14–18	<418	108–280 days/generation	[79]
Cosmopolites sordidus	5–8	14–21	5–7	<730	1–6 months/generation	[31,80]
Curculio caryae	n/a	30	270–1080	n/a	2–3 years/generation	[29,81]
Curculio elephas	n/a	730–1095	90–150	n/a	1 generation/year in Italy	[50,82]
Curculio nucum	>7	28–35	< 365	90	1 generation/year in Turkey	[39,83]
Cylas formicarius	3–7	7–11	5–7	<240	5–8 generations/year in United States	[31,55]
Cylas puncticollis	< 5	<23	<14	<141	20–25 days/generation	[56]
Diaprepes abbreviatus	7–10	240–450	15–30	<147	5–18 months/generation	[84]
Heilipus lauri	<13	<49	<15	n/a	76 days/generation	[58]
Hypothenemus hampei	5–9	10–26	4–9	<157	25–35 days/generation, >8 generations/year in African countries, 2–3 generations/year in Colombia	[31,85]
Kuschelorhynchus macadamiae	6	28	4	n/a	At least 3 generations/year	[86]
Odoiporus longicollis	3–8	30–60	17–22	50–95	53–95 days/generation	[87]
Otiorhynchus sulcatus	>8	84–211	10–50	n/a	1 generation/year	[64]
Pantorhytes plutus	n/a	90–270	14	365–730	4–11 months/generation	[88]
Phlyctinus callosus	6–15	n/a	7–21	n/a	1–2 generations/year	[89]
Pityophthorus juglandis	n/a	n/a	n/a	n/a	7 weeks/generation, 2 generations/year	[90]
Rhynchophorus ferrugineus	1–6	25–105	11–45	n/a	45 days/generation in the Philippines, 139 days/generation in Spain; 3–4 generations/year in India, up to 21 generations/year in Egypt	[71]
Scolytus amygdali	n/a	n/a	n/a	n/a	>3 generations/year in the Mediterranean area	[91]

Note: *Aegorhinus superciliosus*, *Blosyrus asellus*, *Curculio sikkimensis*, *Rhynchophorus bilineatus* and *Xyleborus affinis* were not included in this table as specific data are not available. n/a = specific data not available. Ref. = References.

3. Life Cycle Patterns of Weevils Affecting Horticultural Crops

The three patterns of weevil life cycles which occur in association with horticultural crops are summarised in Figure 1 and Table 3. Adult weevils are normally active on the host plant during feeding and mating. Three locations on or around the host plant are potentially suitable sites for weevils to lay eggs, depending on the species' biology; (1) in/on the fruit, berry or nut; (2) in/on the bud, leaf, branch, vine, stem, pseudostem, corm, or storage root and (3) in the soil or at the base of the plant. As larvae hatch from the eggs they move to, or are already positioned at the location of the larval food source; (1) in the bud, branch, stem, pseudostem, corm, storage root or root; (2) in the fruit, berry or nut. The larval and pupal habitats never leave the immature stages exposed where they could be directly sprayed with either entomopathogens or contact insecticides. The mature larvae pupate either (1) in the berry, nut, bud, branch, vine, stem, pseudostem, corm or storage root of the host plant, or (2) under the ground. Some species need to diapause or overwinter in the soil as either larvae (pecan weevil, chestnut weevil, hazelnut weevil, black vine weevil, banded fruit weevil) or adults (walnut twig beetle, strawberry bud weevil, macadamia seed weevil, chestnut weevil, hazelnut weevil, black vine weevil). After days to months (Table 2), the adults emerge from the host plant or the ground and establish the next generation. Studies on the impacts of EPF on weevils are grouped together based on these life cycle models and discussed in the following sections of this review. Model 1: larvae and pupae both in the host plant; Model 2: larvae in the host plant and pupae under the ground; Model 3: larvae and pupae both under the ground (Table 3).

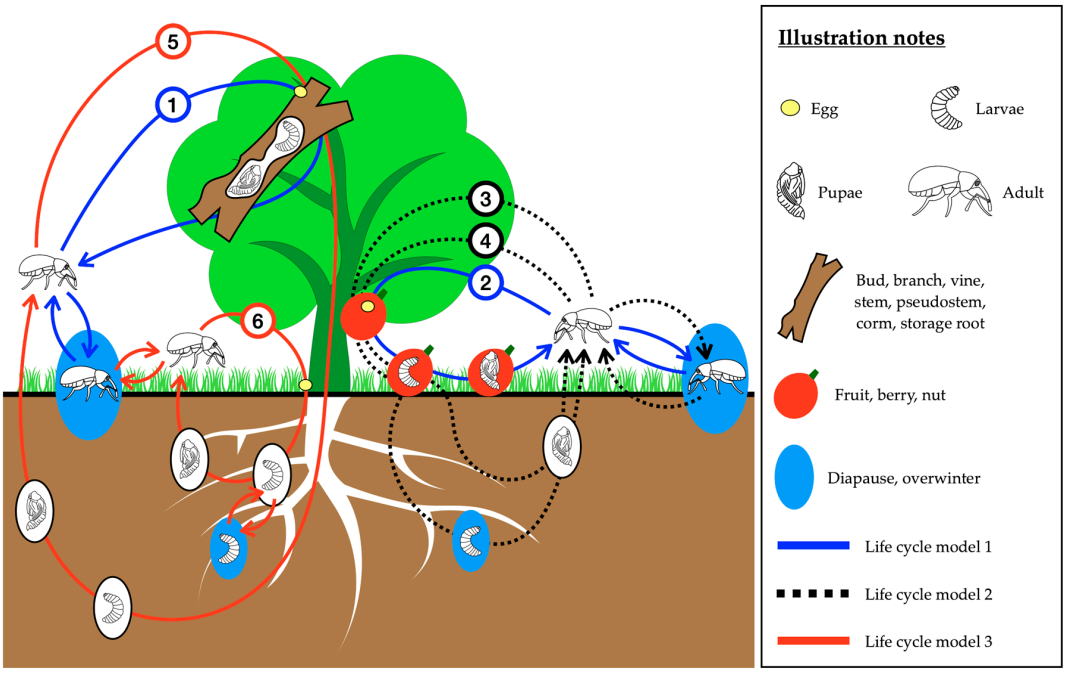


Figure 1. Overview of the life cycles of weevils attacking horticultural crops. The weevil species which were classified into subcategories 1–6 and life cycle models 1–3 in this figure are listed in Table 3.

Table 3. Major horticultural weevil species used in experiments with EPF grouped according to their different life cycle habitat utilisation models. Subcategories 1–6 correspond to those shown in Figure 1.

Life Cycle Model	Subcategory	Weevil Species	Common Name
Model 1: Larvae and Pupae in the Host Plant	**1**	*Aclees* sp. cf. *foveatus*	Fig weevil
		Anthonomus signatus	Strawberry bud weevil
		Blosyrus asellus	Rough sweetpotato weevil
		Cosmopolites sordidus	Banana weevil
		Cylas formicarius	Sweetpotato weevil
		Cylas puncticollis	African Sweetpotato weevil
		Odoiporus longicollis	Banana stem weevil
		Pantorhytes plutus	Cacao weevil
		Pityophthorus juglandis	Walnut twig beetle
		Rhynchophorus ferrugineus	Red palm weevil
		Rhynchophorus bilineatus	Black palm weevil
		Scolytus amygdali	Almond bark beetle
		Xyleborus affinis	Ambrosia beetle
	2	*Heilipus lauri*	Avocado seed weevil
		Hypothenemus hampei	Coffee berry borer
		Kuschelorhynchus macadamiae	Macadamia seed weevil
Model 2: Larvae in the Host Plant and Pupae under the Ground	**3**	*Curculio caryae*	Pecan weevil
		Curculio elephas	Chestnut weevil
		Curculio sikkimensis	Chestnut weevil
		Curculio nucum	Hazelnut weevil
	4	*Conotrachelus nenuphar*	Plum curculio
		Conotrachelus psidii	Guava weevil
Model 3: Larvae and Pupae under the Ground	**5**	*Diaprepes abbreviatus*	Citrus root weevil
	6	*Aegorhinus superciliosus*	Raspberry weevil
		Otiorhynchus sulcatus	Black vine weevil
		Phlyctinus callosus	Banded fruit weevil

4. Effect of Fungal Entomopathogens on Weevils with Life Cycle Model 1: Larvae and Pupae in the Host Plant

In total, 61 screening studies have demonstrated the efficacy of *Metarhizium* spp. and *Beauveria* spp. on weevils with Model 1 habitat utilisation. The aim of these studies was to find the most effective isolate of each EPF through the evaluation of either commercial strains/products or local isolates. In total, 98 isolates of *Metarhizium* spp., 275 isolates of *Beauveria* spp. and 16 commercial strains/products were used for the bioassays. Of the 61 published papers, 55 examined the effects of EPF using aqueous conidial suspensions and only 6 papers examined efficacy using dried conidia applied to different substrates. For bioassays with aqueous conidia, adults or larvae of *Aclees* sp. cf. *foveatus* [92], *Anthonomus signatus* [93], *Cosmopolites sordidus* [94–102], *Cylas formicarius* [103,104], *C. puncticollis* [105], *Heilipus lauri* [106], *Hypothenemus hampei* [107–118], *Kuschelorhynchus macadamiae* [119], *Odoiporus longicollis* [120–122], *Pantorhytes plutus* [65], *Pityophthorus juglandis* [123], *Rhynchophorus ferrugineus* [124–143], *Scolytus amygdali* [144] and *Xyleborus affinis* [76] were immersed for 3–90 s or sprayed with conidial suspensions at varying concentrations. For the studies with dried conidia, *C. sordidus* [145,146] and *R. ferrugineus* [147–150] were rolled in (5 min) or allowed to walk on dried conidia on substrates such as fungal media, rice or wheat (15 min to 24 h).

Efficacy comparisons between EPF species showed that *B. bassiana* performed better than *M. anisopliae* in killing *Aclees* sp. cf. *foveatus* [92], *C. sordidus* [145], *H. hampei* [111] and *H. lauri* [106], whilst the opposite result was found with *R. ferrugineus* [126,147,148]. Other studies showed that *M. anisopliae* and *B. bassiana* were equally effective in killing *C. sordidus* [95,97,102], *C. puncticollis* [105], *K. macadamiae* [119], *P. juglandis* [123] and *R. ferrugineus* [129,134,143]. The majority of highly virulent isolates and/or the highest conidial concentrations tested in each study resulted in moderate (60–80%) to high levels (>80%) of mortality in the target weevils, except for *C. sordidus* [98,100], *H. hampei* [115] and *X. affinis* [76] where only a low level of mortality was obtained. This could be the result of poor virulence

of the fungal isolates since the number of conidia used in these studies was high (adults immersed in 10^8 conidia mL^{-1} [98,100] or spray application at 10^7–10^8 conidia mL^{-1} [76,115]). Two studies showed that using biosynthesised silver nanoparticles for disseminating *M. anisopliae* or *B. bassiana* could improve efficacy against *R. ferrugineus* by 10% in comparison to traditional spray applications of conidial suspensions [136,140]. Formulated EPF [92,119,124,127] and non-formulated commercial strains [107,108,123] provided strong and consistent control of weevils with Model 1 habitat patterns under laboratory conditions. For example, Abdel-Samad et al. [124] and Hajjar et al. [127] found that Agronova® and Broadband® products which contain *B. bassiana* in an oil formulation caused 86–100% mortality of *R. ferrugineus* when the recommended rate of 10^9 conidia mL^{-1} was used to treat adults. Castrillo et al. [123] reported that *B. bassiana* strain GHA and *M. brunneum* strain F52 at 10^6 conidia mL^{-1} were highly virulent and caused high mortality (>90%) of *P. juglandis* 5 days after treatment.

Although the majority of these strains induced moderate (60–80%) to high (>80%) levels of mortality to weevils in controlled environments, economic control of using EPF in the field may not be achieved as readily. As EPF can take at least 15 days to cause weevil mortality of more than 80% under field conditions, the targeted weevils are likely to cause at least some damage to the crops either by feeding or laying viable eggs during the intervening period. Twenty-two studies have evaluated the efficacy of EPF on weevils of walnuts, almonds, bananas, coffee, strawberries, sweetpotatoes and palms in the glasshouse or under field conditions. Of these, fourteen examined spray application of EPF onto the plant and four evaluated either the injection of EPF into the space between the stem and petiole insertion point, or application of the dried formulated fungi onto the plant crown before or after weevil establishment. Only four papers discussed the natural occurrence and prevalence of EPF in the field, and these papers involved the control of either *C. sordidus* [151], *H. hampei* [152,153] or *R. ferrugineus* [154]. The stem injection technique was only applied against *R. ferrugineus* larvae and pupae, which remain inside the plant [131,132,155,156], while the spray applications were invariably targeting adult weevils such as *A. signatus* [157], *C. sordidus* [97,158], *Cylas* spp. [159], *H. hampei* [109,160–163], *P. juglandis* [123], *R. ferrugineus* [125,164,165] and *S. amygdali* [144], which are exposed outside the plant as adults. Variable control was achieved according to the fungal species used [160], fungal persistence [155,157], application technique [125], frequency of the application [163], weather conditions [109,151–153] and insect species. Overall, spray application of the most virulent isolates caused low (<60%) to moderate (60–80%) levels of mortality to the target weevils, whilst application by stem injection led to moderate to high (>80%) mortality of *R. ferrugineus* larvae and pupae.

Eight studies evaluated the effects of incorporating conidia within or on top of topsoil and plant growing media (compost, sawdust) on the mortality of weevils with Model 1 habitat utilisation. These studies aimed to use EPF to control weevils moving through or across the topsoil, plant growing media or across infective fungal substrates used to create a protective barrier around host plants. Of the eight papers, three examined mortality under laboratory conditions and five assessed efficacy in the glasshouse or field. In the laboratory, EPF were sprayed or applied to the soil or growing media before introducing the weevils. High mortality (>80%) of the weevils (*C. formicarius* and *R. ferrugineus*) occurred [126,166], except in the study by Francardi et al. [149]. The low mortality (12–20%) of *R. ferrugineus* recorded by Francardi et al. [149] could be the result of there being insufficient conidia in the soil to adhere to and infect the adults. This was confirmed by the same authors who replaced soil with conidiated rice for adults to move across, improving the mortality rate to more than 85% [149]. However, only low (<60%) to moderate (60–80%) mortality of weevils was achieved in the field when this treatment method was used (24% to 63%, 8% to 75% and 25 to 62% for *C. sordidus* [167,168], *H. hampei* [113,169] and *P. juglandis* [90], respectively). This may have been a consequence of insufficient amounts of conidia being applied, the long period between fungal application and the emergence of adult weevil populations, and/or the effect of the unstable microclimate near the topsoil where there would have been large fluctuations in temperature and humidity. This issue is discussed further in the section on weevils with Model 2 and 3 habitat utilisations.

In order to improve weevil management, combinations of EPF with other biological control agents (BCAs) and attractants have also been explored. Three studies evaluated synergistic interactions of EPF with entomopathogenic nematodes (EPNs) and *Bacillus thuringiensis* against *R. ferrugineus* [170–172]. Saleh et al. [171] reported that *B. bassiana* behaved synergistically with *Steinernema carpocapsae* (EPNs) and killed *R. ferrugineus* adults in just 24 h when both BCAs were co-applied. In contrast, Wakil et al. [172] reported that EPF and EPNs could not be applied at the same time. They found that 72–89% mortality of *R. ferrugineus* larvae could be obtained when EPNs (*Heterorhabditis bacteriophora*) were applied two weeks after *B. bassiana* or *M. anisopliae*. However, low mortality (below 30%) was observed when EPF or EPNs were applied alone, or 45–61% mortality when EPF and EPNs were applied simultaneously, supporting the synergy between EPF and EPNs when used with appropriate timings [172]. Malik et al. [170] reported that *B. thuringiensis* behaved synergistically with *B. bassiana* for managing *R. ferrugineus*. When both entomopathogens were co-applied on *R. ferrugineus* larvae they caused substantially more mortality and reduced the percentages of pupation and adult emergence than *B. bassiana* or *B. thuringiensis* used alone [170].

Combinations of EPF with attractants such as a methanol/ethanol mixture, aggregation pheromones or sex pheromones were tested against *C. sordidus* [173–175], *C. formicarius* [176], *H. hampei* [115] and *R. ferrugineus* [127,177–180]. The term "sex pheromone" is commonly defined as the chemical signals from a female to attract males of the same species for initiation of courtship or mating, whereas an "aggregation pheromone" is a male-produced attractant which draws both sexes of the same species to a calling site to increase mating likelihood [181]. The aims of these studies were to infect adult weevils with EPF by integrating the EPF with attractants as an "attract-and-infect" technique. They showed that the integration of *B. bassiana* with an aggregation pheromone component (ferrugineol or 4-methyl-5-nonanol) in the infective trap could cause high mortality to *R. ferrugineus* adults in the laboratory, but only low to moderate mortality was observed in the field [127,177–180]. Despite this apparent limitation, studies have found that the combination of *B. bassiana* with ferrugineol as part of an attract-and-infect strategy reduced infestations of *R. ferrugineus* [178,179] more effectively than the application of the insecticide chlorpyrifos alone, or the combination of chlorpyrifos with ferrugineol in an attract-and-kill system [178]. Moderate to high mortality of *C. sordidus* adults was also observed in the laboratory when *B. bassiana* was combined with an aggregation pheromone (Cosmolure®—sordidin or (1*S*,3*R*,5*R*,7*S*)-1-ethyl-3,5,7-trimethyl-2,8-dioxabicyclo [3.2.1] octane) [174], but again only low to moderate mortality was obtained in the field [173,175]. Similarly, Mota et al. [115] reported that only moderate mortality of *H. hampei* was observed in the auto inoculation trap containing methanol/ethanol mixture (at 1:1 *v*/*v*) and a *B. bassiana* suspension. In contrast, Yasuda [176] demonstrated high levels of control by combining *B. bassiana* conidia with sex pheromones inside a trap for controlling *C. formicarius* in the field. Although this study showed the potential for combining EPF with attractants, many others have failed to provide good control of adult weevils in the field. In the case of *H. hampei*, poor trap design [182], attractant compound selection, and inappropriate timing in relation to the emergence period of the adults [115,183] contributed to the failure of this technique. Pereira et al. [183] found that methanol/ethanol mixture is not specific to *H. hampei* and many scolytids including "false *H. hampei*"were also captured in the trap. Mota et al. [115] reported that the number of *H. hampei* captured in the trap fluctuated over 22 weeks of the experiment with the noticeable peaks of adult captures at the 5th and 7th week of the trap placement in the field. The same issues with inappropriate timing in relation to the emergence period of the adults were also raised by Sewify et al. [184] and Vacas et al. [185] in their studies on *R. ferrugineus*. Dembilio et al. [179] reported that conidia viability inside the trap significantly reduced over time, from 100% on day 1 to less than 50% at day 67, and, as a consequence, only low to moderate mortality was observed in the field. From these studies, it is obvious that to be effective combinations of EPF with attractants designed to enhance infection rates must be used when adult weevil activity is high, and utilise reliable attractants with good persistence in the field. Other key areas of work needed to optimise attract-and-infect systems include the improvement of EPF persistence in the trap, as well as enhancing the capacity of EPF conidia to adhere to the weevils.

An advantage of the attract-and-infect technique with EPF is the ability to generate local transmission between adults in the first cycle [127,173,179] and to some extent between adults and conidiated cadavers in the second cycle. The mortality of the "recipients" was around 16% for *C. sordidus* and 45% for *R. ferrugineus* when adults that had previously visited traps and served as "donors" had physical contact with them [127,173,179]. Studies showed that copulation is the main basis for disease transmission between adults via physical contact. Many studies have shown that an infected male is able to transmit EPF and subsequently cause mortality to the females, or vice versa [94,124,165,186,187]. Horizontal transmission did not just kill the female adults, but also reduced the number of eggs produced and egg viability by 44 to 68% and by 45 to 55%, respectively, for *C. formicarius* [186] and *R. ferrugineus* [125] before the females died. Interestingly, the percentage of egg viability of *R. ferrugineus* was reduced by 86–100%, after the female mated with the reproductively sterile male (gamma irradiated) carrying *B. bassiana* [188]. After adults were killed by EPF, the conidiated cadavers were also found to generate a second cycle of disease transmission. Dotaona et al. [186] reported that one conidiated cadaver could cause 63% mortality (of 10 adults) to *C. formicarius* under laboratory conditions. From these findings, infected adult weevils and conidiated cadavers have an important role in recycling and transmitting EPF within pest populations.

In addition to the capacity for horizontal transmission, EPF were also found to produce volatile organic compounds (1-octen-3-ol, 2-octen-1-ol, 3-octanol, 3-octanone) and acetic acid, which behave as repellent volatiles [189,190]. Dotaona et al. [191] found that *C. formicarius* showed avoidance behaviour toward the most virulent isolates of *M. anisopliae* when compared to controls or low virulence isolates. In contrast, Leng and Reddy [192] found that *C. formicarius* showed no avoidance behaviour toward *B. bassiana*, but avoidance was observed toward neem (a botanical insecticide), petroleum oil and the insecticide spinosad. The variation between these two studies could be explained by the work of Bojke et al. [189] who demonstrated that *M. anisopliae* was able to produce volatile organic compounds and acetic acid, whereas *B. bassiana* could not. In addition to producing potentially repellent volatiles, some EPF have been reported to have the ability to become endophytes within host plants and decrease the survivorship of weevils feeding on these hosts. Akello et al. [193,194] found that *B. bassiana* was a symbiont with banana plants and caused 53–58% mortality to *C. sordidus* adults. This reduced the population of the next generation by about 23–89%, leading to a reduction of crop damage by 42–87%. Similarly, Prabhavathi and Ghosh [87] also found that *B. bassiana* could colonise banana tissue for at least four months after dipping the corm in a conidial suspension and caused 50–70% mortality to *O. longicollis.* Date palm seedlings can also be endophytically colonised by *B. bassiana*, leading to 70–80% mortality of *R. ferrugineus* larvae when they fed on the endophytic plant in the laboratory [195].

Although numerous studies have confirmed the potential of EPF to suppress weevils in the laboratory, their variable results on horticultural crops under field conditions could lead to confusion amongst end-users or those seeking to develop and register commercial products. In order to give a better understanding of the overall potential of EPF, eleven studies have compared EPF with synthetic insecticides individually or evaluated their simultaneous use. Of the eleven papers, two showed significantly better control by EPF in comparison to synthetic insecticides alone [196,197], but another five showed the opposite result—synthetic insecticides provided superior control [43,96,192,198,199]. Only four papers have discussed synergistic interactions of EPF with sublethal doses of botanical and/or synthetic insecticides [139,200–202]. The combination of EPF with sublethal doses of neem and spinosad killed 100% of *C. formicarius* within 48–72 h; however, the application of the full recommended doses of either the insecticides or EPF alone took more than 72 h to kill 100% of adult weevils in the laboratory [200]. Malik et al. [202] found that the combination of *B. bassiana* with a sublethal dose of imidacloprid killed 100% of *R. ferrugineus* larvae within 20 days, whereas the same sublethal dose of imidacloprid or *B. bassiana* alone killed only 84% and 54–77% of the larvae, respectively. Again, Saleem et al. [201] and Qayyum et al. [139] found that *B. bassiana* showed synergy with a sublethal dose of nitenpyram for the control of *R. ferrugineus* adults and larvae and provided superior control to either treatment applied alone.

5. Effect of Fungal Entomopathogens on Weevils with Life Cycle Model 2: Larvae in the Host Plant and Pupation under the Ground

Seven screening studies have demonstrated the efficacy of *Metarhizium* spp. and *Beauveria* spp. against weevils with Model 2 habitat utilisation. In total, 28 isolates of *Metarhizium* spp. and 13 isolates of *Beauveria* spp. were used for these screening studies. Of the seven papers, five examined the effect of EPF using aqueous conidial suspensions [203–207] and two evaluated the use of dried conidia previously cultured on fungal media [208,209]. For the tests with aqueous conidia, adults or larvae of *Conotrachelus nenuphar* [203], *Curculio caryae* [204], *Curculio nucum* [205] and *Curculio sikkimensis* [206,207] were immersed for 8–60 s or sprayed with conidial suspensions at different concentrations. For the tests with dried conidia, *C. caryae* and *C. nenuphar* were infected by being allowed to walk or crawl for several minutes on a conidiated fungal culture [208,209]. The overall results indicated that the most virulent isolates of *B. bassiana* and *M. anisopliae* induced high mortality (>80%) to the population of weevils treated with aqueous conidia whereas 74–83% mortality of *C. caryae* and 98–99% mortality of *C. nenuphar* were obtained with the dried conidia treatments. In terms of the efficacy comparison between *B. bassiana* and *M. anisopliae*, *B. bassiana* was more active against *C. caryae* [208]; however, the opposite result was obtained with *C. sikkimensis* [207] and *C. nucum* [205].

Seventeen further papers evaluated the effect of fungal conidia applied onto or incorporated into topsoil and plant growing media (vermiculite, soybean straw) on the mortality of weevils with Model 2 habitat utilisation. These studies aimed to evaluate EPF for control of either larvae below the ground, or adult weevils moving on the ground or on plant growing media. Nine studies were conducted in the laboratory and the remaining studies assessed efficacy under field conditions with the most virulent isolates and commercial strains/products (*M. brunneum* strain F52, *B. bassiana* strain GHA—Mycotrol®, Botanigard®, *B. bassiana* strain ATCC 74040—Naturalis®). In the laboratory, EPF were sprayed or applied as a drench onto the soil or plant growing media and left for 1–24 h before the introduction of larvae or adults onto the sprayed surface. Moderate (60–80%) to high (>80%) mortality of weevils such as *C. nenuphar*, *C. caryae*, *C. elephas* and *C. nucum* was obtained after treatment with virulent isolates [209–212], Mycotrol® and Naturalis® [213–215] but only low (<60%) to moderate (60–80%) levels of control were achieved when adult weevils or larvae (e.g., *C. caryae*) were introduced four days after EPF application [208,216]. Low to moderate mortality of *C. caryae* [204,217,218], *C. sikkimensis* [206,207], *C. nucum* [219] and *C. nenuphar* [220] was also achieved in the field after the application of virulent EPF isolates or commercial products (Beaupro®, Metapro® and Botanigard®), suggesting that the weevils moved onto the ground several days after fungal application [208,216]. To mitigate poor infectivity of EPF in the field, Shapiro-Ilan and Brown [215] suggested that using earthworms (*Lumbricus terrestris*) as phoretic hosts for *B. bassiana* in the soil could improve fungal infectivity in comparison to the more traditional applications of EPF directly to the topsoil. Other studies found challenges still remain that complicate efforts to control pecan weevils successfully with EPF. Shapiro-Ilan et al. [221] found that high infectivity of EPF did not persist in the field for longer than one week after application. The number of conidia recovered from field soil declined significantly one week after application, from around 6.5×10^3 CFU/g of soil at day 1 to around 3×10^3 CFU/g of soil at day 8 in a 2009 trial and from 9×10^2 CFU/g of soil at day 1 to 1×10^2 CFU/g of soil at day 8 in a trial conducted during 2010 [218]. The number of conidia recovered from the soil continued to drop to almost zero by day 29 in both years [218]. The authors suggested that conidial densities declined rapidly because there was no mulch or cover crop to provide protection from UV radiation penetrating the crop canopy and contribute towards stabilising topsoil temperature and humidity [218]. Shapiro-Ilan and Mizell [222] also found that the pupal cell of *C. caryae* had antimicrobial properties that had the potential to inhibit penetration and infection by the fungi. Long gaps between fungal application and weevil activity, poor persistence and uneven distribution of EPF in the field, and antimicrobial properties of the pupal cell are all factors that can contribute to the reduced efficacy of fungal applications in the field.

Only four studies have evaluated the efficacy of EPF on weevils of pecan in the field. Spray applications targeting *C. caryae* adults on the plant showed that the most virulent isolates of *M. anisopliae* and Botanigard® (containing *B. bassiana* strain GHA) caused moderate (60–80%) to high (>80%) levels of mortality to *C. caryae* whilst *M. brunneum* strain F52 caused low (<60%) to moderate (60–80%) mortality [217,223–225]. Interestingly, spray applications of EPF on the plant caused slightly higher mortality of *C. caryae* than the application of EPF on the ground [217,223,225]. Although moderate to high mortality of *C. caryae* was achieved, the authors noted that economic control was not achieved as the EPF required weeks to kill the weevils, during which time the weevils continued to cause damage to the crop. Although mortality may have been delayed, these infected adults may play an important role in the horizontal transmission of EPF, providing more effective control in the longer term. In the case of *C. caryae*, infected males or females were able to transmit EPF via contact during mating, leading to 50% mortality in their partners [216].

As fungal applications to the ground and foliage have not achieved the optimum level of control, EPF have been tested and integrated with other components of IPM programs including chemicals and other biological control agents. Combinations of EPF with EPNs (*Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *S. feltiae*) induced moderate (60–80%) to high (>80%) levels of mortality of *C. caryae*, *C. elephas* and *C. nucum* larvae [210,226,227]. These combinations (EPF + EPNs) did not, however, provide any significant advantages over individual treatments (EPF or EPNs alone), which also caused good levels of mortality in the targeted weevils. This was confirmed by Shapiro-Ilan et al. [228] who found that EPF + EPNs did not result in mortality of *C. caryae* higher than that caused by EPF or EPNs alone, and by Batalla-Carrera et al. [210] and Asan et al. [227] who reported that the mortality of *C. elephas* and *C. nucum* caused by EPF + EPNs and EPF alone did not differ. It is difficult to draw direct comparisons between EPF and EPNs because their mode of action and their effective concentrations are different. Studies show that when EPF or EPNs are applied on the topsoil before introducing weevil larvae, the EPNs provide better control of both *C. caryae* [226] and *C. nenuphar* [220] compared to EPF. However, when larvae are immersed in the fungal suspension and compared with EPNs (applied on the topsoil), the mortality of both *C. elephas* [227] and *C. nenuphar* [203] caused by EPF was always higher than that caused by EPNs. These studies suggest that in many cases EPNs are likely to provide better control of weevils active below the soil surface than EPF. This is discussed further in the following sections.

Combinations of EPF with synthetic insecticides have shown their potential synergy, with 100% control of weevils including *C. caryae* [229] and *C. psidii* [230]. When *B. bassiana* was applied together with a sublethal dose of imidacloprid to *C. psidii*, 100% mortality of adults was recorded; however, the application of *B. bassiana* alone killed only 62% of weevils and the sublethal dose of imidacloprid alone did not kill any [230]. The sublethal dose of imidacloprid increased the vulnerability of weevils to EPF, presumably by diverting metabolic activity to insecticide detoxification and thereby reducing the insect's capacity to resist fungal infection. The authors also observed that the sublethal dose of insecticide had a substantial impact on the insect's grooming behaviour [230]. The impact of sublethal doses of insecticides on the grooming behaviour of weevils and its implications for EPF efficacy will be discussed further in the section on weevils with Model 3 habitat utilisation.

6. Effect of Fungal Entomopathogens on Weevils with Life Cycle Model 3: Larvae and Pupae under the Ground

Twelve screening studies have demonstrated the efficacy of *Metarhizium* spp. and *Beauveria* spp. on four weevils (*Aegorhinus superciliosus*, *Diaprepes abbreviatus*, *Otiorhynchus sulcatus* and *Phlyctinus callosus*) with Model 3 habitat utilisation. In total, 56 isolates of *Metarhizium* spp., 29 isolates of *Beauveria* spp. and 7 commercial strains/products were used for these screening studies. All studies examined the effects of EPF using aqueous conidial suspensions. Larvae or adults of *A. superciliosus* [231], *D. abbreviatus* [232], *O. sulcatus* [233–241] and *P. callosus* [242] were immersed for 10–60 s, exposed to a topical application, or sprayed with conidial suspensions at different concentrations. The comparison

of *Metarhizium* spp. and *Beauveria* spp. showed that *Metarhizium* spp. often performed better than *Beauveria* spp. against *O. sulcatus* [235–237], although some studies found that both fungi were equally effective in killing *O. sulcatus* and *P. callosus* [238,240,242]. In general, the majority of the most virulent isolates or commercial strains/products and/or the highest concentration used in each study caused moderate (60–80%) to high (>80%) level of mortality to the target weevils.

At least 17 papers have evaluated the effect of application or incorporation of fungal conidia into or onto topsoil or plant growing media (bark-based potting medium, peat-based media, spent mushroom compost, peat moss, peat compost, turkey grit) on weevil mortality. These studies aimed to use EPF to control target insects as below-ground larvae, or as adults dispersing across the soil surface towards a plant host. Conidia were sprayed onto or drenched into the soil or plant growing media before introducing larvae or adults onto the treated surface under laboratory conditions. Moderate (60–80%) to high (>80%) mortality of the weevils was observed [236,243–254]. The commercial strains/products Met52® and Naturalis® showed strong and consistent control of weevils and caused high (>80%) levels of mortality [248,250,254]. However, application of Met52® as a topsoil drench in the field provided only low mortality of *O. sulcatus* larvae [255]. Moderate to high mortality of the weevils resulted when conidiated rice was applied directly on the topsoil, providing an infective layer for controlling the larvae of this species [256,257] suggesting this approach may provide sufficient conidia on the topsoil to adhere to and cause mortality to *O. sulcatus*. The mortality of *O. sulcatus* was high in the first week after EPF were incorporated with a bark-based potting medium, but mortality decreased to moderate (60–80%) levels after 77 days [249] and zero after 1 year [258]. This is likely the result of conidia degradation, as observed by Bruck [248] who reported that the number of conidia recovered from peat-based and bark-based potting media reduced gradually; from around $1 \times 10^{6.5}$ CFU/g dry potting media at week 2 to around $1 \times 10^{5.5}$ CFU/g dry potting media at week 48. Shapiro-Ilan et al. [218,225] reported the number of conidia recovered from topsoil dropped to almost zero 7 weeks after an EPF application in the field. To minimise rapid conidial degradation in the field, several studies have recommended that EPF should be incorporated with pasteurised potting media (such as peat-based and bark-based potting media) for at least one week before use [248,250]. This allows the EPF to adjust to the media and grow in controlled conditions with good moisture levels and nutrients before being used in the field.

Pope et al. [238] found that the use of *M. brunneum* (F52) conidial powder in Roguard refuges (black plastic crawling insect stations) provided at least 93% control of *O. sulcatus* after 28 days. Deployment of *B. bassiana* (GHA strain) under the same conditions provided only 27–67% weevil mortality. Other studies have also demonstrated that *Metarhizium* spp. perform better than *Beauveria* spp. against *O. sulcatus* [235–237]. Baits or attractants are not essential in Roguard refuges for *O. sulcatus* control since adults of this weevil are nocturnal and move inside the station during the day to avoid exposure to sunlight [64]. The recent development of lures ((Z)-2-pentenol + methyl eugenol) for *O. sulcatus* [259] has, however, improved the attractiveness of the refuges, and deploying the lures with an EPF formulated in linseed oil within the refuges has provided very effective control of *O. sulcatus* in the field [260].

The behaviour of weevils in response to volatiles produced by EPF has also been explored. Rondot and Reineke [261] found that *O. sulcatus* has the ability to detect EPF and avoids the commercial product Naturalis® and Kepler and Bruck [262] showed that whilst *O. sulcatus* does not avoid *M. brunneum* (strain F52), it does avoid the insecticide bifenthrin. Although *O. sulcatus* showed avoidance behaviour in response to Naturalis® [261], this could be a response to additives in the commercial product rather than to *B. bassiana* itself. A recent study found that *B. bassiana* does not produce repellent volatiles [189].

Entomopathogenic fungi synergism with other entomopathogens and insecticides has also been studied in relation to weevils with Model 3 habitat utilisation. Fungal entomopathogens were applied alone or together with EPNs (*Heterorhabditis bacteriophora*, *Steinernema kraussei* and *S. feltiae*) on the topsoil and plant growing media (peat-based media) before introduction of *O. sulcatus* larvae. High mortality of larvae was obtained when *Metarhizium* spp. were combined with EPNs in the

laboratory, glasshouse and to an extent in the field [263–265]. *Metarhizium* spp. seemed to dominate the detrimental effects on the larvae; when used individually, the mortality of larvae caused by *Metarhizium* spp. alone was 40–88% in the glasshouse and 88–94% in the field, and the mortality of the larvae caused by the EPNs was 30–69% in the glasshouse and 20–75% in the field [263–265]. High levels of mortality were also found when EPF were applied together with synthetic insecticides against weevils in this group. More than 90% mortality of *D. abbreviatus* and *O. sulcatus* occurred when EPF were applied together with sublethal doses of imidacloprid, fipronil or neem either directly or via peat-based plant media (a combination of peat, bark, coir and compost) before introduction of the larvae [266–270]. However, the application of either EPF or sublethal doses of insecticides alone caused only low (<60%) to moderate (60–80%) mortality of the target weevils. The lower mortality of the larvae treated with EPF alone is attributed to below-ground movement of the larvae leading to the passive removal of conidia as the larvae moved against soil particles, or active removal associated with grooming behaviour [266–268]. The removal of fungal conidia during grooming has also been observed in adults [271]. A sublethal dose of imidacloprid led to reduced or temporary loss of mobility by the larvae, which were then unable to remove fungal conidia from their cuticle [266–268].

7. Integration of Fungal Entomopathogens in the Integrated Pest Management Programs and Future Research Directions

Entomopathogenic fungi have shown potential to control many weevil species associated with horticultural crops under laboratory conditions, but wide variations in weevil mortality are commonly seen across different fungal species, isolates and strains. In some cases, the fungal strains which were isolated from particular weevils have shown limited capacity to control that species (e.g., coffee berry borer, *H. hampei* [108,112], banana weevil, *C. sordidus* [96,97] and red palm weevil, *R. ferrugineus* [128]). In contrast, other studies have shown that strains of EPF which naturally infect target weevils work better against those species than strains baited from the soil, commercial strains, or commercial formulated products [94,95,115,132]. As there are no consistent patterns in these studies, it is most appropriate to use a registered commercial strain as a reference strain and compare this with any newly isolated strains in screening studies. This will provide more useful baseline data on the relative virulence of new strains, which should be assessed on their potential to provide improvements relative to existing commercial products rather than relative to other, often randomly selected experimental isolates.

Although there were only four studies on the response of weevils to *B. bassiana* deployed as fungal endophytes in plants (all life cycle habitat utilisation Model 1 species) [87,193–195], the establishment of endophytic plants is an effective preventive tactic, and a practical solution for managing weevils of horticultural crops. In addition to causing mortality of the weevils, endophytic plants may have less damage and yield loss, as the fungi that colonise the host plant [193,194] probably produce insecticidal metabolites which may improve the resistance of the host plant to attack [16,272,273]. The establishment of fungal endophytes within annual crops has often been noted [272,273]; however, no studies have been performed on perennial crops that have extended beyond the seedling stage. Studies on seedlings have included those on pecan [274], cacao [275], coffee [276], and those on isolated plant parts [277–279]. Further research on the protection provided by fungal endophytes in mature perennial crops is needed, with a focus on the persistence of endophyte activity in the plant and correlating this to effects on pest populations. The methodology for confirming endophytic activity is crucial for separating the effects of endophytes from those associated with epiphytic fungi [16].

The application of EPF incorporated into plant growing media around and below crops to produce a "contamination layer" or "infective zone" has been shown to provide long-term control of adult and larval weevils. Incorporating EPF with pasteurised organic fertilizers, compost or growing media [248–250] in combination with zero-tillage [280,281] may improve not just the abundance, but also the persistence of EPF in the soil. This approach may help compensate for the problems associated with the limited durability and infectivity of EPF in horticultural crops. Although EPF can in some cases persist in the soil for long periods [282] (up to 15 years in exceptional cases [283]), a single application

of EPF on the topsoil may have only short-term benefits for pest management, as the fungal density usually decreases gradually after application. This theory is supported by many studies [237,284,285]; however, despite its transient nature, the application of compost, organic fertilizer or plant growing media colonised by EPF around crops probably represents the best EPF-based technique to control weevils that have a predominantly subterranean pattern of habitat utilisation. More importantly, this solution is suitable for organic growers who are required to use only compost or organic fertilizer on their crops.

Identification of effective attractants for additional weevil species should allow further development of attract-and-infect or attract-and-kill techniques utilising the most virulent strains of EPF, helping to minimise application and management costs. Integration of EPF (particularly *B. bassiana* and *M. brunneum*) with an attractant was far more effective than combining the attractant with insecticides [178] because weevils were able to detect and avoid many insecticides (e.g., bifenthrin [262], spinosad, neem, petroleum oil [192]), whereas *B. bassiana* and *M. brunneum* do not produce repellent compounds [189] and are consequently suitable to integrate with attract-and-kill systems. The use of adhesive carriers for conidia, such as electrostatically charged powders, will also help to improve the success of attract-and-infect and attract-and-kill techniques. This approach has been successfully integrated with both EPF and synthetic pesticides to control stored product pests [286–288], varroa mites [289], and mosquitoes [290]. Carriers improve the ability of conidia to transfer more easily to the insect and in sufficient numbers to cause mortality, both directly or by subsequent transfer to other individuals. Although attract-and-infect and attract-and-kill systems are good in theory, these techniques may not be applicable to all species, since attractants may be difficult to identify and synthesise, and some species may not utilise pheromones for aggregation or mate location to begin with. Where pheromones or other attractants are known, however, their integration with EPF in these sorts of systems represents a great opportunity for reducing dependence on synthetic insecticides.

One of the most interesting techniques for utilising EPF involves the horizontal transmission of conidia from male weevils sterilised using ionising radiation (Figure 2). Of the journal papers examined in this review, only one paper tested this technique. Significant control of the red palm weevil *R. ferrugineus* was reported [188] and the sexual competitiveness of sterile males was not reduced by sterilisation when compared to non-sterile males [291]. The combination of the sterile insect technique (SIT) with EPF has also been tested on fruit flies including Mexican fruit fly (*Anastrepha ludens*) [21], Mediterranean fruit fly (*Ceratitis capitata*) [19], melon fly (*Bactrocera cucurbitae*) [20], and peach fruit fly (*Bactrocera zonata*) [292]. The sterile insect technique alone or used in combination with EPF has shown potential for safe and selective pest control, but, since sterilisation may have a negative impact on sexual competitiveness [21], further research on optimising sterilisation procedures is needed for each species being targeted.

Although commercial strains of EPF have been regularly used in the field, only moderate levels of control have been obtained [217,218,220,223,225]. This is largely attributable to the negative impact of unfavourable weather conditions [293]. There have been many efforts to improve the formulation of EPF to withstand unfavourable environmental conditions including high temperatures and UV radiation [294,295], but recent efforts have been focussed on finding weather tolerant strains [296,297] and understanding and improving the tolerance of the fungi themselves to heat and sunlight [298,299].

To the best of our knowledge, EPNs and *B. thuringiensis* are the only other biological control agents to be experimentally integrated with EPF. As the mode of action of *B. thuringiensis* is by ingestion, it is suitable for integration with EPF for application to aerial parts of the host plant rather than to the topsoil, and this represents a useful approach for controlling weevil adults feeding on the crops. Several studies have shown the potential of *B. thuringiensis* toxins for controlling weevils of horticultural crops including *C. puncticollis*, *C. brunneus*, and *D. abbreviatus* [300,301] and stem from the findings of Malik et al. [170] that *B. thuringiensis* is suitable to integrate with EPF for controlling other weevil species. Entomopathogenic nematodes are suitable to integrate with EPF for application to the ground rather than to the trunk or foliage of the plant, and this represents a useful approach for controlling

larval weevils with life cycle habitat utilisation Models 2 and 3 (Figure 2). Almost all combinations of EPF with EPNs have proven to be positive and caused significant mortality to the target weevils (e.g., *R. ferrugineus*, *C. nenuphar*, *C. caryae*, *C. elephas*, *C. nucum* and *O. sulcatus*) which they were tested against. In addition, the rotational application of EPF and EPNs at two-week intervals was found to be effective against weevils, especially by Anbesse et al. [302] who also found that three-week intervals were effective. The simultaneous or sequential applications of EPNs and EPF on the soil surface or onto plant growing media produces a "contamination layer" or "infective zone" that brings larval weevils and the biological control agents into close contact, facilitating infection. Entomopathogenic nematodes seem to have an advantage for controlling larvae with Model 2 and 3 habitat utilisation patterns, as they are active entomopathogens, able to move freely in the soil and ambush their hosts which are active below the soil surface. In contrast, EPF are passive entomopathogens and insect infection relies on movement of the host to provide contact with the conidia, particularly when the larvae exit from plant tissues and move into the soil. Achieving EPF infection in weevil larvae living more than a few centimetres below the soil surface is particularly difficult and highlights the need for control methodologies to be chosen based on a thorough knowledge of pest biology and the persistence of entomopathogens in the rhizosphere.

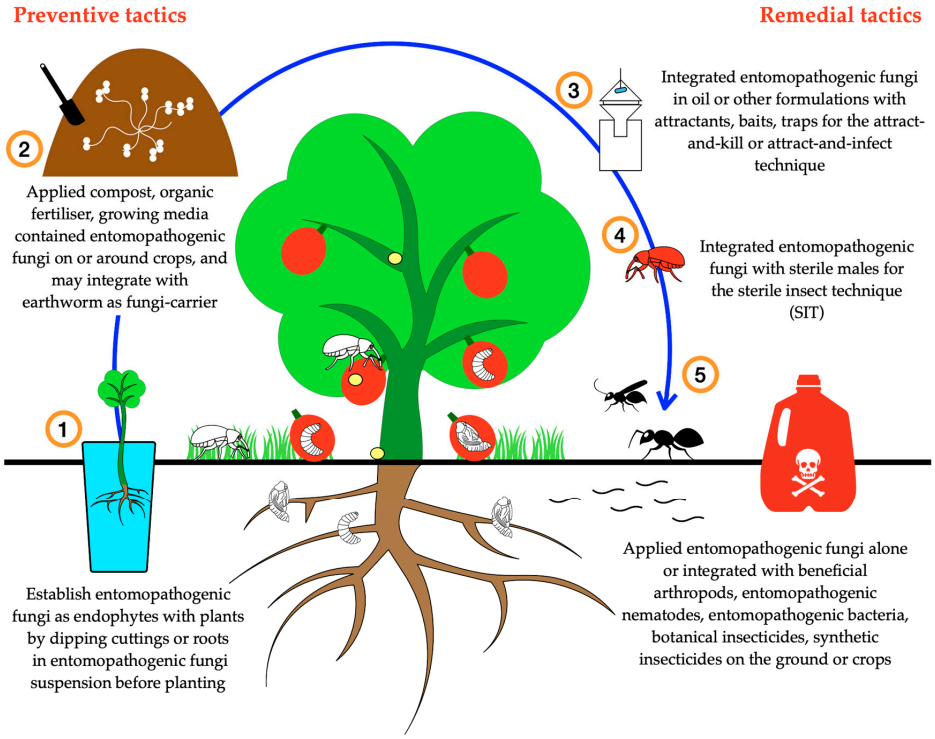


Figure 2. Conceptual illustration of potential uses of entomopathogenic fungi in IPM programs for managing weevils of horticultural crops.

Several studies have focused on the direct effect of EPF on predators of *A. signatus* (e.g., the generalist predatory bug *Anthocoris nemorum* [303]), *C. sordidus* and *R. ferrugineus* (e.g., the predatory earwig *Euborellia annulipes* [304]), *D. abbreviatus* (e.g., Asian lady beetle *Harmonia axyridis* and the generalist predatory lady beetle *Olla v-nigrum* [305–307]) and parasitoids of *H. hampei* (e.g., the bethylid ectoparasitoid *Prorops nasuta* [308], egg parasitoid *Trichogramma pretiosum* [309–311], eulophid endoparasitoid *Phymastichus coffea* [312] and bethylid ectoparasitoid *Cephalonomia stephanoderis* [313]). These studies indicate that the integration of EPF with predators and parasitoids should be feasible, but EPF should be applied at different times relative to any supplementary releases of beneficial insect

species. For example, to effectively integrate EPF with *T. pretiosum* (an egg parasitoid), studies suggest that *T. pretiosum* should be released around three days prior to the application of EPF on crops and the second application of EPF should be delayed for a minimum of seven days after the first application. This timing ensures that when the EPF is applied the majority of parasitoids are developing within host eggs, rather than being exposed to the EPF as adults, since the development of *T. pretiosum* from egg to adult takes around one week [309,310]. Application of EPF to host eggs already parasitised by *T. pretiosum* did not have any negative impact on subsequent emergence of the parasitoid [309,310]. In contrast, the application of EPF to crops before releasing adult *T. pretiosum* may lead to *T. pretiosum* avoiding oviposition into host eggs already infected by the EPF [309,310]. The generalist predatory bug *A. nemorum* is known to avoid prey that are already infected by EPF and the avoidance behaviour was more pronounced towards conidiated cadavers. In addition, adults also avoided laying eggs on the plants that had already been treated with EPF [303]. Although these examples recommend releasing parasitoids and predators before the application of EPF, the optimum timing of EPF applications relative to releases of predators or parasitoids is likely to be specific to each combination of pest, EPF and beneficial species involved, and further studies in this area are required.

Some combinations of EPF with sublethal doses of botanical and synthetic insecticides have been shown to be synergistic and this interaction can also provide an effective solution for the management of weevils on horticultural crops. Combination treatments may work better than applications of either EPF or insecticide alone because the insecticide may disrupt insect grooming behavior that would otherwise lead to the removal of conidia before their germination [266–268,271]. Vulnerability to fungal infection in the insects may also be increased as a consequence of stress caused by insecticide exposure [230]. Although these combinations often show positive results, the use of sublethal insecticide doses may not be possible in field applications due to regulatory requirements designed to specifically combat resistance to standalone insecticide treatments caused by underdosing. In addition, not all synthetic insecticides are synergistic with EPF. In some studies, synthetic insecticides were toxic to EPF in tank mixes [314] and combined applications cannot be recommended. Adverse interactions may be a consequence of either the active ingredient or formulation additives being toxic to the entomopathogens [314]. Modifying the insecticide formulation may help avoid this problem; however, if the active ingredient is toxic to the fungus, the only viable option may be to separate the applications in time, and this requirement may be particularly significant with regard to the potential development of pesticide resistance.

8. Conclusions

In conclusion, entomopathogenic fungi are amongst the most promising biological control agents for use against weevils affecting horticultural crops. Based on the 175 peer-reviewed studies we examined, it is clear that the success of weevil IPM programs relies on having detailed knowledge of the biology of the species involved. Three groups of life cycles based on the weevils' developmental habitats have been recognised in this study and the susceptibility of each group to EPF has been reviewed in the context of their possible pathways of exposure. The integration of EPF into both preventive and remedial aspects of IPM programs using the methods discussed in this review and targeting developmental stages in habitats that make them most vulnerable to EPF infection will help reduce dependence on synthetic insecticides for weevil management in many of the world's major horticultural crops.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/10/659/s1. Figure S1: Flow diagram illustrating the selection process for publications include in this review. Figure S2: (A) The number of published studies using fungal entomopathogens on each weevil species affecting horticultural crops and included in this review, and (B) published studies using fungal entomopathogens for controlling weevils affecting horticultural crops and included in this review from 1973 to 2020.

Author Contributions: Conceptualisation, methodology, writing—review and editing, K.K.K., B.A.L.W., M.M.S., R.K.H., and G.J.A.; investigation, formal analysis, visualisation, and writing—original draft preparation, K.K.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation Australia with the Australian macadamia industry's research and development levy and contributions from the Australian government (Grant No. MC16004).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hill, D.S. *The Economic Importance of Insects*; Springer: London, UK, 1997; pp. 1–5.
2. Culliney, T.W. Chapter 8: Crop losses to arthropods. In *Integrated Pest Management: Pesticide Problems*; Pimentel, D., Peshin, R., Eds.; Springer: Dordrecht, The Netherlands, 2014; Volume 3, pp. 201–225.
3. Atwood, D.; Paisley-Jones, C. *Pesticides Industry Sales and Usage: 2008–2012 Market Estimates*; United States Environmental Protection Agency: Washington, DC, USA, 2017. Available online: https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf (accessed on 16 July 2020).
4. Nicholas, A.H.; Spooner-Hart, R.N.; Vickers, R.A. Abundance and natural control of the woolly aphid *Eriosoma lanigerum* in an Australian apple orchard IPM program. *Biocontrol* **2005**, *50*, 271–291. [CrossRef]
5. Zalucki, M.P.; Adamson, D.; Furlong, M.J. The future of IPM: Whither or wither? *Aust. J. Entomol.* **2009**, *48*, 85–96. [CrossRef]
6. Dutcher, J.D. A review of resurgence and replacement causing pest outbreaks in IPM. In *General Concepts in Integrated Pest and Disease Management*; Ciancio, A., Mukerji, K.G., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 27–43.
7. Pimentel, D.; Acquay, H.; Biltonen, M.; Rice, P.; Silva, M.; Nelson, J.; Lipner, V.; Giordano, S.; Horowitz, A.; D'Amore, M. Environmental and economic costs of pesticide use. *Bioscience* **1992**, *42*, 750–760. [CrossRef]
8. Pickering, J.; Dutcher, J.D.; Ekbom, B.S. The effect of a fungicide on fungal-induced mortality of pecan aphids (Homoptera: Aphididae) in the field. *J. Econ. Entomol.* **1990**, *83*, 1801–1805. [CrossRef]
9. Chandler, D.; Bailey, A.S.; Tatchell, G.M.; Davidson, G.; Greaves, J.; Grant, W.P. The development, regulation and use of biopesticides for integrated pest management. *Philos. Trans. R. Soc. B* **2011**, *366*, 1987–1998. [CrossRef]
10. Leahy, J.; Mendelsohn, M.; Kough, J.; Jones, R.; Berckes, N. Biopesticide oversight and registration at the US Environmental Protection Agency. In *Biopesticides: State of the Art and Future Opportunities*; Gross, A.D., Coats, J.R., Duke, S.O., Seiber, J.N., Eds.; ACS Publications: Washington, DC, USA, 2014; pp. 3–18.
11. Glare, T.; Caradus, J.; Gelernter, W.; Jackson, T.; Keyhani, N.; Köhl, J.; Marrone, P.; Morin, L.; Stewart, A. Have biopesticides come of age? *Trends Biotechnol.* **2012**, *30*, 250–258. [CrossRef]
12. Olson, S. An analysis of the biopesticide market now and where it is going. *Outlooks Pest Manag.* **2015**, *26*, 203–206. [CrossRef]
13. Dolinski, C.; Lacey, L.A. Microbial control of arthropod pests of tropical tree fruits. *Neotrop. Entomol.* **2007**, *36*, 161–179. [CrossRef]
14. Lacey, L.A.; Shapiro-Ilan, D.I. Microbial control of insect pests in temperate orchard systems: Potential for incorporation into IPM. *Annu. Rev. Entomol.* **2008**, *53*, 121–144. [CrossRef]
15. Lacey, L.A.; Grzywacz, D.; Shapiro-Ilan, D.I.; Frutos, R.; Brownbridge, M.; Goettel, M.S. Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* **2015**, *132*, 1–41. [CrossRef]
16. McKinnon, A.C.; Saari, S.; Moran-Diez, M.E.; Meyling, N.V.; Raad, M.; Glare, T.R. *Beauveria bassiana* as an endophyte: A critical review on associated methodology and biocontrol potential. *Biocontrol* **2017**, *62*, 1–17. [CrossRef]
17. Navarro-Llopis, V.; Ayala, I.; Sanchis, J.; Primo, J.; Moya, P. Field efficacy of a *Metarhizium anisopliae*-based attractant-contaminant device to control *Ceratitis capitata* (Diptera: Tephritidae). *J. Econ. Entomol.* **2015**, *108*, 1570–1578. [CrossRef] [PubMed]
18. Brandl, M.A.; Schumann, M.; Przyklenk, M.; Patel, A.; Vidal, S. Wireworm damage reduction in potatoes with an attract-and-kill strategy using *Metarhizium brunneum*. *J. Pest Sci.* **2017**, *90*, 479–493. [CrossRef]
19. Toledo, J.; Flores, S.; Campos, S.; Villaseñor, A.; Enkerlin, W.; Liedo, P.; Valle, Á.; Montoya, P. Pathogenicity of three formulations of *Beauveria bassiana* and efficacy of autoinoculation devices and sterile fruit fly males for dissemination of conidia for the control of *Ceratitis capitata*. *Entomol. Exp. Appl.* **2017**, *164*, 340–349. [CrossRef]

20. Sookar, P.; Bhagwant, S.; Khayrattee, F.B.; Chooneea, Y.; Ekesi, S. Mating compatibility of wild and sterile melon flies, *Bactrocera cucurbitae* (Diptera: Tephritidae) treated with entomopathogenic fungi. *J. Appl. Entomol.* **2014**, *138*, 409–417. [CrossRef]
21. Novelo-Rincón, L.F.; Montoya, P.; Hernández-Ortiz, V.; Liedo, P.; Toledo, J. Mating performance of sterile Mexican fruit fly *Anastrepha ludens* (Dipt., Tephritidae) males used as vectors of *Beauveria bassiana* (Bals.) Vuill. *J. Appl. Entomol.* **2009**, *133*, 702–710. [CrossRef]
22. Rossoni, C.; Kassab, S.O.; Loureiro, E.D.; Pereira, F.F.; Costa, D.P.; Barbosa, R.H.; Zanuncio, J.C. *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) are compatible with *Cotesia flavipes* (Hymenoptera: Braconidae). *Fla. Entomol.* **2014**, *97*, 1794–1804. [CrossRef]
23. Labbé, R.M.; Gillespie, D.R.; Cloutier, C.; Brodeur, J. Compatibility of an entomopathogenic fungus with a predator and a parasitoid in the biological control of greenhouse whitefly. *Biocontrol Sci. Technol.* **2009**, *19*, 429–446. [CrossRef]
24. Al Mazra'awi, M.S.; Shipp, J.L.; Broadbent, A.B.; Kevan, P.G. Dissemination of *Beauveria bassiana* by honey bees (Hymenoptera: Apidae) for control of tarnished plant bug (Hemiptera: Miridae) on canola. *Environ. Entomol.* **2006**, *35*, 1569–1577. [CrossRef]
25. Wraight, S.P.; Ramos, M.E. Characterization of the synergistic interaction between *Beauveria bassiana* strain GHA and *Bacillus thuringiensis morrisoni* strain tenebrionis applied against Colorado potato beetle larvae. *J. Invertebr. Pathol.* **2017**, *144*, 47–57. [CrossRef]
26. Sayed, A.M.M.; Behle, R.W. Evaluating a dual microbial agent biopesticide with *Bacillus thuringiensis* var. *kurstaki and Beauveria bassiana blastospores. Biocontrol Sci. Technol.* **2017**, *27*, 461–474.
27. Duarte, R.T.; Gonçalves, K.C.; Espinosa, D.J.L.; Moreira, L.F.; De Bortoli, S.A.; Humber, R.A.; Polanczyk, R.A. Potential of entomopathogenic fungi as biological control agents of diamondback moth (Lepidoptera: Plutellidae) and compatibility with chemical insecticides. *J. Econ. Entomol.* **2016**, *109*, 594–601. [CrossRef] [PubMed]
28. Niassy, S.; Maniania, N.K.; Subramanian, S.; Gitonga, M.L.; Maranga, R.; Obonyo, A.B.; Ekesi, S. Compatibility of *Metarhizium anisopliae* isolate ICIPE 69 with agrochemicals used in French bean production. *Int. J. Pest Manag.* **2012**, *58*, 131–137. [CrossRef]
29. Ree, B.; Knutson, A.E.; Harris, M. Controlling the Pecan Weevil. Texas Extension E-343. 2005. Available online: http://gregg.agrilife.org/files/2011/09/controllingthepecanweevil_1.pdf (accessed on 16 July 2020).
30. Mulder, P.G.; Harris, M.K.; Grantham, R.A. Biology and management of the pecan weevil (Coleoptera: Curculionidae). *J. Integr. Pest Manag.* **2012**, *3*, A1–A9. [CrossRef]
31. Muniappan, R.; Shepard, B.M.; Carner, G.R.; Ooi, P.A.C. *Arthropod Pests of Horticultural Crops in Tropical Asia*; CABI: Wallingford, UK, 2012; pp. 52–134.
32. Infante, F.; Pérez, J.; Vega, F.E. Redirect research to control coffee pest. *Nature* **2012**, *489*, 502. [CrossRef] [PubMed]
33. Gullan, P.J.; Cranston, P.S. *The Insects: An. Outline of Entomology*, 5th ed.; John Wiley & Sons: Oxford, UK, 2014; pp. 418–456.
34. Oberprieler, R.G.; Marvaldi, A.E.; Anderson, R.S. Weevils, weevils, weevils everywhere. *Zootaxa* **2007**, *1668*, 491–520. [CrossRef]
35. Benelli, G.; Meregalli, M.; Canale, A. Field observations on the mating behavior of *Aclees* sp. cf. *foveatus Voss (Coleoptera: Curculionidae), an exotic pest noxious to fig orchards. J. Insect Behav.* **2014**, *27*, 419–427.
36. Parra, L.; Mutis, A.; Ceballos, R.; Lizama, M.; Pardo, F.; Perich, F.; Quiroz, A. Volatiles released from *Vaccinium corymbosum* were attractive to *Aegorhinus superciliosus* (Coleoptera: Curculionidae) in an olfactometric bioassay. *Environ. Entomol.* **2009**, *38*, 781–789. [CrossRef]
37. Espinoza, J.; Urzúa, A.; Tampe, J.; Parra, L.; Quiroz, A. Repellent activity of the essential oil from the heartwood of *Pilgerodendron uviferum* (D. Don) Florin against *Aegorhinus superciliosus* (Coleoptera: Curculionidae). *Molecules* **2016**, *21*, 533. [CrossRef]
38. Szendrei, Z.; Averill, A.; Alborn, H.; Rodriguez-Saona, C. Identification and field evaluation of attractants for the cranberry weevil, *Anthonomus musculus* Say. *J. Chem. Ecol.* **2011**, *37*, 387–397. [CrossRef]
39. Alford, D.V. *Pest of Fruit Crops: A Colour Handbook*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2014; pp. 152–174.

40. Cross, J.V.; Burgess, C.M. Strawberry fruit yield and quality responses to flower bud removal: A simulation of damage by strawberry blossom weevil (*Anthonomus rubi*). *J. Hortic. Sci. Biotechnol.* **1998**, *73*, 676–680. [CrossRef]
41. Cross, J.V.; Easterbrook, M.A.; Crook, A.M.; Crook, D.; Fitz Gerald, J.D.; Innocenzi, P.J.; Jay, C.N.; Solomon, M.G. Review: Natural enemies and biocontrol of pests of strawberry in northern and central Europe. *Biocontrol Sci. Technol.* **2001**, *11*, 165–216.
42. Jeger, M.; Bragard, C.; Caffier, D.; Candresse, T.; Chatzivassiliou, E.; Dehnen-Schmutz, K.; Gilioli, G.; Gregoire, J.C.; Miret, J.A.J.; Navarro, M.N.; et al. Pest categorisation of *Anthonomus signatus*. *EFSA J.* **2017**, *15*, 4882.
43. Pulakkatu-thodi, I.; Motomura-Wages, S.; Miyasaka, S. Evaluation of insecticides for the management of rough sweetpotato weevil, *Blosyrus asellus* (Coleoptera: Curculionidae) in Hawai'i island. *Crop Prot.* **2018**, *114*, 223–227.
44. Racette, G.; Chouinard, G.; Vincent, C.; Hill, S.B. Ecology and management of plum curculio, *Conotrachelus nenuphar* [Coleoptera: Curculionidae], in apple orchards. *Phytoprotection* **1992**, *73*, 85–100.
45. Leskey, T.C.; Wright, S.E. Monitoring plum curculio, *Conotrachelus nenuphar* (Coleoptera: Curculionidae), populations in apple and peach orchards in the mid-Atlantic. *J. Econ. Entomol.* **2004**, *97*, 79–88.
46. Da Rosa, J.M.; Boff, M.I.C.; Nunes, M.Z.; Agostinetto, L.; Boff, P. Damage caused by *Conotrachelus psidii* (Coleoptera: Curculionidae) to the fruits of feijoa (*Acca sellowiana*). *Rev. Colomb. Entomol.* **2015**, *41*, 12–17.
47. Del Valle, E.E.; Dolinski, C.; Barreto, E.L.S.; Souza, R.M.; Samuels, R.I. Efficacy of *Heterorhabditis baujardi* LPP7 (Nematoda: Rhabditida) applied in *Galleria mellonella* (Lepidoptera: Pyralidae) insect cadavers to *Conotrachelus psidii*, (Coleoptera: Curculionidae) larvae. *Biocontrol Sci. Technol.* **2008**, *18*, 33–41.
48. Rukazambuga, N.D.T.M.; Gold, C.S.; Gowen, S.R. Yield loss in East African highland banana (*Musa* spp., AAA-EA group) caused by the banana weevil, *Cosmopolites sordidus* Germar. *Crop Prot.* **1998**, *17*, 581–589.
49. Keesey, I.W.; Barrett, B.A. Seasonal occurrence and soil distribution of the lesser chestnut weevil, *Curculio sayi* (Coleoptera: Curculionidae) in Mid-Missouri. *J. Kans. Entomol. Soc.* **2008**, *81*, 345–354.
50. Paparatti, B.; Speranza, S. Biological control of chestnut weevil (*Curculio elephas* Gyll.; Coleoptera, Curculionidae) with the entomopathogen fungus *Beauveria bassiana* (Balsamo) Vuill. (Deuteromycotina, Hyphomycetes). In Proceedings of the 2nd International Symposium on Chestnut, Bordeaux, France, 19 October 1998; Salesses, G., Ed.; International Society for Horticultural Science: Leuven, Belgium, 1999; pp. 459–464.
51. Guidone, L.; Valentini, N.; Rolle, L.; Me, G.; Tavella, L. Early nut development as a resistance factor to the attacks of *Curculio nucum* (Coleoptera: Curculionidae). *Ann. Appl. Biol.* **2007**, *150*, 323–329. [CrossRef]
52. Batalla-Carrera, L.; Morton, A.; Garcia-del-Pino, F. Field efficacy against the hazelnut weevil, *Curculio nucum* and short-term persistence of entomopathogenic nematodes. *Span. J. Agric. Res.* **2013**, *11*, 1112–1119. [CrossRef]
53. Pelsue, F.W.; Zhang, R.Z. A review of the Genus *Curculio* from China with descriptions of fourteen new species. Part IV. The *Curculio sikkimensis* (Heller) group (Coleoptera: Curculionidae: Curculioninae: Curculionini). *Coleopt. Bull.* **2003**, *57*, 311–333. [CrossRef]
54. Kim, Y.J.; Yoon, C.M.; Shin, S.C.; Choi, K.S.; Kim, G.H. Seasonal occurrence of the larvae and adults of chestnut weevil, *Curculio sikkimensis* (Coleoptera: Curculionidae). *Korean J. Appl. Entomol.* **2008**, *47*, 9–15. [CrossRef]
55. Reddy, P.P. *Plant Protection in Tropical Root and Tuber Crops*; Springer: New Delhi, India, 2015; pp. 87–98.
56. Smit, N.E.J.M.; van Huis, A. Biology of the African sweetpotato weevil species *Cylas puncticollis* (Boheman) and *C. brunneus* (Fabricius) (Coleoptera: Apionidae). *Int. J. Trop. Insect Sci.* **1998**, *18*, 93–100. [CrossRef]
57. Weissling, T.J.; Peña, J.E.; Giblin-Davis, R.M.; Knapp, J.L., Jr. Diaprepes root weevil, Diaprepes abbreviatus (Linnaeus) (Insecta: Coleoptera: Curculionidae). IFAS Extension EENY-024. 2009. Available online: https://edis.ifas.ufl.edu/pdffiles/IN/IN15100.pdf (accessed on 16 July 2020).
58. Diaz, V.; Caicedo, A.M.; Carabali, A. Life cycle and morphological description of *Heilipus lauri* Boheman (Coleoptera: Curculionidae) in Colombia. *Acta Zool. Mex.* **2017**, *33*, 231–242.
59. Castañeda-Vildozola, Á.; Franco-Mora, O.; De Jesús Pérez-Lopez, D.; Nava-Díaz, C.; Carrasco, J.V.; Vargas-Rojas, L. Association of *Heilipus lauri* Boheman and *Conotrachelus perseae* Barber (Coleoptera: Curculionidae) on avocado in Mexico. *Coleopt. Bull.* **2013**, *67*, 116–118. [CrossRef]

60. Oliveira, C.M.; Auad, A.M.; Mendes, S.M.; Frizzas, M.R. Economic impact of exotic insect pests in Brazilian agriculture. *J. Appl. Entomol.* **2013**, *137*, 1–15. [CrossRef]
61. Bright, J. Macadamia Seed Weevil (*Kuschelorhynchus macadamiae*) Orchard Management. Primefact 1585. 2017. Available online: https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0008/731987/Macadamia-seed-weevil-update-orchard-management_2.pdf (accessed on 16 July 2020).
62. Huwer, R. *Ecology and Management of Sigastus Weevil in Macadamias*; Horticulture Innovation Australia Limited: Sydney, Australia, 2016. Available online: https://www.horticulture.com.au/globalassets/laserfiche/assets/project-reports/mc15010/mc15010-final-report-514.pdf (accessed on 16 July 2020).
63. Padmanaban, B.; Sathiamoorthy, S. The Banana Stem Weevil *Odoiporus longicollis*. Musa Pest Fact Sheet No. 5. 2001. Available online: https://www.bioversityinternational.org/fileadmin/_migrated/uploads/tx_news/The_Banana_stem_weevil_Odoiporus_longicollis_756.pdf (accessed on 16 July 2020).
64. Moorhouse, E.R.; Charnley, A.K.; Gillespie, A.T. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Ann. Appl. Biol.* **1992**, *121*, 431–454. [CrossRef]
65. Prior, C.; Jollands, P.; Le Patourel, G. Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) to the cocoa weevil pest *Pantorhytes plutus* (Coleoptera: Curculionidae). *J. Invertebr. Pathol.* **1988**, *52*, 66–72. [CrossRef]
66. Gressitt, J.L. The weevil genus *Pantorhytes* (Coleoptera) involving cacao pests and epizoic symbiosis with cryptogamic plants and microfauna. *Pac. Insects* **1966**, *8*, 915–965.
67. Ferreira, T.; Malan, A.P. Potential of entomopathogenic nematodes for the control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae). *J. Helminthol.* **2014**, *88*, 293–301. [CrossRef] [PubMed]
68. Witt, A.B.R.; Little, R.M.; Crowe, T.M. The effectiveness of helmeted guineafowl *Numida meleagris* (Linnaeus 1766) in controlling the banded fruit weevil *Phlyctinus callosus* (Schönherr 1826), and their impact on other invertebrates in apple orchards in the Western Cape Province, South Africa. *Agric. Ecosyst. Environ.* **1995**, *55*, 169–179. [CrossRef]
69. Seybold, S.J.; Coleman, T.W.; Dallara, P.L.; Dart, N.L.; Graves, A.D.; Pederson, L.A.; Spichiger, S.E. Recent collecting reveals new state records and geographic extremes in the distribution of the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Scolytidae), in the United States. *Pan-Pac. Entomol.* **2012**, *88*, 277–280. [CrossRef]
70. Wattanapongsiri, A. A Revision to the Genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). Ph.D. Thesis, Oregon State University, Corvallis, OR, USA, 1965.
71. Faleiro, J.R. A review of the issues and management of the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Rhynchophoridae) in coconut and date palm during the last one hundred years. *Int. J. Trop. Insect Sci.* **2006**, *26*, 135–154.
72. El-Sabea, A.M.R.; Faleiro, J.R.; Abo-El-Saad, M.M. The threat of red palm weevil *Rhynchophorus ferrugineus* to date plantations of the Gulf region in the Middle-East: An economic perspective. *Outlooks Pest Manag.* **2009**, *20*, 131–134. [CrossRef]
73. Oehlschlager, A.C.; Chinchilla, C.; Castillo, G.; Gonzalez, L. Control of red ring disease by mass trapping of *Rhynchophorus palmarum* (Coleoptera: Curculionidae). *Fla. Entomol.* **2002**, *85*, 507–513. [CrossRef]
74. Chang, V.C.S. Macadamia quick decline and *Xyleborus* beetles (Coleoptera: Scolytidae). *Int. J. Pest Manag.* **1993**, *39*, 144–148. [CrossRef]
75. Lona, I.D.; Miller, D.G., III; Hatfield, C.A.; Rosecrance, R.C.; Nelson, L.J.; Audley, J.P.; Siefker, M.A.; Chen, Y.; Seybold, S.J. Host selection behavior mediated by differential landing rates of the walnut twig beetle, *Pityophthorus juglandis*, and associated subcortical insect species, on two western North American walnut species, *Juglans californica* and *J. major*. *Entomol. Exp. Appl.* **2020**, *168*, 240–258. [CrossRef]
76. Castrejón-Antonio, J.E.; Tamez-Guerra, P.; Montesinos-Matias, R.; Ek-Ramos, M.J.; Garza-López, P.M.; Arredondo-Bernal, H.C. Selection of *Beauveria bassiana* (Hypocreales: Cordycipitaceae) strains to control *Xyleborus affinis* (Curculionidae: Scolytinae) females. *PeerJ* **2020**, *8*, e9472. [CrossRef]
77. Mailloux, G.; Bostanian, N.J. Development of the strawberry bud weevil (Coleoptera: Curculionidae) in strawberry fields. *Ann. Entomol. Soc Am.* **1993**, *86*, 384–393. [CrossRef]
78. Eaton, A.T. Plum Curculio. UNH Cooperative Extension. 2018. Available online: https://extension.unh.edu/resources/files/Resource002799_Rep4154.pdf (accessed on 16 July 2020).

79. Bailez, O.E.; Viana-Bailez, A.M.; de Lima, J.O.G.; Moreira, D.D.O. Life-history of the guava weevil, *Conotrachelus psidii* Marshall (Coleoptera: Curculionidae), under laboratory conditions. *Neotrop. Entomol.* **2003**, *32*, 203–207. [CrossRef]
80. Hill, D.S. *Pests of Crops in Warmer Climates and Their Control*; Springer: London, UK, 2008; p. 329.
81. Cottrell, T.E.; Wood, B.W. Movement of adult pecan weevils *Curculio caryae* within pecan orchards. *Agric. For. Entomol.* **2008**, *10*, 363–373. [CrossRef]
82. Venette, R.; Davis, E.; Heisler, H.; Larson, M. Mini Risk Assessment, Chestnut Weevil, Curculio elephas (Gyllenhal), [Coleoptera: Curculionidae]. 2003. Available online: http://download.ceris.purdue.edu/file/336 (accessed on 16 July 2020).
83. Tuncer, C.; Ecevit, O. Current status of hazelnut pests in Turkey. In Proceedings of the 4th International Symposium on Hazelnut, Ordu, Turkey, 30 July 1996; Köksal, A.I., Okay, Y., Günes, N.T., Eds.; International Society for Horticultural Science: Leuven, Belgium, 1997; pp. 545–552.
84. Grafton-Cardwell, E.; Godfrey, K.; Peña, J.; McCoy, C.; Luck, R. Diaprepes Root Weevil. ANR Publication 8131. 2004. Available online: https://ucanr.edu/datastoreFiles/391-265.pdf (accessed on 16 July 2020).
85. Damon, A. A review of the biology and control of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). *Bull. Entomol. Res.* **2000**, *90*, 453–465. [CrossRef] [PubMed]
86. Bright, J. *Sigastus Weevil Update. Part 1. Life Cycle and Monitoring Keys to Control*; Australian Macadamia Society Ltd.: Lismore, Australia, 2017. Available online: https://www.horticulture.com.au/globalassets/hort-innovation/resource-assets/mc-ipm-program-sigastus-weevil-fact-sheet.pdf (accessed on 16 July 2020).
87. Prabhavathi, M.K.; Ghosh, S.K. Studies on the interaction between *Odoiporous longicollis* and endophytic *Beauveria bassiana* by establishing fungal infection to bsw in the plant system. *Int. J. Plant Prot.* **2014**, *7*, 312–317. [CrossRef]
88. Tsatsia, H.; Jackson, G. Cocoa Weevil Borer. Available online: http://www.pestnet.org/fact_sheets/cocoa_weevil_borer_061.htm (accessed on 23 May 2018).
89. Dlamini, B.E.; Addison, P.; Malan, A.P. A review of the biology and control of *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), with special reference to biological control using entomopathogenic nematodes and fungi. *Afr. Entomol.* **2019**, *27*, 279–288. [CrossRef]
90. Mayfield, A.E.; Juzwik, J.; Scholer, J.; Vandenberg, J.D.; Taylor, A. Effect of bark application with *Beauveria bassiana* and permethrin insecticide on the walnut twig beetle (Coleoptera: Curculionidae) in black walnut bolts. *J. Econ. Entomol.* **2019**, *112*, 2493–2496. [CrossRef]
91. Mendel, Z.; Ben-Yehuda, S.; Marcus, R.; Nestel, D. Distribution and extent of damage by *Scolytus* spp. to stone and pome fruit orchards in Israel. *Int. J. Trop. Insect Sci.* **1997**, *17*, 175–181. [CrossRef]
92. Gargani, E.; Mazza, G.; Benvenuti, C.; Torrini, G.; Strangi, A.; Pennacchio, F.; Roversi, P.F. Biological control of *Aclees* sp. cf. *foveatus* and first recovery of an associate *Beauveria bassiana* strain. *Redia* **2016**, *99*, 29–33.
93. Sabbahi, R.; Merzouki, A.; Guertin, C. Efficacy of *Beauveria bassiana* against the strawberry pests, *Lygus lineolaris*, *Anthonomus signatus* and *Otiorhynchus ovatus*. *J. Appl. Entomol.* **2008**, *132*, 151–160. [CrossRef]
94. Lopes, R.B.; Michereff-Filho, M.; Tigano, M.S.; Neves, P.M.O.J.; López, E.L.; Fancelli, M.; da Silva, J.P. Virulence and horizontal transmission of selected Brazilian strains of *Beauveria bassiana* against *Cosmopolites sordidus* under laboratory conditions. *Bull. Insectol.* **2011**, *64*, 201–208.
95. Lopes, R.B.; Mesquita, A.L.M.; Tigano, M.S.; Souza, D.A.; Martins, I.; Faria, M. Diversity of indigenous *Beauveria* and *Metarhizium* spp. in a commercial banana field and their virulence toward *Cosmopolites sordidus* (Coleoptera: Curculionidae). *Fungal Ecol.* **2013**, *6*, 356–364. [CrossRef]
96. Fancelli, M.; Dias, A.B.; Delalibera, I.; de Jesus, S.C.; do Nascimento, A.S.; de Oliveira e Silva, S.; Caldas, R.C.; Ledo, C.A.S. *Beauveria bassiana* strains for biological control of *Cosmopolites sordidus* (Germ.) (Coleoptera: Curculionidae) in plantain. *Biomed Res. Int.* **2013**, *2013*, 184756. [CrossRef] [PubMed]
97. González, D.N.; Chávez, M.A.A.; Gutiérrez, R.L.; Cupul, W.C.; Ochoa, J.M.; Velasco, E.G. Suitability of *Cordyceps bassiana* and *Metarhizium anisopliae* for biological control of *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) in an organic Mexican banana plantation: Laboratory and field trials. *J. Plant Dis. Prot.* **2018**, *125*, 73–81. [CrossRef]
98. Omukoko, C.A.; Maniania, K.N.; Wesonga, J.M.; Kahangi, E.M.; Wamocho, L.S. Pathogenicity of isolates of *beauveria bassiana* to the banana weevil *Cosmopolites sordidus*. *J. Agric. Sci. Technol.* **2011**, *13*, 3–14.
99. Omukoko, C.A.; Maniania, K.N.; Wesonga, J.M.; Kahangi, E.M.; Wamocho, L.S. Virulence of three strains of *Beauveria bassiana* against the banana weevil. *J. Agric. Biol. Sci.* **2014**, *9*, 333–336.

100. Omukoko, C.A.; Wesonga, J.M.; Maniania, K.N.; Kahangi, E.M.; Wamocho, L.S. Screening of *Beauveria bassiana* isolates to the banana weevil and horizontal transmission under laboratory conditions. *J. Agric. Sci. Technol.* **2014**, *16*, 1–12.
101. Maharaj, K.; Khan, A. Efficacy of banana spray oil, mineral oil and water formulations of *Beauveria bassiana* Balsamo for the control of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) in *Musa* spp. *Int. J. Trop. Agric.* **2016**, *34*, 1455–1460.
102. Membang, G.; Ambang, Z.; Mahot, H.C.; Kuate, A.F.; Fiaboe, K.K.M.; Hanna, R. *Cosmopolites sordidus* (Germar) susceptibility to indigenous Cameroonian *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) isolates. *J. Appl. Entomol.* **2020**, *144*, 468–480. [CrossRef]
103. Dotaona, R.; Wilson, B.A.L.; Stevens, M.M.; Holloway, J.; Ash, G.J. Screening of tropical isolates of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) for virulence to the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Int. J. Trop. Insect Sci.* **2015**, *35*, 153–163. [CrossRef]
104. Saputro, T.B.; Prayogo, Y.; Rohman, F.L.; Alami, N.H. The virulence improvement of *Beauveria bassiana* in infecting *Cylas formicarius* modulated by various chitin based compounds. *Biodiversitas* **2019**, *20*, 2486–2493. [CrossRef]
105. Ondiaka, S.; Maniania, N.K.; Nyamasyo, G.H.N.; Nderitu, J.H. Virulence of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to sweetpotato weevil *Cylas puncticollis* and effects on fecundity and egg viability. *Ann. Appl. Biol.* **2008**, *153*, 41–48. [CrossRef]
106. Clavijo, A.P.; Holguin, C.M. Pathogenicity of commercial entomopathogenic fungal strains on the avocado seed borer (ASB), *Heilipus lauri* (Coleoptera: Curculionidae) under laboratory conditions. *Int. J. Trop. Insect Sci.* **2020**. [CrossRef]
107. De la Rosa-Reyes, W.; Godinez-Aguilar, J.L.; Alatorre-Rosas, R. Biological activity of five strains of *Metarhizium anisopliae*, upon the coffee berry borer *Hypothenemus hampei* (Col.: Scolytidae). *Entomophaga* **1995**, *40*, 403–412. [CrossRef]
108. De la Rosa, W.; Alatorre, R.; Trujillo, J.; Barrera, J.F. Virulence of *Beauveria bassiana* (Deuteromycetes) strains against the coffee berry borer (Coleoptera: Scolytidae). *J. Econ. Entomol.* **1997**, *90*, 1534–1538. [CrossRef]
109. Haraprasad, N.; Niranjana, S.R.; Prakash, H.S.; Shetty, H.S.; Wahab, S. *Beauveria bassiana*—A potential mycopesticide for the efficient control of coffee berry borer, *Hypothenemus hampei* (Ferrari) in India. *Biocontrol Sci. Technol.* **2001**, *11*, 251–260. [CrossRef]
110. Pava-Ripoll, M.; Posada, F.J.; Momen, B.; Wang, C.; St. Leger, R.J. Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene. *J. Invertebr. Pathol.* **2008**, *99*, 220–226. [CrossRef]
111. Samuels, R.I.; Pereira, R.C.; Gava, C.A.T. Infection of the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae) by Brazilian isolates of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Biocontrol Sci. Technol.* **2002**, *12*, 631–635. [CrossRef]
112. Varela, A.; Morales, E. Characterization of some *Beauveria bassiana* isolates and their virulence toward the coffee berry borer *Hypothenemus hampei*. *J. Invertebr. Pathol.* **1996**, *67*, 147–152. [CrossRef]
113. Vera, J.T.; Montoya, E.C.; Benavides, P.; Góngora, C.E. Evaluation of *Beauveria bassiana* (Ascomycota: Hypocreales) as a control of the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae) emerging from fallen, infested coffee berries on the ground. *Biocontrol Sci. Technol.* **2011**, *21*, 1–14. [CrossRef]
114. Cruz, L.P.; Gaitan, A.L.; Gongora, C.E. Exploiting the genetic diversity of *Beauveria bassiana* for improving the biological control of the coffee berry borer through the use of strain mixtures. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 918–926. [CrossRef] [PubMed]
115. Mota, L.H.C.; Silva, W.D.; Sermarini, R.A.; Demétrio, C.G.B.; Bento, J.M.S.; Delalibera, I. Autoinoculation trap for management of *Hypothenemus hampei* (Ferrari) with *Beauveria bassiana* (Bals.) in coffee crops. *Biol. Control* **2017**, *111*, 32–39. [CrossRef]
116. Posada-Flórez, F.J. Production of *Beauveria bassiana* fungal spores on rice to control the coffee berry borer, *Hypothenemus hampei*, in Colombia. *J. Insect Sci.* **2008**, *8*, 41. [CrossRef]
117. Balakrishnan, M.M.; Prakash, R.N. Infectivity of ten *Metarhizium anisopliae* isolates to the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae). *J. Entomol. Zool. Stud.* **2014**, *2*, 246–249.

118. Belay, Y.C.; Tenkegna, T.A. Bioassay and pilot mass production of entomopathogenic fungus, *Beauveria bassiana* for the control of coffee berry borer (*Hypothenemus hampei*: Scolytidae), Ferrari. *J. Appl. Biosci.* **2017**, *117*, 11669–11683.
119. Khun, K.K.; Ash, G.J.; Stevens, M.M.; Huwer, R.K.; Wilson, B.A.L. Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays. *J. Invertebr. Pathol.* **2020**, *174*, 107437. [CrossRef] [PubMed]
120. Padmanaban, B.; Thangavelu, R.; Gopi, M.; Mustaffa, M.M. Effect of mass multiplication media on sporulation, field efficacy and shelf life of *Beauveria bassiana* against rhizome and pseudostem weevils of banana. *J. Biol. Control* **2009**, *23*, 277–283.
121. Alagesan, A.; Padmanaban, B.; Tharani, G.; Jawahar, S.; Manivannan, S. An assessment of biological control of the banana pseudostem weevil *Odoiporus longicollis* (Olivier) by entomopathogenic fungi *Beauveria bassiana*. *Biocatal. Agric. Biotechnol.* **2019**, *20*, 101262. [CrossRef]
122. Awasthi, N.S.; Sridharan, S.; Mohankumar, S. In vitro evaluation of native isolate of *Metarhizium anisopliae* (Metchinkoff) Sorokin and its oil in water formulations against *Odoiporus longicollis* Olivier. *J. Biol. Control* **2017**, *31*, 248–252. [CrossRef]
123. Castrillo, L.A.; Mayfield, A.E.; Griggs, M.H.; Camp, R.; Mudder, B.; Taylor, A.; Vandenberg, J.D. Mortality and reduced brood production in walnut twig beetles, *Pityophthorus juglandis* (Coleoptera: Curculionidae), following exposure to commercial strains of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum*. *Biol. Control* **2017**, *114*, 79–86. [CrossRef]
124. Abdel-Samad, S.S.M.; Mahmoud, B.A.; Abbas, M.S.T. Evaluation of the fungus, *Beauveria bassiana* (Bals.) Vuill as a bio-control agent against the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) (Coleoptera: Curculionidae). *Egypt J. Biol. Pest Control* **2011**, *21*, 125–129.
125. Dembilio, Ó.; Quesada-Moraga, E.; Santiago-Álvarez, C.; Jacas, J.A. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil, *Rhynchophorus ferrugineus*. *J. Invertebr. Pathol.* **2010**, *104*, 214–221. [CrossRef] [PubMed]
126. Gindin, G.; Levski, S.; Glazer, I.; Soroker, V. Evaluation of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus*. *Phytoparasitica* **2006**, *34*, 370–379. [CrossRef]
127. Hajjar, M.J.; Ajlan, A.M.; Al-Ahmad, M.H. New approach of *Beauveria bassiana* to control the red palm weevil (Coleoptera: Curculionidae) by trapping technique. *J. Econ. Entomol.* **2015**, *108*, 425–432. [CrossRef] [PubMed]
128. Hussain, A.; Rizwan-ul-Haq, M.; Al-Ayedh, H.; Ahmed, S.; Al-Jabr, A.M. Effect of *Beauveria bassiana* infection on the feeding performance and antioxidant defence of red palm weevil, *Rhynchophorus ferrugineus*. *Biocontrol* **2015**, *60*, 849–859. [CrossRef]
129. Hussain, A.; Rizwan-ul-Haq, M.; Al-Ayedh, H.; AlJabr, A.M. Susceptibility and immune defence mechanisms of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) against entomopathogenic fungal infections. *Int. J. Mol. Sci.* **2016**, *17*, 1518. [CrossRef] [PubMed]
130. Lo Verde, G.; Torta, L.; Mondello, V.; Caldarella, C.G.; Burruano, S.; Caleca, V. Pathogenicity bioassays of isolates of *Beauveria bassiana* on *Rhynchophorus ferrugineus*. *Pest Manag. Sci.* **2015**, *71*, 323–328. [CrossRef]
131. Merghem, A. Susceptibility of the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) to the green muscardine fungus, *Metarhizium anisopliae* (Metsch.) in the laboratory and in palm trees orchards. *Egypt J. Biol. Pest Control* **2011**, *21*, 179–183.
132. Ricaño, J.; Güerri-Agulló, B.; Serna-Sarriás, M.J.; Rubio-Llorca, G.; Asensio, L.; Barranco, P.; Lopez-Llorca, L.V. Evaluation of the pathogenicity of multiple isolates of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) for the assessment of a solid formulation under simulated field conditions. *Fla. Entomol.* **2013**, *96*, 1311–1324. [CrossRef]
133. Sun, X.D.; Yan, W.; Qin, W.Q.; Zhang, J.; Niu, X.Q.; Ma, G.C.; Li, F.H. Screening of tropical isolates of *Metarhizium anisopliae* for virulence to the red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *SpringerPlus* **2016**, *5*, 1100. [CrossRef]
134. Yasin, M.; Wakil, W.; Ghazanfar, M.U.; Qayyum, M.A.; Tahir, M.; Bedford, G.O. Virulence of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against red palm weevil, *Rhynchophorus ferrugineus* (Olivier). *Entomol. Res.* **2019**, *49*, 3–12. [CrossRef]

135. El Husseini, M.M. Efficacy of the fungus *Beauveria bassiana* (Balsamo) Vuillemin on the red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) larvae and adults under laboratory conditions. *Egypt J. Biol. Pest Control* **2019**, *29*, 58. [CrossRef]
136. Abdel-Raheem, M.A.; Alghamdi, H.A.; Reyad, N.F. Virulence of fungal spores and silver nanoparticles from entomopathogenic fungi on the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *Egypt J. Biol. Pest Control* **2019**, *29*, 97. [CrossRef]
137. Aldossary, A.A.; Shehata, S.T.; Hegazy, G.; Salem, M.A.; Faiza, M.A.M. Assessment of the entomopathogenic fungus *Beauveria bassiana* Saudi Arabian isolate (B-SA3) against the developmental stages of the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.). *Arab Univ. J. Agric. Sci.* **2009**, *17*, 227–237.
138. Hou, F.J.; Addis, S.N.K.; Azmi, W.A. Virulence evaluation of entomopathogenic fungi against the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Dryopthoridae). *Malays. Appl. Biol.* **2018**, *47*, 25–30.
139. Qayyum, M.A.; Saleem, M.A.; Saeed, S.; Wakil, W.; Ishtiaq, M.; Ashraf, W.; Ahmed, N.; Ali, M.; Ikram, R.M.; Yasin, M.; et al. Integration of entomopathogenic fungi and eco-friendly insecticides for management of red palm weevil, *Rhynchophorus ferrugineus* (Olivier). *Saudi J. Biol. Sci.* **2020**, *27*, 1811–1817. [CrossRef]
140. Abdel-Raheem, M.A.; Reyad, N.F.; Alghamdi, H.A. Virulence of nanoparticle preparation of entomopathogenic fungi and entomopathogenic bacteria against red palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae). *Rom. Biotechnol. Lett.* **2020**, *25*, 1151–1159. [CrossRef]
141. Ishak, I.; Ng, L.C.; Haris-Hussain, M.; Jalinas, J.; Idris, A.B.; Azlina, Z.; Samsudin, A.; Wahizatul, A.A. Pathogenicity of an indigenous strain of the entomopathogenic fungus *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) (MET-GRA4 Strain) as a potential biological control agent against the red palm weevil (Coleoptera: Dryophthoridae). *J. Econ. Entomol.* **2020**, *113*, 43–49. [CrossRef]
142. Cheong, J.L.; Azmi, W.A. Dataset on the influence of relative humidity on the pathogenicity of *Metarhizium anisopliae* isolates from Thailand and Malaysia against red palm weevil (*Rhynchophorus ferrugineus*, Olivier) adult. *Data Brief* **2020**, *30*, 105482. [CrossRef]
143. Al-Keridis, L.A.; Gaber, N.M.; Aldawood, A.S. Pathogenicity of Saudi Arabian fungal isolates against egg and larval stages of *Rhynchophorus ferrugineus* under laboratory conditions. *Int. J. Trop. Insect Sci.* **2020**. [CrossRef]
144. Batta, Y.A. Biocontrol of almond bark beetle (*Scolytus amygdali* Geurin-Meneville, Coleoptera: Scolytidae) using *Beauveria bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes). *J. Appl. Microbiol.* **2007**, *103*, 1406–1414. [CrossRef] [PubMed]
145. Kaaya, G.P.; Seshu-Reddy, K.V.; Kokwaro, E.D.; Munyinyi, D.M. Pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Serratia marcescens* to the banana weevil *Cosmopolites sordidus*. *Biocontrol Sci. Technol.* **1993**, *3*, 177–187. [CrossRef]
146. Magara, E.; Nankinga, C.M.K.; Gold, C.S.; Kyamanywa, S.; Ragama, P.; Tushemereirwe, W.K.; Moore, D.; Gowen, S.R. Efficacy of *Beauveria bassiana* substrates and formulations for the control of banana weevil. *Uganda J. Agric. Sci.* **2004**, *9*, 900–905.
147. Francardi, V.; Benvenuti, C.; Roversi, P.F.; Rumine, P.; Barzanti, G. Entomopathogenicity of *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorokin isolated from different sources in the control of *Rhynchophorus ferrugineus* (Oliver) (Coleoptera Curculionidae). *Redia* **2012**, *95*, 49–55.
148. Francardi, V.; Benvenuti, C.; Barzanti, G.P.; Roversi, P.F. Autocontamination trap with entomopathogenic fungi: A possible strategy in the control of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Curculionidae). *Redia* **2013**, *96*, 57–67.
149. Francardi, V.; Benvenuti, C.; Barzanti, G.P.; Roversi, P.F. *Metarhizium anisopliae* biopesticides and fungus isolates: Control efficacy against *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae) on different contamination substrata. *Redia* **2015**, *98*, 25–29.
150. Cito, A.; Mazza, G.; Strangi, A.; Benvenuti, C.; Barzanti, G.P.; Dreassi, E.; Turchetti, T.; Francardi, V.; Roversi, P.F. Characterization and comparison of *Metarhizium* strains isolated from *Rhynchophorus ferrugineus*. *FEMS Microbiol. Lett.* **2014**, *355*, 108–115. [CrossRef]
151. Peña, J.E.; Gilbin-Davis, R.M.; Duncan, R. Impact of indigenous *Beauveria bassiana* (Balsamo) Vuillemin on banana weevil and rotten sugarcane weevil (Coleoptera: Curculionidae) populations in banana in Florida. *J. Agric. Entomol.* **1995**, *12*, 163–167.

152. Monzón, A.J.; Guharay, F.; Klingen, I. Natural occurrence of *Beauveria bassiana* in *Hypothenemus hampei* (Coleoptera: Curculionidae) populations in unsprayed coffee fields. *J. Invertebr. Pathol.* **2008**, *97*, 134–141. [CrossRef]
153. Wraight, S.P.; Galaini-Wraight, S.; Howes, R.L.; Castrillo, L.A.; Carruthers, R.I.; Smith, R.H.; Matsumoto, T.K.; Keith, L.M. Prevalence of naturally-occurring strains of *Beauveria bassiana* in populations of coffee berry borer *Hypothenemus hampei* on Hawai'i Island, with observations on coffee plant-*H. hampei*-*B. bassiana* interactions. *J. Invertebr. Pathol.* **2018**, *156*, 54–72. [CrossRef]
154. Asiry, K.A.; Sulieman, A.M.E.; Al-Anazi, N.A.; Veettil, V.N.; Abdelgadir, M.; Alkhregi, I. Isolation, phenotypic and genotypic characterization of indigenous *Beauveria bassiana* isolates from date palm infested with *Rhynchophorus ferrugineus* in Hail region, Saudi Arabia. *Biosci. Biotechnol. Res. Commun.* **2018**, *11*, 393–401. [CrossRef]
155. Güerri-Agulló, B.; López-Follana, R.; Asensio, L.; Barranco, P.; Lopez-Llorca, L.V. Use of a solid formulation of *Beauveria bassiana* for biocontrol of the red palm weevil (*Rhynchophorus ferrugineus*) (Coleoptera: Dryophthoridae) under field conditions in SE Spain. *Fla. Entomol.* **2011**, *94*, 737–747. [CrossRef]
156. Prior, C.; Arura, M. The infectivity of *Metarhizium anisopliae* to two insect pests of coconuts. *J. Invertebr. Pathol.* **1985**, *45*, 187–194. [CrossRef]
157. Sabbahi, R.; Merzouki, A.; Guertin, C. Potential effect of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Anthonomus signatus* (Coleoptera: Curculionidae) in strawberries. *Biocontrol Sci. Technol.* **2009**, *19*, 729–741. [CrossRef]
158. Schoeman, P.S.; Botha, H. Field management of the banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), with *Beauveria bassiana*. *Afr. Plant Prot.* **2003**, *9*, 1–3.
159. Hlerema, I.; Laurie, S.; Eiasu, B. Preliminary observations on use of *Beauveria bassiana* for the control of the sweetpotato weevil (*Cylas* sp.) in South Africa. *Open Agric.* **2017**, *2*, 595–599.
160. de la Rosa, W.; Alatorre, R.; Barrera, J.F.; Toriello, C. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycetes) upon the coffee berry borer (Coleoptera: Scolytidae) under field conditions. *J. Econ. Entomol.* **2000**, *93*, 1409–1414. [CrossRef]
161. Edgington, S.; Segura, H.; de la Rosa, W.; Williams, T. Photoprotection of *Beauveria bassiana*: Testing simple formulations for control of the coffee berry borer. *Int. J. Pest Manag.* **2000**, *46*, 169–176. [CrossRef]
162. Greco, E.B.; Wright, M.G.; Burgueño, J.; Jaronski, S.T. Efficacy of *Beauveria bassiana* applications on coffee berry borer across an elevation gradient in Hawaii. *Biocontrol Sci. Technol.* **2018**, *28*, 995–1013. [CrossRef]
163. Hollingsworth, R.G.; Aristizábal, L.F.; Shriner, S.; Mascarin, G.M.; Moral, R.D.; Arthurs, S.P. Incorporating *Beauveria bassiana* into an integrated pest management plan for coffee berry borer in Hawaii. *Front. Sustain. Food Syst.* **2020**, *4*, 22. [CrossRef]
164. El-Sufty, R.; Al-Awash, S.A.; Al Bgham, S.; Shahdad, A.S.; Al Bathra, A.H. Pathogenicity of the fungus *Beauveria bassiana* (Bals.) Vuill to the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) (Col.: Curculionidae) under laboratory and field conditions. *Egypt J. Biol. Pest Control* **2009**, *19*, 81–85.
165. Sewify, G.H.; Belal, M.H.; Al-Awash, S.A. Use of the entomopathogenic fungus, *Beauveria bassiana* for the biological control of the red palm weevil, *Rhynchophorus ferrugineus* Olivier. *Egypt J. Biol. Pest Control* **2009**, *19*, 157–163.
166. Su, C.Y.; Tzean, S.S.; Ko, W.H. *Beauveria bassiana* as the lethal factor in a Taiwanese soil pernicious to sweetpotato weevil, *Cylas formicarius*. *J. Invertebr. Pathol.* **1988**, *52*, 195–197. [CrossRef]
167. Nankinga, C.M.; Moore, D. Reduction of banana weevil populations using different formulations of the entomopathogenic fungus *Beauveria bassiana*. *Biocontrol Sci. Technol.* **2000**, *10*, 645–657. [CrossRef]
168. Godonou, I.; Green, K.R.; Oduro, K.A.; Lomer, C.J.; Afreh-Nuamah, K. Field evaluation of selected formulations of *Beauveria bassiana* for the management of the banana weevil (*Cosmopolites sordidus*) on plantain (*Musa* spp., AAB group). *Biocontrol Sci. Technol.* **2000**, *10*, 779–788. [CrossRef]
169. Bustillo, A.E.; Bernal, M.G.; Benavides, P.; Chaves, B. Dynamics of *Beauveria bassiana* and *Metarhizium anisopliae* infecting *Hypothenemus hampei* (Coleoptera: Scolytidae) populations emerging from fallen coffee berries. *Fla. Entomol.* **1999**, *82*, 491–498. [CrossRef]
170. Malik, M.A.; Ahmad, S.J.N.; Ahmad, J.N.; Abbasi, A.; Sufyan, M.; Arif, M.J. Efficacy of *Bacillus thuringiensis* and *Beauveria bassiana* against red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *Afr. Entomol.* **2019**, *27*, 386–394. [CrossRef]

171. Saleh, M.M.E.; Hegazy, G.; Salem, M.; Hanounik, S.B.; Al Mohanna, O.; Alheji, M.A. Persistence of *Steinernema carpocapsae* (Nematoda: Steinernematidae) and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soil around date palm trunks and their effect on adults of *Rhynchophorus ferrugineus*. *Egypt J. Biol. Pest Control* **2004**, *14*, 141–145.
172. Wakil, W.; Yasin, M.; Shapiro-Ilan, D.I. Effects of single and combined applications of entomopathogenic fungi and nematodes against *Rhynchophorus ferrugineus* (Olivier). *Sci. Rep.* **2017**, *7*, 5971. [CrossRef]
173. Tinzaara, W.; Gold, C.S.; Dicke, M.; Van Huis, A.; Nankinga, C.M.; Kagezi, G.H.; Ragama, P.E. The use of aggregation pheromone to enhance dissemination of *Beauveria bassiana* for the control of the banana weevil in Uganda. *Biocontrol Sci. Technol.* **2007**, *17*, 111–124. [CrossRef]
174. Lopes, R.B.; Laumann, R.A.; Moore, D.; Oliveira, M.W.M.; Faria, M. Combination of the fungus *Beauveria bassiana* and pheromone in an attract-and-kill strategy against the banana weevil, *Cosmopolites sordidus*. *Entomol. Exp. Appl.* **2014**, *151*, 75–85. [CrossRef]
175. Tinzaara, W.; Emudong, P.; Nankinga, C.; Tushemereirwe, W.; Kagezi, G.; Gold, C.S.; Dicke, M.; Van Huis, A.; Karamura, E. Enhancing dissemination of *Beauveria bassiana* with host plant base incision trap for the management of the banana weevil *Cosmopolites sordidus*. *Afr. J. Agric. Res.* **2015**, *10*, 3878–3884. [CrossRef]
176. Yasuda, K. Auto-infection system for the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Curculionidae) with entomopathogenic fungi, *Beauveria bassiana* using a modified sex pheromone trap in the field. *Appl. Entomol. Zool.* **1999**, *34*, 501–505. [CrossRef]
177. El-Sufty, R.; Al Bgham, S.; Al-Awash, S.; Shahdad, A.; Al Bathra, A. A Trap for auto-dissemination of the entomopathogenic fungus *Beauveria bassiana* by red palm weevil adults in date palm plantations. *Egypt J. Biol. Pest Control* **2011**, *21*, 271–276.
178. Sewify, G.H.; Belal, M.H.; Saeed, M.Q. Using pheromone mass-trapping and the entomopathogenic fungus *Beauveria bassiana* in IPM programs for controlling the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Rhynchophoridae). *Egypt J. Biol. Pest Control* **2014**, *24*, 197–202.
179. Dembilio, Ó.; Moya, P.; Vacas, S.; Ortega-Garcia, L.; Quesada-Moraga, E.; Jaques, J.A.; Navarro-Llopis, V. Development of an attract-and-infect system to control *Rhynchophorus ferrugineus* with the entomopathogenic fungus *Beauveria bassiana*. *Pest. Manag. Sci.* **2018**, *74*, 1861–1869. [CrossRef]
180. El-Sufty, R.; Al Bgham, S.; Al-Awash, S.; Shahdad, A.; Al Bathra, A. A study on a trap for autodissemination of the entomopathogenic fungus *Beauveria bassiana* by red palm weevil adults in date palm plantations. *J. Basic. Appl. Mycol.* **2010**, *1*, 61–65.
181. Landolt, P.J. Sex attractant and aggregation pheromones of male phytophagous insects. *Am. Entomol.* **1997**, *43*, 12–22. [CrossRef]
182. Uemura-Lima, D.H.; Ventura, M.U.; Mikami, A.Y.; da Silva, F.C.; Morales, L. Responses of coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), to vertical distribution of methanol:ethanol traps. *Neotrop. Entomol.* **2010**, *39*, 930–933. [CrossRef]
183. Pereira, A.E.; Vilela, E.F.; Tinoco, R.S.; de Lima, J.O.G.; Fantine, A.K.; Morais, E.G.F.; França, C.F.M. Correlation between numbers captured and infestation levels of the coffee berry-borer, *Hypothenemus hampei*: A preliminary basis for an action threshold using baited traps. *Int. J. Pest Manag.* **2012**, *58*, 183–190. [CrossRef]
184. Sewify, G.H.; Belal, M.H.; Qaed, M.S. Food-baited aggregation pheromone traps for management of the red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *Egypt J. Biol. Pest Control* **2014**, *24*, 431–436.
185. Vacas, S.; Abad-Payá, M.; Primo, J.; Navarro-Llopis, V. Identification of pheromone synergists for *Rhynchophorus ferrugineus* trapping systems from *Phoenix canariensis* palm volatiles. *J. Agric. Food Chem.* **2014**, *62*, 6053–6064. [CrossRef]
186. Dotaona, R.; Wilson, B.A.L.; Stevens, M.M.; Holloway, J.; Ash, G.J. Chronic effects and horizontal transmission of *Metarhizium anisopliae* strain QS155 infection in the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Biol. Control* **2017**, *114*, 24–29. [CrossRef]
187. Schoeman, P.S.; Schoeman, M.H. Transmission of *Beauveria bassiana* from infected to uninfected adults of the banana weevil *Cosmopolites sordidus* (Coleoptera: Curculionidae). *Afr. Plant Prot.* **1999**, *5*, 53–54.
188. Llácer, E.; Santiago-Álvarez, C.; Jacas, J.A. Could sterile males be used to vector a microbiological control agent? The case of *Rhynchophorus ferrugineus* and *Beauveria bassiana*. *Bull. Entomol. Res.* **2013**, *103*, 241–250. [CrossRef]

189. Bojke, A.; Tkaczuk, C.; Stepnowski, P.; Golebiowski, M. Comparison of volatile compounds released by entomopathogenic fungi. *Microbiol. Res.* **2018**, *214*, 129–136. [CrossRef]
190. Herrera, J.M.; Pizzolitto, R.P.; Zunino, M.P.; Dambolena, J.S.; Zygadlo, J.A. Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize. *J. Stored Prod. Res.* **2015**, *62*, 74–80. [CrossRef]
191. Dotaona, R.; Wilson, B.A.L.; Ash, G.J.; Holloway, J.; Stevens, M.M. Sweetpotato weevil, *Cylas formicarius* (Fab.) (Coleoptera: Brentidae) avoids its host plant when a virulent *Metarhizium anisopliae* isolate is present. *J. Invertebr. Pathol.* **2017**, *148*, 67–72. [CrossRef]
192. Leng, P.H.; Reddy, G.V.P. Bioactivity of selected eco-friendly pesticides against *Cylas formicarius* (Coleoptera: Brentidae). *Fla. Entomol.* **2012**, *95*, 1040–1047. [CrossRef]
193. Akello, J.; Dubois, T.; Coyne, D.; Kyamanywa, S. Effect of endophytic *Beauveria bassiana* on populations of the banana weevil, *Cosmopolites sordidus*, and their damage in tissue-cultured banana plants. *Entomol. Exp. Appl.* **2008**, *129*, 157–165. [CrossRef]
194. Akello, J.; Dubois, T.; Coyne, D.; Kyamanywa, S. Endophytic *Beauveria bassiana* in banana (*Musa* spp.) reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. *Crop Prot.* **2008**, *27*, 1437–1441. [CrossRef]
195. Arab, Y.A.; El-Deeb, H.M. The use of endophyte *Beauveria bassiana* for bio-protection of date palm seedlings against red palm weevil and rhizoctonia root-rot disease. *Sci. J. King Faisal Univ.* **2012**, *13*, 91–101.
196. Villacarlos, L.T.; Granados-Polo, M.F.U. Potential of *Metarhizium anisopliae* for the control of the sweetpotato weevil, *Cylas formicarius* (F.) (Curculionidae: Coleoptera). *Philipp. J. Crop Sci.* **1989**, *14*, 109–114.
197. El Kichaoui, A.Y.; Abu Asaker, B.A.; El-Hindi, M.W. Isolation, molecular identification and under lab evaluation of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* against the red palm weevil *R. ferrugineus* in Gaza Strip. *Adv. Microbiol.* **2017**, *7*, 109–124. [CrossRef]
198. McPhie, D.; Burrack, H.J. Effects of microbial, organically acceptable, and reduced risk insecticides on *Anthonomus signatus* (Curculionidae: Coleoptera) in strawberries (*Fragaria* x *ananassa*). *Crop Prot.* **2016**, *89*, 255–258. [CrossRef]
199. Irulandi, S.; Aiyanathan, K.E.A.; Bhuvaneswari, S.S.B. Assessment of biopesticides and insecticide against pseudostem weevil *Odoiporus longicollis* Oliver in red banana. *J. Biopestic.* **2012**, *5*, 68–71.
200. Reddy, G.V.P.; Zhao, Z.H.; Humber, R.A. Laboratory and field efficacy of entomopathogenic fungi for the management of the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *J. Invertebr. Pathol.* **2014**, *122*, 10–15. [CrossRef]
201. Saleem, M.A.; Qayyum, M.A.; Ali, M.; Amin, M.; Tayyab, M.; Maqsood, S. Effect of sub-lethal doses of *Beauveria bassiana* and nitenpyram on the development of red palm weevil, *Rhynchophorus ferrugineus* (Olivier). *Pak. J. Zool.* **2019**, *51*, 559–565. [CrossRef]
202. Malik, M.A.; Manzoor, M.; Ali, H.; Muhammad, A.; ul Islam, S.; Qasim, M.; Ahmad, N.; Idrees, A.; Muhammad, A.; Saqib, H.S.A. Evaluation of imidacloprid and entomopathogenic fungi, *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). *J. Entomol. Zool. Stud.* **2016**, *4*, 262–268.
203. Alston, D.G.; Rangel, D.E.N.; Lacey, L.A.; Golez, H.G.; Kim, J.J.; Roberts, D.W. Evaluation of novel fungal and nematode isolates for control of *Conotrachelus nenuphar* (Coleoptera: Curculionidae) larvae. *Biol. Control* **2005**, *35*, 163–171. [CrossRef]
204. Harrison, R.D.; Gardner, W.A.; Kinard, D.J. Relative susceptibility of pecan weevil fourth instars and adults to selected isolates of *Beauveria bassiana*. *Biol. Control* **1993**, *3*, 34–38. [CrossRef]
205. Cheng, Y.Q.; Liu, T.; Zhao, Y.X.; Geng, W.T.; Chen, L.T.; Liu, J.F. Evaluation of pathogenicity of the fungi *Metarhizium anisopliae* and *Beauveria bassiana* in hazelnut weevil (*Curculio nucum* L., Coleoptera, Curculionidae) larvae. *Indian J. Microbiol.* **2016**, *56*, 405–410. [CrossRef] [PubMed]
206. Ihara, F.; Toyama, M.; Sato, T. Pathogenicity of *Metarhizium anisopliae* to the chestnut weevil larvae under laboratory and field conditions. *Appl. Entomol. Zool.* **2003**, *38*, 461–465. [CrossRef]
207. Ihara, F.; Toyama, M.; Higaki, M.; Mishwo, K.; Yaginuma, K. Comparison of pathogenicities of *Beauveria bassiana* and *Metarhizium anisopliae* to chestnut pests. *Appl. Entomol. Zool.* **2009**, *44*, 127–132. [CrossRef]
208. Tedders, W.L.; Weaver, D.J.; Wehunt, E.J. Pecan weevil: Suppression of larvae with the fungi *Metarhizium anisopliae* and *Beauveria bassiana* and the nematode *Neoaplectana dutkyi*. *J. Econ. Entomol.* **1973**, *66*, 723–725. [CrossRef]

209. Tedders, W.L.; Weaver, D.J.; Wehunt, E.J.; Gentry, C.R. Bioassay of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Neoaplectana carpocapsae* against larvae of the plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae). *Environ. Entomol.* **1982**, *11*, 901–904. [CrossRef]
210. Batalla-Carrera, L.; Morton, A.; Santamaria, S.; Garcia-del-Pino, F. Isolation and virulence of entomopathogenic fungi against larvae of hazelnut weevil *Curculio nucum* (Coleoptera, Curculionidae) and the effects of combining *Metarhizium anisopliae* with entomopathogenic nematodes in the laboratory. *Biocontrol Sci. Technol.* **2013**, *23*, 101–125. [CrossRef]
211. Champlin, F.R.; Cheung, P.Y.K.; Pekrul, S.; Smith, R.J.; Burton, R.L.; Grula, E.A. Virulence of *Beauveria bassiana* mutants for the pecan weevil. *J. Econ. Entomol.* **1981**, *74*, 617–621. [CrossRef]
212. Gottwald, T.R.; Tedders, W.L. Colonization, transmission, and longevity of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina: Hypomycetes) on pecan weevil larvae (Coleoptera: Curculionidae) in the soil. *Environ. Entomol.* **1984**, *13*, 557–560. [CrossRef]
213. Torrini, G.; Benvenuti, C.; Binazzi, F.; Marianelli, L.; Paoli, F.; Peverieri, G.S.; Roversi, P.F. Entomopathogenic fungi and nematodes against larvae of the chestnut weevil, *Curculio elephas* (Coleoptera: Curculionidae): A laboratory evaluation. *Int. J. Pest Manag.* **2018**, *64*, 287–293. [CrossRef]
214. Shapiro-Ilan, D.I.; Gardner, W.A.; Fuxa, J.R.; Wood, B.W.; Nguyen, K.B.; Adams, B.J.; Humber, R.A.; Hall, M.J. Survey of entomopathogenic nematodes and fungi endemic to pecan orchards of the Southeastern United States and their virulence to the pecan weevil (Coleoptera: Curculionidae). *Environ. Entomol.* **2003**, *32*, 187–195. [CrossRef]
215. Shapiro-Ilan, D.I.; Brown, I. Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control. *Biol. Control* **2013**, *66*, 41–48. [CrossRef]
216. Gottwald, T.R.; Tedders, W.L. Suppression of pecan weevil (Coleoptera: Curculionidae) populations with entomopathogenic fungi. *Environ. Entomol.* **1983**, *12*, 471–474. [CrossRef]
217. Shapiro-Ilan, D.I.; Cottrell, T.E.; Gardner, W.A.; Leland, J.; Behles, R.W. Laboratory mortality and mycosis of adult *Curculio caryae* (Coleoptera: Curculionidae) following application of *Metarhizium anisopliae* in the laboratory or field. *J. Entomol. Sci.* **2009**, *44*, 24–36. [CrossRef]
218. Shapiro-Ilan, D.I.; Gardner, W.A.; Wells, L.; Wood, B.W. Cumulative impact of a clover cover crop on the persistence and efficacy of *Beauveria bassiana* in suppressing the pecan weevil (Coleoptera: Curculionidae). *Environ. Entomol.* **2012**, *41*, 298–307. [CrossRef] [PubMed]
219. Sarraquigne, J.P.; Couturié, E.; Fernandez, M.M. Integrated control of hazelnut weevil (*Curculio nucum*): An evaluation of entomopathogenic nematodes and parasitic fungi. *Acta Hortic.* **2009**, *845*, 555–560. [CrossRef]
220. Pereault, R.J.; Whalon, M.E.; Alston, D.G. Field efficacy of entomopathogenic fungi and nematodes targeting caged last-instar plum curculio (Coleoptera: Curculionidae) in Michigan cherry and apple orchards. *Environ. Entomol.* **2009**, *38*, 1126–1134. [CrossRef]
221. Shapiro-Ilan, D.I.; Cottrell, T.E.; Gardner, W.A. Trunk perimeter applications of *Beauveria bassiana* to suppress adult *Curculio caryae* (Coleoptera: Curculionidae). *J. Entomol. Sci.* **2004**, *39*, 337–349. [CrossRef]
222. Shapiro-Ilan, D.I.; Mizell, R.F. An insect pupal cell with antimicrobial properties that suppress an entomopathogenic fungus. *J. Invertebr. Pathol.* **2015**, *124*, 114–116. [CrossRef]
223. Shapiro-Ilan, D.I.; Gardner, W.A.; Cottrell, T.E.; Behle, R.W.; Wood, B.W. Comparison of application methods for suppressing the pecan weevil (Coleoptera: Curculionidae) with *Beauveria bassiana* under field conditions. *Environ. Entomol.* **2008**, *37*, 162–171. [CrossRef]
224. Shapiro-Ilan, D.I.; Cottrell, T.E.; Gardner, W.A.; Behle, R.W.; Ree, B.; Harris, M.K. Efficacy of entomopathogenic fungi in suppressing pecan weevil, *Curculio caryae* (Coleoptera: Curculionidae), in commercial pecan orchards. *Southwest Entomol.* **2009**, *34*, 111–120. [CrossRef]
225. Shapiro-Ilan, D.I.; Gardner, W.A.; Wells, L.; Cottrell, T.E.; Behle, R.W.; Wood, B.W. Effects of entomopathogenic fungus species, and impact of fertilizers, on biological control of pecan weevil (Coleoptera: Curculionidae). *Environ. Entomol.* **2013**, *42*, 253–261. [CrossRef] [PubMed]
226. Shapiro-Ilan, D.I.; Jackson, M.; Reilly, C.C.; Hotchkiss, M.W. Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biol. Control* **2004**, *30*, 119–126. [CrossRef]

227. Asan, C.; Hazir, S.; Cimen, H.; Ulug, D.; Taylor, J.; Butt, T.; Karagoz, M. An innovative strategy for control of the chestnut weevil *Curculio elephas* (Coleoptera: Curculionidae) using *Metarhizium brunneum*. *Crop Prot.* **2017**, *102*, 147–153. [CrossRef]
228. Shapiro-Ilan, D.I.; Cottrell, T.E.; Bock, C.; Mai, K.; Boykin, D.; Wells, L.; Hudson, W.G.; Mizell, R.F. Control of pecan weevil with microbial biopesticides. *Environ. Entomol.* **2017**, *46*, 1299–1304. [CrossRef]
229. Shapiro-Ilan, D.I.; Cottrell, T.E.; Wood, B.W. Effects of combining microbial and chemical insecticides on mortality of the pecan weevil (Coleoptera: Curculionidae). *J. Econ. Entomol.* **2011**, *104*, 14–20. [CrossRef]
230. Brito, E.S.; de Paula, A.R.; Vieira, L.P.; Dolinski, C.; Samuels, R.I. Combining vegetable oil and sub-lethal concentrations of imidacloprid with *Beauveria bassiana* and *Metarhizium anisopliae* against adult guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae). *Biocontrol Sci. Technol.* **2008**, *18*, 665–673. [CrossRef]
231. Sepulveda, M.; Vargas, M.; Gerding, M.; Ceballos, R.; Oyarzua, P. Molecular, morphological and pathogenic characterization of six strains of *Metarhizium* spp. (Deuteromycotina: Hyphomycetes) for the control of *Aegorhinus superciliosus* (Coleoptera: Curculionidae). *Chil. J. Agric. Res.* **2016**, *76*, 77–83. [CrossRef]
232. McCoy, C.W.; Boucias, D.G. Selection of *Beauveria bassiana* pathotypes as potential microbial control agents of soil-inhabiting citrus weevils. *Mem. Inst. Oswaldo Cruz* **1989**, *84*, 75–80. [CrossRef]
233. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. Laboratory selection of *Metarhizium* spp. isolates for control of vine weevil larvae (*Otiorhynchus sulcatus*). *J. Invertebr. Pathol.* **1993**, *62*, 15–21. [CrossRef]
234. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. The influence of temperature on the susceptibility of vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), larvae to *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Ann. Appl. Biol.* **1994**, *124*, 185–193. [CrossRef]
235. Bruck, D.J. Natural occurrence of entomopathogens in pacific northwest nursery soils and their virulence to the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae). *Environ. Entomol.* **2004**, *33*, 1335–1343. [CrossRef]
236. Hirsch, J.; Reineke, A. Efficiency of commercial entomopathogenic fungal species against different members of the genus *Otiorhynchus* (Coleoptera: Curculionidae) under laboratory and semi-field conditions. *J. Plant Dis. Prot.* **2014**, *121*, 211–218. [CrossRef]
237. Klingen, I.; Westrum, K.; Meyling, N.V. Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres. *Biol. Control* **2015**, *81*, 1–7. [CrossRef]
238. Pope, T.W.; Hough, G.; Arbona, C.; Roberts, H.; Bennison, J.; Buxton, J.; Prince, G.; Chandler, D. Investigating the potential of an autodissemination system for managing populations of vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic fungi. *J. Invertebr. Pathol.* **2018**, *154*, 79–84. [CrossRef] [PubMed]
239. Morera-Margarit, P.; Karley, A.J.; Mitchell, C.; Graham, R.I.; Pope, T.W. Geographic origin may not influence vine weevil *Otiorhynchus sulcatus* (Fabricius) susceptibility to the entomopathogenic fungus *Metarhizium brunneum* (Petch). *Biocontrol Sci. Technol.* **2020**. [CrossRef]
240. Poprawski, T.J.; Marchal, M.; Robert, P.H. Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic Hyphomycetes. *Environ. Entomol.* **1985**, *14*, 247–253. [CrossRef]
241. Soares Jr, G.G.; Marchal, M.; Ferron, P. Susceptibility of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) larvae to *Metarhizium anisopliae* and *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) at two different temperatures. *Environ. Entomol.* **1983**, *12*, 1887–1891. [CrossRef]
242. Dlamini, B.E.; Malan, A.P.; Addison, P. Control of the banded fruit weevil, *Phlyctinus callosus* (Schonherr) (Coleoptera: Curculionidae), using entomopathogenic fungi. *Afr. Entomol.* **2020**, *28*, 106–114. [CrossRef]
243. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. Effect of potting media on the control of *Otiorhynchus sulcatus* larvae on outdoor strawberry plants using the entomogenous fungus *Metarhizium anisopliae*. *Biol. Control* **1992**, *2*, 238–243. [CrossRef]
244. Moorhouse, E.R.; Easterbrook, M.A.; Gillespie, A.T.; Charnley, A.K. Control of *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) larvae on a range of hardy ornamental nursery stock species using the entomogenous fungus *Metarhizium anisopliae*. *Biocontrol Sci. Technol.* **1993**, *3*, 63–72. [CrossRef]
245. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. Application of *Metarhizium anisopliae* (Metsch.) Sor. conidia to control *Otiorhynchus sulcatus* (F) (Coleoptera: Curculionidae) larvae on glasshouse pot plants. *Ann. Appl. Biol.* **1993**, *122*, 623–636. [CrossRef]

246. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. The development of *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) larvae on a range of ornamental pot-plant species and the potential for control using *Metarhizium anisopliae*. *J. Hortic. Sci.* **1993**, *68*, 627–635. [CrossRef]
247. Moorhouse, E.R.; Gillespie, A.T.; Charnley, A.K. Selection of virulent and persistent *Metarhizium anisopliae* isolates to control black vine weevil (*Otiorhynchus sulcatus*) larvae on glasshouse Begonia. *J. Invertebr. Pathol.* **1993**, *62*, 47–52. [CrossRef]
248. Bruck, D.J. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: Implications for pest management. *Biol. Control* **2005**, *32*, 155–163. [CrossRef]
249. Bruck, D.J.; Donahue, K.M. Persistence of *Metarhizium anisopliae* incorporated into soilless potting media for control of the black vine weevil, *Otiorhynchus sulcatus* in container-grown ornamentals. *J. Invertebr. Pathol.* **2007**, *95*, 146–150. [CrossRef] [PubMed]
250. Noble, R.; Dobrovin-Pennington, A.; Fitzgerald, J.; Dew, K.; Wilson, C.; Ross, K.; Perkins, C. Improving biocontrol of black vine weevil (*Otiorhynchus sulcatus*) with entomopathogenic fungi in growing media by incorporating spent mushroom compost. *Biocontrol* **2018**, *63*, 697–706. [CrossRef]
251. Cross, J.V.; Burgess, C.M. Localised insecticide treatment for the control of vine weevil larvae (*Otiorhynchus sulcatus*) on field-grown strawberry. *Crop Prot.* **1997**, *16*, 565–574. [CrossRef]
252. Oddsdottir, E.S.; Eilenberg, J.; Sen, R.; Halldorsson, G. The effects of insect pathogenic soil fungi and ectomycorrhizal inoculation of birch seedlings on the survival of *Otiorhynchus* larvae. *Agric. For. Entomol.* **2010**, *12*, 319–324. [CrossRef]
253. Vainio, A.; Hokkanen, H.M.T. The potential of entomopathogenic fungi and nematodes against *Otiorhynchus ovatus* L. and *O. dubius* Ström (Col., Curculionidae) in the field. *J. Appl. Entomol.* **1993**, *115*, 379–387. [CrossRef]
254. Bruck, D.J. Effect of potting media components on the infectivity of *Metarhizium anisopliae* against the black vine weevil (Coleoptera: Curculionidae). *J. Environ. Hortic.* **2006**, *24*, 91–94. [CrossRef]
255. Bruck, D.J. Efficacy of *Metarhizium anisopliae* as a curative application for black vine weevil (*Otiorhynchus sulcatus*) infesting container-grown nursery crops. *J. Environ. Hortic.* **2007**, *25*, 150–156. [CrossRef]
256. Booth, S.R.; Shanks Jr, C.H. Potential of a dried rice/mycelium formulation of entomopathogenic fungi to suppress subterranean pests in small fruits. *Biocontrol Sci. Technol.* **1998**, *8*, 197–206. [CrossRef]
257. Booth, S.R.; Tanigoshi, L.; Dewes, I. Potential of a dried mycelium formulation of an indigenous strain of *Metarhizium anisopliae* against subterranean pests of cranberry. *Biocontrol Sci. Technol.* **2000**, *10*, 659–668. [CrossRef]
258. Easter-brook, M.A.; Cantwell, M.P.; Chandler, D. Control of the black vine weevil, *Otiorhynchus sulcatus*, with the fungus *Metarhizium anisopliae*. *Phytoparasitica* **1992**, *20*, S17–S19. [CrossRef]
259. Roberts, J.M.; Jahir, A.; Graham, J.; Pope, T.W. Catch me if you can: The influence of refuge/trap design, previous feeding experience, and semiochemical lures on vine weevil (Coleoptera: Curculionidae) monitoring success. *Pest Manag. Sci.* **2019**, *76*, 553–560. [CrossRef]
260. van Tol, R.W.H.M.; Elberse, I.A.M.; Bruck, D.J. Development of a refuge-kairomone device for monitoring and control of the vine weevil, *Otiorhynchus sulcatus*, by lure-and-kill and lure-and-infect. *Crop Prot.* **2020**, *129*, 105045. [CrossRef]
261. Rondot, Y.; Reineke, A. Association of *Beauveria bassiana* with grapevine plants deters adult black vine weevils, *Otiorhynchus sulcatus*. *Biocontrol Sci. Technol.* **2017**, *27*, 811–820. [CrossRef]
262. Kepler, R.M.; Bruck, D.J. Examination of the interaction between the black vine weevil (Coleoptera: Curculionidae) and an entomopathogenic fungus reveals a new tritrophic interaction. *Environ. Entomol.* **2006**, *35*, 1021–1029. [CrossRef]
263. Ansari, M.A.; Shah, F.A.; Butt, T.M. Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus*, control. *Entomol. Exp. Appl.* **2008**, *129*, 340–347. [CrossRef]
264. Ansari, M.A.; Shah, F.A.; Butt, T.M. The entomopathogenic nematode *Steinernema kraussei* and *Metarhizium anisopliae* work synergistically in controlling overwintering larvae of the black vine weevil, *Otiorhynchus sulcatus*, in strawberry growbags. *Biocontrol Sci. Technol.* **2010**, *20*, 99–105. [CrossRef]
265. Ansari, M.A.; Butt, T.M. Influence of the application methods and doses on the susceptibility of black vine weevil larvae *Otiorhynchus sulcatus* to *Metarhizium anisopliae* in field-grown strawberries. *Biocontrol* **2013**, *58*, 257–267. [CrossRef]

266. Quintela, E.D.; McCoy, C.W. Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environ. Entomol.* **1997**, *26*, 1173–1182. [CrossRef]
267. Quintela, E.D.; McCoy, C.W. Conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) treated with imidacloprid. *J. Invertebr. Pathol.* **1998**, *72*, 220–230. [CrossRef] [PubMed]
268. Quintela, E.D.; McCoy, C.W. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *J. Econ. Entomol.* **1998**, *91*, 110–122. [CrossRef]
269. Shah, F.A.; Ansari, M.A.; Prasad, M.; Butt, T.M. Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. *Biol. Control* **2007**, *40*, 246–252. [CrossRef]
270. Shah, F.A.; Gaffney, M.; Ansari, M.A.; Prasad, M.; Butt, T.M. Neem seed cake enhances the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for the control of black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Biol. Control* **2008**, *44*, 111–115. [CrossRef]
271. Gillett-Kaufman, J.L.; Kimbrough, J.W. A modified method to visualize infection sites of spores of the entomopathogen *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) on the exoskeleton of citrus root weevil *Diaprepes abbreviatus* (Coleoptera: Curculionidae) adults. *Fla. Entomol.* **2009**, *92*, 623–628.
272. Jaber, L.R.; Ownley, B.H. Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control* **2018**, *116*, 36–45. [CrossRef]
273. Bamisile, B.S.; Dash, C.K.; Akutse, K.S.; Keppanan, R.; Wang, L.D. Fungal endophytes: Beyond herbivore management. *Front. Microbiol.* **2018**, *9*, 544. [CrossRef]
274. Ramakuwela, T.; Hatting, J.; Bock, C.; Vega, F.E.; Wells, L.; Mbata, G.N.; Shapiro-Ilan, D.I. Establishment of *Beauveria bassiana* as a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests. *Biol. Control* **2020**, *140*, 104102. [CrossRef]
275. Posada, F.; Vega, F.E. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia* **2005**, *97*, 1195–1200. [CrossRef] [PubMed]
276. Posada, F.; Aime, M.C.; Peterson, S.W.; Rehner, S.A.; Vega, F.E. Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycol. Res.* **2007**, *111*, 748–757. [CrossRef] [PubMed]
277. Gómez-Vidal, S.; Lopez-Llorca, L.V.; Jansson, H.B.; Salinas, J. Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. *Micron* **2006**, *37*, 624–632. [CrossRef]
278. Bamisile, B.S.; Dash, C.K.; Akutse, K.S.; Qasim, M.; Aguila, L.C.R.; Wang, F.F.; Keppanan, R.; Wang, L.D. Endophytic *Beauveria bassiana* in foliar-treated *Citrus limon* plants acting as a growth suppressor to three successive generations of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). *Insects* **2019**, *10*, 176. [CrossRef]
279. Rondot, Y.; Reineke, A. Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects. *Biol. Control* **2018**, *116*, 82–89. [CrossRef]
280. Bing, L.A.; Lewis, L.C. Occurrence of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimes and in *Zea mays* L and virulence towards *Ostrinia nubilalis* (Hubner). *Agric. Ecosyst. Environ.* **1993**, *45*, 147–156. [CrossRef]
281. Oliveira, I.; Pereira, J.A.; Quesada-Moraga, E.; Lino-Neto, T.; Bento, A.; Baptista, P. Effect of soil tillage on natural occurrence of fungal entomopathogens associated to *Prays oleae* Bern. *Sci. Hortic.* **2013**, *159*, 190–196. [CrossRef]
282. Milner, R.J.; Samson, P.; Morton, R. Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: Effect of isolate and formulation on persistence over 3.5 years. *Biocontrol Sci. Technol.* **2003**, *13*, 507–516. [CrossRef]
283. Mayerhofer, J.; Enkerli, J.; Zelger, R.; Strasser, H. Biological control of the European cockchafer: Persistence of *Beauveria brongniartii* after long-term applications in the Euroregion Tyrol. *Biocontrol* **2015**, *60*, 617–629. [CrossRef]
284. Swiergiel, W.; Meyling, N.V.; Porcel, M.; Rämert, B. Soil application of *Beauveria bassiana* GHA against apple sawfly, *Hoplocampa testudinea* (Hymenoptera: Tenthredinidae): Field mortality and fungal persistence. *Insect Sci.* **2016**, *23*, 854–868. [CrossRef] [PubMed]

285. Coombes, C.A.; Hill, M.P.; Moore, S.D.; Dames, J.F. Entomopathogenic fungi as control agents of *Thaumatotibia leucotreta* in citrus orchards: Field efficacy and persistence. *Biocontrol* **2016**, *61*, 729–739. [CrossRef]
286. Baxter, I.H.; Howard, N.; Armsworth, C.G.; Barton, L.E.E.; Jackson, C. The potential of two electrostatic powders as the basis for an auto dissemination control method of *Plodia interpunctella* (Hubner). *J. Stored Prod. Res.* **2008**, *44*, 152–161. [CrossRef]
287. Athanassiou, C.G.; Vassilakos, T.N.; Dutton, A.C.; Jessop, N.; Sherwood, D.; Pease, G.; Brglez, A.; Storm, C.; Trdan, S. Combining electrostatic powder with an insecticide: Effect on stored-product beetles and on the commodity. *Pest Manag. Sci.* **2016**, *72*, 2208–2217. [CrossRef] [PubMed]
288. Athanassiou, C.G.; Rumbos, C.I.; Sakka, M.; Potin, O.; Storm, C.; Dillon, A.B. Delivering *Beauveria bassiana* with electrostatic powder for the control of stored-product beetles. *Pest Manag. Sci.* **2017**, *73*, 1725–1736. [CrossRef] [PubMed]
289. Meikle, W.G.; Mercadier, G.; Holst, N.; Nansen, C.; Girod, V. Duration and spread of an entomopathogenic fungus, *Beauveria bassiana* (Deuteromycota: Hyphomycetes), used to treat varroa mites (Acari: Varroidae) in honey bee (Hymenoptera: Apidae) hives. *J. Econ. Entomol.* **2007**, *100*, 1–10. [CrossRef]
290. Andriessen, R.; Snetselaar, J.; Suer, R.A.; Osinga, A.J.; Deschietere, J.; Lyimo, I.N.; Mnyone, L.L.; Brooke, B.D.; Ranson, H.; Knols, B.G.J.; et al. Electrostatic coating enhances bioavailability of insecticides and breaks pyrethroid resistance in mosquitoes. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 12081–12086. [CrossRef]
291. Mazza, G.; Inghilesi, A.F.; Stasolla, G.; Cini, A.; Cervo, R.; Benvenuti, C.; Francardi, V.; Cristofaro, M.; Arnone, S.; Roversi, P.F. Sterile *Rhynchophorus ferrugineus* males efficiently impair reproduction while maintaining their sexual competitiveness in a social context. *J. Pest Sci.* **2016**, *89*, 459–468. [CrossRef]
292. Sookar, P.; Alleck, M.; Ahseek, N.; Bhagwant, S. Sterile male peach fruit flies, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), as a potential vector of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in a SIT programme. *Afr. Entomol.* **2014**, *22*, 488–498. [CrossRef]
293. Jaronski, S.T. Ecological factors in the inundative use of fungal entomopathogens. *Biocontrol* **2010**, *55*, 159–185. [CrossRef]
294. Hedimbi, M.; Kaaya, G.P.; Singh, S.; Chimwamurombe, P.M.; Gindin, G.; Glazer, I.; Samish, M. Protection of *Metarhizium anisopliae* conidia from ultra-violet radiation and their pathogenicity to *Rhipicephalus evertsi evertsi* ticks. *Exp. Appl. Acarol.* **2008**, *46*, 149–156. [CrossRef] [PubMed]
295. de Oliveira, D.G.P.; Lopes, R.B.; Rezende, J.M.; Delalibera, I. Increased tolerance of *Beauveria bassiana* and *Metarhizium anisopliae* conidia to high temperature provided by oil-based formulations. *J. Invertebr. Pathol.* **2018**, *151*, 151–157. [CrossRef]
296. Rangel, D.E.N.; Braga, G.U.L.; Anderson, A.J.; Roberts, D.W. Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *J. Invertebr. Pathol.* **2005**, *88*, 116–125. [CrossRef] [PubMed]
297. Fernandes, È.K.K.; Rangel, D.E.N.; Moraes, Á.M.L.; Bittencourt, V.R.E.P.; Roberts, D.W. Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. *J. Invertebr. Pathol.* **2007**, *96*, 237–243. [CrossRef] [PubMed]
298. Rangel, D.E.N.; Braga, G.U.L.; Fernandes, È.K.K.; Keyser, C.A.; Hallsworth, J.E.; Roberts, D.W. Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. *Curr. Genet.* **2015**, *61*, 383–404. [CrossRef] [PubMed]
299. Rangel, D.E.N.; Roberts, D.W. Possible source of the high UV-B and heat tolerance of *Metarhizium acridum* (isolate ARSEF 324). *J. Invertebr. Pathol.* **2018**, *157*, 32–35. [CrossRef] [PubMed]
300. Ekobu, M.; Solera, M.; Kyamanywa, S.; Mwanga, R.O.M.; Odongo, B.; Ghislain, M.; Moar, W.J. Toxicity of seven *Bacillus thuringiensis* cry proteins against *Cylas puncticollis* and *Cylas brunneus* (Coleoptera: Brentidae) using a novel artificial diet. *J. Econ. Entomol.* **2010**, *103*, 1493–1502. [CrossRef]
301. Mahmoud, S.B.; Ramos, J.E.; Shatters, R.G., Jr.; Hall, D.G.; Lapointe, S.L.; Niedz, R.P.; Rougé, P.; Cave, R.D.; Borovsky, D. Expression of *Bacillus thuringiensis* cytolytic toxin (Cyt2Ca1) in citrus roots to control *Diaprepes abbreviatus* larvae. *Pestic. Biochem. Phys.* **2017**, *136*, 1–11. [CrossRef]
302. Anbesse, S.A.; Adge, B.J.; Gebru, W.M. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. *Nematology* **2008**, *10*, 701–709.
303. Meyling, N.V.; Pell, J.K. Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecol. Entomol.* **2006**, *31*, 162–171. [CrossRef]

304. de Oliveira, F.Q.; Batista, J.D.; Malaquias, J.B.; de Brito, C.H.; Dos Santos, E.P. Susceptibility of the predator *Euborellia annulipes* (Dermaptera: Anisolabididae) to mycoinsecticides. *Rev. Colomb. Entomol.* **2011**, *37*, 234–237.
305. Cottrell, T.E.; Shapiro-Ilan, D.I. Susceptibility of endemic and exotic North American ladybirds (Coleoptera: Coccinellidae) to endemic fungal entomopathogens. *Eur. J. Entomol.* **2008**, *105*, 455–460. [CrossRef]
306. Zhu, H.; Kim, J.J. Target-oriented dissemination of *Beauveria bassiana* conidia by the predators, *Harmonia axyridis* (Coleoptera: Coccinellidae) and *Chrysoperla carnea* (Neuroptera: Chrysopidae) for biocontrol of *Myzus persicae*. *Biocontrol Sci. Technol.* **2012**, *22*, 393–406. [CrossRef]
307. Cottrell, T.E.; Shapiro-Ilan, D.I. Susceptibility of a native and an exotic lady beetle (Coleoptera: Coccinellidae) to *Beauveria bassiana*. *J. Invertebr. Pathol.* **2003**, *84*, 137–144. [CrossRef]
308. de la Rosa, W.; Segura, H.R.; Barrera, J.F.; Williams, T. Laboratory evaluation of the impact of entomopathogenic fungi on *Prorops nasuta* (Hymenoptera: Bethylidae), a parasitoid of the coffee berry borer. *Environ. Entomol.* **2000**, *29*, 126–131. [CrossRef]
309. Potrich, M.; Alves, L.F.A.; Lozano, E.; Roman, J.C.; Pietrowski, V.; Neves, P.M.O.J. Interactions between *Beauveria bassiana* and *Trichogramma pretiosum* under laboratory conditions. *Entomol. Exp. Appl.* **2015**, *154*, 213–221. [CrossRef]
310. Potrich, M.; Alves, L.F.A.; Lozano, E.R.; Bonini, A.K.; Neves, P.M.O.J. Potential side effects of the entomopathogenic fungus *Metarhizium anisopliae* on the egg parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) under controlled conditions. *J. Econ. Entomol.* **2017**, *110*, 2318–2324. [CrossRef]
311. Potrich, M.; Alves, L.F.A.; Haas, J.; da Silva, E.R.L.; Daros, A.; Pietrowski, V.; Neves, P.M.O.J. Selectivity of *Beauveria bassiana* and *Metarhizium anisopliae* to *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae). *Neotrop. Entomol.* **2009**, *38*, 822–826. [CrossRef]
312. Castillo, A.; Gómez, J.; Infante, F.; Vega, F.E. Susceptibility of the parasitoid *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae) to *Beauveria bassiana* under laboratory conditions. *Neotrop. Entomol.* **2009**, *38*, 665–670. [CrossRef]
313. De la Rosa, W.; Godinez, J.L.; Alatorre, R.; Trujillo, J. Susceptibility of the parasitoid *Cephalonomia stephanoderis* to *Beauveria bassiana* and *Metarhizium anisopliae* strains. *Southwest Entomol.* **1997**, *22*, 233–242.
314. Khun, K.K.; Ash, G.J.; Stevens, M.M.; Huwer, R.K.; Wilson, B.A.L. Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia. *Pest Manag. Sci.* **2020**. [CrossRef] [PubMed]

Chapter 3: Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson
Journal of Invertebrate Pathology 174, 107437.
https://doi.org/10.1016/j.jip.2020.107437

Note: Additional data and figure associated to this paper are included in the appendix B and C.

Journal of Invertebrate Pathology 174 (2020) 107437

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays

Kim Khuy Khun[a,b,*], Gavin J. Ash[b], Mark M. Stevens[c,d], Ruth K. Huwer[e], Bree A.L. Wilson[b]

[a] Faculty of Agronomy, Royal University of Agriculture, P.O. Box 2696, Dangkor District, Phnom Penh, Cambodia
[b] Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, Queensland 4350, Australia
[c] Yanco Agricultural Institute, NSW Department of Primary Industries, New South Wales 2703, Australia
[d] Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Australia
[e] Wollongbar Primary Industries Institute, NSW Department of Primary Industries, New South Wales 2477, Australia

ARTICLE INFO

Keywords:
Macadamia
Kuschelorhynchus
Metarhizium
Beauveria
Virulence
Pathogenicity

ABSTRACT

Macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler, is a major pest of macadamia in eastern Australia, causing yield losses of up to 15%. Current control methods involve two applications of acephate per season but more recently have moved to a single application of indoxacarb, combined with the collection and destruction of fallen nuts that contain developing larvae. As a first step towards reducing the dependence of the industry on synthetic insecticides, we tested six isolates of *M. anisopliae,* six isolates of *B. bassiana* and one commercial *B. bassiana* product (Velifer® biological insecticide) against adult macadamia seed weevil under laboratory conditions. All isolates were pathogenic against adult weevils with *M. anisopliae* accession ECS1/BRIP 70272 and *B. bassiana* accession B27/BRIP 70267 causing 97.5% and 92.5% mortality 12 days after being treated at 1×10^7 conidia/mL. Isolates ECS1/BRIP 70272 and B27/BRIP 70267 had the shortest LT_{50} values of 5.13 days and 5.37 days respectively. The median lethal concentrations (LC_{50}) for ECS1/BRIP 70272 and B27/BRIP 70267 were 1.48×10^5 and 1.65×10^5 conidia/mL respectively. Results of this study indicate that *M. anisopliae* accession ECS1/BRIP 70272 and *B. bassiana* accession B27/BRIP 70267 have considerable potential for *K. macadamiae* control, and should be developed into biological insecticides for integration into macadamia pest management programs.

1. Introduction

Macadamias (*Macadamia integrifolia* Maiden and Betche and *M. tetraphylla* L. Johnson) are the second largest nut crop grown in Australia, with 25,000 ha under cultivation and a total farm-gate value of AUD 285 million (ANIC, 2016; AMS, 2018). Australia and South Africa are the largest macadamia producers, and together are responsible for around 48% of global production (INDFC, 2018). In Australia, several important insect pests have been reported to affect macadamias, with macadamia seed weevil being regarded as the greatest threat to the industry (QDAF, 2018). Macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler (Coleoptera: Curculionidae), formerly known as '*Sigastus* weevil' (Jennings and Oberprieler, 2018), is a native Australian insect, which was initially found in macadamias on the Atherton Tablelands, Queensland in 1994 (Fay et al., 2001) and later in the Northern Rivers region of New South Wales (NSW) (Bright, 2017a, 2017c). The weevil is a major pest of macadamias at the nut setting stage (Bright, 2017a, 2017c) with the female weevil ovipositing inside the nut, inducing premature nut drop (Fay et al., 2001). This premature nut drop has been estimated to lead to crop losses of around 15% (Huwer, 2016). Adults feed on young leaves and can completely remove the bark from seedlings, sometimes killing young plants within a few days (Kim Khuy Khun, personal observations).

The life cycle of the macadamia seed weevil from egg to adult emergence takes around 40 days at 25 °C (Bright, 2017a, 2017c). Adult females lay up to 280 eggs each (Bright, 2017a, 2017c), but only a few eggs are laid each day (Fay et al., 2001). Eggs are laid singly inside individual nuts when they are about 10 mm in diameter, in the tissue between the shell and the husk of the fruit (Fay et al., 2001). The eggs hatch in 6 days under typical ambient temperature and the larvae develop inside the nuts, feeding on the kernel. The larval stage lasts 4

* Corresponding author at: Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, QLD 4350, Australia.
E-mail address: KimKhuy.Khun@usq.edu.au (K.K. Khun).

https://doi.org/10.1016/j.jip.2020.107437
Received 27 September 2019; Received in revised form 29 May 2020; Accepted 20 June 2020
Available online 25 June 2020

weeks, passing through four instars and adult weevils emerge after a prepupal period of 2 days and pupal period of 4 days. The weevil passes through three generations in a year, with the first and second generations in November and December and the third and overwintering generation from March to October (Bright, 2017a, 2017c).

Chemical control and orchard floor hygiene have been the basis of macadamia seed weevil management programs. The broad-spectrum organophosphate insecticide acephate is currently used (Bright, 2017b, 2017c, 2018), despite being detrimental to beneficial insects (Singh, 1984). However, more recently, the oxadiazine insecticide indoxacarb has been permitted in macadamia orchards for weevil management (APVMA, 2018).

The entomopathogenic fungi *M. anisopliae* (Metschnikoff) Sorokin and *B. bassiana* (Balsamo) Vuillemin have cosmopolitan distributions (Roberts and St. Leger, 2004; Rehner and Buckley, 2005) and have shown potential for controlling many economically important insect pests in horticultural crops (Dolinski and Lacey, 2007; Lacey and Shapiro-Ilan, 2008; Lacey et al., 2015). Whilst preliminary reports (e.g. Bright, 2018) have foreshadowed the potential of entomopathogenic fungi for macadamia seed weevil control, detailed and systematic studies had not yet been conducted, and the objective of our study was to evaluate several strains of *M. anisopliae* and *B. bassiana* against adults of the macadamia seed weevil using laboratory bioassays.

2. Materials and methods

2.1. Insects

Kuschelorhynchus macadamiae used in these experiments were reared directly from infested nuts. Fallen nuts were collected at 2 week intervals (between October and December 2018) from a macadamia block at the NSW Department of Primary Industries Centre for Tropical Horticulture at Alstonville (28°51′12″S 153°27′37″E) and from an additional orchard (28°48′27″S 153°25′23″E) in the Northern Rivers region, NSW. The infested nuts were stored in 50 L plastic containers (57 × 38 × 32 cm) at 24 ± 2 °C and 61 ± 13% relative humidity (RH) in the laboratory. Small ventilation holes (2 mm in diameter) were made through the lid and on one side of each container to allow air movement and prevent condensation. Adults that emerged from the nuts were collected daily and transferred to Bugdorm® insect rearing cages (60 × 60 × 60 cm, Megaview Science Co. Ltd, Taiwan). Weevils were provided with macadamia nuts and leaves every 2 days for 1–2 weeks. The macadamia nuts used as a food source were gathered from the same locations where the infested nuts were initially collected. Nuts were surface sterilized with 1% sodium hypochlorite (NaOCl) for 5 min, rinsed three times in water for approximately 3 min and allowed to dry in a laminar flow chamber before being frozen at −80 °C. The frozen nuts were brought to room temperature before being supplied to the weevils. Although entomopathogenic fungi have occasionally been found on *K. macadamiae* in the field, there was no evidence of fungal infection in the weevils we obtained from field collected nuts, and we attribute this to the use of surface sterilized nuts as a post-emergence food source, combined with the immediate isolation of groups of adults that emerged at daily intervals.

2.2. Culturing of fungal isolates

Isolates of *Metarhizium* and *Beauveria* used in this study are listed in Table 1. Velifer® biological insecticide (BASF Australia Ltd, Victoria, Australia) is a commercial oil-based *B. bassiana* strain PPRI 5339 formulation containing at least 8×10^9 viable conidia/mL, whereas Velifer®-R is the *B. bassiana* fungal strain we isolated from the Velifer® biological insecticide. To obtain Velifer®-R, the Velifer® biological insecticide was applied to macadamia seed weevils and later the conidia that emerged from cadavers was sampled and cultured on malt extract agar using a single spore technique (Zhang et al., 2013).

Isolates of *Metarhizium* were cultured on Sabouraud dextrose agar (Inglis et al., 2012) supplemented with 1% (w/v) yeast extract (SDAY). Isolates of *Beauveria* were cultured on MEA media. All fungal isolates were incubated in the dark at 25 ± 1 °C for 15 days before harvesting the conidia for experimentation.

2.3. Molecular identification of isolates

Fungal genomic DNA was extracted from 2-week-old mycelia and conidia (20–50 mg) using the method described by Hervás-Aguilar et al. (2007). DNA samples were stored at −20 °C until subjected to PCR. The B locus nuclear intergenic region (Bloc) was used to identify species of *Beauveria* with primers B22U/B822L (Fisher et al., 2011). To identify species of *Metarhizium,* the 5′ region of elongation factor-1 alpha (EFT1) was amplified with primers EF1T/EF2T (Rehner and Buckley, 2005). Each PCR reaction was 50 µl and contained 25 µl GoTaq® 2x Green Master Mix (Promega, Alexandria, NSW, Australia), 2 µl of each forward and reverse primers (10 mmol), 19 µl of nuclease-free water and 2 µl of fungal DNA (at 25–30 ng/µl). The PCR conditions were; denaturation at 94 °C for 3 min, then 34 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, with a final extension of 10 min at 72 °C (Senthil Kumar et al., 2016; Medo et al., 2016). PCR products were sent to Macrogen Inc. (Seoul, South Korea) for PCR purification and DNA sequencing.

The sequence data and sequences of referenced *Beauveria* and *Metarhizium* species retrieved from Genbank using BLAST (Basic Local Alignment Search Tool) were edited and aligned manually using version 7.0 of Molecular Evolutionary Genetics Analysis (MEGA 7) software (Kumar et al., 2016). The overall mean pairwise Jukes-Cantor (JC) distance was calculated, and as JC < 1.0, neighbour joining (NJ) was used to analyse similarities between sequences (Nei and Kumar, 2000). The phylogeny was developed using a bootstrap method with 1000 replications and applying the Kimura 2-parameter model (Kimura, 1980).

2.4. Conidia viability

The viability of conidia was checked prior to all bioassays. A 10^4 conidia/mL suspension was prepared in sterile Tween® 20 (0.05% v/v in distilled water) and 20 µl of the suspension was spread evenly on a 4 cm^2 block of SDAY or MEA media on a sterile glass slide. The slides were placed inside Petri dishes lined with filter paper dampened with sterile distilled water and incubated at 25 ± 1 °C in the dark. After 18 h of incubation, the percentage conidial germination was determined from 100 to 200 conidial counts per slide using an Olympus BX53 compound microscope (400×) equipped with a digital camera (Model DP74, Olympus Australia Pty Ltd, Victoria, Australia). The conidia were considered to have germinated when the germ-tubes were twice the diameter of the propagule (Inglis et al., 2012). All isolates had > 86% germination of conidia.

2.5. Screening for virulence using a single concentration bioassay

A single concentration bioassay was conducted to identify isolates with the greatest virulence. Conidial suspensions of the twelve isolates were prepared by scraping the surface of the cultures with a sterile spatula and suspending the conidia in 10 mL of sterile Tween® 20 (0.05% v/v in distilled water) in a 50 mL centrifuge tube (Labtek Pty Ltd, Queensland, Australia). The suspensions were homogenised by vortexing for 5 min and the conidial concentrations were measured using a haemocytometer (Laboroptik Ltd, Lancing, UK), then adjusted to 1×10^7 conidia/mL. Four conidial suspensions of each isolate were prepared independently from four fungal plates and one was used per replicate.

For each replicate and treatment, ten mixed sex adults were randomly collected from the insect cage in the laboratory and placed in a

Table 1
List of fungal isolates screened against macadamia seed weevil. Known collection localities are all in Australia.

Species	Isolate/Accession[1]	GenBank Accession	Origin/References	Collection Locality	Year	Collector/Provider
M. anisopliae	B4A1/BRIP 70268	MN966532	Soil	Bundaberg	2017	B. Wilson
	DA1/BRIP 70271	MN966531	Soil	Bundaberg	2017	B. Wilson
	ECF1/BRIP 70270	MN966529	Soil	Rockhampton	2017	B. Wilson
	ECS1/BRIP 70272	MN966530	Soil	Rockhampton	2017	B. Wilson
	M81/BRIP 70266*	MN966528	Leemon and McMahon (2009); Leemon (2012)	Yeerongpilly	2007	D. Leemon
	QS155/DAR 82480*	MN973821	Dotaona et al. (2015)	Mapuru	2015	R. Dotaona
B. bassiana	B27/BRIP 70267	MN909971	*Bovicola ovis*	Yeerongpilly	2005	D. Leemon
	B48/BRIP 70269	MN909972	*K. macadamiae*	Alstonville	2016	C. Maddox
	B49/BRIP 70274	MN909973	*Paropsisterna tigrina*	Lismore	2015	C. Maddox
	B50/BRIP 70276	MN909974	*K. macadamiae*	Binna Burra	2017	J. Coates
	B60/BRIP 70275	MN909975	Unknown	Dutton Park	2017	D. Leemon
	Velifer® biological insecticide	–	Oil formulation contained *B. bassiana* strain PPRI 5339			BASF Australia Ltd
	Velifer®-R*	–	Isolated from Velifer® biological insecticide			

[1] BRIP, lodged in the Queensland Plant Pathology Herbarium, Queensland Department of Agriculture and Fisheries, Brisbane; DAR, lodged in the New South Wales Plant Pathology Herbarium, NSW Department of Primary Industries, Orange.
* Referenced virulent isolates.

500 mL plastic container (9.5 cm Ø and 9.5 cm height) with small ventilation holes (2 mm) in the lid of each container. Prior to spray applications, all containers were chilled at 4 °C for 15 min to reduce weevil mobility. Each container was then opened and sprayed with 1 mL of a conidial suspension using an X-Press It® micro-atomiser (X-Press Graph-X Pty. Ltd., Victoria, Australia) calibrated to deposit approximately $1.6 \times 10^4 \pm 3.6 \times 10^3$ conidia/cm^2. For the commercial formulation (Velifer® biological insecticide) the recommended rate was prepared (0.5 mL/L water) and 1 mL was sprayed into the open container. A further ten adults were sprayed with 1 mL of 0.05% (v/v) Tween® 20 in distilled water as a control treatment. After spraying, each container received a single macadamia nut and was incubated at high humidity (> 95%) in the dark for 24 h, followed by incubation at 25 ± 1 °C, 65 ± 3% RH and 16L:8D photoperiod in a Conviron® A1000 growth chamber (Conviron Asia Pacific Pty Ltd., Melbourne, Australia).

The containers were arranged in randomized complete blocks and each treatment had four replicates. Each container was provided with a new macadamia nut every second day for 12 days. Dead weevils were removed daily, placed in Petri plates containing filter paper dampened with sterile distilled water and sealed with Parafilm®. These plates were incubated in the dark at 25 ± 1 °C for 7 days to stimulate mycosis and verify fungal infection. Mortality was calculated based on the number of surviving weevils 12 days after inoculum application. In total, 560 insects were used in this experiment.

2.6. Assessment of conidia production on the mycosed cadavers

The mycosed cadavers from the single concentration bioassay were also assessed for conidial production. After 7 days of incubation two mycosed cadavers were randomly selected from each replicate, oven dried at 35 °C for 30 min, and transferred into separate 2 mL centrifuge tubes containing 1 mL of sterile Tween® 20 (0.05% v/v in distilled water) (Tumuhaise et al., 2015). The tubes were stored at 4 °C until assessment. To quantify the number of colony forming units (CFU) each 2 mL tube was vortexed for 5 min to dislodge conidia from the mycosed weevils and the conidial concentration determined using a haemocytometer.

2.7. Multiple rate bioassays

The two isolates of *M. anisopliae* and *B. bassiana* that caused the highest weevil mortality in the single concentration bioassay were used in this experiment. For both isolates, five concentrations ranging from 1×10^4 to 1×10^8 conidia/mL were prepared in sterile Tween® 20 (0.05% v/v in distilled water). Four fungal suspensions of each isolate were prepared independently from four fungal plates and each was used in one replicate only. To determine LC_{50}, LC_{90} and LC_{95} values, groups of ten mixed sex adult weevils were sprayed with 1 mL of the conidial suspensions with a micro-atomiser. A further ten adults were sprayed with 1 mL of 0.05% (v/v) Tween® 20 in distilled water as a control treatment. All treated weevils were incubated and fed as described previously and dead individuals were evaluated for fungal infection as described in the previous experiment. The containers were placed in a randomized complete block design with four replications. In total, 480 insects were used in this experiment.

2.8. Statistical analysis

All analyses were performed using RStudio Version 1.2.1335. (RStudio Team, 2018) built on R Version 3.5.2. (R Core Team, 2017). The Shapiro-Wilk Test for normality (Shapiro and Wilk, 1965) and Levene's test for homogeneity of variance were applied to all data using the CAR (Companion to Applied Regression, Ver. 3.0-3) package (Fox et al., 2019) before analyses of variance (ANOVA) were conducted.

The numbers of CFU recovered from weevil cadavers were log-transformed to meet the assumptions of ANOVA. Two-way ANOVAs assessing treatment and block effects were conducted on the transformed numbers of CFU obtained from dead weevils in the single-rate mortality bioassays.

Data from the single-rate bioassays could not be normalised by transformation, so a non-parametric analysis was used. The PMCMR (Calculate Pairwise Multiple Comparisons of Mean Rank Sums, Ver. 4.3) package (Pohlert, 2018) in R was used to apply Friedman's test to the mortality data associated with isolates of both genera, with the Nemenyi post-hoc test used to identify significant differences between specific treatments.

Weevil mortality from the multiple rate bioassays was analysed by probit analysis using the Ecotox (Analysis of Ecotoxicology, Ver. 1.4.0) package (Hlina, 2019) in R to calculate the lethal concentrations for 50%, 90% and 95% (LC_{50}, LC_{90} and LC_{95}) of the population. The median lethal time (LT_{50}) was calculated for each isolate tested in the single-rate bioassays using the same package. For each isolate, the LT_{50} was calculated for each block and the resultant LT_{50} values were subjected to ANOVA to separate means. No control weevil mortality occurred in either the single or multiple-rate bioassays, so adjustment of treatment mortalities using Abbott's formula (Abbott, 1925) was not required.

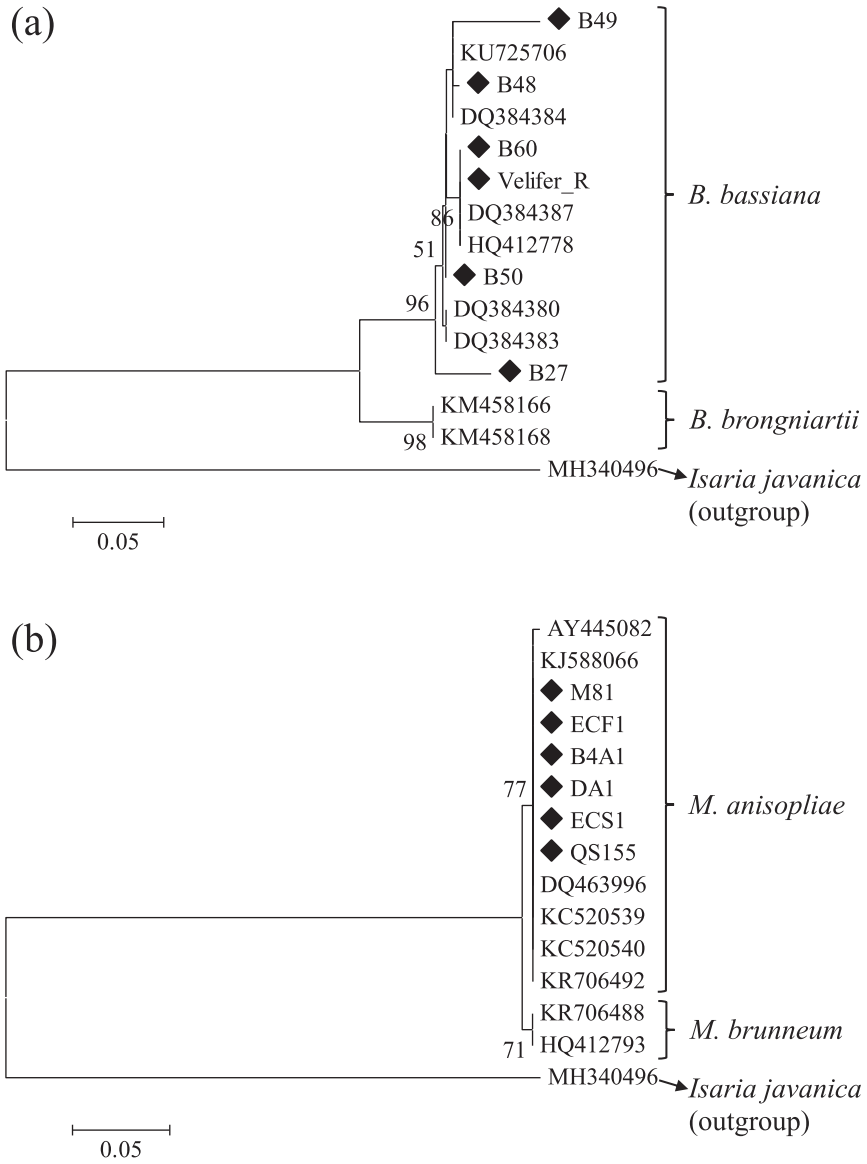


Fig. 1. Phylogenetic trees constructed using Kimura 2-parameter neighbour-joining (NJ) based on (a) Bloc sequences of *Beauveria* isolates, and (b) EFT1 sequences of *Metarhizium* isolates. Numbers on branches indicate the percentage of replicate trees in which associated taxa clustered together (values > 50 shown). ♦ indicates isolates used in the current study.

3. Results

3.1. Identification of fungal isolates

Using BLAST, the Bloc sequence from isolate B50/BRIP 70276 displayed 97–99.6% homology to *B. bassiana* whereas the EFT1 sequence from isolate QS155/DAR 82480 displayed 97–100% homology to some species of *Metarhizium* including *M. anisopliae* and *M. brunneum*. The final datasets revealed that the Bloc sequences of the six isolates of *Beauveria* were together in the clade of *B. bassiana* (Fig. 1a) whereas the six EFT1 sequences of *Metarhizium* were together in the clade of *M. anisopliae* (Fig. 1b), confirming that all our isolates of *Beauveria* and *Metarhizium* were *B. bassiana* and *M. anisopliae*, respectively.

3.2. Single concentration bioassay assessment

Twelve days after inoculation, significant differences in mortality were identified amongst isolates of *M. anisopliae* and *B. bassiana* (Friedman's test, $\chi^2 = 22.95$ df = 12, $P = 0.028$) (Table 2). For *M. anisopliae*, the adult weevil mortality caused by ECS1/BRIP 70272 (97.5%) was significantly greater than that caused by B4A1/BRIP 70268 and M81/BRIP 70266 (both 72.5% mortality), but not significantly different to other isolates of *M. anisopliae*. For *B. bassiana*, B27/BRIP 70267 induced the highest rate of mortality (92.5%), which was significantly greater than that caused by Velifer®-R. There were no significant differences between B27/BRIP 70267 and the remaining *B. bassiana* isolates. Block effects were not observed (Friedman's test; $\chi^2 = 1.21$, df = 3, $P = 0.75$).

For median lethal time (LT_{50}), *M. anisopliae* isolate ECS1/BRIP

Table 2
Response of adult macadamia seed weevils to isolates of entomopathogenic fungi and a commercial *B. bassiana* formulation (Velifer® biological insecticide) applied at its recommended application rate. Velifer®-R isolated from Velifer® biological insecticide represents a positive control. Mortality at 12 days post-treatment, median lethal times (LT_{50}) and mean number of colony-forming units (CFU) recovered from infected weevils seven days after death.

Species	Isolate/Accession	% Mortality ± SE[1]	LT_{50} (days) ± SE[2]	CFU ($\times 10^7$) ± SE[2]
M. anisopliae	ECS1/BRIP 70272	97.5 ± 2.50 a	5.13 ± 0.62	11.71 ± 0.92
	DA1/BRIP 70271	85.0 ± 6.45 abc	6.09 ± 0.70	11.63 ± 0.82
	ECF1/BRIP 70270	82.5 ± 4.79 abc	6.56 ± 0.51	11.04 ± 0.72
	QS155/DAR 82480	80.0 ± 4.08 abc	6.90 ± 1.29	11.51 ± 1.53
	B4A1/BRIP 70268	72.5 ± 4.79 bc	6.73 ± 0.74	10.56 ± 1.32
	M81/BRIP 70266	72.5 ± 7.50 bc	7.92 ± 1.01	10.51 ± 0.81
B. bassiana	B27/BRIP 70267	92.5 ± 2.50 ab	5.37 ± 0.67	12.22 ± 1.58
	B48/BRIP 70269	80.0 ± 4.08 abc	5.86 ± 0.92	12.18 ± 1.01
	B49/BRIP 70274	80.0 ± 4.08 abc	7.18 ± 0.80	11.94 ± 0.82
	Velifer® biological insecticide	80.0 ± 5.77 abc	5.95 ± 0.32	12.00 ± 1.77
	B60/BRIP 70275	75.0 ± 2.89 abc	6.34 ± 1.18	11.90 ± 1.42
	B50/BRIP 70276	72.5 ± 2.50 bc	6.89 ± 0.69	12.09 ± 1.16
	Velifer®-R	70.0 ± 4.08 c	8.71 ± 0.47	11.74 ± 1.23

[1] Both fungal species analysed collectively but grouped by species. Means followed by different letters are significantly different (Friedman's Test, Nemenyi's Post-hoc Test, $P < 0.05$).
[2] LT_{50} and mean CFU values calculated separately for each replicate (block). There were no significant differences between mean LT_{50} values or between the mean numbers of CFU recovered from weevil cadavers (ANOVA, $P > 0.05$).

70272 had the lowest LT_{50} in absolute terms (5.13 days); however, there were no significant differences between this isolate and any of the other isolates of either species, or Velifer® biological insecticide ($F_{12, 36} = 1.40$, $P = 0.21$). Block effects were not significant ($F_{3, 36} = 0.16$, $P = 0.92$).

3.3. Assessment of conidia production on mycosed cadavers

No significant differences were observed in conidial production from mycosed cadavers killed by any of the twelve fungal isolates or by Velifer® biological insecticide ($F_{12, 36} = 0.24$, $P = 0.99$) (Table 2). No block effects were significant ($F_{3, 36} = 1.93$, $P = 0.14$). On average, the number of conidia produced per mycosed cadaver killed by *M. anisopliae* and *B. bassiana* were around 1.12×10^8 and 1.2×10^8 conidia, respectively.

3.4. Multiple rate bioassays

After 12 days, the LC_{50}, LC_{90} and LC_{95} for *M. anisopliae* accession ECS1/BRIP 70272 were 1.48×10^5, 8.02×10^6, and 2.49×10^7 conidia/mL, respectively. For *B. bassiana* accession B27/BRIP 70267, the corresponding values were higher at 1.65×10^5, 1.34×10^7 and 4.64×10^7 conidia/mL (Table 3).

4. Discussion

In this study, 12 fungal isolates and a commercial fungal biopesticide were screened on macadamia seed weevils; at least 7 of these isolates were highly virulent and just as effective as the Velifer® biopesticide. Amplifying and sequencing specific regions of the *Beauveria* and *Metarhizium* genomes using published primers confirmed all our tested isolates to be either *B. bassiana* (Rehner et al., 2006) or *M. anisopliae* (Bischoff et al., 2006).

Table 3
Probit analysis results from the multiple-rate bioassays of *M. anisopliae* ECS1/BRIP 70272 and *B. bassiana* B27/BRIP 70267 against adult macadamia seed weevils. FL, fiducial limits.

LC	Rate (conidia/mL)	95% FL
M. anisopliae ECS1/BRIP 70272, $\chi^2 = 12.4$ (18 df), slope 0.74 ± 0.09		
LC_{50}	1.48×10^5	6.88×10^4 to 2.89×10^5
LC_{90}	8.02×10^6	3.30×10^6 to 3.01×10^7
LC_{95}	2.49×10^7	8.58×10^6 to 1.29×10^8
B. bassiana B27/BRIP 70267, $\chi^2 = 9.3$ (18 df), slope 0.67 ± 0.09		
LC_{50}	1.65×10^5	7.22×10^4 to 3.36×10^5
LC_{90}	1.34×10^7	5.07×10^6 to 5.79×10^7
LC_{95}	4.64×10^7	1.44×10^7 to 2.92×10^8

Among the highly virulent isolates, *B. bassiana* accession B27/BRIP 70267 and *M. anisopliae* accession ECS1/BRIP 7072 caused the highest mortality of adults, with > 90% mortality recorded 12 days post-treatment and a LT_{50} of around 5 days. We recorded greater mortality with the application of our isolates on the macadamia seed weevil compared to other studies on red palm weevil (*Rhynchophorus ferrugineus*) (Yasin et al., 2019), banana weevil (*Cosmopolites sordidus*) (González et al., 2018) and black vine weevil (*Otiorhynchus sulcatus*) (Pope et al., 2018). In their work on the much larger red palm weevil, Yasin et al., 2019 reported their most virulent isolates of *M. anisopliae* and *B. bassiana* caused 50% mortality 12 days post-treatment when inoculated with 1×10^7 conidia/mL. González et al., 2018 reported that even at higher conidial concentration (1×10^8 conidia/mL), the mortality of banana weevils did not exceed 85% at 22 days post-treatment. The LC_{50} of the virulent isolates in our study ($LC_{50} < 1.65 \times 10^5$ conidia/mL) was lower than that observed by González et al., 2018, Yasin et al., 2019 ($LC_{50} > 10^6$ conidia/mL), confirming that the isolates in our study were highly virulent. In contrast to the other weevil studies above, Dotaona et al., 2015 often found > 98% mortality to sweetpotato weevil (*Cylas formicarius*), 10 days after application of *M. anisopliae* by dipping (1×10^7 conidia/mL), and the LC_{50} for the most virulent isolate was 1.7×10^5 conidia/mL.

Beauveria bassiana accession B27/BRIP 70267, isolated from sheep lice (*Bovicola ovis*), induced higher mortality in the macadamia seed weevil than B50/BRIP 70276, which was originally isolated from our target insect. Other studies have demonstrated similar findings, with *B. bassiana* isolated from soil or other host insects performing better than isolates found naturally infecting the target species. Examples include studies on banana weevil (Lopes et al., 2011; Fancelli et al., 2013) and red palm weevil (Hussain et al., 2015). Fancelli et al. (2013) found that *B. bassiana* isolated from silky cane weevil (*Metamasius hemipterus*) induced high mortality (96%) in the banana weevil, whereas another four *B. bassiana* isolates, which naturally infected banana weevil, only caused 48–56% mortality. However, other studies such as those by Kaaya et al. (1993), Ricaño et al. (2013) and Mota et al. (2017) have provided contrasting results, with target-derived *B. bassiana* strains found to be superior to those recovered from other insect taxa. Overall,

there is a lack of direct correlation between the use of target-derived isolates and high virulence; however, in some instances the preferential use of target-derived strains from the natural distribution of a pest species may confer benefits. Indigenous strains may have the ability to better tolerate local weather conditions than introduced strains. Klingen et al. (2015) found that indigenous isolates did not induce greater mortality to the target insects when compared with exotic or introduced isolates, but the number of recovered conidia from the soil two years after inoculation was higher when indigenous isolates were applied.

It is difficult to draw direct comparisons between the commercial Velifer® biological insecticide and the fungal isolates tested because of variations in the conidial concentrations. The actual concentration of conidia in the Velifer® biological insecticide was not determined; however, unless the Velifer® biological insecticide was more concentrated than specified, it was assumed that the tested conidial concentration was below 1×10^7 conidia/mL. The activity of Velifer® biological insecticide was not significantly different to any of the tested *B. bassiana* isolates, but the activity of Velifer®-R, the isolate derived from Velifer® biological insecticide, was significantly below that of B27/BIRP 70267, the most virulent *B. bassiana* isolate we assessed. This confirms the critical role that formulation plays in enhancing the activity of fungal entomopathogens.

The *B. bassiana* accession B27/BRIP 70267 performed better than both the strain isolated directly from macadamia seed weevil (B50/BRIP 70276) and the commercial *B. bassiana* strain (Velifer®-R). By contrast, Leemon and McMahon (2009) found that B27/BRIP 70267 was only moderately virulent against the small hive beetle (*Aethina tumida*), producing mortality of around 60% under their experimental conditions. Whilst this could reflect target-specific variations in response, it could also be the result of differences in inoculation technique: here the macadamia seed weevils were sprayed with a conidial suspension, but the adult small hive beetles were rolled directly on *B. bassiana* conidia (Leemon and McMahon, 2009). Dotaona et al. (2015) found that *M. anisopliae* accession QS155/DAR 82480 was the most virulent isolate to sweetpotato weevil (ca. 64% and 100% mortality at 5 and 10 days after inoculation respectively). Whilst *M. anisopliae* M81/BRIP 70266 was found to be one of the best for control (ca. 80%) of the larvae of small hive beetle (Leemon and McMahon, 2009; Leemon, 2012). In this, study neither of these isolates performed as well as ECS1/BRIP 70272. Again, this could be either a consequence of different inoculation techniques or species-specific responses by the different target insects.

An important aspect of using entomopathogenic fungi in controlling insect pests in agricultural systems is the ability of the pathogen to continue suppressing the pest population after its application and after its exposure to various abiotic and biotic factors (Hajek and St. Leger, 1994). The success of the fungi continuing to be effective is dependent on the capacity of the entomopathogenic fungus to reach the second part of the cycle; that is the fungus first needs to kill the insect before it can produce conidia for dispersal among other individuals (Chouvenc and Su, 2010). This study demonstrated that all isolates produced large amounts of conidia on the cadavers and could have the ability to cause secondary infections among other individuals, although further investigation is required to confirm this. The number of conidia on the macadamia seed weevil mycosed cadaver in our study ($> 10^8$ conidia/cadaver) was higher than observed in other studies. For examples on various aphid species and citrus psyllids (*Diaphorina citri*) $< 4.5 \times 10^7$ conidia/cadaver was observed (Conceschi et al., 2016, Bayissa et al., 2017, Mweke et al., 2018). Although this could be the result of the size of insect host, this could be an important factor when considering commercialisation.

Clearly, further screening may identify more effective entomopathogenic fungi to manage *K. macadamiae*. However, the next and most valuable steps in integrating entomopathogenic fungi into the management of *K. macadamiae* involves the application of formulated conidial sprays of these strains in orchards when the weevils are active and assessing the reduction in premature nut drop caused by infestation of *K. macadamiae*.

Acknowledgements

Ian Purdue, Jarrah Coates and Mark Hickey are thanked for fieldwork support and Diana Leemon is thanked for providing us with several of the fungal isolates. This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation, Australia with the Australian macadamia industry's research and development levy and contributions from the Australian Government.

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.
APVMA, 2018. Permit to allow minor use of an agvet chemical product for the control of seed weevil in macadamia (Permit PER86827). Australian Pesticides and Veterinary Medicines Authority, Canberra.
AMS, 2018. Yearbook 2018. Australian Macadamia Society Ltd, Lismore, Australia.
ANIC, 2016. Australia's Tree Nut Industry: growing for success 2016. Australian Nut Industry Council, Queensland, Australia.
Bayissa, W., Ekesi, S., Mohamed, S.A., Kaaya, G.P., Wagacha, J.M., Hanna, R., Maniania, N.K., 2017. Selection of fungal isolates for virulence against three aphid pest species of crucifers and okra. J. Pest Sci. 90, 355–368. https://doi.org/10.1007/s10340-016-0781-4.
Bischoff, J.F., Rehner, S.A., Humber, R.A., 2006. *Metarhizium frigidum* sp nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. Mycologia 98, 737–745. https://doi.org/10.3852/mycologia.98.5.737.
Bright, J., 2017a. Macadamia seed weevil (*Kuschelorhynchus macadamiae*) life cycle and monitoring. Primefact 1586. https://www.dpi.nsw.gov.au/_data/assets/pdf_file/0003/731982/Macadamia-seed-weevil-update-lifecycle_2.pdf.
Bright, J., 2017b. Macadamia seed weevil (*Kuschelorhynchus macadamiae*) orchard management. Primefact 1585. https://www.dpi.nsw.gov.au/_data/assets/pdf_file/0008/731987/Macadamia-seed-weevil-update-orchard-management_2.pdf.
Bright, J., 2017c. *Sigastus* weevil update. Part 1. In: Life cycle and monitoring keys to control. Australian Macadamia Society Ltd, Lismore, Australia. https://www.horticulture.com.au/globalassets/hort-innovation/resource-assets/mc-ipm-program-sigastus-weevil-fact-sheet.pdf.
Bright, J., 2018. Macadamia Plant Protection Guide 2018–19. NSW Department of Primary Industries, Wollongbar, Australia.
Chouvenc, T., Su, N.Y., 2010. Apparent synergy among defense mechanisms in subterranean termites (Rhinotermitidae) against epizootic events: limits and potential for biological control. J. Econ. Entomol. 103, 1327–1337. https://doi.org/10.1603/ec09407.
Conceschi, M.R., D'Alessandro, C.P., Moral, R.D., Demetrio, C.G.B., Delalibera, I., 2016. Transmission potential of the entomopathogenic fungi *Isaria fumosorosea* and *Beauveria bassiana* from sporulated cadavers of *Diaphorina citri* and *Toxoptera citricida* to uninfected *D. citri* adults. Biocontrol 61, 567–577. https://doi.org/10.1007/s10526-016-9733-4.
Dolinski, C., Lacey, L.A., 2007. Microbial control of arthropod pests of tropical tree fruits. Neotrop. Entomol. 36, 161–179. https://doi.org/10.1590/s1519-566x2007000200001.
Dotaona, R., Wilson, B.A.L., Stevens, M.M., Holloway, J., Ash, G.J., 2015. Screening of tropical isolates of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) for virulence to the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). Int. J. Trop. Insect Sci. 35, 153–163. https://doi.org/10.1017/s1742758415000211.
Fancelli, Marilene, Dias, Alex Batista, Delalibera Júnior, Italo, Jesus, Sandra Cerqueira de, Nascimento, Antonio Souza do, Silva, Sebastião de Oliveira e, Caldas, Ranulfo Correa, Ledo, Carlos Alberto da Silva, 2013. *Beauveria bassiana* Strains for Biological Control of *Cosmopolites sordidus* (Germ.) (Coleoptera: Curculionidae) in Plantain. Biomed Res. Int. 2013, 1–7. https://doi.org/10.1155/2013/184756.
Fay, H.A.C., De Faveri, S.G., Storey, R.I., Watson, J., 2001. *Sigastus* weevil – an emerging pest of macadamias in north Queensland. In: Proceedings of the Sixth Workshop for Tropical Agricultural Entomology, Darwin, Australia, 11–15 May 1998.
Fisher, J.J., Rehner, S.A., Bruck, D.J., 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. J. Invertebr. Pathol. 106, 289–295. https://doi.org/10.1016/j.jip.2010.11.001.
Fox, J., Weisberg, S., Price, B., 2019. CAR: companion to applied regression. Ver. 3.0-3. R package. Functions to Accompany J. Fox and S. Weisberg, An R Companion to Applied Regression, Third Edn, Sage, Thousand Oaks CA (in press). https://cran.r-project.org/package=car.
González, D.N., Chávez, M.A.Á., Gutiérrez, R.L., Cupul, W.C., Ochoa, J.M., Velasco, E.G., 2018. Suitability of *Cordyceps bassiana* and *Metarhizium anisopliae* for biological control of *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) in an organic Mexican banana plantation: laboratory and field trials. J. Plant Dis. Prot. 125, 73–81. https://doi.org/10.1007/s41348-017-0126-4.
Hajek, A.E., St. Leger, R.J., 1994. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39, 293–322. https://doi.org/10.1146/annurev.en.39.010194.

001453.
Hervás-Aguilar, A., Rodríguez, J.M., Tilburn, J., Arst, H.N., Peñalva, M.A., 2007. Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. J. Biol. Chem. 282, 34735–34747. https://doi.org/10.1074/jbc.M706723200.
Hlina, B.L., 2019. Ecotox: analysis of ecotoxicology. Ver. 1.4.0. R package. https://cran.r-project.org/package=ecotox.
Hussain, A., Rizwan-ul-Haq, M., Al-Ayedh, H., Ahmed, S., Al-Jabr, A.M., 2015. Effect of *Beauveria bassiana* infection on the feeding performance and antioxidant defence of red palm weevil, *Rhynchophorus ferrugineus*. BioControl 60, 849–859. https://doi.org/10.1007/s10526-015-9682-3.
Huwer, R., 2016. Ecology and management of *Sigastus* weevil in macadamias. Final report, Project MC15010. Horticulture Innovation. Australia Limited, Sydney, Australia.
Inglis, G.D., Enkerli, J., Goettel, M.S., 2012. Laboratory techniques used for entomopathogenic fungi: Hypocreales. In: Lacey, L. (Ed.), Manual of Techniques in Invertebrate Pathology. Academic Press, London, UK, pp. 189–253.
INDFC, 2018. Statistical review. Macadamias. Nutfruit, 74(2), 76. International Nut and Dried Fruit Council, REUS, Spain.
Jennings, D., Oberprieler, R.G., 2018. A Review of the Tribe Cryptoplini (Coleoptera: Curculioninae), with revision of the Genus *Menechirus* Hartmann, 1901 and description of a new genus associated with macadamia. In: Diversity 10. pp. 34.
Kaaya, G.P., Seshu-Reddy, K.V., Kokwaro, E.D., Munyinyi, D.M., 1993. Pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Serratia marcescens* to the banana weevil *Cosmopolites sordidus*. Biocontrol Sci. Technol. 3, 177–187. https://doi.org/10.1080/09583159309355274.
Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16 (2), 111–120. https://doi.org/10.1007/BF01731581.
Klingen, I., Westrum, K., Meyling, N.V., 2015. Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres. Biol. Control 81, 1–7. https://doi.org/10.1016/j.biocontrol.2014.10.006.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.
Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009.
Lacey, L.A., Shapiro-Ilan, D.I., 2008. Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. Ann. Rev. Entomol. 53, 121–144. https://doi.org/10.1146/annurev.ento.53.103106.093419.
Leemon, D.M., 2012. In-hive fungal biocontrol of small hive beetle. Final report, Project PRJ-004150 (RIRDC Publication No. 12/012), 69 pp. Rural Industries Research and Development Corporation, Canberra, Australia. https://www.agrifutures.com.au/wp-content/uploads/publications/12-012.pdf.
Leemon, D.M., McMahon, J., 2009. Feasibility study into in-hive fungal bio-control of small hive beetle. Final report, Project PRJ- 000037 (RIRDC Publication No. 09/090), 30 pp. Rural Industries Research and Development Corporation, Canberra, Australia. https://www.agrifutures.com.au/wp-content/uploads/publications/09-090.pdf.
Lopes, R.B., Michereff-Filho, M., Tigano, M.S., Neves, P.M.O.J., López, E.L., Fancelli, M., da Silva, J.P., 2011. Virulence and horizontal transmission of selected Brazilian strains of *Beauveria bassiana* against *Cosmopolites sordidus* under laboratory conditions. Bull. Insectol. 64, 201–208.
Medo, J., Michalko, J., Medová, J., Cagáň, L., 2016. Phylogenetic structure and habitat associations of *Beauveria* species isolated from soils in Slovakia. J. Invertebr. Pathol. 140, 46–50. https://doi.org/10.1016/j.jip.2016.08.009.
Mota, L.H.C., Silva, W.D., Sermarini, R.A., Demétrio, C.G.B., Bento, J.M.S., Delalibera Jr., I., 2017. Autoinoculation trap for management of *Hypothenemus hampei* (Ferrari) with *Beauveria bassiana* (Bals.) in coffee crops. Biol. Control 111, 32–39. https://doi.org/10.1016/j.biocontrol.2017.05.007.
Mweke, A., Ulrichs, C., Nana, P., Akutse, K.S., Fiaboe, K.K.M., Maniania, N.K., Ekesi, S., 2018. Evaluation of the entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria* sp for the management of *Aphis craccivora* (Hemiptera: Aphididdae). J. Econ. Entomol. 111, 1587–1594. https://doi.org/10.1093/jee/toy135.
Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, UK.
Pohlert, T., 2018. PMCMR: calculate pairwise multiple comparisons of mean rank sums. Ver. 4.3. R package. http://CRAN.R-project.org/package=PMCMR.
Pope, T.W., Hough, G., Arbona, C., Roberts, H., Bennison, J., Buxton, J., Prince, G., Chandler, D., 2018. Investigating the potential of an autodissemination system for managing populations of vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic fungi. J. Invertebr. Pathol. 154, 79–84. https://doi.org/10.1016/j.jip.2018.04.002.
QDAF, 2018. Macadamia industry interim benchmark report: 2009 to 2017 seasons. Project MC15005. Queensland Department of Agriculture and Fisheries, Brisbane, Australia. https://www.horticulture.com.au/growers/help-your-business-grow/research-reports-publications-fact-sheets-and-more/macadamia-industry-benchmark-report-2009-2017/.
R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rehner, S.A., Buckley, E., 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97, 84–98. https://doi.org/10.1080/15572536.2006.11832842.
Rehner, S.A., Posada, F., Buckley, E.P., Infante, F., Castillo, A., Vega, F.E., 2006. Phylogenetic origins of African and Neotropical *Beauveria bassiana* s.l. pathogens of the coffee berry borer, *Hypothenemus hampei*. J. Invertebr. Pathol. 93, 11–21. https://doi.org/10.1016/j.jip.2006.04.005.
Ricaño, J., Güerri-Agulló, B., Serna-Sarriás, M.J., Rubio-Llorca, G., Asensio, L., Barranco, P., Lopez-Llorca, L.V., 2013. Evaluation of the pathogenicity of multiple isolates of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) for the assessment of a solid formulation under simulated field conditions. Florida Entomol. 96, 1311–1324. https://doi.org/10.1653/024.096.0410.
Roberts, D.W., St. Leger, R.J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. In: In: Laskin, A.I., Bennet, J.W., Gadd, G.M. (Eds.), Advances in Applied Microbiology Vol. 54. Elsevier Academic Press Inc, USA, pp. 1–70.
RStudio Team, 2018. RStudio: Integrated Development for R. RStudio Inc, Boston, USA.
Senthil Kumar, C.M., Jacob, T.K., Devasahayam, S., D'Silva, S., Nandeesh, P.G., 2016. Characterization and virulence of *Beauveria bassiana* associated with auger beetle (*Sinoxylon anale*) infesting allspice (*Pimenta dioica*). J. Invertebr. Pathol. 139, 67–73. https://doi.org/10.1016/j.jip.2016.07.016.
Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples). Biometrika 52, 591–611. https://doi.org/10.1093/biomet/52.3-4.591.
Singh, A.K., 1984. Improved analysis of acephate and methamidophos in biological samples by selective ion monitoring gas chromatography—mass spectrometry. J. Chromatogr. A 301, 465–469. https://doi.org/10.1016/S0021-9673(01)89221-9.
Tumuhaise, V., Ekesi, S., Mohamed, S.A., Ndegwa, P.N., Irungu, L.W., Srinivasan, R., Maniania, N.K., 2015. Pathogenicity and performance of two candidate isolates of *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) in four liquid culture media for the management of the legume pod borer *Maruca vitrata* (Lepidoptera: Crambidae). Int. J. Trop. Insect Sci. 35, 34–47. https://doi.org/10.1017/s1742758414000605.
Yasin, M., Wakil, W., Ghazanfar, M.U., Qayyum, M.A., Tahir, M., Bedford, G.O., 2019. Virulence of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against red palm weevil, *Rhynchophorus ferrugineus* (Olivier). Entomol. Res. 49, 3–12. https://doi.org/10.1111/1748-5967.12260.
Zhang, K., Su, Y.Y., Cai, L., 2013. An optimized protocol of single spore isolation for fungi. Cryptogam. Mycol. 34, 349–356. https://doi.org/10.7872/crym.v34.iss4.2013.349.

Chapter 4: Transmission of *Metarhizium anisopliae* and *Beauveria bassiana* to adults of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) from infected adults and conidiated cadavers

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson
Scientific reports 11, 2188
https://doi.org/10.1038/s41598-021-81647-0

Note 1: The total area of each photo at 600x magnification is 0.03 mm^2, thus, the number of conidia attached to the head (Fig. 5) and legs (Fig. 6) were > 1.3 x 10^4 conidia/mm^2 compared to other parts of the body, < 6.7 x 10^3 conidia/mm^2 (Fig. 7).
Note 2: Additional figures associated to this manuscript are included in the appendix C, D and E.

www.nature.com/scientificreports

scientific reports

Check for updates

OPEN

Transmission of *Metarhizium anisopliae* and *Beauveria bassiana* to adults of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) from infected adults and conidiated cadavers

Kim Khuy Khun[1,2]✉, Gavin J. Ash[2], Mark M. Stevens[3,4], Ruth K. Huwer[5] & Bree A. L. Wilson[2]

Kuschelorhynchus macadamiae **is a major pest of macadamias in Australia, causing yield losses of up to 15%. Our previous studies have shown the weevil is susceptible to *Beauveria bassiana* and *Metarhizium anisopliae*. The aim of this study was to investigate horizontal transmission of both fungal species to healthy weevils from both infected adults and weevil cadavers. In a confined environment the mortality of healthy adults caused by the transmission of conidia from live fungus-infected adults was < 50%. Under similar experimental conditions, the mortality of healthy adults reached 100% when exposed to conidiated cadavers. However, when conidiated cadavers were used in more spacious environments (insect cages), the mortality of adults was < 80%. Using scanning electron microscopy, it was observed that all healthy adults had conidia attached to all external parts of the body. This suggests that although the conidia were readily transferred to the adults, the lower mortality in the larger insect cages could be the result of an unfavourable environmental factor such as low humidity. The presence of conidia attached to all the adults indicated that they did not show any discriminatory behaviour such as avoidance of conidiated cadavers infected by these two fungal species. The results from this study show that there is potential for enhanced control of adult *K. macadamiae* via transmission from either fungus-infected adults or conidiated cadavers and this could strengthen sustainable pest management in macadamias.**

Macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler, formerly known as *Sigastus* weevil[1], is a native Australian insect which was initially found in macadamias (*Macadamia integrifolia* Maiden and Betche and *M. tetraphylla* L.A.S. Johnson) on the Atherton Tablelands, Queensland[2] in 1994 and later in the Northern Rivers, New South Wales (NSW)[3,4]. This weevil is a major pest of macadamias at the nut development stage[3,4] with the female weevil ovipositing inside the husk of the macadamias when they are about 10 mm in diameter, and inducing premature nut drop between the months of September and December each year[2,5]. This premature nut drop has been estimated to lead to approximately AU$ 15 million worth of lost production[6]. Adults also feed on young leaves and completely remove the bark from seedlings, leading to plant death within a few days (K. K. Khun, personal observation).

The entomopathogenic fungi, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) have cosmopolitan distributions[7,8] and

[1]Faculty of Agronomy, Royal University of Agriculture, Dangkor District, P.O. Box 2696, Phnom Penh, Cambodia. [2]Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia. [3]NSW Department of Primary Industries, Yanco Agricultural Institute, Yanco, NSW 2703, Australia. [4]Graham Centre for Agricultural Innovation, NSW Department of Primary Industries, Charles Sturt University, Wagga Wagga, NSW 2650, Australia. [5]NSW Department of Primary Industries, Wollongbar Primary Industries Institute, Wollongbar, NSW 2477, Australia. ✉email: Khun.KimKhuy@rua.edu.kh

are commonly isolated from insects and soil using selective media and insect baits (such as *Galleria mellonella* L. and *Tenebrio molitor* L.), respectively[9,10]. Various studies have shown the potential of fungal entomopathogens for controlling many economically important weevils affecting horticultural crops[11–13]. In our previous study, entomopathogenic fungi looked promising for the control of *K. macadamiae*[14]. In the laboratory *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 were the most effective strains, providing better control of *K. macadamiae* than a commercial strain of *B. bassiana* (PPRI 5339) or other tested fungal strains available in Australia[14]. In addition, these strains could conidiate well on weevil cadavers[14], indicating the possibility of horizontal infection by the entomopathogens under suitable conditions.

The natural occurrence of fungal entomopathogens on *K. macadamiae* has been documented in the Northern Rivers[6] and at least three strains of fungal entomopathogens have been isolated from *K. macadamiae* in this region[6,14]. Their activities against *K. macadamiae* in the field were attributed to the suitability of the weather conditions, the dense canopy of the mature macadamias and the agricultural practices in the region. Some studies have suggested that conserving naturally occurring fungal entomopathogens in the field could assist with control of established pests[15,16]. As the macadamia agroecosystem is naturally suitable for fungal entomopathogens, conserving naturally occurring entomopathogens may complement inundative applications of formulated entomopathogens for the control of *K. macadamiae*.

An important aspect of using entomopathogenic fungi for controlling important insect pests in horticultural systems is the capacity of the pathogens to continue to suppress pest populations in the field after their initial application by horizontal transmission or dissemination by abiotic or biotics means[9,10]. As fungal entomopathogens may require several days to cause mortality to insects, the conidia adhering to the insect exoskeleton after application may also be transferred to other adults of the same or different species via physical contact (horizontal transmission)[17–22]. Moreover, contact with conidiated cadavers is also considered a means of on-going suppression of the pest population (horizontal infection). This is mainly due to the number of conidia on insect cadavers being at least 10 times higher than the number of conidia on fungus-infected adults[14], and conidia on the cadavers being easily picked up by other insects[23]. The conidia present on cadavers have also been shown to be more tolerant of solar radiation under field conditions[24]. One study found that around 89% of *B. bassiana* conidia remained viable after cadavers were exposed directly to the sunlight for up to 2 weeks and around 87% of conidia remanined viable when the cadavers were shaded inside a PVC cylinder in the field for up to 20 weeks[24]. High inoculum levels and strong persistence suggest that conidia present on cadavers have the potential to suppress pest populations in the field, however, only a few studies have explored the potential for conidia transmission via physical contact with conidiated cadavers (e.g. diamondback moth, *Plutella xylostella* L.[25], the Asian citrus psyllid, *Diaphorina citri* Kuwayama[26], sweetpotato weevil, *Cylas formicarius* F.[17] and the Colorado potato beetle, *Leptinotarsa decemlineata* Say[27,28]).

No previous studies have examined the transmission of entomopathogens between *K. macadamiae* individuals or the ability of conidiated cadavers to cause disease transmission in this species. Our goals in this study were to investigate and understand fungal infection in weevil populations driven by the proportion of fungus-infected adults or conidiated cadavers, and document the behaviour of adults toward conidiated cadavers killed by different fungal species.

Results

Horizontal transmission from fungus-infected adults to healthy adults. The mortality of all fungus-infected adults or donors (marked with red ink) including positive controls was 90–100% and 88–100% for *M. anisopliae* strain ECS1 and *B. bassiana* strain B27, respectively. The mortality of healthy adult weevils was significantly increased by higher ratios of the fungus-infected adults to healthy individuals ($P<0.05$) and over time ($P<0.05$) for both fungal species. A significant interaction between the ratio of the B27 infected adults and the measured times on the mortality of healthy adults was also observed ($P<0.05$), but no significant interaction was observed between the ratio of the ECS1 infected adults and the measured times ($P=0.4$).

The pairwise Wilcoxon rank-sum test for multiple comparisons revealed that the highest ratio of the B27 infected adults (1:1) caused the highest mortality to healthy adults at all measured time points and was significantly higher than that observed in the three lowest ratios (1:5, 1:10, 1:20) at 6 days, 9 days and 12 days post-introduction (Fig. 1A, $P<0.05$). For ECS1, the highest ratio of fungus-infected adults (1:1) also caused the highest mortality to healthy adults across all measured time points and was significantly higher than the mortality observed at the three lowest ratios at 9 days and 12 days post-introduction (Fig. 1B, $P<0.05$), though there were no statistically significant differences at 6 days. Within individual ratios, only ECS1 at the 1:1 ratio produced significantly higher mortalities across time periods.

Horizontal infection from conidiated cadavers to healthy adults in a confined environment. The mean total number of ECS1 and B27 conidia from each conidiated cadaver was 1.27×10^8 and 1.35×10^8 respectively. The mortality of healthy adults was significantly affected by the ratio of conidiated cadavers to healthy weevils ($P<0.05$) and over time ($P<0.05$) for both fungal species. No significant interaction between the ratio of the *B. bassiana* strain B27 conidiated cadavers and the measured times on the mortality of the healthy adults was found ($P=0.22$), but a significant interaction was observed between the ratio of the *M. anisopliae* strain ECS1 conidiated cadavers and the measured times ($P<0.05$).

The pairwise Wilcoxon rank-sum test for multiple comparisons showed that the highest ratio of B27 conidiated cadavers to healthy adults (1:1) caused the highest mortality to healthy adults across all measured times but mortality was significantly higher than at the two lowest ratios (1:10 and 1:20) only at 3 days and 6 days post-introduction (Fig. 2A, $P<0.05$). The highest ratio of ECS1 conidiated cadavers (1:1) caused the highest

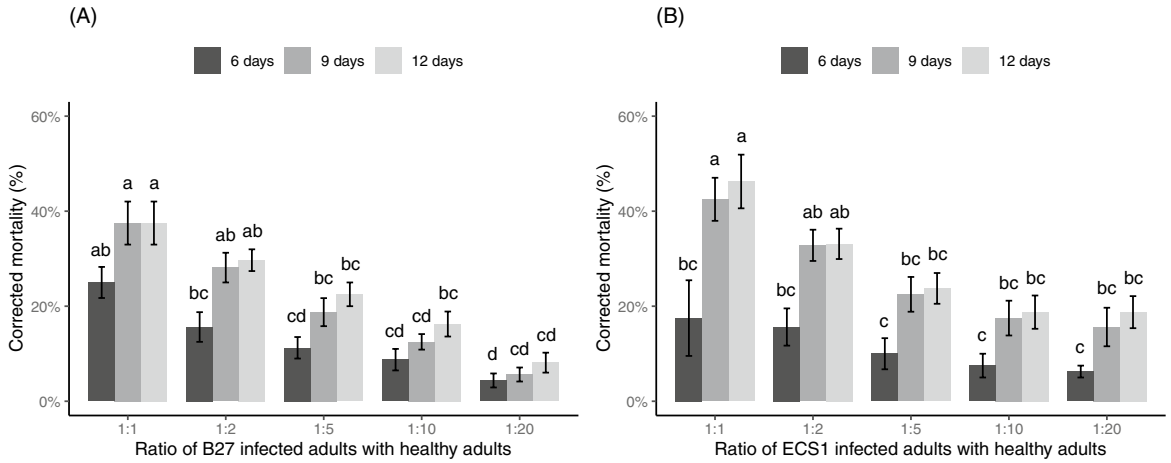


Figure 1. The mortality of healthy adult weevils at 6 days, 9 days and 12 days post-introduction of (**A**) B27 infected adults and (**B**) ECS1 infected adults at different ratios inside a 500 mL container. Results of multifactorial "F1-LD-F1" non-parametric analyses: (**A**) Wald-type statistics (WTS) = 126.13, df = 4, $P < 0.001$ (for ratios), WTS = 41.55, df = 2, $P < 0.001$ (for measured times), WTS = 42.19, df = 8, $P < 0.001$ (for interactions), (**B**) WTS = 34.48, df = 4, $P < 0.001$ (for ratios), WTS = 62.59, df = 2, $P < 0.001$ (for measured times), WTS = 8.31, df = 8, $P = 0.4$ (for interactions). Columns with different letters are significantly different from each other (pairwise Wilcoxon rank-sum test, $P < 0.05$). (**A**,**B**) were analysed separately. Error bars represent standard errors.

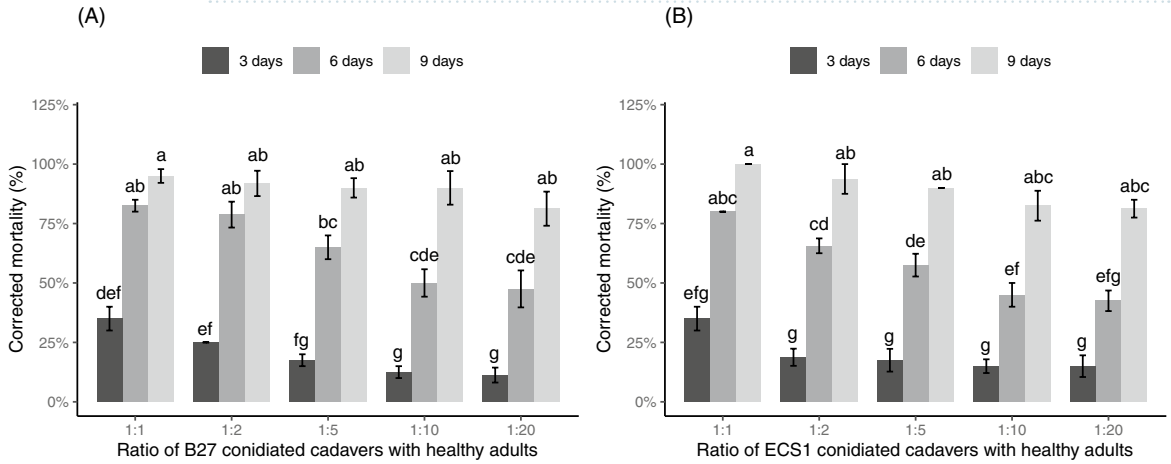


Figure 2. The mortality of healthy adult weevils at 3 days, 6 days and 9 days post-introduction of (**A**) B27 conidiated cadavers and (**B**) ECS1 conidiated cadavers at different ratios inside a 500 mL container. Results of multifactorial "F1-LD-F1" non-parametric analyses: (**A**) Wald-type statistics (WTS) = 47.57, df = 4, $P < 0.001$ (for ratios), WTS = 436.53, df = 2, $P < 0.001$ (for measured times), WTS = 10.64, df = 8, $P = 0.22$ (for interactions), (**B**) WTS = 93.85, df = 4, $P < 0.001$ (for ratios), WTS = 581.02, df = 2, $P < 0.001$ (for measured times), WTS = 24.45, df = 8, $P < 0.01$ (for interactions). Columns with different letters are significantly different from each other (pairwise Wilcoxon rank-sum test, $P < 0.05$). (**A**,**B**) were analysed separately. Error bars represent standard errors.

mortality to healthy adults at all measured times but in contrast to B27 mortality was significantly higher than the three lowest ratios only at 6 days post-introduction (Fig. 2B, $P < 0.05$).

Horizontal infection from conidiated cadavers to healthy adults in an insect cage. The mortality of healthy adults was significantly influenced by the ratio of the conidiated cadavers ($P < 0.05$), time ($P < 0.05$) and their interactions ($P < 0.05$) for both fungal species. The pairwise Wilcoxon rank-sum test for multiple comparisons showed that the highest ratio of *B. bassiana* strain B27 conidiated cadavers (ratio 1:1) caused the highest mortality to healthy adults across all measured times, and was significantly higher than the three lowest ratios (1:5, 1:10, 1:20) at 12 days and the two lowest ratios at 18 days post-introduction (Fig. 3A, $P < 0.05$). For *M. anisopliae* strain ECS1, the highest ratio of the conidiated cadavers (1:1) also caused the highest mortality to

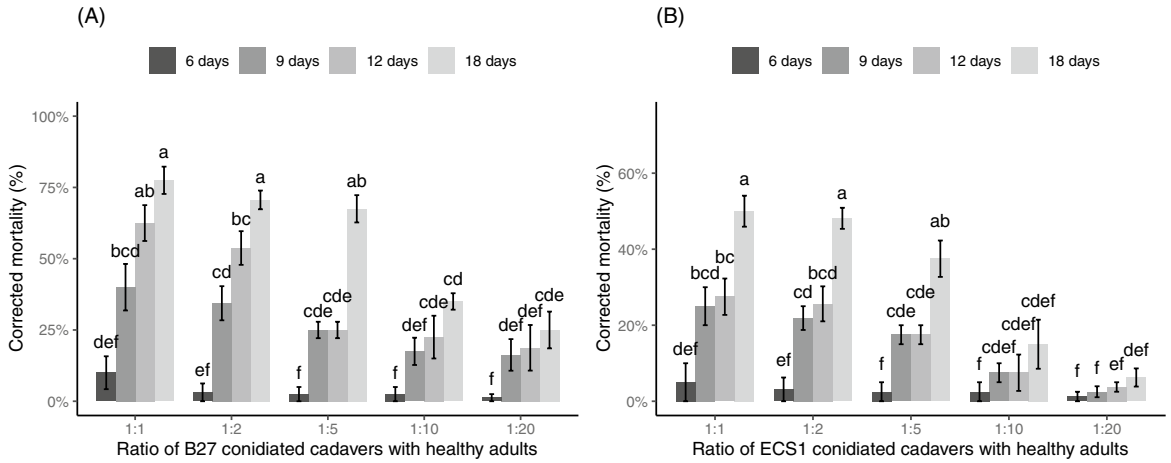


Figure 3. The mortality of healthy adult weevils at 6 days, 9 days 12 days and 18 days post-introduction of (**A**) B27 conidiated cadaver and (**B**) ECS1 conidiated cadaver at different ratios inside an insect cage. Results of multifactorial "F1-LD-F1" non-parametric analyses: (**A**) Wald-type statistics (WTS) = 45.42, df = 4, $P < 0.001$ (for ratios), WTS = 413.89, df = 3, $P < 0.001$ (for measured times), WTS = 72.66, df = 12, $P < 0.001$ (for interactions), (**B**) WTS = 135.16, df = 4, $P < 0.001$ (for ratios), WTS = 107.64, df = 3, $P < 0.001$ (for measured times), WTS = 97.11, df = 12, $P < 0.001$ (for interactions). Columns with different letters are significantly different from each other (pairwise Wilcoxon rank-sum test, $P < 0.05$). (**A**,**B**) were analysed separately. Error bars represent standard errors.

healthy adults across all measured times and was significantly higher than the ratio 1:20 at 9 days and 12 days and the two lowest ratios at 18 days post-introduction (Fig. 3B, $P < 0.05$).

Relationships between the proportion of fungus-infected adults or conidiated cadavers to the mortality of healthy adults at 9 days post-introduction. For *B. bassiana* strain B27, there were positive non-linear relationships between the mortality of healthy adults and the proportion of both fungus-infected adults and conidiated cadavers (Fig. 4A). Consistent responses of the adults to either B27 infected adults or B27 conidiated cadavers in either set of experimental conditions (500 mL containers and insect cages) were found, where the best models for the three different experiments were fitted with a two-parameter log-logistic model (LL.2, Fig. 4A). The curves of these three models did not have any inflection points and this suggested that the mortality of adults continued increasing when the proportion of conidiated cadavers or fungus-infected adults increased in the population.

For *M. anisopliae* strain ECS1, there were also positive non-linear relationships between the mortality of healthy adults and the proportion of fungus-infected adults or the conidiated cadavers (Fig. 4B). The responses of the adults to either ECS1 infected adults or ECS1 conidiated cadavers in the confined environment (500 mL containers) were the same and their relationships were fitted with two-parameter log-logistic models (LL.2, Fig. 4B). However, the relationship between the mortality of adults and ECS1 conidiated cadavers in the insect cage was better described with a three-parameter log-logistic model (LL.3, Fig. 4B). The curve of the LL.3 model suggested that adult mortality reached an inflection point when the proportion of ECS1 conidiated cadavers inside the cage reached 17% (ratio 1:5). Although mortality increased with the proportion of conidiated cadavers up to 50% (ratio 1:1), based on this model the mortality of healthy adults is not expected to increase to above 27.5%.

Scanning electron microscopy observation on the horizonal infection to healthy adults from conidiated cadavers in an insect cage. All examined adults (5 adults/cage) had fungal conidia attached to all parts of their bodies at all times for both fungal species (Fig. 5, 6, 7). The number of conidia attached to the head (Fig. 5) and legs (Fig. 6) were very high (more than 400 conidia per photo at 600 × magnification) compared to other parts of the body (less than 200 conidia per photo at the same magnification) (Fig. 7). Most of the B27 conidia that were attached to hairs of the tarsal pad, tibial comb and head (particularly the rostrum and eyes) started to germinate at 6 days post-introduction, whereas the germination of ECS1 conidia was delayed until 9 days post-introduction.

Discussion

In this study physical contact with fungus-infected adults caused low to moderate levels of mortality in initially healthy weevils. Even at the highest ratio of fungus-infected adults (1:1) in the population, the mortality of healthy adults at 12 days post-introduction was only 37.5% and 46.3%, for *B. bassiana* strain B27 and *M. anisopliae* strain ECS1, respectively. This low mortality indicates the importance of the form of the fungal conidia that are applied to the adults, with dry conidia showing improved performance relative to those sprayed as liquid formulations. Studies of conidial transmission in other insect species without the involvement of mating have shown that

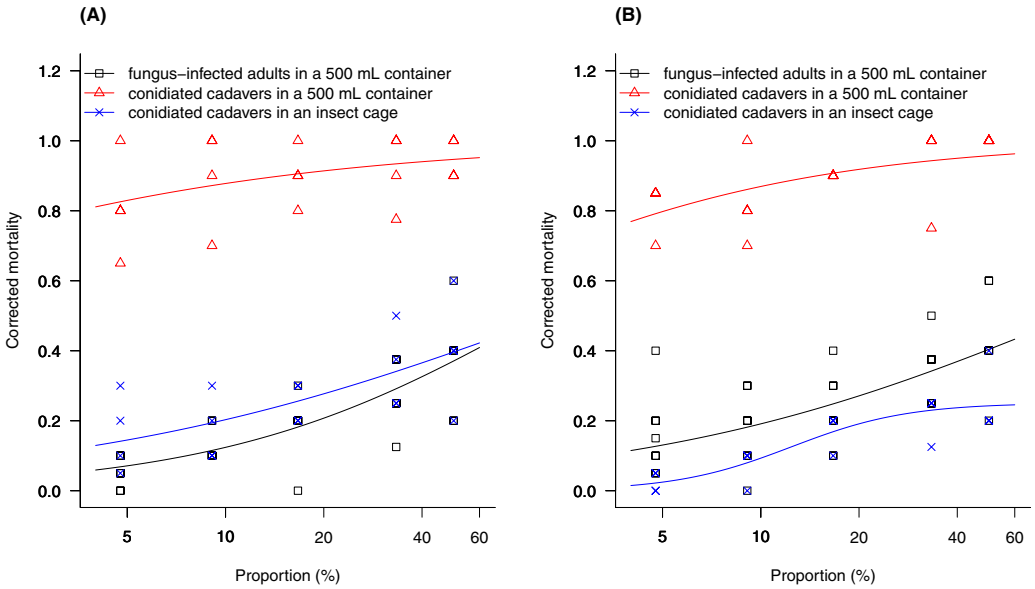


Figure 4. The non-linear relationship curves between the mortality of healthy adults at 9 days post-introduction and the proportion of (**A**) B27 or (**B**) ECS1 infected adults or conidiated cadavers in different experimental conditions. Individual symbol presents data of each replication and some were overlapped (bolded symbols) as shown in the figures. Models for (**A**): (Δ) $y = \frac{1}{1+\exp(-0.56*(\log(x)-\log(0.3)))}$, (×) $y = \frac{1}{1+\exp(-0.58*(\log(x)-\log(102.22)))}$ and (□) $y = \frac{1}{1+\exp(-0.88*(\log(x)-\log(90.84)))}$, Models for (B): (Δ) $y = \frac{1}{1+\exp(-0.75*(\log(x)-\log(0.81)))}$, (□) $y = \frac{1}{1+\exp(-0.65*(\log(x)-\log(90.64)))}$, and (×) $y = \frac{0.25}{1+\exp(-2.44*(\log(x)-\log(12.4)))}$.

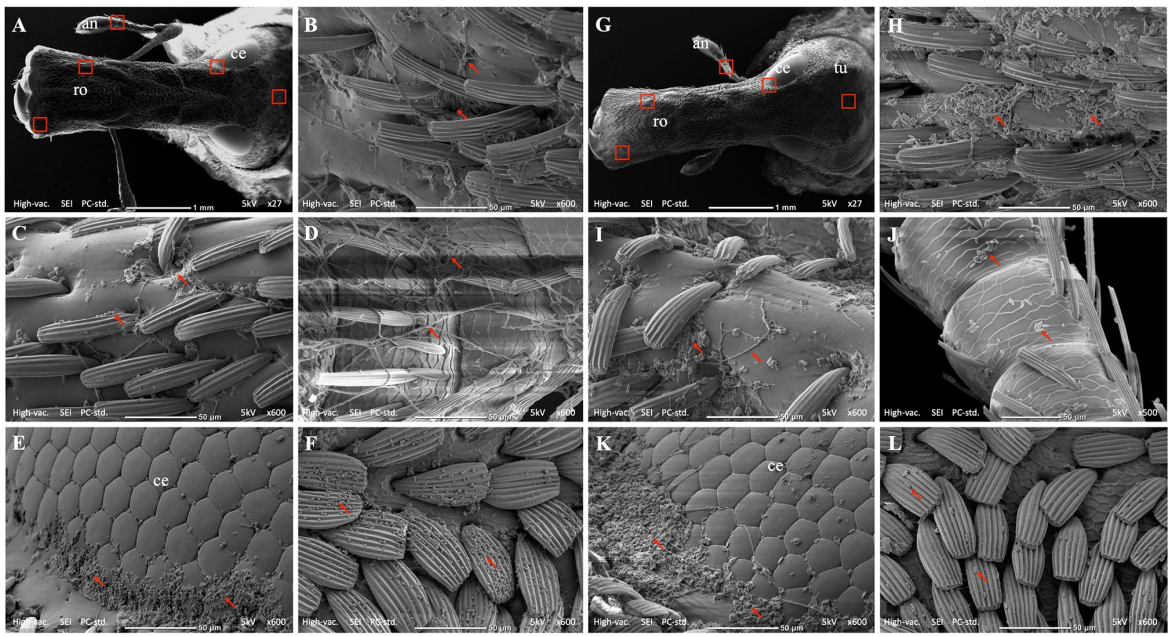


Figure 5. (**A**,**G**) Dorsal view of the head capsule showing antenna (an), compound eyes (ce), rostrum (ro) and tubercles (tu). Fungal conidia attached and/or germinated on (**B**,**H**) left side of rostrum, (**C**,**I**) right side of rostrum, (**D**,**J**) funicles of the antenna, (**E**,**K**) compound eyes and (**F**,**L**) scales on the head. The red arrows point at the conidia and/or the germinated conidia of B27 (**B**–**F**) and ECS1 (**H**–**L**); the red boxes in image A and G illustrates the parts of head capsule shown at higher magnification in images **B**–**F** and **H**–**L**, respectively. As there were numerous conidia either attached to, or germinated on the weevil, the arrows are used to indicate examples.

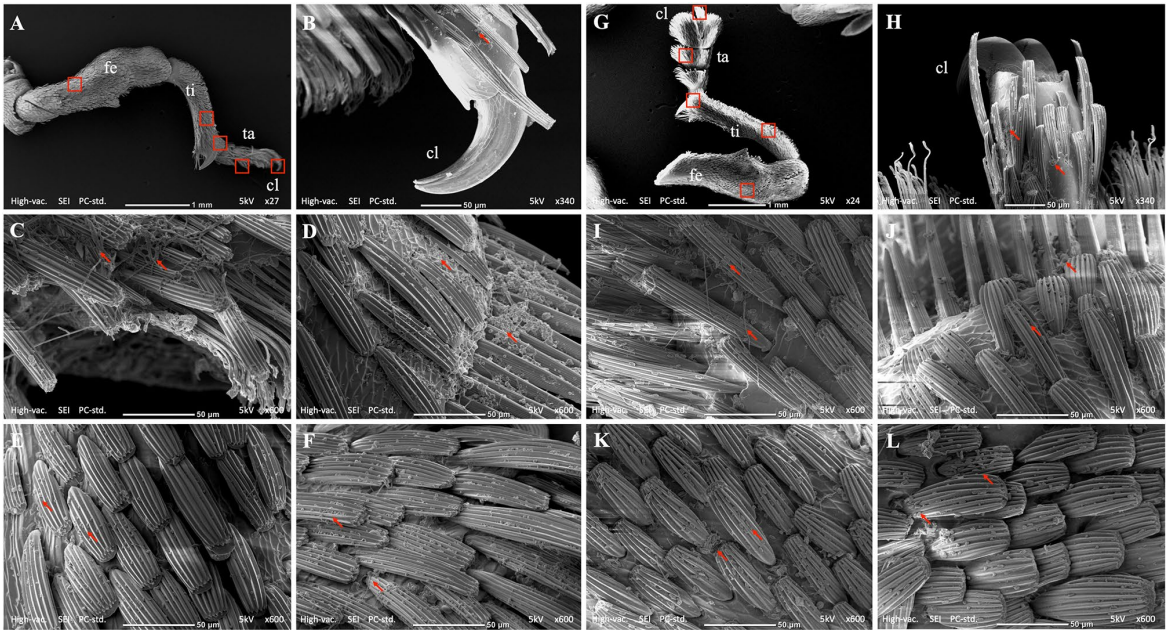


Figure 6. (**A**,**G**) Lateral view of the foreleg showing claws (cl), tarsus (ta), tibia (ti) and femur (fe). Fungal conidia attached and/or germinated on (**B**,**H**) claws, (**C**,**I**) tarsus, (**D**,**J**) tibial comb, (**E**,**K**) scales on the tibia and (**F**,**L**) scales on the femur. The red arrows point at the conidia and/or germinated conidia of B27 (**B**–**F**) and ECS1 (**H**–**L**); the red boxes in image A and G indicate the leg parts shown at higher magnification in images **B**–**F** and **H**–**L**, respectively. As there were numerous conidia either attached to, or germinated on the weevil, the arrows are used to indicate examples.

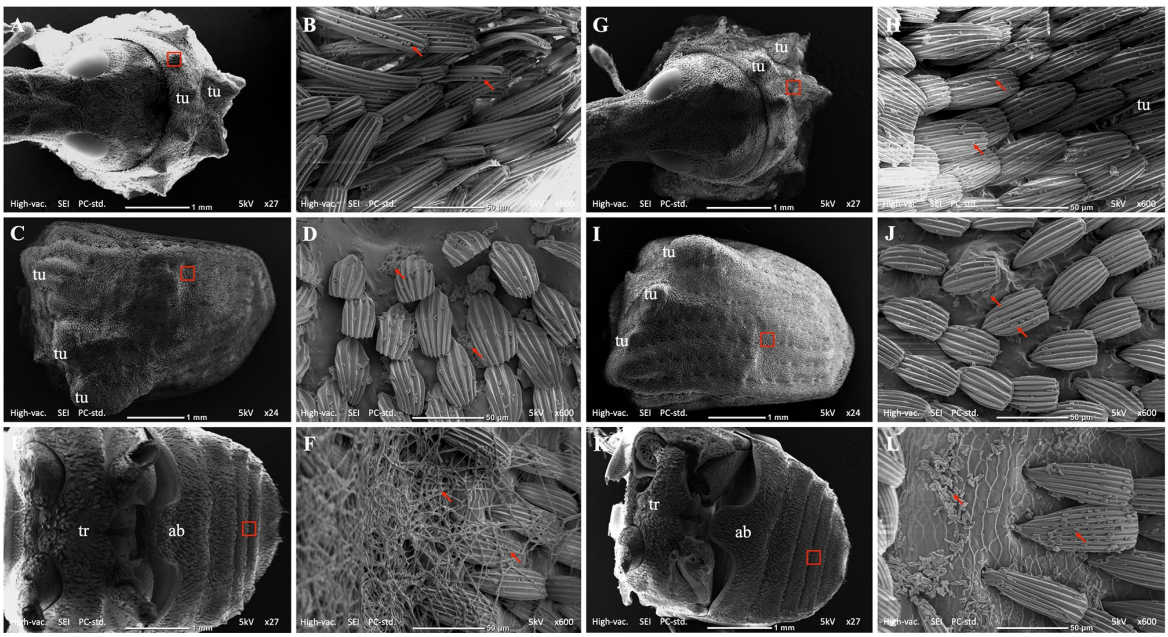


Figure 7. (**A**,**G**) Anterior dorsal view of the pronotum showing tubercles, (**B**,**H**) fungal conidia attached on scales around the tubercle, (**C**,**I**) Dorsal view of the elytron showing tubercles; (**D**,**J**) fungal conidia attached to the scales of the elytron, (**E**,**K**) Ventral view of the thorax (tr) and abdomen (ab), (**F**,**L**) fungal conidia germinated or attached to the scales of the abdomen. The red arrows point at the conidia or the germinated conidia of B27 (**B**,**D**,**F**) and ECS1 (**H**,**J**,**L**). As there were numerous conidia either attached to, or germinated on the weevil, the arrows are used to indicate examples. Red boxes indicate areas shown at higher magnification in other images.

adults carrying conidia in a dry form could deliver infective propagules easily and cause high mortality to the recipients[22,29]. More than 60% mortality of healthy Japanese beetle (*Popillia japonica* Newman) occurred after 9 days when adults infected with conidia of *M. anisopliae* or *B. bassiana* were introduced to healthy adults at the ratio of 1:3 inside an insect cage (50 × 50 × 50 cm)[22]. Similarly, around 77% mortality of initially healthy beet webworm moth (*Spoladea recurvalis* F.) was obtained after 10 days when moths infected with conidia of *M. anisopliae* were introduced to healthy moths at the ratio 1:1 inside an insect cage (30 × 30 × 30 cm)[29]. Although dried conidia appear to be effectively transmitted and are capable of causing high mortality to the recipients[22,29], the application of dried conidia onto crops in the field may not be viable unless they are combined with attractants of some sort. The integration of dried *B. bassiana* conidia with the sex pheromone for *C. formicarius* showed that high mortality of adults (> 90%) can be obtained in the field after three weeks of trap deployment[30]. Similarly, black vine weevil, *Otiorhynchus sulcatus* F., can be successfully controlled in the field by integrating an attractant ((Z)-2-pentenol + methyl eugenol) with *B. bassiana*[31]. Dry fungal entomopathogens have also been integrated with other attractants such as aggregation pheromones for controlling other weevil species including banana weevil, *Cosmopolites sordidus* Germar[32–34] and red palm weevil, *Rhynchophorus ferrugineus* Olivier[35,36].

Although some studies have shown that dried conidia can be highly effective for fungal disease transmission[22,29], the dried conidia may also be easily removed by grooming behaviour or in the environment, and the efficacy of dried conidia may be greatest when infected adults are introduced to healthy weevils before the loss of conidia occurs. This was demonstrated in a study on *P. japonica* where the 9 day mortality fell from over 60 to 40% when exposure of fungus-infected adults to healthy beetles was delayed for 24 h[22].

Earlier studies have suggested that conidia formed on cadavers could be a potential inoculum source and could readily deliver ongoing inoculum to the pest population[17,25–28]. In our study we tested the potential of conidiated cadavers to control live adults under two sets of experimental conditions. In the confined environment experiment we observed close physical contact between adults and conidiated cadavers and consequently high mortality was observed, around 95% (B27) and 100% (ECS1) at the 1:1 ratio and around 81% (B27 and ECS1) at the 1:20 ratio at 9 days post-introduction. However, high mortality of adults exposed to the same treatments was not observed in the insect cage experiment. Even at 12 days post-introduction, the mortality of adults inside the insect cage was only 62.5% (B27) and 27.5% (ECS1) at the ratio 1:1 and 18.8% (B27) and 3.8% (ECS1) at the ratio 1:20. Clearly these differences could be the result of the disparity in volume of the space being occupied by the insects (148 times greater in the cage), affecting the frequency and duration of contact between healthy, infected and dead weevils and hence the transmission of conidia. Differences in relative humidity (RH) in the test environments may also be involved.

A similar study to ours found that the corrected mortality of healthy *D. citri* at 10 days post-introduction to *B. bassiana* conidiated cadavers at the ratios 1:2 and 1:20 in 500 mL containers was around 70% and 39% respectively, whereas under field conditions the corresponding mortalities of initially healthy *D. citri* were reduced to only 48% and 17%[26]. The response of *D. citri* to *Isaria fumosorosea* Wize conidiated cadavers was also evaluated where the corrected mortality was around 56% and 24% at the ratios of 1:2 and 1:20 respectively in 500 mL containers and 47% and 7% respectively in the field[26].

Relative humidity (RH) is a major factor influencing the successful use of fungal entomopathogens as pest control agents[9,10]. Our insect cages were maintained at 56% RH in the insectary, whereas the Conviron A1000 growth chamber used to house the 500 mL containers was maintained at 65% RH. Some studies have shown that slight increases RH can improve the activity of fungal entomopathogens on their hosts[37–40]. By increasing RH from 50 to 70% mortality of the coffee berry borer *Hypothenemus hampei* Ferrari previously infected with *B. bassiana* at 1×10^6 conidia/mL increased from 69 to 87%[40]. By increasing RH from 60 to 70% mortality of apple rust mite (*Aculus schlechtendali* Nal.) increased from 39 to 53% after treatment with *Paecilomyces lilacinus* (Thom) Samson at 1×10^5 conidia/mL and from 76 to 89% after treatment at 1×10^8 conidia/mL[39].

Although the mortality of weevils inside the insect cages was not as high as in the 500 mL containers, there was clear evidence that after 12 days of the experiment live adults all had attached fungal conidia from the cadavers. At this time all live adults from the insect cages were incubated at high humidity (> 95%) for 24 h. The mortality of initially healthy adults 5 days later increased to around 77.5% (for B27) and 50% (for ECS1) at the ratio 1:1 and around 25% (for B27) and 6.25% (for ECS1) at the ratio 1:20 (Fig. 3). SEM evidence showed that at the 1:5 ratio all adults had physical contact with conidiated cadavers based on the high number of conidia on hairs on the tarsal pad and tibial comb (Fig. 6). These infected adults subsequently contacted other adults, as shown by the conidia found on the elytra and pronota of other individuals (Fig. 7). High densities of conidia were also found on the compound eyes and rostrums (Fig. 5), suggesting that infected adults used their forelegs to which conidia are attached to groom these body parts. Overall, our results suggest that while the weevils inside the cage had numerous attached conidia acquired via physical contact with conidiated cadavers or fungus-infected adults, the conidia could not germinate and infect adults quickly when the RH was below a certain level.

Between *B. bassiana* strain B27 and *M. anisopliae* strain ECS1, we often found that the conidia of B27 germinated on the weevil's cuticle at 6 days post-introduction whereas ECS1 conidia germinated at 9 days. This suggests that *M. anisopliae* conidia may be more sensitive to low RH than those of *B. bassiana*. Supporting this theory, an earlier study found that conidia of all tested strains of *B. bassiana* germinated faster and with higher total percentage germination than most strains of *M. anisopliae* when the incubation conditions were unfavourable (water activity was around 0.93 a_w)[41]. When the water activity was high (> 0.99 a_w) the conidia of most strains of *M. anisopliae* germinated faster and with higher total percentage germination than strains of *B. bassiana*[41].

In this study adult weevils did not show avoidance behaviour towards conidiated cadavers killed by either *B. bassiana* or *M. anisopliae*. This is supported by the results of the SEM investigation where fungal conidia were found on all the specimens examined, and is similar to the results of other studies, where coleopteran species showed no avoidance behaviour toward *B. bassiana*[27,28,42]. However, our results contrast with some studies where coleopterans showed avoidance behaviour toward both *M. anisopliae*[43–45] and *B. bassiana*[46]. A recent study has

shown that *M. anisopliae* is able to produce volatile organic compounds (1-octen-3-ol, 2-octen-1-ol, 3-octanol, 3-octanone) and acetic acid. These compounds act as repellents for many insect species such as *C. formicarius*[43], *P. japonica*[44] and groundnut bruchid, *Caryedon serratus* Olivier[45]. *Beauveria bassiana* however, is not capable of producing these compounds[47]. The reported deterrent effect of *B. bassiana* on *O. sulcatus* could be the result of formulation additives rather than the entomopathogen itself[46]. A study similar to ours showed that the presence of *B. bassiana* conidiated cadavers on the topsoil may result in horizontal infection to *L. decemlineata* in open environments[27]. The adults of *L. decemlineata* did not show any avoidance behaviour toward the cadavers and they tended to have higher infection levels when the number of conidiated cadavers on the topsoil was increased[27]. Our results also show that the number of adults infected by *B. bassiana* can be increased by increasing the proportion of cadavers on the seedlings relative to the number of healthy weevils present (Fig. 4A). The lack of avoidance behaviour in our study could be due to the production of only non-repellent volatiles by strain ECS1, the complete absence of volatile production in this strain, or the dilution of volatiles by increased air movement in the insect cages. Further work could investigate volatile production by ECS1 and incorporate olfactometer studies to further assess the effect of any volatiles produced on *K. macadamiae* behaviour under controlled conditions.

In this study we have demonstrated that fungal entomopathogens could provide an additional means of sustainable control of adult weevils through horizontal transmission from fungus-infected adults to healthy adults and horizontal infection arising as a consequence of physical contact with conidiated cadavers. During the period of *K. macadamiae* activity between September and December temperatures are < 27 °C with RH of 65–75% (Supplementary Fig. 1)[48], suggesting that the entomopathogens could be very effective in the orchard at this time. We believe that the microclimate in the macadamia orchards is more suitable for the fungal entomopathogens than indicated by the data from the nearest meteorology station (lower temperatures and higher RH). This assumption is based on the thick foliage and dense shade within the canopies of mature macadamia trees. As weather conditions in the Northern Rivers are ideal for the persistence of entomopathogenic fungi, the two strains used in this study are strong candidates for macadamia seed weevil control. Additional research is required to optimise biopesticide formulations to best suit application to tree crops and enhance fungal persistence, and to develop an attract-and-infect technique for field use.

Materials and methods

Insects and seedlings. *Kuschelorhynchus macadamiae* cannot currently be reared on artificial media, therefore weevil infested nuts were collected at 2 week intervals from three locations (28° 51′ 12″ S 153° 27′ 37″ E, 28° 48′ 27″ S 153° 25′ 23″ E and 28° 52′ 07″ S 153° 24′ 06″ E) between October and December 2018/2019 in the Northern Rivers. More than 9400 infested nuts were collected from these locations. The weevils were obtained from the infested nuts and fed as described in our previous study[14].

Macadamia seedlings (approximately 30 cm in height, 4-months old, variety H2) for the studies were purchased from Next Block Nursery, Fernleigh, NSW. The seedlings were placed in the glasshouse (26 ± 1 °C and 54 ± 1% RH in the day and 21 ± 1 °C and 65 ± 1% RH at night) for at least 4 weeks before experimentation.

Fungi. In this study two fungal strains were used, ECS1 (*M. anisopliae*) and B27 (*B. bassiana*). These strains have been lodged in the Queensland Plant Pathology Herbarium, Department of Agriculture and Fisheries, Brisbane, with accession numbers BRIP 70,272 (ECS1) and BRIP 70,267 (B27). Strain ECS1 was cultured on sterile Sabouraud dextrose agar supplemented with 1% (w/v) yeast extract (SDAY)[49] and strain B27 was cultured on sterile malt extract agar (MEA) media[49]. All fungal strains were incubated in the dark at 25 ± 1 °C for 15 days before harvesting the conidia for experimentation.

Conidial suspensions of both fungal strains were prepared by scraping the surface of the conidiated cultures with a sterile spatula and suspending the inoculum in 10 mL of sterile Tween 20 (0.05% v/v in distilled water) in a 50 mL centrifuge tube (Labtek Pty Ltd, Brendale, Queensland). The suspensions were homogenised by vortexing for 5 min and the conidial concentrations were determined using a haemocytometer (Laboroptik Ltd, Lancing, UK) and an Olympus BX53 compound microscope (400x) equipped with a digital camera (Model DP74, Olympus Australia Pty Ltd, Macquarie Park, NSW). Conidia concentrations were then adjusted to LC_{95} levels; 2.49×10^7 conidia/mL and 4.64×10^7 conidia/mL for ECS1 and B27, respectively[14]. The germination of both fungal species was checked before experimentation and was always > 90%. The conidia were considered to have germinated when the germ-tubes were twice the diameter of the conidia[50].

Obtaining conidiated cadavers and conidia quantification. A group of ten mixed-sex adults was randomly collected from the insectary and placed in a 500 mL plastic container (9.5 cm diameter and height) with small ventilation holes (2 mm diameter) in the lid of each container. Prior to spray applications of the entomopathogens, all containers were chilled at 4 °C for 15 min to reduce weevil mobility. Each container was then opened and sprayed with 1 mL of LC_{95} conidial suspension using an X-Press It micro-atomiser (X-Press Graph-X Pty Ltd, Moorabbin, Victoria) calibrated to deposit approximately $4 \times 10^4 \pm 9 \times 10^3$ conidia/cm^2 and $7.3 \times 10^4 \pm 1.6 \times 10^4$ conidia/cm^2 for ECS1 and B27, respectively. After spraying, each container received a single macadamia nut and was incubated at high humidity (> 95%) in darkness for 24 h, followed by incubation at 25 ± 1 °C, 65 ± 3% RH with a 16L:8D photoperiod in a Conviron A1000 growth chamber (Conviron Asia Pacific Pty Ltd, Melbourne, Victoria). Each container was provided with a new macadamia nut (nut in husk) every second day for 12 days, and all dead weevils were removed and placed in Petri plates containing filter paper dampened with sterile distilled water and sealed with Parafilm. These plates were incubated in the dark at 25 ± 1 °C for 7 days to stimulate conidiation. Two separate containers of insects were sprayed, each with one of the fungal strains, and this was repeated 8 times (at 3-day intervals).

Treatments	Bioassay I		Bioassays II & III	
	Donors[a]	Recipients	Donors[b]	Recipients
Control	–	10	–	10
1:1	5	5	5	5
1:2	4	8	4	8
1:5	2	10	2	10
1:10	1	10	1	10
1:20	1	20	1	20
Positive control	12	–	–	–

Table 1. Summary of treatments used in bioassays on *Kuschelorhynchus macadamiae* in the laboratory. [a]The number of fungus-infected adults used as donors in the experiment. The fungus-infected adults were initially painted with permanent red pen ink and infected with fungal suspension at their LC_{95} conidial concentrations, followed by high humidity incubation (> 95% RH) for 24 h before being introduced to the recipients. [b]The number of conidiated cadavers used as the donors in the experiments.

The conidiated cadavers from each spraying were assessed for conidial production. After 7 days of incubation, a conidiated cadaver was randomly selected from the Petri plates, dried in an oven at 35 °C for 30 min, and transferred into separate 2 mL centrifuge tube containing 1 mL of sterile Tween 20 (0.05% v/v)[14,51]. To quantify the number of conidia per cadaver, each 2 mL centrifuge tube was vortexed for 5 min to dislodge conidia from the conidiated cadaver, and then the conidia were counted using a haemocytometer and an Olympus BX53 compound microscope (400x).

Horizontal transmission from fungus-infected adults to healthy adults. In this experiment we examined the effect of inoculum transfer from fungus-infected adults which served as donors to healthy adults which served as recipients and determined how infection rates were driven by the proportion of the donors relative to the recipients. To confirm the potential of inoculum transfer, seven treatments were used for each fungal species (Table 1: Bioassay I). Donor weevils were marked on their elytra or pronotum with permanent red pen which was allowed to dry for 1 h so they could be easily differentiated from recipients. Three separate containers which each contained 10 marked weevils were sprayed with 1 mL of each fungal strain at the LC_{95} conidial concentration using an X-Press It micro-atomiser, fed a macadamia nut and incubated at > 95% RH for 24 h before further experimentation in each replicate. All donors were introduced to groups of recipients in a 500 mL container according to their ratios. All containers were incubated as previously described in a Conviron A1000 growth chamber. All insects were fed as described above for 12 days and dead weevils were removed daily and verified for fungal infection as previously described. This experiment was replicated eight times (at 3-day intervals) and a total of 1408 insects were used (704 adults for each fungal species).

Horizontal infection from conidiated cadavers to healthy adults in a confined environment. In this study we examined the potential for conidia transfer from conidiated cadavers to healthy adults. To confirm conidial transfer, a control and five different ratios of conidiated cadavers and healthy adults were used for each fungal species (Table 1: Bioassay II). For each ratio, healthy adults and conidiated cadavers were placed in a 500 mL container. All insects were incubated and fed as described in the previous experiment for 9 days. Dead weevils were removed daily and verified for fungal infection as described in the previous experiment. This experiment was replicated four times (at 3-day intervals) and a total of 504 healthy adults were used (252 adults for each fungal species).

Horizontal infection from conidiated cadavers to healthy adults in an insect cage. A macadamia seedling was placed inside a Bugdorm insect rearing cage (32.5 × 32.5 × 70 cm, Australian Entomological Supplies Pty Ltd, South Murwillumbah, NSW) inside the insectary (25 ± 1 °C, 56 ± 1% RH and 16L:8D photoperiod). Conidiated cadavers killed by ECS1 or B27 were placed on the macadamia leaves (the 2nd to 5th leaves counted from the 1st bottom leave) at different ratios (Table 1: Bioassay III) without the use of pins or adhesives. After 1 h the required number of healthy adults were released into the insect cages. Dead weevils were removed daily for 12 days and verified for fungal infection as described in the previous experiment. As adult weevils killed the seedling by defoliation and ring barking after 12 days, live adults were then transferred to 500 mL plastic containers, incubated at high humidity (> 95%) in the darkness for 24 h, followed by incubation in the insectary. Weevils in each container were provided with a new macadamia nut every second day for another 5 days. This experiment was replicated 4 times (at 3-day intervals) and a total of 504 initially healthy adults were used (252 adults for each fungal species).

Scanning electron microscopy observations on the horizontal infection of healthy adults from conidiated cadavers in insect cages. In the scanning electron microscopy (SEM) studies our aim was to identify the external body parts of the adults which had come into contact with conidiated cadavers on a macadamia seedling. Two conidiated cadavers (ECS1 or B27) were placed on two macadamia leaves (between the 2nd and 5th leaves counted from the 1st bottom leaf) of a seedling previously placed inside a Bugdorm insect

rearing cage (32.5 × 32.5 × 70 cm). After 1 h, ten adults were released inside the insect cage. Four insect cages for each fungal species were used and assigned for the post release periods of 3, 6, 9 and 12 days. In total, 80 adults were used, 40 adults for each fungal species. All the insect rearing cages were maintained in the insectary for the duration of the experiment. After 3, 6, 9 and 12 days post release, all adults in each assigned cage were collected and directly fixed in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and stored at 4 °C. Five of ten fixed insects from each assigned cage were randomly selected and rinsed three times (10 min each) in 0.05 M phosphate buffer (pH 7.3). The samples were then dehydrated through a graded ethanol series (35%, 50%, 75%, 95% and 100% ethanol) with 15 min at each step. The samples were further processed using a Autosamdri 815 series A critical point dryer (Tousimis, Rockville, MD, USA) before being mounted on stubs (25 mm diameter, ProScitech Pty Ltd, Thuringowa, Queensland) using double sided carbon tape (25 mm diameter, ProScitech Pty Ltd) and then sputter coated with gold for 1 min. Specimens were examined with a SEM Neoscope JCM-6000 (JEOL Australiasia Pty Ltd, Frenchs Forest, NSW). The number of conidia was estimated from five photos of each body part at 600 × magnification.

Statistical analysis. All analyses were performed using RStudio[52] Version 1.2.1335. built on R[53] Version 3.5.2. Before analyses the mortality of healthy adults was corrected using Abbott's formula[54] and the mortalities in the corresponding controls. Corrected data were assessed using the Shapiro–Wilk Test for normality[55] and Levene's Test for homogeneity of variance using the CAR (Companion to Applied Regression, Version 3.0-3) package[56].

As the data could not be normalised by transformation, a non-parametric analysis of variance was performed. The multifactorial "F1-LD-F1" non-parametric analysis of longitudinal data in factorial experiments was used to analyse the corrected mortality of healthy adults (recipients) caused by different ratios of fungus-infected adults or conidiated cadavers (donors) over 3 repeated measures in bioassay I (6 days, 9 days and 12 days post-introduction), 3 repeated measures in bioassay II (3 days, 6 days and 9 days post-introduction) and 4 repeated measures in bioassay III (6 days, 9 days 12 days and 18 days post-introduction). Wald-type statistics (WTS) were calculated using the nparLD (Nonparametric analysis of Longitudinal Data, Version 2.1) package[57] to check for significant effects of the ratios, repeated measures and/or their interactions ($P < 0.05$), and the pairwise Wilcoxon rank-sum test was used to separate means. The datasets for B27 and ECS1 were analysed separately. The ggplot2 (Grammar of Graphics, Version 3.2.1) package was used to generate the figures[58].

Since the datasets at 9 days post-introduction were available for all bioassays, the relationships between the proportion of fungus-infected adults or conidiated cadavers and the mortality of healthy adults were determined. The relationships were analysed with functions *drm()* and *mselect()* of the DRC (Dose–Response Curves, Version 3.0-1) package[59] in order to find the best fitted models by comparing the log-likelihood values, Akaike's Information Criteria (AIC), lack of fit and residual variance of all models was evaluated against linear, quadratic and cubic regression models. All datasets were fitted to the non-linear 2-parameter log-logistic model (LL.2), $y = \frac{1}{1+\exp(b*(\log(x)-\log(e)))}$, except the dataset for adult mortality caused by ECS1 cadavers inside insect cages which was fitted to the non-linear 3-parameter log-logistic model (LL.3), $y = \frac{d}{1+\exp(b*(\log(x)-\log(e)))}$. For both models, d is the upper limit, b is the slope, e is the median effective pressure (EP50) and x is the proportion of cadavers or donor adults.

Data availability

All raw and processed data for this study are provided as a supplementary file.

Received: 25 November 2020; Accepted: 8 January 2021
Published online: 26 January 2021

References

1. Jennings, D. & Oberprieler, R. G. A review of the Tribe Cryptoplini (Coleoptera: Curculioninae), with revision of the Genus *Menechirus* Hartmann, 1901 and description of a new Genus associated with *Macadamia*. *Diversity* **10**, 71. https://doi.org/10.3390/d10030071 (2018).
2. Fay, H. A. C., De Faveri, S. G., Storey, R. I. & Watson, J. *Sigastus* weevil—an emerging pest of macadamias in north Queensland. *Proceedings of the 6th Workshop for Tropical Agricultural Entomology*, Darwin, Australia, 11–15 May 1998. pp. 137–140 (2001)
3. Bright, J. *Sigastus* weevil update. Part 1. Life cycle and monitoring keys to control. Australian Macadamia Society Ltd, Lismore, Australia (2017). [Retrieved from: https://www.horticulture.com.au/globalassets/hort-innovation/resource-assets/mc-ipm-program-sigastus-weevil-fact-sheet.pdf. Accessed 4 August, 2017]
4. Bright, J. Macadamia seed weevil (*Kuschelorhynchus macadamiae*) life cycle and monitoring. Primefact 1586, Wollongbar, Australia (2017). [Retrieved from: https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/731982/Macadamia-seed-weevil-update-lifecycle_2.pdf. Accessed 15 August, 2017]
5. Lee, S. *Sigastus* weevil. The Nutshell - MPC's newsletter for macadamia growers, Lismore, Australia (2014). [Retrieved from: https://mpcmacs.com.au/media/Nutshell-May-2014.pdf. Accessed 16 August, 2017]
6. Huwer, R. Ecology and management of *Sigastus* weevil in macadamias. Horticulture Innovation Australia Limited, Sydney, Australia (2016). [Retrieved from: https://www.horticulture.com.au/globalassets/laserfiche/assets/project-reports/mc15010/mc15010-final-report-514.pdf. Accessed 23 March, 2019]
7. Rehner, S. A. & Buckley, E. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: Evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* **97**, 84–98. https://doi.org/10.1080/15572536.2006.11832842 (2005).
8. Roberts, D. W. & Leger, R. J. *Metarhizium* spp, cosmopolitan insect-pathogenic fungi: Mycological aspects. In *Adv Appl Microbiol* Vol. 54 (eds Laskin, A. I. *et al.*) 1–70 (Elsevier Academic Press Inc, Amsterdam, 2004).
9. Zimmermann, G. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Sci. Technol.* **17**, 553–596. https://doi.org/10.1080/09583150701309006 (2007).
10. Zimmermann, G. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Technol.* **17**, 879–920. https://doi.org/10.1080/09583150701593963 (2007).

11. Dolinski, C. & Lacey, L. A. Microbial control of arthropod pests of tropical tree fruits. *Neotrop. Entomol.* **36**, 161–179. https://doi.org/10.1590/s1519-566x2007000200001 (2007).
12. Lacey, L. A. & Shapiro-Ilan, D. I. Microbial control of insect pests in temperate orchard systems: Potential for incorporation into IPM. *Annu. Rev. Entomol.* **53**, 121–144. https://doi.org/10.1146/annurev.ento.53.103106.093419 (2008).
13. Khun, K. K., Wilson, B. A. L., Stevens, M. M., Huwer, R. K. & Ash, G. J. Integration of entomopathogenic fungi into IPM programs: Studies involving weevils (Coleoptera: Curculionoidea) affecting horticultural crops. *Insects* **11**, 659. https://doi.org/10.3390/insects11100659 (2020).
14. Khun, K. K., Ash, G. J., Stevens, M. M., Huwer, R. K. & Wilson, B. A. L. Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays. *J. Invertebr. Pathol.* **174**, 107437. https://doi.org/10.1016/j.jip.2020.107437 (2020).
15. Meyling, N. V. & Eilenberg, J. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biol. Control* **43**, 145–155. https://doi.org/10.1016/j.biocontrol.2007.07.007 (2007).
16. Pell, J. K., Hannam, J. J. & Steinkraus, D. C. Conservation biological control using fungal entomopathogens. *Biocontrol* **55**, 187–198. https://doi.org/10.1007/s10526-009-9245-6 (2010).
17. Dotaona, R., Wilson, B. A. L., Stevens, M. M., Holloway, J. & Ash, G. J. Chronic effects and horizontal transmission of *Metarhizium anisopliae* strain QS155 infection in the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Biol. Control* **114**, 24–29. https://doi.org/10.1016/j.biocontrol.2017.07.008 (2017).
18. Kocacevik, S., Sevim, A., Eroglu, M., Demirbag, Z. & Demir, I. Virulence and horizontal transmission of *Beauveria pseudobassiana* S.A Rehner & Humber in *Ips sexdentatus* and *Ips typographus* (Coleoptera: Curculionidae). *Turk. J. Agric. For.* **40**, 241–248. https://doi.org/10.3906/tar-1504-64 (2016).
19. Lopes, R. B. *et al.* Virulence and horizontal transmission of selected Brazilian strains of *Beauveria bassiana* against *Cosmopolites sordidus* under laboratory conditions. *Bull. Insectol.* **64**, 201–208 (2011).
20. Ugine, T. A., Peters, K. E., Gardescu, S. & Hajek, A. E. The effect of time postexposure and sex on the horizontal transmission of *Metarhizium brunneum* conidia between Asian longhorned beetle (Coleoptera: Cerambycidae) mates. *Environ. Entomol.* **43**, 1552–1560. https://doi.org/10.1603/EN14116 (2014).
21. Dembilio, Ó., Quesada-Moraga, E., Santiago-Álvarez, C. & Jacas, J. A. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil *Rhynchophorus ferrugineus*. *J. Invertebr. Pathol.* **104**, 214–221. https://doi.org/10.1016/j.jip.2010.04.006 (2010).
22. Lacey, L. A., Martins, A. & Ribeiro, C. The pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* for adults of the Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae). *Eur. J. Entomol.* **91**, 313–319 (1994).
23. Hajek, A. E. & Leger, R. J. Interactions between fungal pathogens and insect hosts. *Annu. Rev. Entomol.* **39**, 293–322. https://doi.org/10.1146/annurev.en.39.010194.001453 (1994).
24. Daoust, R. A. & Pereira, R. M. Survival of *Beauveria bassiana* (Deuteromycetes, Moniliales) conidia on cadavers of cowpea pests stored outdoors and in laboratory in Brazil. *Environ. Entomol.* **15**, 642–647. https://doi.org/10.1093/ee/15.3.642 (1986).
25. Furlong, M. J. & Pell, J. K. Horizontal transmission of entomopathogenic fungi by the diamondback moth. *Biol. Control* **22**, 288–299. https://doi.org/10.1006/bcon.2001.0981 (2001).
26. Conceschi, M. R., D'Alessandro, C. P., Moral, R. D., Demétrio, C. G. B. & Delalibera, I. Transmission potential of the entomopathogenic fungi *Isaria fumosorosea* and *Beauveria bassiana* from sporulated cadavers of *Diaphorina citri* and *Toxoptera citricida* to uninfected *D. citri* adults. *Biocontrol* **61**, 567–577. https://doi.org/10.1007/s10526-016-9733-4 (2016).
27. Klinger, E., Groden, E. & Drummond, F. *Beauveria bassiana* horizontal infection between cadavers and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Environ. Entomol.* **35**, 992–1000. https://doi.org/10.1603/0046-225x-35.4.992 (2006).
28. Long, D. W., Groden, E. & Drummond, F. A. Horizontal transmission of *Beauveria bassiana* (Bals.) Vuill. *Agric. For. Entomol.* **2**, 11–17. https://doi.org/10.1046/j.1461-9563.2000.00046.x (2000).
29. Opisa, S., du Plessis, H., Akutse, K. S., Fiaboe, K. K. M. & Ekesi, S. Horizontal transmission of *Metarhizium anisopliae* between *Spoladea recurvalis* (Lepidoptera: Crambidae) adults and compatibility of the fungus with the attractant phenylacetaldehyde. *Microb. Pathog.* **131**, 197–204. https://doi.org/10.1016/j.micpath.2019.04.010 (2019).
30. Yasuda, K. Auto-infection system for the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Curculionidae) with entomopathogenic fungi, *Beauveria bassiana* using a modified sex pheromone trap in the field. *Appl. Entomol. Zool.* **34**, 501–505. https://doi.org/10.1303/aez.34.501 (1999).
31. van Tol, R. W. H. M., Elberse, I. A. M. & Bruck, D. J. Development of a refuge-kairomone device for monitoring and control of the vine weevil, *Otiorhynchus sulcatus*, by lure-and-kill and lure-and-infect. *Crop Prot.* **129**, 105045. https://doi.org/10.1016/j.cropro.2019.105045 (2020).
32. Tinzaara, W. *et al.* The use of aggregation pheromone to enhance dissemination of *Beauveria bassiana* for the control of the banana weevil in Uganda. *Biocontrol Sci. Technol.* **17**, 111–124. https://doi.org/10.1080/09583150600937089 (2007).
33. Lopes, R. B., Laumann, R. A., Moore, D., Oliveira, M. W. M. & Faria, M. Combination of the fungus *Beauveria bassiana* and pheromone in an attract-and-kill strategy against the banana weevil, *Cosmopolites sordidus*. *Entomol. Exp. Appl.* **151**, 75–85. https://doi.org/10.1111/eea.12171 (2014).
34. Tinzaara, W. *et al.* Enhancing dissemination of *Beauveria bassiana* with host plant base incision trap for the management of the banana weevil *Cosmopolites sordidus*. *Afr. J. Agric. Res.* **10**, 3878–3884. https://doi.org/10.5897/AJAR2015.9882 (2015).
35. Hajjar, M. J., Ajlan, A. M. & Al-Ahmad, M. H. New approach of *Beauveria bassiana* to control the red palm weevil (Coleoptera: Curculionidae) by trapping technique. *J. Econ. Entomol.* **108**, 425–432. https://doi.org/10.1093/jee/tou055 (2015).
36. Dembilio, Ó. *et al.* Development of an attract-and-infect system to control *Rhynchophorus ferrugineus* with the entomopathogenic fungus *Beauveria bassiana*. *Pest Manag. Sci* **74**, 1861–1869. https://doi.org/10.1002/ps.4888 (2018).
37. Athanassiou, C. G., Kavallieratos, N. G., Rumbos, C. I. & Kontodimas, D. C. Influence of temperature and relative humidity on the insecticidal efficacy of *Metarhizium anisopliae* against larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on wheat. *J. Insect Sci.* **17**, 1–7. https://doi.org/10.1093/jisesa/iew107 (2017).
38. Mustu, M., Demirci, F., Kaydan, M. B. & Ülgentürk, S. Laboratory assay of the effectiveness of the entomopathogenic fungus *Isaria farinosa* (Holmsk.) Fries (Sordariomycetes: Hypocreales) against the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), even under the use of fungicides. *Int. J. Pest Manag.* **61**, 264–271. https://doi.org/10.1080/09670874.2015.1047811 (2015).
39. Demirci, F. & Denizhan, E. *Paecilomyces lilacinus*, a potential biocontrol agent on apple rust mite *Aculus schlechtendali* and interactions with some fungicides *in vitro*. *Phytoparasitica* **38**, 125–132. https://doi.org/10.1007/s12600-010-0082-z (2010).
40. Haraprasad, N., Niranjana, S. R., Prakash, H. S., Shetty, H. S. & Wahab, S. *Beauveria bassiana*—a potential mycopesticide for the efficient control of coffee berry borer, *Hypothenemus hampei* (Ferrari) in India. *Biocontrol Sci. Technol.* **11**, 251–260. https://doi.org/10.1080/09583150120035675 (2001).
41. Lazzarini, G. M. J., Rocha, L. F. N. & Luz, C. Impact of moisture on *in vitro* germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. *Mycol. Res.* **110**, 485–492. https://doi.org/10.1016/j.mycres.2005.12.001 (2006).
42. Leng, P. H. & Reddy, G. V. P. Bioactivity of selected eco-friendly pesticides against *Cylas formicarius* (Coleoptera: Brentidae). *Fla. Entomol* **95**, 1040–1047. https://doi.org/10.1653/024.095.0433 (2012).

43. Dotaona, R., Wilson, B. A. L., Ash, G. J., Holloway, J. & Stevens, M. M. Sweetpotato weevil, *Cylas formicarius* (Fab.) (Coleoptera: Brentidae) avoids its host plant when a virulent *Metarhizium anisopliae* isolate is present. *J. Invertebr. Pathol.* **148**, 67–72. https://doi.org/10.1016/j.jip.2017.05.010 (2017).
44. Villani, M. G. *et al.* Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environ. Entomol.* **23**, 502–513. https://doi.org/10.1093/ee/23.2.502 (1994).
45. Ekesi, S., Egwurube, E. A., Akpa, A. D. & Onu, I. Laboratory evaluation of the entomopathogenic fungus, *Metarhizium anisopliae* for the control of the groundnut bruchid, *Caryedon serratus* on groundnut. *J. Stored Prod. Res.* **37**, 313–321. https://doi.org/10.1016/s0022-474x(00)00028-x (2001).
46. Rondot, Y. & Reineke, A. Association of *Beauveria bassiana* with grapevine plants deters adult black vine weevils, *Otiorhynchus sulcatus*. *Biocontrol Sci. Technol.* **27**, 811–820. https://doi.org/10.1080/09583157.2017.1347604 (2017).
47. Bojke, A., Tkaczuk, C., Stepnowski, P. & Golebiowski, M. Comparison of volatile compounds released by entomopathogenic fungi. *Microbiol. Res.* **214**, 129–136. https://doi.org/10.1016/j.micres.2018.06.011 (2018).
48. BOM. *Monthly climate statistics: Period 1991–2020 at Alstonville Tropical Fruit Research Station* (2020). [Retrieved from http://www.bom.gov.au/jsp/ncc/cdio/cvg/av?p_stn_num=058131&p_prim_element_index=0&p_comp_element_index=0&redraw=null&p_display_type=statistics_summary&normals_years=1991-2020&tablesizebutt=normal. Accessed 8 September, 2020]
49. Khun, K. K., Ash, G. J., Stevens, M. M., Huwer, R. K. & Wilson, B. A. L. Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia. *Pest Manag. Sci* https://doi.org/10.1002/ps.6065 (2020).
50. Inglis, G. D., Enkerli, J. & Goettel, M. S. Chapter 7: Laboratory techniques used for entomopathogenic fungi: Hypocreales. In *Manual of Techniques in Invertebrate Pathology* (ed. Lacey, L. A.) 189–253 (Elsevier Academic Press Inc, Amsterdam, 2012).
51. Tumuhaise, V. *et al.* Pathogenicity and performance of two candidate isolates of *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) in four liquid culture media for the management of the legume pod borer *Maruca vitrata* (Lepidoptera: Crambidae). *Int. J. Trop. Insect Sci.* **35**, 34–47. https://doi.org/10.1017/s1742758414000605 (2015).
52. RStudio Team. RStudio: Integrated development for R. RStudio, Inc., Boston, MA (2018). [Retrieved from: https://rstudio.com. Accessed 21 October 2018]
53. R Core Team. R: a language and environment for statistical computing. R Foundation for statistical computing, Vienna, Austria (2018). [Retrieved from: https://www.r-project.org. Accessed 21 October 2018]
54. Abbott, W. S. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **18**, 265–267. https://doi.org/10.1093/jee/18.2.265a (1925).
55. Shapiro, S. S. & Wilk, M. B. An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611. https://doi.org/10.1093/biomet/52.3-4.591 (1965).
56. Fox, J. & Weisberg, S. (eds) *An R Companion to Applied Regression* (Sage publications, Thousand Oaks, 2019).
57. Noguchi, K., Gel, Y. R., Brunner, E. & Konietschke, F. nparLD: An R software package for the nonparametric analysis of longitudinal data in factorial experiments. *J. Stat. Softw.* **50**, 23. https://doi.org/10.18637/jss.v050.i12 (2012).
58. Wickham, H. (ed.) *ggplot2: Elegant Graphics for Data Analysis* (Springer, New York, 2016).
59. Ritz, C., Baty, F., Streibig, J. C. & Gerhard, D. Dose-response analysis using R. *PLoS ONE* **10**, e0146021. https://doi.org/10.1371/journal.pone.0146021 (2015).

Acknowledgements

Ian Purdue, Jarrah Coates, David Robertson and Mark Hickey are thanked for fieldwork support. Dr. Diana Leemon is thanked for providing us with *B. bassiana* strain B27. Dr. Susette Eberhard and Mohan Trada are thanked for assistance with scanning electron microscopy. This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation, Australia with the Australian macadamia industry's research and development levy and contributions from the Australian Government.

Author contributions

K.K.K. conceived and designed the research, conducted the experiments, analysed the data, prepared all figures and wrote the manuscript; G.J.A., M.M.S., R.K.H. and B.A.L.W. conceived and designed the research, contributed to the data interpretation, and revised and gave critical input on the manuscript. All authors agreed with the final version of the manuscript for the publication.

Competing interests

The authors declare no competing interests.

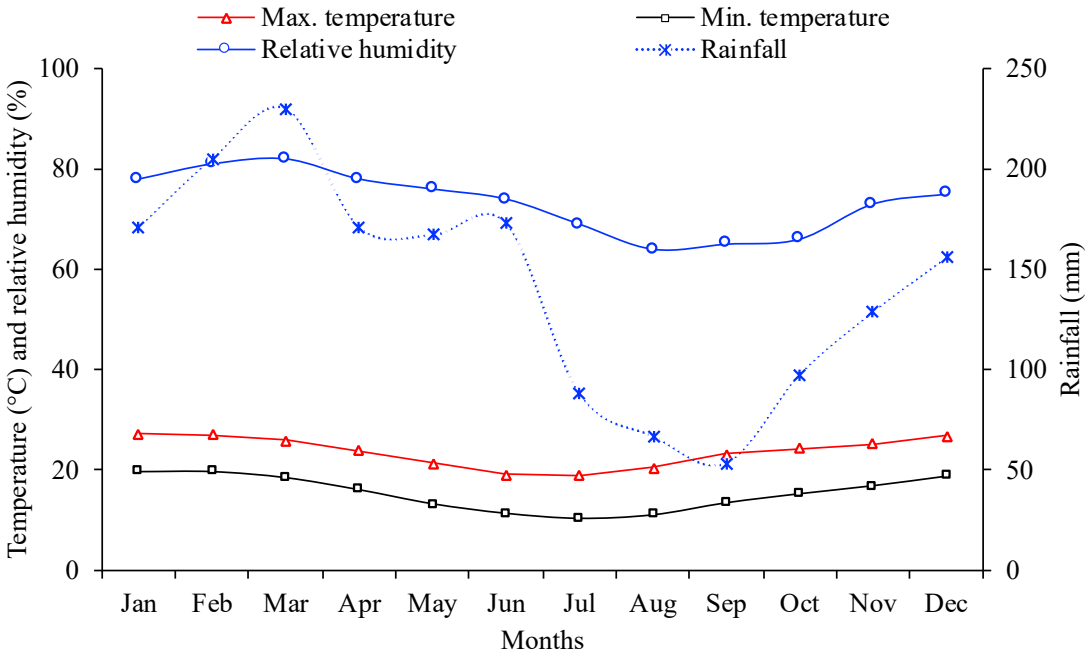
Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-81647-0.

Correspondence and requests for materials should be addressed to K.K.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Supplementary Figure 1: Mean monthly temperature, relative humidity and rainfall at the Centre for Tropical Horticulture in Alstonville, Northern Rivers, between 1991 and 2011 (compiled data from Australian Bureau of Meteorology[48]).

Chapter 5: Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* to insecticides and fungicides used in macadamia production in Australia

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson

Pest Management Science 77, 709-718

https://doi.org/10.1002/ps.6065

Note: Additional figures associated to this paper are included in the appendix F. All raw and processed data for this Chapter can be accessed at: https://cloudstor.aarnet.edu.au/plus/s/SX1WAQ2VTQsf3KF

Research Article

Received: 5 June 2020 Revised: 25 August 2020 Accepted article published: 31 August 2020 Published online in Wiley Online Library: 28 September 2020

(wileyonlinelibrary.com) DOI 10.1002/ps.6065

Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia

Kim Khuy Khun,[a,b*] Gavin J Ash,[b] Mark M Stevens,[c,d] Ruth K Huwer[e] and Bree AL Wilson[b]

Abstract

Background: Integrating fungal biocontrol agents into crop protection programs dominated by synthetic pesticides is an important first step towards developing an integrated pest management (IPM) program; however, their successful integration relies on an understanding of how their performance may be impacted by the remaining agrochemicals deployed for managing other pests and diseases. In this study we tested 10 formulated pesticides used in macadamia production at different concentrations to determine their effects on the germination, mycelial growth and sporulation of *Metarhizium anisopliae* and *Beauveria bassiana in vitro*. Further tests with laboratory-grade actives of the noncompatible pesticides were conducted to determine whether any antagonistic effects were caused by the active constituent or by formulation additives.

Results: At their registered concentrations, formulated trichlorfon, acephate and indoxacarb were compatible with *M. anisopliae*, whereas *B. bassiana* showed compatibility with formulated trichlorfon, acephate, indoxacarb, sulfoxaflor and spinetoram. Bioassays using laboratory-grade active constituents indicated that the adverse impact of formulated beta-cyfluthrin on both fungal species and that of formulated methidathion on *B. bassiana* is probably due to components of the emulsifiable concentrate formulations rather than their active constituents. Diazinon was the only insecticidal active that showed high toxicity to both fungal species. The two fungicides, carbendazim and pyraclostrobin, were toxic to both fungal species at all tested concentrations.

Conclusion: Our results identify which pesticides used on macadamias in Australia are compatible and incompatible with entomopathogenic fungi. Future studies on pesticide degradation rates will help define the spray intervals required to eliminate these adverse effects.

Keywords: biological index; compatibility; entomopathogenic fungi; fungicides; insecticides

1 INTRODUCTION

Macadamias (*Macadamia integrifolia* Maiden and Betche and *M. tetraphylla* L. Johnson) are the second largest nut crop grown in Australia with a total farm-gate value of AUD 285 million and retail value of more than AUD 850 million.[1,2] The crop is susceptible to various pests and diseases and to control them a number of insecticides and fungicides have been registered.[3] Although these agrochemicals are widely used, the Australian macadamia industry is committed to the development of an integrated pest and disease management (IPDM) program, reducing the use of broad-spectrum chemicals and integrating biological control agents (BCAs) into pest management practices in order to conserve beneficial insects and protect the environment in the macadamia agro-ecosystem.[2]

The entomopathogenic fungi *Metarhizium anisopliae* (Metschn.) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill. are among the main fungal BCAs with cosmopolitan distributions[4,5] and they have shown potential for controlling many economically important insect pests in horticultural crops.[6–8] However, to achieve effective control (>90%) high inoculum rates are required

* *Correspondence to: KK Khun, Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, QLD 4350 Australia, E-mail: kimkhuy.khun@usq.edu.au*

a *Faculty of Agronomy, Royal University of Agriculture, Phnom Penh, Cambodia*

b *Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, Toowoomba, QLD, Australia*

c *NSW Department of Primary Industries, Yanco Agricultural Institute, Yanco, NSW, Australia*

d *Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga, NSW, Australia*

e *NSW Department of Primary Industries, Wollongbar Primary Industries Institute, Wollongbar, NSW, Australia*

to cause sufficient levels of infection within the pest population. The integration of entomopathogenic fungi with low application rates of insecticides has been shown to improve their efficacy,[9–11] and several mechanisms have been suggested for this interaction. The insecticides could be acting as a general stressor by weakening the insect cuticle,[12,13] reducing the target pest's mobility due to paralysis caused by the insecticides or disrupting the removal of fungal conidia via grooming behaviour[14,15] and causing the insect to be more vulnerable to the attachment and entry of fungal entomopathogens.

Many insecticides have been recognised as compatible with entomopathogenic fungi[9–11] but some have been shown to be antagonistic.[16–18] However, most studies that have identified antagonistic interactions have been unable to identify the underlying cause of these adverse responses. Morris[19] found that components of insecticide formulations may play an important role in compatibility with bacterial entomopathogens, especially with respect to emulsifiers and similar additives. Similarly, Anderson and Roberts[20] found that emulsifiable concentrate (EC) formulations of commercial insecticides had negative impacts on *B. bassiana*. Components of the EC formulations, particularly toluene and similar aromatic solvents, were identified as toxic to *B. bassiana*.[20] In contrast to insecticides, fungicides (regardless of formulation type) are always toxic to fungal entomopathogens.[21–23]

Recent findings have demonstrated the potential of entomopathogenic fungi for controlling insect pests on macadamias (e.g. macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler),[24] but as the industry still relies heavily on pesticides to minimise pest and disease problems,[3,25] the use of entomopathogens in the field requires an understanding of the impact of each of these pesticides on the fungi. In this study, we evaluated the impact of eight common insecticides and two fungicides used in macadamia production in Australia on the germination, mycelial growth and sporulation of the entomopathogenic fungi *M. anisopliae* and *B. bassiana*, and sought to identify the cause of fungal inhibition by testing laboratory-grade actives of the incompatible formulated insecticides and fungicides.

2 MATERIALS AND METHODS

2.1 Fungal isolates

The isolates of *M. anisopliae* and *B. bassiana* used in this study are listed in Table 1. The Velifer biological insecticide (BASF Australia Ltd, Melbourne, Australia) is a commercial oil-based *B. bassiana* strain PPRI 5339 formulation containing at least 8×10^9 viable conidia mL^{-1}, whereas PPRI 5339 is the *B. bassiana* fungal strain isolated from Velifer biological insecticide. To obtain PPRI 5339, Velifer biological insecticide was applied to macadamia seed weevils and later the conidia that emerged from cadavers were sampled and cultured on malt extract agar [MEA, 30 g of malt extract (Merck Pty Ltd, Melbourne, Australia), 10 g of peptone (Bio-Strategy Ltd, Melbourne, Australia), 15 g of agar (Bio-Strategy Ltd) and 1000 mL of water] media using a single conidium technique.[29]

Isolates of *M. anisopliae* were cultured on sterile Sabouraud dextrose agar [10 g of peptone, 40 g of dextrose (Bio-Strategy Ltd), 15 g of agar and 1000 mL of water],[30] supplemented with 1% (w/v) yeast extract (Merck Pty Ltd) (SDAY) and isolates of *B. bassiana* were cultured on MEA. Malt extract agar and SDAY media are routinely used to grow *B. bassiana* and *M. anisopliae*,[31] and in our study isolates of *M. anisopliae* and *B. bassiana* grew best on SDAY and MEA, respectively. These media were consequently used for all our cultures, ensuring that each fungal species responded appropriately to the insecticides and fungicides in the *in vitro* study, while avoiding any indirect negative effects of potentially suboptimal media. All fungal isolates were incubated in the dark at 25 ± 1° C for 15 days before harvesting the conidia for experimentation.

2.2 Response of *M. anisopliae* and *B. bassiana* isolates to spinetoram-treated media

The formulated insecticides and fungicides used in macadamia production in Australia that we assessed are listed in Table 2. The insecticide spinetoram (Success Neo, Dow Agrosciences Australia Limited, Sydney, Australia) was selected at random for testing the response of a number of isolates of *M. anisopliae* and *B. bassiana*, with a view to determining if all isolates were likely to respond to insecticide exposure in a uniform way. As fungicides often have severe detrimental effects on fungal entomopathogens[32,33] these were avoided in this experiment as their use could have obscured more subtle variations in the response of different isolates.

The test method used in this study was based on established guidelines for testing the side effects of pesticides on entomopathogenic fungi.[34] First, a stock suspension of spinetoram was prepared at the concentration of 50 times the full field concentration (FFC) of 24 mg AI L^{-1}. The selective media for each fungus (SDAY media for *M. anisopliae* and MEA media for *B. bassiana*) was sterilized (121° C for 15 min) and cooled to 45–55° C. Spinetoram stock suspension was then added at either 1/50 or 1/100 the total volume of media in order to create toxic media at 100% and 50% of the FFC, respectively. The liquid media was gently inverted for 20 s then poured into 90-mm diameter sterile Petri dishes.

To test the response of *M. anisopliae* and *B. bassiana* isolates to spinetoram, the germination, mycelial growth and sporulation of each fungal isolate was measured. Spinetoram at concentrations of 100%, 50% and 0% (control) of its FFC were used to evaluate the response of 12 fungal isolates (six of each fungal species). This experiment was replicated five times at 24 h intervals. For each replicate, the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

Prior to inoculation of each replicate, the fungal conidia were harvested from sporulated cultures by scraping the surface of the agar plates with sterile spatulas and dispersing the conidia in sterile water containing 0.05% v/v Tween20 (Sigma-Aldrich, Sydney, Australia). Each suspension was homogenised by vortexing for 5 min and the conidial concentration was calculated using a haemocytometer (Laboroptik Ltd, Lancing, UK) and an Olympus BX53 compound microscope (400×) equipped with a digital camera (DP74, Olympus Australia, Melbourne, Australia). The suspensions were adjusted to 1×10^4 conidia mL^{-1} by dilution with Tween20 (0.05% v/v).

For conidia germination, 20 µL of conidial suspension at a concentration of 1×10^4 conidia mL^{-1} was spread evenly on a block (4 cm^2) of SDAY or MEA toxic media on a sterile glass slide. The slides were placed inside sterile Petri dishes lined with filter paper dampened with sterile distilled water and incubated at 25 ± 1° C in the dark. After 18 h of incubation, percentage conidial germination was determined from 100–200 conidial counts per slide using an Olympus BX53 compound microscope (400×). The conidia were considered to have germinated if the germ-tubes were twice the diameter of the propagule.[30]

For mycelial growth, 10 µL of conidial suspension at the concentration of 1×10^4 conidia mL^{-1} was inoculated in the centre of

Table 1. Fungal isolates used in this study and screened against formulated spinetoram

Fungal species	Isolate/accession	Origin/references	Collection locality	Year	Collector /provider
Metarhizium anisopliae	B4A1/BRIP 70268	Soil	Bundaberg	2017	B. Wilson
	DA1/BRIP 70271	Soil	Bundaberg	2017	B. Wilson
	ECF1/BRIP 70270	Soil	Rockhampton	2017	B. Wilson
	ECS1/BRIP 70272	Soil	Rockhampton	2017	B. Wilson
	M81/BRIP 70266	26, 27	Yeerongpilly	2007	D. Leemon
	QS155/DAR 82480	28	Mapuru	2015	R. Dotaona
Beauveria bassiana	B27/BRIP 70267	*Bovicola ovis*	Yeerongpilly	2005	D. Leemon
	B48/BRIP 70269	*Kuschelorhynchus macadamiae*	Alstonville	2016	C. Maddox
	B49/BRIP 70274	*Paropsisterna tigrina*	Lismore	2015	C. Maddox
	B50/BRIP 70276	*Kuschelorhynchus macadamiae*	Binna Burra	2017	J. Coates
	B60/BRIP 70275	Unknown	Dutton Park	2017	D. Leemon
	PPRI 5339	Isolated from Velifer biological insecticide			

Known collection localities are all in Australia.DAR, lodged in the New South Wales Plant Pathology Herbarium, NSW Department of Primary Industries, Orange; BRIP, lodged in the Queensland Plant Pathology Herbarium, Queensland Department of Agriculture and Fisheries, Brisbane.

SDAY or MEA toxic media, double sealed with Parafilm and incubated at 25 ± 1°C for 15 days. Radial growth of the colony was measured on days 5, 10 and 15 after inoculation.

To determine sporulation levels, the mycelial mat was harvested 15 days after inoculation by scraping the entire surface of the colony with a sterile spatula, suspending the dislodged conidia in 10 mL of sterile Tween20 (0.05% v/v) and homogenising by vortexing for 5 min. The conidial concentration was determined using a haemocytometer as described previously.

2.3 Response of QS155 and B50 to media containing pesticides registered for use on macadamia in Australia

M. anisopliae QS155 and *B. bassiana* B50 were selected for this experiment because they showed similar responses to spinetoram when compared to the other tested *M. anisopliae* and *B. bassiana* isolates. In this experiment, 10 pesticides (eight insecticides and two fungicides; Table 2) at concentrations of 100%, 50%, 25%, 12.5%, 6.25% and 0% (control) of their FFCs were used to check the response of the two fungal species. Conidial germination, mycelial growth and sporulation assessments were conducted as described in section 2.2. Sabouraud dextrose agar with yeast was used for *M. anisopliae* QS155 and MEA media was used for *B. bassiana* B50. This experiment was replicated five times at 24 h intervals. For each replicate the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

The toxic media containing pesticides at different concentrations was prepared as described in section 2.2. Stock suspensions of pesticides were prepared at concentrations 50 times that of each FFC and added to the warm media (45–55° C) at 1/50, 1/100, 1/200, 1/400 and 1/800 times the total volume of the media in order to achieve toxic media at 100%, 50%, 25%, 12.5% and 6.25% of each FFC, respectively.

2.4 Response of QS155 and B50 to acetone-treated media

As commercial formulations of methidathion, diazinon, beta-cyfluthrin, carbendazim and pyraclostrobin were not compatible with either fungal species, the laboratory-grade active constituents of these pesticides were used to verify that their antagonistic effects were due to the active ingredients and no other formulation components. These actives needed to be dissolved in

Table 2. Agrochemical treatments used on macadamias in Australia[3] and evaluated in this study

Pesticides	Trade name and formulation type	IRAC or FRAC codes	Active ingredient (AI)	Application rate (amount.100 L^{-1})	Spray concentration (FFC, mg AI L^{-1})	Manufacturer
Insecticides	Lancer® GR	1B	Acephate 970 g kg^{-1}	80 g	776	UPL Australia Limited
	Diazinon® EC	1B	Diazinon 800 g L^{-1}	125 mL	1000	Amgrow Pty Ltd
	Suprathion® EC	1B	Methidathion 400 g L^{-1}	125 mL	500	Adama Australia Pty Limited
	Tyranex® SL	1B	Trichlorfon 500 g L^{-1}	100 mL	500	Imtrade Australia Pty Ltd
	Bulldock® EC	3A	Beta-cyfluthrin 25 g L^{-1}	50 mL	12.5	Bayer Crop Science Pty Ltd
	Transform® SC	4C	Sulfoxaflor 240 g L^{-1}	40 mL	96	Dow Agrosciences Australia Limited
	Success® Neo SC	5	Spinetoram 120 g L^{-1}	20 mL	24	Dow Agrosciences Australia Limited
	Avatar® WG	22A	Indoxacarb 300 g kg^{-1}	25 g	75	FMC Australia Pty Ltd
Fungicides	Howzat® SC	1	Carbendazim 500 g L^{-1}	50 mL	250	Adama Australia Pty Limited
	Cabrio® EC	11	Pyraclostrobin 250 g L^{-1}	40 mL	100	BASF Australia Ltd

GR, granular; EC, emulsifiable concentrate; SL, suspension liquid; SC, suspension concentrate; WG, wettable granule; IRAC, Insecticides Resistance Action Committee; FRAC, Fungicide Resistance Action Committee; FFC, full field concentration.

acetone to be dispersed into the culture media, and as 3% (v/v) acetone is known to have a negative impact on *B. bassiana*,[20] acetone at lower concentrations was tested for its effects on the fungal cultures prior to the evaluation of laboratory grade actives. Acetone (HPLC grade, ≥99.8%, Sigma-Aldrich) was added to sterile distilled water to achieve a 25% v/v acetone stock solution, which was added to warm media at 1/12.5, 1/25 and 1/50 of the total volume of media in order to achieve 2%, 1% and 0.5% v/v acetone media, respectively. Media without acetone was used in the control treatment. Five conidial suspensions of each isolate were prepared independently from five fungal plates and one was used per replicate. Five Petri dishes (replicates) were used per concentration. The germination observations were performed as described in section 2.2. For mycelial growth, the process was similar to that described in section 2.2, except that the sterile Petri dishes had a diameter of 60 mm and mycelial growth was measured three times at 4, 8 and 12 days post-inoculation. For sporulation, the mycelial mat was harvested 12 days after inoculation by scraping the entire sporulation surface with a sterile spatula and the conidia were counted as described in section 2.2.

2.5 Response of QS155 and B50 to media containing laboratory-grade active ingredients of incompatible pesticides

Laboratory-grade analytical standards of methidathion, diazinon, beta-cyfluthrin, carbendazim and pyraclostrobin were used in this experiment. All five compounds were obtained from Sigma-Aldrich and had purity levels between 96.3% and 99.9%. Five pesticides (three insecticides and two fungicides) at concentrations of 100%, 50% and 25% of their respective FFCs and a control (no pesticide and 0.5% v/v acetone) were used to evaluate the response of *M. anisopliae* QS155 and *B. bassiana* B50. This experiment was replicated five times at 24 h intervals. For each replicate the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

A stock solution of each pesticide was prepared by dissolving the laboratory-grade active in acetone to achieve 200 times its FFC. This stock solution was then diluted with sterile distilled water to 50 times its FFC. This was in turn added to the warm media at 1/50, 1/100 and 1/200 times the total volume of the media to provide toxic media at concentrations of 100%, 50% and 25% of the FFC, respectively. As the toxic media at 50% and 25% of FFC contained acetone at only 0.25% and 0.125%, respectively, 25% acetone stock solution was added to the media to achieve a consistent acetone concentration of 0.5% in each treatment. The observations for conidia germination, mycelial growth and sporulation were made as described in section 2.4.

2.6 Statistical analysis

To determine the compatibility of entomopathogenic fungi with formulated commercial pesticides (section 2.2 and 2.3), acetone (section 2.4) and laboratory-grade pesticides (section 2.5), the biological index (BI) proposed by Rossi-Zalaf *et al.*[35] as cited in Alves *et al.*[16] and others[36,37] was used, calculated as

$$BI = \frac{(47*VG) + (43*SP) + (10*GER)}{100}$$

where *VG* is the percentage of vegetative growth of fungal colony, *SP* is the percentage of colony sporulation and *GER* is the percentage of conidia germination relative to the control. The value of *BI* indicates the level of compatibility where a *BI* value of 0 to 41 = toxic, 42 to 66 = moderately toxic, and more than 66 = compatible. All subsequent analyses were performed in Rstudio[38] Version 1.2.1335 built on R[39] Version 3.5.2.

2.6.1 *Analyses of the biological index for M. anisopliae*

For the experiments in section 2.2 and 2.4, the Shapiro–Wilk Test[40] for normality and Levene's test for homogeneity of variance using the CAR[41] (Companion to Applied Regression) Version 3.0–3 package were applied, and as data conformed to the assumption of normality, two-way analysis of variance (ANOVA) was used for experiments in section 2.2 and one-way ANOVA was used for experiments in section 2.4. Significant differences between treatment means were identified with a Tukey adjustment for multiple comparisons using the Lsmeans[42] (Least-Squares means) Version 2.30-0 package.

For the experiments in section 2.3 and 2.5, the assumption of normality was not met, so we used generalised linear mixed models (GLMMs) in order to accommodate data with mixed and random effects.[43,44] We evaluated the effects of pesticides, concentrations, and their interactions (fixed factors) and replicates (as a random factor) on the biological index of *M. anisopliae* QS155. GLMMs with beta binomial distribution and log-link function were used (following Akaike's Information Criterion) with the glmmTMB[45] (Generalised Linear Mixed Models using Template Model Builder) Version 0.2.3 and BRMS[46, 47] (Bayesian Regression Models using Stan) Version 2.9.0 packages; means were compared with a Tukey adjustment for multiple comparisons using the Lsmeans[42] Version 2.30-0 package. As the values for pyraclostrobin were zero for all concentrations it was excluded from the analyses.

2.6.2 *Analyses of the biological index for B. bassiana*

In the experiments of section 2.2, 2.3 and 2.5, the assumption of data normality was not met, so GLMMs were used to accommodate data with mixed and random effects.[43,44] We assessed the main effects and interactions of spinetoram concentrations and fungal isolates (fixed factors) in the experiment in section 2.2, and pesticides and concentrations (fixed factors) in the experiments of sections 2.3 and 2.5. Replicates were treated as random factors in all analyses. Analyses were conducted with beta binomial distributions and log-link functions using the same protocols and analysis packages used for analysis of the *M. anisopliae* data.

In the section 2.4 experiment the biological index was analysed using a non-parametric one-way ANOVA (Kruskal–Wallis test) followed by Dunn's *post hoc* test using the FSA[48] (Fisheries Stock Analysis) Version 0.8.25 package with a Bonferroni correction for multiple comparisons, since the data did not fulfil the assumption for an analysis of variance even after transformation.

3 RESULTS

3.1 Response of *M. anisopliae* and *B. bassiana* isolates to spinetoram-treated media

There were significant differences in the response of *M. anisopliae* isolates to spinetoram at 50% of FFC (12 mg AI L^{-1}), with the BI of all isolates between 67 and 81 and showing that formulated spinetoram at this concentration is compatible with *M. anisopliae* ($P < 0.05$, Table 3). At 100% of FFC (24 mg AI L^{-1}) the BI values fell to between 50 and 62, indicating incompatibility at this concentration. No significant differences were observed between isolates ($P > 0.05$) at this concentration. However, significant differences

Table 3. Biological index (BI) of the response of *M. anisopliae* and *B. bassiana* to spinetoram at 50% and 100% of full field concentration (FFC) (12 and 24 mg AI L^{-1}, respectively)

		Spinetoram concentrations	
Fungal species	Isolate/accession	50% of FFC (%) ± SE	100% of FFC (%) ± SE
Metarhizium anisopliae[a]	QS155/DAR 82480	81.34 ± 5.25 Aa	61.38 ± 3.63 Ab
	B4A1/BRIP 70268	78.55 ± 2.85 ABa	62.48 ± 4.75 Ab
	ECS1/BRIP 70272	76.74 ± 1.32 ABa	54.33 ± 1.20 Ab
	ECF1/BRIP 70270	71.41 ± 1.42 ABa	60.07 ± 2.60 Ab
	M81/BRIP 70266	67.08 ± 2.71 Ba	54.37 ± 1.39 Ab
	DA1/BRIP 70271	67.50 ± 4.56 Ba	50.79 ± 2.76 Ab
Beauveria bassiana[b]	PPRI 5339	94.30 ± 1.69 Aa	82.77 ± 3.68 Ab
	B49/BRIP 70274	86.91 ± 5.54 Aa	64.12 ± 4.81 Bb
	B50/BRIP 70276	85.55 ± 5.44 Aa	69.00 ± 0.94 ABb
	B60/BRIP 70275	82.75 ± 4.34 Aa	78.23 ± 5.26 ABa
	B27/BRIP 70267	80.62 ± 4.90 ABa	68.13 ± 2.19 ABb
	B48/BRIP 70269	64.31 ± 3.37 Ba	60.93 ± 2.96 Ba

[a] $F_{(5,48)} = 4.86$, $P < 0.01$ (for isolate factor), $F_{(1,48)} = 81.32$, $P < 0.001$ (for concentration factor), $F_{(5,48)} = 0.87$, $P > 0.05$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).
[b] Wald $\chi^2 = 39.67$, df = 5, $P < 0.01$ (for isolate factor), Wald $\chi^2 = 5.47$, df = 1, $P < 0.05$ (for concentration factor), Wald $\chi^2 = 13.8$, df = 5, $P < 0.05$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).

between means were observed due to spinetoram concentrations ($P < 0.05$).

In contrast, *B. bassiana* isolates showed more variability in their response to spinetoram at the two test concentrations. B48 was not compatible to spinetoram at either 50% or 100% of the FFC (12 and 24 mg AI L^{-1} respectively), whereas B49 was not compatible with spinetoram only at the higher rate (Table 3). B27, B50, B60 and PPRI 5339 had BI values above 66, showing that they were compatible to spinetoram at both concentrations. B50 showed no significant differences from any of the other isolates at 50% of FFC, except B48 ($P < 0.05$), or from any of the isolates at 100% of FFC ($P > 0.05$). Again, significant differences between means were observed on PPRI 5339, B27, B49 and B50 isolates due to spinetoram concentrations ($P < 0.05$).

3.2 Response of QS155 and B50 to media containing pesticides registered for use on macadamia in Australia

Significant differences were observed when *M. anisopliae* QS155 was exposed to different pesticides and concentrations ($P < 0.05$, Table 4). Trichlorfon, acephate and indoxacarb at all concentrations were compatible with QS155 whereas beta-cyfluthrin and spinetoram were compatible with QS155 at 50% of their FFCs or lower. Sulfoxaflor was compatible with QS155 at 25% of FFC or lower. However, methidathion and diazinon were moderately toxic at 6.25% and 12.5% of FFC and very toxic to QS155 at higher concentrations. Increasing the concentration of insecticides in the media from 6.25% to 100% of their respective FFCs significantly reduced BI values ($P < 0.05$) for all insecticides except trichlorfon and indoxacarb. Both fungicides (carbendazim and pyraclostrobin) were very toxic to QS155 even at the lowest concentration, 6.25% of FFC.

Differences between the BI values for *B. bassiana* B50 exposed to different pesticides and concentrations were also statistically significant ($P < 0.05$, Table 4). Trichlorfon, acephate, indoxacarb, sulfoxaflor and spinetoram were compatible with B50 at all concentrations, whereas beta-cyfluthrin and methidathion were compatible with B50 only at 25% of their FFCs or lower. Diazinon was compatible with B50 only at 12.5% of its FFC or below. Increasing the concentrations of insecticides in the media from 6.25% to 100% of their respective FFCs significantly reduced the BI values of all insecticides ($P < 0.05$). Both fungicides at all tested concentrations were highly toxic to B50.

3.3 Response of QS155 and B50 to acetone-treated media

Acetone at 2% showed a strong toxic effect on both fungal species with the BI values of *M. anisopliae* QS155 and *B. bassiana* B50 decreasing to 44 and 46, respectively. At 1% acetone B50 responded positively with the BI increasing to 84 but QS155 was still quite sensitive (BI = 65). At 0.5% acetone, both fungal species were compatible (BI values 80–94). At 2% acetone, BI values were significantly ($P < 0.05$) reduced relative to the 0.5% concentration for both fungal isolates (Table 5).

3.4 Response of QS155 and B50 to media containing laboratory-grade active ingredients of incompatible pesticides

The BI of *M. anisopliae* QS155 exposed to different laboratory-grade pesticides and concentrations varied significantly ($P < 0.05$, Table 6). Laboratory grade beta-cyfluthrin at all concentrations was compatible with QS155 whereas methidathion showed compatibility with QS155 at 50% of FFC or lower. However, laboratory-grade diazinon was toxic to QS155 at all tested concentrations. Both laboratory-grade fungicides (carbendazim and pyraclostrobin) were toxic to QS155 even at the lowest concentration (25% of FFC). BI values for beta-cyfluthrin and methidathion were significantly reduced by higher toxicant concentrations ($P < 0.05$).

Significant differences were observed when *B. bassiana* B50 was exposed to different laboratory-grade pesticides and concentrations ($P < 0.05$, Table 6). In contrast to the results from QS155,

Table 4. Biological index of the response of *M. anisopliae* QS155 and *B. bassiana* B50 to pesticides at 6.25%, 12.5%, 25%, 50% and 100% of their respective full field concentrations (FFCs)

		Pesticide concentrations				
Isolate	Pesticide	6.25% of FFC ± SE	12.5% of FFC ± SE	25% of FFC ± SE	50% of FFC ± SE	100% of FFC ± SE
Metarhizium anisopliae QS155[a]	Trichlorfon	91.24 ± 2.04 Aa	90.23 ± 1.40 ABa	87.75 ± 1.18 Aa	88.52 ± 2.98 Aa	87.47 ± 3.21 Aa
	Acephate	93.28 ± 1.94 Aa	91.30 ± 1.64 Aab	86.35 ± 2.68 Abc	82.40 ± 2.57 Ac	80.94 ± 2.07 Ac
	Indoxacarb	86.03 ± 1.15 Aa	82.93 ± 2.95 BCa	81.74 ± 2.32 Aa	81.16 ± 2.54 ABa	79.90 ± 1.48 Aa
	Beta-cyfluthrin	89.92 ± 1.95 Aa	85.34 ± 2.60 ABCab	78.99 ± 1.48 ABbc	69.97 ± 1.80 CDcd	62.07 ± 4.86 Bd
	Spinetoram	89.94 ± 2.13 Aa	84.47 ± 1.82 ABCab	80.38 ± 2.59 Ab	71.33 ± 2.34 BCc	55.38 ± 4.77 BCd
	Sulfoxaflor	87.57 ± 3.26 Aa	78.42 ± 3.03 Cb	68.84 ± 3.24 Bc	59.45 ± 4.71 Dcd	49.48 ± 6.12 Cd
	Methidathion	64.79 ± 2.32 Ba	48.67 ± 2.49 Db	37.93 ± 1.84 Cc	24.99 ± 1.06 Ed	20.61 ± 1.48 Dd
	Diazinon	63.68 ± 1.83 Ba	48.41 ± 1.82 Db	33.74 ± 1.05 Cc	22.20 ± 3.13 Ed	17.91 ± 0.12 Dd
	Carbendazim	8.26 ± 0.53 Ca	8.21 ± 0.43 Ea	7.59 ± 0.33 Da	7.16 ± 0.40 Fa	5.52 ± 0.20 Ea
	Pyraclostrobin	0.00	0.00	0.00	0.00	0.00
Beauveria bassiana B50[b]	Trichlorfon	92.52 ± 1.91 ABa	86.57 ± 1.72 ABb	85.87 ± 2.00 Ab	83.49 ± 1.33 ABb	80.66 ± 1.55 Ab
	Acephate	95.22 ± 0.98 Aa	90.84 ± 1.80 Aab	90.18 ± 1.07 Ab	86.56 ± 1.66 Abc	80.53 ± 2.72 Ac
	Indoxacarb	93.40 ± 2.62 Aa	88.36 ± 1.22 ABab	87.88 ± 1.34 Ab	81.24 ± 1.21 ABc	79.15 ± 2.18 ABc
	Sulfoxaflor	94.65 ± 1.30 Aa	91.75 ± 1.62 Aab	88.47 ± 1.77 Abc	84.15 ± 2.41 ABc	76.58 ± 1.67 ABd
	Spinetoram	92.51 ± 1.93 ABa	87.81 ± 2.40 ABab	83.89 ± 1.15 Abc	77.75 ± 2.83 Bcd	70.14 ± 2.30 Bd
	Beta-cyfluthrin	94.88 ± 0.95 Aa	90.59 ± 2.55 Aa	83.03 ± 2.32 Ab	64.03 ± 2.72 Cc	50.48 ± 4.94 Cd
	Methidathion	87.10 ± 1.66 BCa	81.12 ± 0.88 BCa	68.54 ± 1.45 Bb	56.90 ± 0.79 Cc	45.33 ± 3.21 Cd
	Diazinon	81.89 ± 4.11 Ca	73.21 ± 4.22 Cb	57.13 ± 1.90 Cc	38.36 ± 1.89 Dd	29.00 ± 0.81 De
	Pyraclostrobin	21.76 ± 0.41 Da	20.96 ± 0.40 Da	20.83 ± 0.46 Da	17.65 ± 0.78 Ea	8.88 ± 2.23 Eb
	Carbendazim	9.37 ± 0.16 Ea	9.42 ± 0.12 Ea	9.07 ± 0.15 Ea	8.98 ± 0.08 Fa	8.62 ± 0.17 Ea

[a] Wald $\chi^2 = 582.59$, df = 8, $P < 0.01$ (for pesticide factor), Wald $\chi^2 = 33.46$, df = 4, $P < 0.01$ (for concentration factor), Wald $\chi^2 = 137.94$, df = 32, $P < 0.01$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).
[b] Wald $\chi^2 = 1354.75$, df = 9, $P < 0.01$ (for pesticide factor), Wald $\chi^2 = 50.72$, df = 4, $P < 0.01$ (for concentration factor), Wald $\chi^2 = 253.44$, df = 36, $P < 0.01$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

beta-cyfluthrin and methidathion showed compatibility with B50 at all concentrations, but diazinon was compatible with B50 only at 25% of its FFC. However, both laboratory-grade fungicides at all concentrations were still toxic to B50. The BI values of all insecticides were significantly reduced by higher test concentrations ($P < 0.05$).

Table 5. Biological index of the response of *M. anisopliae* QS155 and *B. bassiana* B50 to media impregnated with acetone at 0.5%, 1% and 2%

	Acetone concentration (v/v)		
Isolate	0.5% (%) ± SE	1% (%) ± SE	2% (%) ± SE
Metarhizium anisopliae QS155[a]	80.19 ± 3.25 a	65.98 ± 1.32 b	44.48 ± 1.65 c
Beauveria bassiana B50[b]	94.10 ± 1.03 a	84.71 ± 1.78 ab	46.13 ± 0.57 b

[a] $F_{(2,12)} = 64.63$, $P < 0.001$. Means followed by different lowercase letters are significant different (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).
[b] Kruskal–Wallis test; $\chi^2 = 12.5$, df = 2, $P < 0.01$. Means followed by different lowercase letters are significant different (Dunn's *post hoc* test with Bonferroni correction, $P < 0.05$).

4 DISCUSSION

There were very few differences between the BI values of different *M. anisopliae* isolates or *B. bassiana* isolates to formulated spinetoram at 12 and 24 mg AI L^{-1}, demonstrating the general similarity of responses to this representative agrochemical across isolates. In a study on *B. bassiana* isolates from diverse geographic areas, formulated piperonyl butoxide and permethrin adversely affected all isolates, whilst formulated carbaryl and oxamyl had no adverse impact on any of them.[20] Similarly, Duarte *et al.*[49] found that four *B. bassiana* isolates responded similarly to five pesticides (neem, acephate, thiamethoxam, deltamethrin and methomyl) and Pires *et al.*[50] found that two isolates of *M. anisopliae* showed similar responses to neem, indoxacarb and spinosad, supporting our decision to use single isolates of each fungal species for further testing.

The results of all compatibility tests using *M. anisopliae* QS155 and *B. bassiana* B50 are summarised in Table 7 and show that the tested chemicals fit into several clear categories. The fungicides carbendazim and pyraclostrobin were, perhaps unsurprisingly, highly toxic to both fungal species at all rates tested down to 6.25% of FFC levels (15.6 and 6.2 mg AI L^{-1}, respectively). Relative to their field rates, carbendazim appeared more active than pyraclostrobin against *B. bassiana*, whilst the opposite response occurred with *M. anisopliae*. Pyraclostrobin reduced the BI of *M. anisopliae* to zero at all concentrations tested. Our results agrees with the findings of Moorhouse *et al.*[51] and others[52–54]

Table 6. Biological index measuring the response of *M. anisopliae* QS155 and *B. bassiana* B50 to laboratory-grade pesticides at 25%, 50% and 100% of their respective FFC (full field concentration) values

		Pesticide concentration (laboratory grade)		
Isolate	Pesticide	25% of FFC ± SE	50% of FFC ± SE	100% of FFC ± SE
Metarhizium anisopliae QS155[a]	Beta-cyfluthrin	92.61 ± 2.92 Aa	90.57 ± 3.46 Aab	85.53 ± 2.49 Ab
	Methidathion	93.27 ± 2.99 Aa	82.86 ± 5.05 Ab	55.23 ± 1.15 Bc
	Diazinon	41.00 ± 0.80 Ba	39.96 ± 1.26 Ba	35.60 ± 1.17 Ca
	Carbendazim	9.37 ± 0.07 Ca	8.62 ± 0.26 Ca	8.18 ± 0.35 Da
	Pyraclostrobin	0.00	0.00	0.00
Beauveria bassiana B50[b]	Beta-cyfluthrin	98.40 ± 0.35 Aa	91.74 ± 0.69 Ab	87.30 ± 0.94 Ab
	Methidathion	91.76 ± 0.55 Ba	88.37 ± 0.68 Aa	81.19 ± 1.15 Ab
	Diazinon	78.21 ± 1.43 Ca	59.79 ± 1.15 Bb	45.39 ± 1.64 Bc
	Pyraclostrobin	14.06 ± 0.25 Da	13.99 ± 0.16 Ca	12.87 ± 0.11 Ca
	Carbendazim	9.29 ± 0.07 Da	8.53 ± 0.23 Ca	8.03 ± 0.15 Ca

[a] Wald $\chi^2 = 291.78$, df = 3, $P < 0.01$ (for pesticide factor), Wald $\chi^2 = 7.91$, df = 2, $P < 0.05$ (for concentration factor), Wald $\chi^2 = 49.43$, df = 6, $P < 0.01$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).
[b] Wald $\chi^2 = 906.04$, df = 4, $P < 0.01$ (for pesticide factor), Wald $\chi^2 = 34.1$, df = 2, $P < 0.01$ (for concentration factor), Wald $\chi^2 = 58.36$, df = 8, $P < 0.01$ (for interaction). Means followed by different uppercase letters in columns and lowercase letters in rows indicate significant differences (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

who found that carbendazim had post-germination fungicidal effects on both species. They found that the conidia of *M. anisopliae* and *B. bassiana* normally germinated in 24 h when they were cultured on media containing carbendazim at concentrations between 55 and 5500 mg AI L^{-1} but that mycelial growth was totally inhibited. This agreed with our observation that the

Table 7. Summary of the responses of *M. anisopliae* QS155 and *B. bassiana* B50 to formulated and laboratory-grade pesticides used for macadamia crop protection in Australia

		Commercial formulation (see Table 2)					Laboratory-grade material		
		6.25% FFC	12.5% FFC	25% FFC	50% FFC	100% FFC	25% FFC	50% FFC	100% FFC
Active	FFC (mg AI L^{-1})	*Metarhizium anisopliae* QS155							
Acephate	776	93	91	86	82	81			
Methidathion	500	65	49	38	25	21	93	83	55
Diazinon	1000	64	48	34	22	18	41	40	36
Trichlorfon	500	91	90	88	89	87			
Indoxacarb	75	86	83	82	81	80			
Beta-cyfluthrin	12.5	90	85	79	70	62	93	91	86
Sulfoxaflor	96	88	78	69	59	49			
Spinetoram	24	90	84	80	71	55			
Carbendazim	250	8	8	8	7	6	9	9	8
Pyraclostrobin	100	0	0	0	0	0	0	0	0
		Beauveria bassiana B50							
Acephate	776	95	91	90	87	81			
Methidathion	500	87	81	69	57	45	92	88	81
Diazinon	1000	82	73	57	38	29	78	60	45
Trichlorfon	500	93	87	86	83	81			
Indoxacarb	75	93	88	88	81	79			
Beta-cyfluthrin	12.5	95	91	83	64	50	98	92	87
Sulfoxaflor	96	95	92	88	84	77			
Spinetoram	24	93	88	84	78	70			
Carbendazim	250	9	9	9	9	9	9	9	8
Pyraclostrobin	100	22	21	21	18	9	14	14	13

Data are biological index (BI) values. FFC, full field concentration. Orange cells, highly toxic (BI ≤ 41); yellow cells, moderately toxic (BI = 42–66); green cells, compatible (BI ≥ 66).

conidia produced abnormal, distorted, swollen and stunted germlings after exposure to carbendazim at <50% of FFC for 48 h, but the cell walls ruptured after exposure to carbendazim at 100% of the FFC for the same period. In contrast, studies on pyraclostrobin have shown fungistatic effects on both species at 67–600 mg AI L^{-1} with no conidia germinating in the first 24 h but some poor mycelial growth occuring.[32,33] Again, this conforms with our observations in which *B. bassiana* conidia enlarged and germinated after exposure to pyraclostrobin at all tested concentrations for 72 h or longer, but that mycelial growth remained stunted after 5 days of incubation. The detrimental effect of fungicides was also observed on other fungal taxa pathogenic to invertebrate pests, including *Isaria fumosorosea* Wize, *Isaria farinosa* (Holmsk.) Fr. and *Paecilomyces lilacinus* (Thom) Samson.[55–57]

The second category of compounds are those where the formulated products were compatible (BI ≥ 66) with the fungi at rates up to 100% of their full field concentrations. These included acephate, trichlorfon and indoxacarb for both species, and sulfoxaflor and spinetoram for *B. bassiana* only. Our results are in accordance with the results of Saito,[58] who found that acephate was not toxic to *B. bassiana* even at 1000 mg AI L^{-1}. Akbar *et al.*[18] and others[50,54,59] found indoxacarb was compatible with *M. anisopliae* and *I. fumosorosea*. To our knowledge, no literature is available on the direct effect of sulfoxaflor and spinetoram on *M. anisopliae* or *B. bassiana*, although Wari *et al.*[60] have conducted bioassays assessing the impact of spinetoram alone and in combination with *B. bassiana* strain GHA against the whitefly *Bemisia tabaci* (Gennadius). Other studies have found that spinosad, which belongs to the same insecticide group as spinetoram, is not toxic to *M. anisopliae*[18] or *B. bassiana*.[50,61]

The compatibility of trichlorfon with both fungal species that we found in this study contrasts with the findings of other workers. Saito[58] found that trichlorfon at 1000 mg AI L^{-1} reduced mycelial growth of *B. bassiana* by 43% and Ayala-Zermeño *et al.*[62] found that trichlorfon at 5000 mg AI L^{-1} reduced mycelial growth of *M. anisopliae* and *Paecilomyces fumosoroseus* (Wize) A.H.S. Br. & G. Sm. by 27% and 38%, respectively. At a higher concentration of 8750 mg AI L^{-1} trichlorfon decreased the mycelial growth of *M. anisopliae* by 41% and *P. fumosoroseus* by 70%.[62] Our results show that trichlorfon at 500 mg AI L^{-1} reduced the mycelial growth of *B. bassiana* B50 and *M. anisopliae* QS155 by only 14% and 6%, respectively, but this relatively minor impact could reflect the relatively low concentrations we tested compared to those evaluated by other workers, particularly Ayala-Zermeño *et al.*[62]

Compounds in the third category are those in emulsifiable concentrate (EC) formulations where the formulated products were moderately to highly toxic at 100% of their FFC levels but showed reduced toxicity when the laboratory-grade materials were tested alone. These included beta-cyfluthrin, methidathion and diazinon. There is extensive evidence to show that EC pesticide formulations can have adverse impacts on entomopathogenic fungi. Anderson and Roberts[20] found that EC formulations of permethrin and piperonyl butoxide had negative impacts on six isolates of *B. bassiana* sourced from three separate countries. These formulations were found to contain toluene and similar aromatic solvents, which were toxic to the fungal entomopathogens. Similar negative effects of commercial EC insecticide formulations (chlorpyrifos, indoxacarb, emamectin benzoate, lufenuron, profenophos, abamectin, diazinon, L-cyhalothrin, cypermethrin and methidathion) were also observed on *M. anisopliae* by Asi *et al.*[17] and a number of other authors[37,52,61,63]. The adverse effects of EC formulation components on entomopathogens is not confined to those used with insecticides. Emulsifiable concentrate formulations of acaricides (amitraz, pyridaphenthion and pyridine) are very toxic to *B. bassiana*,[64] and herbicides formulated as ECs (e.g. flurochloridone and pendimethalin) are also antagonistic to this species.[21,37]

Integrating entomopathogens into the pest management plan for any crop requires an understanding of the potential adverse effects of agrochemicals that may be applied before, after or with the entomopathogen. It is also important to understand whether these adverse effects are caused by the active ingredient (and are therefore probably intractable) or whether they are associated with other components of the formulation and have the potential to be reduced or eliminated through the substitution of particular additives or the development of alternative formulation types. This study has shown that the fungicides carbendazim and pyraclostrobin are inherently detrimental to *M. anisopliae* and *B. bassiana* and their application to control fungal diseases of macadamias will largely eliminate these entomopathogens if they have been previously applied or are present naturally. Residual concentrations of these compounds on plant surfaces will need to fall by well over 93% before the application of entomopathogens will be likely to provide reasonable levels of insect infection, and field studies on residue dynamics will be needed to determine the time periods required to achieve these levels of chemical breakdown.

Whilst our bioassays have reinforced earlier findings that components of EC formulations can have adverse impacts on entomopathogenic fungi, these components are used for specific reasons such as enhancing product efficacy and surface wetting, and for providing uniform spray mixtures with active ingredients that often have very low water solubilities. The use of formulated products is therefore unavoidable, however our data provides the basis for selecting formulated products that, when timed appropriately, can be used to target various macadamia pests without compromising the benefits derived from *M. anisopliae* and *B. bassiana* applications.

Our data show that EC formulations of methidathion and diazinon remain moderately toxic to *M. anisopliae* even at 6.25% of their FFC values. In contrast, formulated acephate, indoxacarb and trichlorfon are compatible with *M. anisopliae* at rates up to and including their FFCs of 776, 75 and 500 mg AI L^{-1}, respectively, and could be applied at the same time as *M. anisopliae*, although further work would be required to determine their compatibility in tank mixes. Applications of the remaining insecticides (beta-cyfluthrin, sulfoxaflor and spinetoram) will be likely to need a buffer period for at least partial breakdown before *M. anisopliae* is applied unless the fungus can be formulated in a way that provides the conidia with some protection.

B. bassiana was generally less affected by the formulated insecticides than *M. anisopliae*, but diazinon remains problematic for both fungal species due to the toxicity of the active and the high spray concentration routinely used against macadamia pests in Australia. Five insecticides, acephate, trichlorfon, indoxacarb, sulfoxaflor and spinetoram, were all compatible with *B. bassiana* at 100% of their full field concentrations.

If there is sufficient market incentive the substitution of EC formulation additives with alternative emulsifiers and adjuvants may lower the impact of formulated products on entomopathogenic fungi. However, formulation changes can be made by manufacturers for other reasons and initiate the reverse effect, effectively making a formulation more toxic to an entomopathogen rather than reducing its toxicity. There is generally only

limited disclosure of formulation components on product labels, and in many jurisdictions there is no requirement to advise end-users of a change in formulation constituents other than those involving the active ingredient. As a consequence, industries integrating entomopathogens into crop protection programs need to monitor potential adverse pesticide impacts on BCAs and develop crop protection calendars that reflect both the interactions between biological and chemical control agents and the weathering profiles of chemicals under field conditions. Our data and the published literature indicate that emulsifiable concentrate formulations, insecticides applied at high application rates with actives inherently detrimental to fungal germination and growth (such as diazinon), and particularly fungicides pose the greatest risk to successfully introducing entomopathogenic fungi into crop protection programs dominated by agrochemicals.

5 CONCLUSION

This study has identified the crop protection compounds that can be safely applied to Australian macadamia orchards where the entomopathogens *M. anisopliae* and/or *B. bassiana* are active, either as natural populations or as a consequence of deliberate application. Some treatments were identified as antagonistic to these fungi, and residue breakdown studies need to be conducted to determine the necessary periods between the application of these treatments and any subsequent entomopathogen applications. With this information it will be possible to conduct more detailed studies on the response of pests such as the macadamia seed weevil to sequential or combination treatments of insecticides and entomopathogens that have the potential to reduce total insecticide inputs and delay or prevent the development of insecticide resistance.

ACKNOWLEDGEMENT

Craig Maddox of Wollongbar Primary Industries Institute, NSW Department of Primary Industries is thanked for providing us with pesticide samples. Rachel King, USQ statistician, is thanked for assistance with RStudio. This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation, Australia with the Australian macadamia industry's research and development levy and contributions from the Australian Government.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 ANIC. *Growing for success: Australian's Tree Nut Industry 2019*. Australian Nut Industry Council, VIC. Available: https://nutindustry.org.au/wp-content/uploads/2019/05/Growing_Success_2019_Email_LoRes.pdf [February 2020].

2 AMS. Yearbook 2019. Australian Macadamia Society Ltd, Lismore, Australia. Available: https://d1bel7n84kyh0s.cloudfront.net/uploads/2019/11/2019-AUSTRALIAN-MACADAMIAS-YEARBOOK-FINAL-V1.0.pdf [26 February 2020].

3 Bright J. *Macadamia plant protection guide 2019–20*. NSW Department of Primary Industries, Wollongbar. Available: https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/529161/Macadamia-plant-protection-guide-2019.pdf [3 March 2020].

4 Rehner SA and Buckley E, A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* **97**:84–98 (2005).

5 Roberts DW and St Leger RJ, *Metarhizium spp.*, cosmopolitan insect-pathogenic fungi: mycological aspects, in Advances in Applied Microbiology, ed. by Laskin AI, Bennet JW and Gadd GM. Elsevier Academic Press Inc, Cambridge, MA, pp. 1-70 (2004).

6 Dolinski C and Lacey LA, Microbial control of arthropod pests of tropical tree fruits. *Neotrop Entomol* **36**:161–179 (2007).

7 Lacey LA and Shapiro-Ilan DI, Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. *Annu Rev Entomol* **53**:121–144 (2008).

8 Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M and Goettel MS, Insect pathogens as biological control agents: back to the future. *J Invertebr Pathol* **132**:1–41 (2015).

9 Younas A, Wakil W, Khan Z, Shaaban M and Prager SM, The efficacy of *Beauveria bassiana*, jasmonic acid and chlorantraniliprole on larval populations of *Helicoverpa armigera* in chickpea crop ecosystems. *Pest Manag Sci* **73**:418–424 (2017).

10 Rivero-Borja M, Guzmán-Franco AW, Rodriguez-Leyva E, Santillán-Ortega C and Pérez-Panduro A, Interaction of *Beauveria bassiana* and *Metarhizium anisopliae* with chlorpyrifos ethyl and spinosad in *Spodoptera frugiperda* larvae. *Pest Manag Sci* **74**:2047–2052 (2018).

11 Meyling NV, Arthur S, Pedersen KE, Dhakal S, Cedergreen N and Fredensborg BL, Implications of sequence and timing of exposure for synergy between the pyrethroid insecticide alpha-cypermethrin and the entomopathogenic fungus *Beauveria bassiana*. *Pest Manag Sci* **74**:2488–2495 (2018).

12 dos Santos TTM, Quintela ED, Mascarin GM and Santana MV, Enhanced mortality of *Bemisia tabaci* nymphs by *Isaria javanica* combined with sublethal doses of chemical insecticides. *J Appl Entomol* **142**: 598–609 (2018).

13 Kumar V, Francis A, Avery PB, McKenzie CL and Osborne LS, Assessing compatibility of *Isaria fumosorosea* and buprofezin for mitigation of *Aleurodicus rugioperculatus* (Hemiptera: Aleyrodidae): an invasive pest in the Florida landscape. *J Econ Entomol* **111**:1069–1079 (2018).

14 Quintela ED and McCoy CW, Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environ Entomol* **26**:1173–1182 (1997).

15 Brito ES, de Paula AR, Vieira LP, Dolinski C and Samuels RI, Combining vegetable oil and sub-lethal concentrations of imidacloprid with *Beauveria bassiana* and *Metarhizium anisopliae* against adult guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae). *Biocontrol Sci Technol* **18**:665–673 (2008).

16 Alves LFA, Mamprim AP, Formentini MA, Martins CC and Pinto FG, Effect of disinfectants and pesticides used in poultry houses on *Beauveria bassiana* (Bals.) Vuill. fungus. *Braz J Poultry Sci* **18**: 283–290 (2016).

17 Asi MR, Bashir MH, Afzal M, Ashfaq M and Sahi ST, Compatibility of entomopathogenic fungi, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* with selective insecticides. *Pak J Bot* **42**:4207–4214 (2010).

18 Akbar S, Freed S, Hameed A, Gul HT, Akmal M, Malik MN *et al*., Compatibility of *Metarhizium anisopliae* with different insecticides and fungicides. *Afr J Microbiol Res* **6**:3956–3962 (2012).

19 Morris ON, Compatibility of 27 chemical insecticides with *Bacillus thuringiensis* var. *kurstaki*. *Can Entomol* **109**:855–864 (1977).

20 Anderson TE and Roberts DW, Compatibility of *Beauveria bassiana* isolates with insecticide formulations used in Colorado potato beetle (Coleoptera: Chrysomelidae) control. *J Econ Entomol* **76**:1437–1441 (1983).

21 Celar FA and Kos K, Effects of selected herbicides and fungicides on growth, sporulation and conidial germination of entomopathogenic fungus *Beauveria bassiana*. *Pest Manag Sci* **72**:2110–2117 (2016).

22 Shah FA, Ansari MA, Watkins J, Phelps Z, Cross J and Butt TM, Influence of commercial fungicides on the germination, growth and virulence of four species of entomopathogenic fungi. *Biocontrol Sci Technol* **19**:743–753 (2009).

23 Kouassi M, Coderre D and Todorova SI, Effects of the timing of applications on the incompatibility of three fungicides and one isolate of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina). *J Appl Entomol* **127**:421–426 (2003).

24 Khun KK, Ash GJ, Stevens MM, Huwer RK and Wilson BAL, Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays. *J Invertebr Pathol* **174**:107437 (2020). https://doi.org/10.1016/j.jip.2020.107437.

25 Akinsanmi OA, Miles AK and Drenth A, Alternative fungicides for controlling husk spot caused by *Pseudocercospora macadamiae* in macadamia. *Australas Plant Pathol* **37**:141–147 (2008).
26 Leemon DM and McMahon J, *Feasibility Study into in-Hive Fungal Bio-Control of Small Hive Beetle*. Rural Industries Research and Development Corporation, Canberra. (2009). Available: https://www.agrifutures.com.au/wp-content/uploads/publications/09-090.pdf [10 November 2019].
27 Leemon DM, *In-Hive Fungal Biocontrol of Small Hive Beetle*. Rural Industries Research and Development Corporation, Canberra. (2012). Available: https://www.agrifutures.com.au/wp-content/uploads/publications/12-012.pdf [10 November 2019].
28 Dotaona R, Wilson BAL, Stevens MM, Holloway J and Ash GJ, Screening of tropical isolates of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) for virulence to the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Int J Trop Insect Sci* **35**:153–163 (2015).
29 Zhang K, Su YY and Cai L, An optimized protocol of single spore isolation for fungi. *Cryptogam Mycol* **34**:349–356 (2013).
30 Inglis GD, Enkerli J and Goettel MS, Chapter 7: Laboratory techniques used for entomopathogenic fungi: Hypocreales, in *Manual of Techniques in Invertebrate Pathology*, ed. by Lacey LA. Academic Press, London, pp. 189–253 (2012).
31 Kamp AM and Bidochka MJ, Conidium production by insect pathogenic fungi on commercially available agars. *Lett Appl Microbiol* **35**: 74–77 (2002).
32 Bruck DJ, Impact of fungicides on *Metarhizium anisopliae* in the rhizosphere, bulk soil and *in vitro*. *Biocontrol* **54**:597–606 (2009).
33 Roberti R, Righini H, Masetti A and Maini S, Compatibility of *Beauveria bassiana* with fungicides *in vitro* and on zucchini plants infested with *Trialeurodes vaporariorum*. *Biol Control* **113**:39–44 (2017).
34 Coremans-Pelseneer J, Laboratory tests on the entomopathogenic fungus *Beauveria*. *IOBC/WPRS Bull* **17**:147–155 (1994).
35 Rossi-Zalaf LS, Alves SB, Lopes RB, Silveira Neto S and Tanzini MR, Interação de microrganismos com outros agentes de controle de pragas e doenças, in *Controle microbiano de pragas na América Latina: avanços e desafios*, ed. by Alves SB and Lopes RB. FEALQ, Piracicaba, SP, pp. 279–302 (2008).
36 Ribeiro LP, Blume E, Bogorni PC, Dequech STB, Brand SC and Junges E, Compatibility of *Beauveria bassiana* commercial isolate with botanical insecticides utilized in organic crops in southern Brazil. *Biol Agric Hortic* **28**:223–240 (2012).
37 da Silva RA, Quintela ED, Mascarin GM, Barrigossi JAF and Lião LM, Compatibility of conventional agrochemicals used in rice crops with the entomopathogenic fungus *Metarhizium anisopliae*. *Sci Agric* **70**: 152–160 (2013).
38 RStudio Team. *RStudio: Integrated development for R*. RStudio, Inc., Boston, MA. Available: https://rstudio.com [21 October 2018].
39 R Core Team. *R: a language and environment for statistical computing*. R Foundation for statistical computing, Vienna. Available: https://www.r-project.org [21 October 2018].
40 Shapiro SS and Wilk MB, An analysis of variance test for normality (complete samples). *Biometrika* **52**:591–611 (1965).
41 Fox J and Weisberg S, *An R Companion to Applied Regression*. Sage Publications, Thousand Oaks, CA (2019).
42 Lenth RV, Least-squares means: the R package lsmeans. *J Stat Softw* **69**: 1–33 (2016).
43 Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH *et al*., Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol Evol* **24**:127–135 (2009).
44 Stroup WW, Rethinking the analysis of non-normal data in plant and soil science. *Agron J* **107**:811–827 (2015).
45 Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A *et al*., glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J* **9**:378–400 (2017).
46 Bürkner PC, Advanced bayesian multilevel modeling with the R package brms. *R J* **10**:395–411 (2018).
47 Bürkner PC, brms: an R package for bayesian multilevel models using stan. *J Stat Softw* **80**:1–28 (2017).
48 Ogle D, Wheeler P and Dinno A. FSA*: Simple fisheries stock assessment methods. Version 0.8.25*. R package. Available: https://CRAN.R-project.org/package=FSA [21 October 2019].
49 Duarte RT, Gonçalves KC, Espinosa DJL, Moreira LF, De Bortoli SA, Humber RA *et al*., Potential of entomopathogenic fungi as biological control agents of diamondback moth (Lepidoptera: Plutellidae) and compatibility with chemical insecticides. *J Econ Entomol* **109**: 594–601 (2016).
50 Pires LM, Marques EJ, de Oliveira JV and Alves SB, Selection of isolates of entomopathogenic fungi for controlling *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and their compatibility with insecticides used in tomato crop. *Neotrop Entomol* **39**:977–984 (2010).
51 Moorhouse ER, Gillespie AT, Sellers EK and Charnley AK, Influence of fungicides and insecticides on the entomogenous fungus *Metarhizium anisopliae* a pathogen of the vine weevil, *Otiorhynchus sulcatus*. *Biocontrol Sci Technol* **2**:49–58 (1992).
52 Niassy S, Maniania NK, Subramanian S, Gitonga ML, Maranga R, Obonyo AB *et al*., Compatibility of *Metarhizium anisopliae* isolate ICIPE 69 with agrochemicals used in French bean production. *Int J Pest Manage* **58**:131–137 (2012).
53 Oliveira DGP, Pauli G, Mascarin GM and Delalibera I, A protocol for determination of conidial viability of the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* from commercial products. *J Microbiol Methods* **119**:44–52 (2015).
54 Rachappa V, Lingappa S and Patil RK, Effect of agrochemicals on growth and sporulation of *Metarhizium anisopliae* (Metschnikoff) Sorokin. *Karnataka J Agric Sci* **20**:410–413 (2007).
55 D'Alessandro CP, Padin S, Urrutia MI and López Lastra CC, Interaction of fungicides with the entomopathogenic fungus *Isaria fumosorosea*. *Biocontrol Sci Technol* **21**:189–197 (2011).
56 Demirci F, Mustu M, Kaydan MB and Ülgenturk S, Effects of some fungicides on *Isaria farinosa*, and *in vitro* growth and infection rate on *Planococcus citri*. *Phytoparasitica* **39**:353–360 (2011).
57 Demirci F and Denizhan E, *Paecilomyces lilacinus*, a potential biocontrol agent on apple rust mite *Aculus schlechtendali* and interactions with some fungicides *in vitro*. *Phytoparasitica* **38**:125–132 (2010).
58 Saito T, Effect of pesticides on conidial germination and hyphal growth of the entomopathogenic fungus *Beauveria bassiana*. *Jpn J Appl Entomol Zool* **28**:87–89 (1984).
59 Ali S, Huang Z, Zou SX, Bashir MH, Wang ZQ and Ren SX, The effect of insecticides on growth, germination and cuticle-degrading enzyme production by *Isaria fumosorosea*. *Biocontrol Sci Technol* **22**: 1047–1058 (2012).
60 Wari D, Okada R, Takagi M, Yaguchi M, Kashima T and Ogawara T, Augmentation and compatibility of *Beauveria bassiana* with pesticides against different growth stages of *Bemisia tabaci* (Gennadius); an *in vitro* and field approach. *Pest Manag Sci* **76**:3236–3252 (2020).
61 Amutha M, Gulsar Banu J, Surulivelu T and Gopalakrishnan N, Effect of commonly used insecticides on the growth of white Muscardine fungus, *Beauveria bassiana* under laboratory conditions. *J Biopestic* **3**:143–146 (2010).
62 Ayala-Zermeño MA, Navarro-Barranco H, Mier T and Toriello C, Effect of agro-chemicals on *in vitro* growth of the entomopathogenic fungi *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith. *Rev Latinoam Microbiol* **41**: 223–229 (1999).
63 Golshan H, Saber M, Majidi-Shilsar F, Bagheri M and Mahdavi V, Effects of common pesticides used in rice fields on the conidial germination of several isolates of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin. *J Entomol Res Soc* **15**:17–22 (2013).
64 de Oliveira RC and Neves PMOJ, Compatibility of *Beauveria bassiana* with acaricides. *Neotrop Entomol* **33**:353–358 (2004).

Chapter 6: Interactions of fungal entomopathogens with synthetic insecticides for the control of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae)

Kim Khuy Khun, Gavin J. Ash, Mark M. Stevens, Ruth K. Huwer, Bree A.L. Wilson

Journal of Applied Entomology
https://doi.org/10.1111/jen.12879

Note: Additional figures associated to this manuscript are included in the appendix C and G.

Received: 24 November 2020 | Revised: 28 February 2021 | Accepted: 1 March 2021

DOI: 10.1111/jen.12879

ORIGINAL CONTRIBUTION

JOURNAL OF APPLIED ENTOMOLOGY WILEY

Interactions of fungal entomopathogens with synthetic insecticides for the control of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae)

Kim Khuy Khun[1,2] | Gavin J. Ash[2] | Mark M. Stevens[3,4] | Ruth K. Huwer[5] | Bree A. L. Wilson[2]

[1]Faculty of Agronomy, Royal University of Agriculture, Phnom Penh, Cambodia

[2]Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, Toowoomba, QLD, Australia

[3]NSW Department of Primary Industries, Yanco Agricultural Institute, Yanco, NSW, Australia

[4]Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga, NSW, Australia

[5]NSW Department of Primary Industries, Wollongbar Primary Industries Institute, Wollongbar, NSW, Australia

Correspondence
Kim Khuy Khun, Centre for Crop Health, Institute of Life Sciences and the Environment, University of Southern Queensland, QLD 4350 Australia.
Emails: KimKhuy.Khun@usq.edu.au; Khun.KimKhuy@rua.edu.kh

Abstract

This study investigated the interactions between insecticides (acephate and indoxacarb) and fungal entomopathogens (*Beauveria bassiana* [Bals.-Criv.] Vuill. strain B27, *Metarhizium anisopliae* [Metschn.] Sorokin strain ECS1, and a commercial *B. bassiana* product, Velifer® Biological Insecticide) for controlling the macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler, in the laboratory and glasshouse. In the laboratory, additive interactions between insecticides at their full field concentrations (776 mg AI/L of acephate and 75 mg AI/L of indoxacarb) and fungal entomopathogens at 10^7 conidia/ml (ECS1 and B27) or at full field concentration (0.5 ml of Velifer®/L) were seen at 6 days and 12 days post-application. Under the same experimental conditions, synergistic interactions against *K. macadamiae* were observed 6 days post-application when fungal entomopathogens at 2.5×10^6 conidia/ml or at 25% of full field concentration (Velifer®) were co-applied with insecticides at 25% of their full field concentrations, whilst additive interactions were again observed at 12 days post-application. In the glasshouse, additive interactions between insecticides (at full field concentrations) and fungal entomopathogens (at 10^7 conidia/ml, or at full field concentration for Velifer®) were obtained at 6 days and 12 days post-application. The results from this study suggest that acephate and indoxacarb have both synergistic and additive effects against *K. macadamiae* when deployed together with fungal entomopathogens, depending on the initial concentrations of mixture components. Combined application of entomopathogens with compatible insecticides promises to provide more effective management of *K. macadamiae* than individual chemical applications.

KEYWORDS

acephate, *Beauveria bassiana*, combined application, indoxacarb, *Metarhizium anisopliae*, synergy

1 | INTRODUCTION

Macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler (Coleoptera: Curculionidae), formerly known as '*Sigastus* weevil' (Jennings & Oberprieler, 2018), is a native Australian insect, which has been categorized as one of the key pests of macadamias (*Macadamia integrifolia* Maiden and Betche and *M. tetraphylla* L.A.S. Johnson) (QDAF, 2019). Adult females lay eggs inside the husk of

macadamia nuts when they are about 10 mm in diameter and induce premature nut to drop between late September (spring) and early December (summer) (Bright, 2019). This nut drop has been estimated to lead to crop losses of approximately 15% (Huwer, 2016). Adults also defoliate young seedlings and remove the bark, leading to rapid seedling death after 2–3 days in the glasshouse, depending on the number of adults present. Ten adults can completely remove the bark of a seedling within 12 days (K. K. Khun, personal observations).

The life cycle of *K. macadamiae* from egg to adult emergence takes around 40 days at 25°C (Bright, 2017a). Adult females lay up to 280 eggs each, but only 20 eggs are laid each week (Bright, 2017a). The larvae hatch from the egg in 6 days and develop inside the nuts by feeding on the developing shell tissue and kernel. The larval stage lasts 4 weeks, passing through four instars. The adult weevils emerge after a prepupal period of 2 days and a pupal period of 4 days. Most adults live for around 100 days; however, some may live for over a year (Bright, 2017a). The weevil has three generations in a year, with the first and second generations in November and December and the third and overwintering generation from March to October (Bright, 2017b).

Acephate and indoxacarb are currently the only insecticides available for the management of *K. macadamiae* in Australian macadamia orchards, and no commercial entomopathogenic fungi (EPF) formulations are currently registered for use against this pest. Chemical control of *K. macadamiae* mainly occurs during the first generation of the weevil; however, it may also be necessary to target the second generation if populations remain high (Bright, 2019). Non-target impacts associated with using synthetic insecticides in Australian macadamias, specifically with regard to the management of latania scale (*Hemiberlesia lataniae* Signoret), have been reported (Treverrow, 1987). Reliance on broad-spectrum insecticides such as acephate and indoxacarb has put selection pressure on pest populations. This has led to the development of resistant populations that are not controlled by the chemical applications that were formerly effective against susceptible individuals (Nehare et al., 2010; Snodgrass et al., 2009). Some studies have suggested that the integration of EPF with synthetic insecticides could provide a sustainable solution to control pests and suppress insecticide-resistant populations (Bahiense et al., 2006; Farenhorst et al., 2010; Farooq et al., 2018) as insects are less likely to become resistant to the fungi when compared to other microbial controls (Lacey et al., 2001).

Beauveria bassiana (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) are EPF with the potential to control many economically important insect pests of horticultural crops (Dolinski & Lacey, 2007; Khun, Wilson, et al., 2020; Lacey & Shapiro-Ilan, 2008). In our previous work, *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 were shown to be effective for the control of *K. macadamiae* in the laboratory, and the mortality of adults caused by both strains was higher than other tested strains and equivalent to that caused by the commercial *B. bassiana* product, Velifer® Biological Insecticide (Khun, Ash, et al., 2020). In line with the Australian macadamia industry's commitment to reducing the use of broad-spectrum chemicals and integrating biological control agents into pest management practices (AMS, 2019), the aim of this study was to determine the feasibility of combining EPF with insecticides (acephate and indoxacarb) currently used for *K. macadamiae* control. Because *B. bassiana* strain B50 and *M. anisopliae* strain QS155 were compatible with acephate and indoxacarb in our previous *in vitro* study (Khun et al., 2021a), we hypothesized that these insecticides would not have any inhibitory effect on the germination and growth of B27 and ECS1 when prepared and applied as spray mixtures on *K. macadamiae* and consequently, the combined insecticide/EPF applications would be expected to improve the efficacy of *K. macadamiae* management in commercial macadamia orchards.

2 | MATERIALS AND METHODS

2.1 | Insects, seedlings, fungi and insecticides

Kuschelorhynchus macadamiae cannot currently be reared on artificial media, so nuts infested by the weevils were collected at 2-week intervals from three orchards in the Northern Rivers region, New South Wales (NSW) (28°51′12″S 153°27′37″E, 28°48′27″S 153°25′23″E and 28°52′07″S 153°24′06″E) between October and December 2018/19. The weevils were obtained from the infested nuts and fed as described by Khun, Ash, et al. (2020).

Macadamia seedlings (approximately 30 cm height, 4 months old, variety H2) used in these studies were purchased from Next Block Nursery, Fernleigh, NSW. They were placed in the glasshouse (26 ± 1°C and 54 ± 1% relative humidity (RH) during the day and 21 ± 1°C and 65 ± 1% RH at night) for 4 weeks before experimentation.

Two fungal strains, ECS1 (*M. anisopliae*) and B27 (*B. bassiana*) and a commercial *B. bassiana* product (Velifer® Biological Insecticide, BASF Australia Ltd) were used in the experiments. Both fungal strains have been lodged in the Queensland Plant Pathology Herbarium, Brisbane, Australia, with the accessions BRIP 70272 (ECS1) and BRIP 70267 (B27). The DNA sequences of strains ECS1 and B27 were deposited in GenBank under accessions MN966530 (the 5′ region of elongation factor-1 alpha) and MN909971 (B locus nuclear intergenic region), respectively. Velifer® is a commercial oil-based *B. bassiana* strain PPRI 5339 formulation containing at least 8×10^9 viable conidia/ml and is recommended to be used at 0.5 ml/L of water. Fungal strains ECS1 and B27 were cultured on sterile Sabouraud dextrose agar (SDA: 10 g peptone, 40 g dextrose, 15 g agar (all from Bio-Strategy Ltd) and 1,000 ml of water) (Inglis et al., 2012). Both fungal strains were incubated in the dark at 25 ± 1°C for 15 days before harvesting the conidia for experimentation.

The insecticides used in this study were indoxacarb (Avatar®, FMC Australasia Pty Ltd) and acephate (Lancer®970, UPL Australia Limited). Avatar® is a 300 g/kg wettable granule formulation of indoxacarb containing a 75% *S*:25% *R* mixture of indoxacarb enantiomers; however, only the *S* enantiomer is insecticidally active (Wing et al., 2000), whilst Lancer® is a granular formulation containing

TABLE 1 Summary of treatments used in bioassays on *Kuschelorhynchus macadamiae* under laboratory and glasshouse conditions

	Bioassay 1	Bioassay 2		Bioassay 3		Bioassay 4	Bioassay 5	
Treatment number	Insecticide concentration[a]	Insecticides at 100% FFC	Fungi[b]	Insecticides at 25% of FFC	Fungi[c]	Fungi[b]	Insecticides at 100% FFC	Fungi[b]
1	Water	Water	Tween®20	Water	Tween®20	Tween®20	Water	Tween®20 + VO
2	0.1%	Water	ECS1	Water	ECS1	ECS1	Water	ECS1 + VO
3	1%	Water	B27	Water	B27	ECS1 + VO	Water	B27 + VO
4	5%	Water	Velifer®	Water	Velifer®	B27	Water	Velifer®
5	10%	Acephate	Tween®20	Acephate	Tween®20	B27 + VO	Acephate	Tween®20 + VO
6	25%	Acephate	ECS1	Acephate	ECS1	VO	Acephate	ECS1 + VO
7	50%	Acephate	B27	Acephate	B27	Velifer®	Acephate	B27 + VO
8	75%	Acephate	Velifer®	Acephate	Velifer®		Acephate	Velifer®
9	100%	Indoxacarb	Tween®20	Indoxacarb	Tween®20		Indoxacarb	Tween®20 + VO
10		Indoxacarb	ECS1	Indoxacarb	ECS1		Indoxacarb	ECS1 + VO
11		Indoxacarb	B27	Indoxacarb	B27		Indoxacarb	B27 + VO
12		Indoxacarb	Velifer®	Indoxacarb	Velifer®		Indoxacarb	Velifer®

[a]Acephate or indoxacarb at different proportions of the full field concentration (FFC). The FFCs of acephate and indoxacarb are 776 mg A.I/L and 75 mg A.I/L, respectively.

[b]Entomopathogenic fungi at the concentration of 1×10^7 conidia/ml, except Velifer® which was at 100% of FFC (= 0.5 ml/L). VO = 10% Vegetable oil (v/v).

[c]Entomopathogenic fungi at the concentration of 2.5×10^6 conidia/ml, except Velifer® which was at 25% of FFC (= 0.125 ml/L).

acephate at 970 g/kg. These insecticides are both available for use against *K. macadamiae* in Australia (Bright, 2019). The registered full field concentrations (FFCs) of indoxacarb and acephate are 75 mg AI/L and 776 mg AI/L of water, respectively.

2.2 | Interaction of EPF with insecticides on *K. macadamiae* in the laboratory

2.2.1 | Effect of insecticides on *K. macadamiae*

Insecticides (indoxacarb and acephate), each at eight different concentrations between 0.1% and 100% of their FFCs, were used in this experiment, with sterile water as the control (Table 1: Bioassay 1). Ten mixed-sex adults were randomly collected from the insect cages and placed in a 500 ml plastic container (9.5 cm diameter and height) with small ventilation holes (2 mm diameter) in the lid of the container. Prior to spray applications, containers containing *K. macadamiae* were chilled at 4°C for 15 min to reduce adult mobility. Each container was then opened and sprayed with 1 ml of insecticide suspension using an X-Press It® micro-atomiser (X-Press Graph-X Pty Ltd). After spraying, each container received a single macadamia nut and was maintained in the fume hood at 23°C and 63% RH. Each container of weevils was provided with a new macadamia nut every second day for 6 days and cumulative mortality was recorded daily over this period. The bioassay was replicated four times at 2-day intervals with a total of 720 insects being used in the experiment (360 insects for each insecticide).

2.2.2 | Effect of insecticides on EPF

Conidial suspensions of B27 and ECS1 were prepared by scraping the surface of the sporulated cultures with sterile spatulas and suspending the inoculum in 10 ml of sterile Tween®20 (0.05% v/v in distilled water, Sigma-Aldrich) in a 50 ml centrifuge tube (Labtek Pty Ltd). The suspensions were homogenized by vortexing for 5 min, and the conidial concentrations were determined using a haemocytometer (Laboroptik Ltd) and a compound microscope at 400× magnification (Olympus BX53, Olympus Australia Pty Ltd).

The effect of mixing insecticides with fungi (simulating a spray tank mix) on the germination and mycelial growth of the fungi was checked prior to testing on *K. macadamiae*. Insecticides (acephate and indoxacarb) at 200% of their FFCs were mixed with *M. anisopliae* strain ECS1 (2×10^5 conidia/ml), *B. bassiana* strain B27 (2×10^5 conidia/ml) and Velifer® (at 50% of FFC) at the volume ratio of 1:1 in order to achieve a 100% FFC insecticide rate. Fungal suspensions without insecticides were used as control treatments. To determine the conidial germination, 20 µl of the fungus-insecticide suspension was spread evenly on a 4 cm^2 block of SDA media on a sterile glass slide. The slides were placed inside Petri plates lined with filter paper dampened with sterile distilled water and incubated at 25 ± 1°C in the dark. After 18 h of incubation, percentage conidial germination was determined from 100 to 200 conidial counts per slide using an Olympus BX53 compound microscope (400×). The conidia were considered to have germinated if the germ tube was twice the diameter of the propagule (Inglis et al., 2012). To determine the fungal growth rate, 10 µl of the fungus-insecticide suspension was inoculated in the centre of SDA media in 90 mm diameter Petri plates, double-sealed with Parafilm® and incubated as above. The radial growth was recorded every 5 days for 15 days on six radii per plate. The growth rate per day was defined as the mean of the daily growth rates on the three measurement days. This experiment was replicated four times at 3-day intervals.

2.2.3 | Co-application of EPF and insecticides on *K. macadamiae*

To examine the interaction between EPF and insecticides on *K. macadamiae*, two bioassays were conducted. In the first bioassay, insecticides (acephate and indoxacarb) at 200% of FFC and water (a control) were mixed with *M. anisopliae* strain ECS1 (2×10^7 conidia/ml), *B. bassiana* strain B27 (2×10^7 conidia/ml), Velifer® (at 200% of FFC) and sterile Tween®20 (0.05% v/v) at the volume ratio of 1:1 to yield 12 treatments (Table 1: Bioassay 2). In the second bioassay, the concentration of insecticides and EPF in the first bioassay was reduced by 75% in order to observe the interactions at the lower dose rate on *K. macadamiae* (Table 1: Bioassay 3).

To examine the effect of the treatments on *K. macadamiae*, 10 adults (mixed sex) were randomly collected from the insect cages and placed in a 500 ml plastic container. Prior to spray applications, all containers were chilled at 4°C for 15 min to reduce weevil mobility. Each container was then opened and sprayed with 1 ml of a test treatment using a micro-atomizer. After spraying, each container received a single macadamia nut and was incubated at high humidity (>95%) in darkness for 24 h, followed by incubation (25 ± 1°C, 65 ± 3% RH and 16L:8D photoperiod) in a Conviron® A1000 growth chamber (Conviron Asia Pacific Pty Ltd). Weevils in each container were provided with a new macadamia nut every second day for 12 days. Dead weevils were removed daily and placed in Petri plates containing filter paper moistened with sterile distilled water and sealed with Parafilm®. These plates were incubated in the dark at 25 ± 1°C for 7 days to stimulate mycosis and to verify fungal infection. Mortality was calculated based on the number of surviving weevils 6 days and 12 days after inoculum application. Bioassay 2 was replicated four times (at 3-day intervals) with a total of 480 insects and Bioassay 3 was replicated five times (at 3-day intervals), using a total of 600 insects.

2.3 | Interaction of EPF with insecticides on *K. macadamiae* in the glasshouse

2.3.1 | Effect of oil formulations as UV protectants for EPF

In this experiment, we compared Codacide® with other potential oil-based protectants. Five treatments, olive oil (Coles®, Coles Pty Ltd),

vegetable oil (Crisco® premium oil, Goodman Fielder Pty Ltd), botanical oil (SynertrolHorti®, Organic Crop Protectants Pty Ltd), canola oil (Codacide®, Microcide Ltd) and Tween®20, were used in this experiment. The test method used in this study was modified from the protocols used to examine the effect of exposing fungal conidia to UV radiation (Ghajar et al., 2006). Nine ml of ECS1 or B27 at the concentration of 1.12×10^6 conidia/ml was mixed with 1 ml of the oil or Tween®20 (0.05% v/v) in order to achieve a concentration of 1×10^6 conidia/ml with or without oil (10% v/v). To test for UV protection, 200 μl of the conidial suspensions in different oil types or in Tween®20 was pipetted onto 60 mm sterile Petri plates, double-sealed with Parafilm® and then left for 1 h to ensure the conidia had settled before placing the plates in the glasshouse to expose them to solar UV radiation. Additional plates with the same fungal suspensions and oil covered with aluminium foil and kept in the same glasshouse conditions served as control treatments. The plates were exposed at midday in the glasshouse (26 ± 1°C and 54 ± 1% RH during the day and 21 ± 1°C and 65 ± 1% RH at night). After 24 h of exposure, the suspension in each Petri plate was spread evenly on a 4 cm^2 block of SDA media on a sterile glass slide and incubated as described previously. Four replicates (Petri plates) were used, where each replicate was prepared from one of four separate original culture plates. The percentage of conidial germination was determined at 24 h, 48 h and 72 h post-incubation.

2.3.2 | Efficacy of oil-formulated EPF against *K. macadamiae*

The effects of ECS1 and B27 in vegetable oil (10% v/v) on *K. macadamiae* were tested in the glasshouse as the vegetable oil provided better UV protection than the other oil types (see results). Groups of 10 mixed-sex adults were randomly collected from insect cages in the laboratory and placed on macadamia seedlings in separate Bugdorm® insect rearing cages (32.5 × 32.5 × 70 cm, Australian Entomological Supplies Pty Ltd) in the glasshouse (26 ± 1°C and 54 ± 1% RH during the day and 21 ± 1°C and 65 ± 1% RH at night). After 24 h, each seedling was sprayed with 5 ml of one treatment using a microatomizer. There were seven treatments (Table 1: Bioassay 4). Dead weevils were evaluated for fungal infection as described in previous experiment. As adult weevils killed the seedling by defoliation and ring barking after 12 days, remaining live adults were transferred to 500 ml plastic containers, incubated at high humidity (>95%) in darkness for 24 h, followed by further incubation in the glasshouse. Weevils in each container were provided with a new macadamia nut every second day for another 5 days. This experiment was replicated four times (at 2-day intervals) using a total of 280 insects.

2.3.3 | Co-application of UV-protected EPF and insecticides on K. macadamiae

In this bioassay, 12 treatments (Table 1: Bioassay 5) were evaluated against *K. macadamiae* in the glasshouse. Four treatments each involved either acephate or indoxacarb (both 100% of FFC) or water as an insecticide control. Within each set, insecticide treatments were combined with ECS1, B27 or Tween® 20 (as an EPF control), all formulated with vegetable oil. The fourth treatment in each set was Velifer® but without additional vegetable oil. In other respects, the experimental protocol was the same as that used in section 2.3.2. Each seedling was sprayed with 5 ml of a treatment using a microatomizer. The mortality of adults was recorded for 12 days after inoculum application and dead weevils were evaluated for fungal infection as described for the previous bioassay. This bioassay was replicated five times (at 3-day intervals) and 600 insects were used.

2.4 | Statistical analysis

All analyses were performed using RStudio ver. 1.2.1335. (RStudio Team, 2018) built on R ver. 3.5.2. (R Core Team, 2018). The mortality of treated weevils was corrected using corresponding control mortalities and Abbott's formula (Abbott, 1925) when required.

Weevil mortality in Bioassay 1 was analysed by probit analysis using the Ecotox (Analysis of Ecotoxicology, ver. 1.4.0) package (Hlina, 2019) in RStudio to calculate the lethal concentration (LC) for 25%, 50%, 75% and 95% of the population using the cumulative mortality data at 6 day post-treatment. The median lethal time (LT_{50}) of both insecticides at FFC was also calculated using the same package.

The Shapiro–Wilk Test for normality (Shapiro & Wilk, 1965) and Levene's Test for homogeneity for variance were applied to all remaining data using the CAR (Companion to Applied Regression, ver. 3.0–3) package (Fox & Weisberg, 2019) before the analyses, except for the data for insecticides and EPF interactions. The effect of insecticides on fungal germination was assessed using a one-way ANOVA followed by a Tukey HSD test to separate means; however, the effect of insecticides on fungal daily growth rate was analysed using a Kruskal–Wallis test followed by Dunn's post hoc test using the FSA (Fisheries Stock Analysis, ver. 0.8.25) package (Ogle et al., 2019) with a Bonferroni correction for multiple comparisons, since the data did not fulfil the assumptions for analysis of variance.

The multifactorial 'F1-LD-F1' non-parametric analysis of longitudinal data in factorial experiments was used to analyse the germination of ECS1 and B27 in different oil types over three repeated measures (24 h, 48 h and 72 h post-incubation). The mortality of weevils in Bioassay 4 was also analysed with the same protocol over three repeated measures (6 days, 12 days and 18 days post-application). The Wald-type statistics (WTS) were computed using the nparLD (Nonparametric Analysis of Longitudinal Data, ver. 2.1) package (Noguchi et al., 2012) to check the significant effect of the treatments, repeated measures and their interactions ($p < .05$), and the pairwise Wilcoxon rank-sum test was used to separate means.

For the interaction between EPF and insecticides on *K. macadamiae* in Bioassays 2, 3, and 5, the expected mortality was calculated at 6 days and 12 days post-application and compared with the observed mortality arising from the combination treatments. The percentage expected mortality (M_E) was obtained using the

formula: $M_E = M_f + M_i(1-M_f)$ (Pachamuthu & Kamble, 2000) where M_i and M_f are the percentages of the observed mortalities caused by the insecticides and fungi, respectively. The expected mortalities (M_E) were compared with the observed mortalities (M_{fi}) using a chi-square test (χ^2) with the formula: $\chi^2 = (M_{fi} - M_E)^2/M_E$ (Pachamuthu & Kamble, 2000) where M_{fi} represents the observed mortality caused by the combination of fungi and insecticides, and then compared to the table value for 1 *df* (3.84). When the calculated $\chi^2 > 3.84$ it indicates that the interaction is synergistic if $M_{fi} - M_E > 0$ or that the interaction is antagonistic if $M_{fi} - M_E < 0$. In contrast, if the calculated $\chi^2 < 3.84$ then an additive effect is occurring (Pachamuthu & Kamble, 2000). The ggplot2 (Grammar of Graphics, ver. 3.2.1) package was used to generate the figures (Wickham, 2016).

3 | RESULTS

3.1 | Interaction of EPF with insecticides on *K. macadamiae* in the laboratory

3.1.1 | Effect of insecticides on *K. macadamiae*

Probit analyses showed that the calculated LC_{25}, LC_{50} and LC_{75} values for indoxacarb were always lower than the corresponding values for acephate (Table 2), both in terms of their absolute concentrations and the proportions of their FFCs. However, the LC_{95} for indoxacarb was calculated at 111 mg AI/L, which is 148% of the currently used FFC for this chemical and it is higher than the LC_{95} for acephate, 97.1% of the FFC (Table 2).

The LT_{50} for acephate was lower than the LT_{50} for indoxacarb when FFCs of both insecticides were applied to the adults. In addition, the response curve for acephate was always above that for indoxacarb (Figure 1).

3.1.2 | Effect of insecticides on EPF

Whilst the germination test showed significant differences between fungal strains ($F_{8,24} = 10.82$, $p < .0001$), neither of the insecticides had any negative effects on germination ($p > .05$, Table 3). Notably, the germination of ECS1 with or without indoxacarb was significantly higher than Velifer® with or without indoxacarb ($p < .05$) whereas ECS1 with acephate was not different to Velifer® with acephate ($p > .05$). The germination of B27 with or without either insecticide was not significantly different to ECS1 with or without either insecticide ($p > .05$).

Similar results were obtained in relation to daily growth rates, where differences in growth rates between treatments occurred (Kruskal–Wallis $\chi^2 = 26.359$, $df = 8$, $p < .001$) but no significant effects were caused by the addition of insecticides to individual EPF strains ($p > .05$, Table 3). The daily growth of Velifer® with indoxacarb was significantly lower than ECS1 with indoxacarb ($p < .05$) whereas Velifer® with or without acephate was not different to ECS1 with or without acephate ($p > .05$). The daily growth rate of B27 with or without either insecticide did not differ from that of either ECS1 or Velifer® with or without either insecticide ($p > .05$).

3.1.3 | Co-application of EPF and insecticides on *K. macadamiae*

After incubation, all dead weevils produced mycoses consistent with the relevant EPF used in each bioassay. In Bioassay 2, the interactions between EPF and insecticides at their FFCs produced only additive effects on *K. macadamiae* ($\chi^2 < 3.84$, Table 4). In Bioassay 3, the interactions between EPF and insecticides both at 25% of the concentrations used in Bioassay 2 consisted of both additive and synergistic effects (Table 5). In treatments where acephate and the three EPF were co-applied to *K. macadamiae*, their interactions were synergistic at 6 days post-application ($\chi^2 > 3.84$, Table 5) whereas at 12 days

TABLE 2 Acute toxicity of acephate (Lancer®) and indoxacarb (Avatar®) to adults of *Kuschelorhynchus macadamiae* assessed at 6 days post-treatment (Bioassay 1)

Compound	LC level	LC value (mg AI/L)	95% FL (mg AI/L)	LC value as % of FFC[a]
Acephate	LC_{25}	82.2	61.2–103.1	10.6 (7.9–13.3)
	LC_{50}	156.6	127.1–189.1	20.2 (16.4–24.4)
	LC_{75}	297.6	244.9–375.1	38.4 (31.6–48.4)
	LC_{95}	752.5	565.8–1,116.0	97.1 (73.0–144.0)
Indoxacarb[b]	LC_{25}	2.8	1.7–4.0	3.7 (2.3–5.3)
	LC_{50}	8.2	6.0–10.7	10.9 (8.0–14.3)
	LC_{75}	23.8	17.9–33.5	31.7 (23.8–44.6)
	LC_{95}	111.0	71.1–209.3	148.0 (94.8–279.0)

Note: Acephate: Slope 2.41 (*SE* 0.24), $\chi^2 = 20.4$ (32 *df*). Indoxacarb: Slope 1.45 (*SE* 0.15), $\chi^2 = 27.3$ (32 *df*).

Abbreviation: FL, fiducial limits.

[a]FFC (Full Field Concentration), acephate 775 mg AI/L, indoxacarb 75 mg AI/L.

[b]75% *S*:25% *R* mixture of enantiomers.

post-application, only the interaction of acephate and Velifer® was identified as synergistic (Table 5). In treatments where indoxacarb and EPF were co-applied to *K. macadamiae*, only the interaction of indoxacarb and B27 was synergistic at 6 days post-application ($\chi^2 > 3.84$). At 12 days post-application, indoxacarb and EPF combinations had only additive effects on *K. macadamiae* ($\chi^2 < 3.84$, Table 5).

3.2 | Interaction of EPF with insecticides on *K. macadamiae* in the glasshouse

3.2.1 | Effect of oil formulations as UV protectants for EPF

The germination in the control treatments was around 89% for both fungal species in all oil types in the first 24 h post-incubation and 100% at 48 h post-incubation, indicating that the tested oil types had no negative effects on either fungal species.

When exposed to solar UV radiation, significant germination differences for B27 were obtained with different oil types (WTS = 160.91, $df = 4$, $p < .001$), time of assessment (WTS = 643.29, $df = 2$, $p < .001$) and their interaction (WTS = 286.13, $df = 8$, $p < .001$). No germination of B27 had occurred at 24 h post-incubation; however, at 48 h and 72 h post-incubation, the germination of B27 in the vegetable oil formulation was significantly higher than in other oil types (Wilcoxon rank-sum test, $p < .001$, Figure 2a).

Similarly to B27, there was a significant difference in the germination of ECS1 with different oil types (WTS = 2,118.41, $df = 3$, $p < .001$), time of assessment (WTS = 2,106.93, $df = 2$, $p < .001$) and their interaction (WTS = 11,119.88, $df = 5$ $p < .001$). At 24 h post-incubation, no germination was recorded for ECS1. At 48 h and 72 h post-incubation, the highest germination was observed for ECS1 in vegetable oil and this was significantly higher than for all other oil types ($p < .001$, Figure 2b).

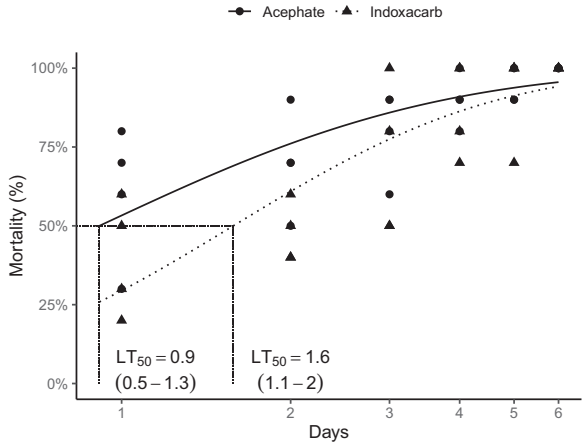


FIGURE 1 Mortality of adult *Kuschelorhynchus macadamiae* over time after application of acephate and indoxacarb at full field concentrations. Acephate 775 mg AI/L, indoxacarb 75 mg AI/L (Bioassay 1)

3.2.2 | Efficacy of oil-formulated EPF against *K. macadamiae*

All dead weevils in the glasshouse produced mycoses consistent with the relevant EPF after incubation. There were significant effects of treatments on the mortality of *K. macadamiae* (WTS = 144.59, $df = 5$, $p < .001$), time of assessment (WTS = 64.24, $df = 2$, $p < .001$) and their interaction (WTS = 274.44, $df = 10$, $p < .001$). The Wilcoxon rank-sum test for multiple comparisons revealed that the mortalities of weevils treated with ECS1 + oil or ECS1 alone did not differ significantly at any time point ($p > .05$, Figure 3), whereas the mortalities of weevils treated with B27 + oil or B27 alone were significantly different, but only at 12 days post-application ($p < .05$, Figure 3). The mortality of weevils treated with Velifer® was significantly lower than ECS1 + oil, B27 + oil and B27 alone at 6 days post-application, and lower than B27 + oil at 12 days post-application ($p < .05$, Figure 3).

TABLE 3 Effect of insecticides on the germination and daily growth of *Metarhizium anisopliae*, *Beauveria bassiana* and Velifer® biological insecticide

Fungi	Insecticide	Germination (%) ± SEM[a]	Growth (cm/day) ± SEM[b]
M. anisopliae ECS1	Control	97.00 ± 0.4 a	0.48 ± 0.007 a
	Acephate at FFC	92.75 ± 0.8 abc	0.46 ± 0.005 ab
	Indoxacarb at FFC	96.00 ± 0.7 a	0.48 ± 0.005 a
B. bassiana B27	Control	93.75 ± 1.5 ab	0.34 ± 0.003 ab
	Acephate at FFC	93.00 ± 1.4 abc	0.34 ± 0.002 ab
	Indoxacarb at FFC	92.50 ± 1.7 abc	0.34 ± 0.002 ab
Velifer®	Control	90.00 ± 0.8 bcd	0.33 ± 0.008 ab
	Acephate at FFC	89.00 ± 0.8 cd	0.33 ± 0.008 ab
	Indoxacarb at FFC	87.50 ± 1.3 d	0.31 ± 0.022 b

[a]Means followed by different letters are significantly different (ANOVA, Tukey's HSD test, $p < .05$).
[b]Means followed by different letters are significantly different (Kruskal–Wallis rank-sum test, Dunn's post hoc test with Bonferroni correction, $p < .05$).

TABLE 4 Interaction of fungal entomopathogens at 10^7 conidia/ml and insecticides at FFC on *Kuschelorhynchus macadamiae* at 6 days and 12 days post-application in the laboratory (Bioassay 2)

Days after application	Treatment	Observed mortality (%) ± SEM[a]	Expected mortality (%)	χ^2	Interaction
6 days	Acephate + ECS1	82.5 ± 2.50	80.50	0.050	Additive
	Acephate + B27	85.0 ± 2.89	80.50	0.252	Additive
	Acephate + Velifer®	77.5 ± 7.50	78.06	0.004	Additive
	Indoxacarb + ECS1	82.5 ± 6.29	79.00	0.155	Additive
	Indoxacarb + B27	80.0 ± 4.08	79.00	0.013	Additive
	Indoxacarb + Velifer®	77.5 ± 2.50	76.38	0.017	Additive
	Acephate	67.5 ± 4.79	–	–	–
	Indoxacarb	65.0 ± 2.89	–	–	–
	ECS1	40.0 ± 8.16	–	–	–
	B27	40.0 ± 4.08	–	–	–
	Velifer®	32.5 ± 16.52	–	–	–
12 days	Acephate + ECS1	90.0 ± 4.1	93.13	0.105	Additive
	Acephate + B27	87.5 ± 2.5	89.69	0.053	Additive
	Acephate + Velifer®	80.0 ± 4.1	84.88	0.280	Additive
	Indoxacarb + ECS1	92.5 ± 2.5	95.00	0.066	Additive
	Indoxacarb + B27	90.0 ± 4.1	92.50	0.068	Additive
	Indoxacarb + Velifer®	85.0 ± 5.0	89.00	0.180	Additive
	Acephate	72.5 ± 7.5	–	–	–
	Indoxacarb	80.0 ± 7.1	–	–	–
	ECS1	75.0 ± 11.9	–	–	–
	B27	62.5 ± 7.5	–	–	–
	Velifer®	45.0 ± 15.5	–	–	–

[a]Observed mortality at 6 days and 12 days post-application was corrected with Abbott's formula (the mortality in the control treatment at 6 days and 12 days post-application was 5% and 7.5%, respectively).

The presence of vegetable oil as a formulation additive for ECS1 and B27 led to weevil mortalities 22%–30% and 25%–35% higher than the corresponding treatments without oil at 12 days and 18 days post-application, respectively (Figure 3), although only one pairwise comparison was statistically significant.

3.2.3 | Co-application of UV-protected EPF and insecticides on *K. macadamiae*

All dead weevils in the insect cages produced mycoses consistent with the relevant EPF after incubation. The interactions between UV-protected EPF and insecticides consisted of additive and synergistic effects on *K. macadamiae* (Table 6). In treatments where acephate and EPF were co-applied on *K. macadamiae*, their interactions were additive at both 6 days and 12 days post-application ($\chi^2 < 3.84$, Table 6). In treatments where indoxacarb and EPF were co-applied on *K. macadamiae*, only ECS1 and B27 were synergistic at 6 days post-application ($\chi^2 > 3.84$, Table 6). Velifer® had only additive interactions with indoxacarb ($\chi^2 < 3.84$, Table 6). At 12 days post-application, indoxacarb had only additive interactions with UV-protected EPF against *K. macadamiae* ($\chi^2 < 3.84$, Table 6).

4 | DISCUSSION

Our previous *in vitro* study examining the effects of the insecticides acephate and indoxacarb at concentrations between 6.25% and 100% of their respective FFCs showed that these insecticides had no detrimental effect on the conidial germination, growth and sporulation of *M. anisopliae* strain QS155 or *B. bassiana* strain B50 (Khun et al., 2021a). In this study, both insecticides were mixed directly with *M. anisopliae* strain ECS1 and *B. bassiana* strain B27 in order to simulate a tank mixture for orchard spraying. No inhibition of fungal germination or growth was observed, and this confirmed that both insecticide formulations are compatible with both fungal species. Many studies have reported similar results, also finding that acephate and indoxacarb are compatible with *B. bassiana* and *M. anisopliae* (Akbar et al., 2012; Pires et al., 2010; Rachappa et al., 2007; Saito, 1984).

However, some studies have found that indoxacarb has antagonistic effects on *M. anisopliae* and *B. bassiana* (Amutha et al., 2010; Asi et al., 2010). This could be the consequence of the type of indoxacarb formulation. In this study, indoxacarb was used as a wettable granule, whereas Amutha et al. (2010) and Asi et al. (2010) tested indoxacarb as emulsifiable concentrate (EC) formulations. Supporting

TABLE 5 Interaction of fungal entomopathogens at 2.5×10^6 conidia/ml and insecticides at 25% of FFC on *Kuschelorhynchus macadamiae* at 6 days and 12 days post-application in the laboratory (Bioassay 3)

Days after application	Treatment	Observed mortality (%) ± SEM[a]	Expected mortality (%)	χ^2	Interaction
6 days	Acephate + ECS1	78.0 ± 10.20	60.04	5.372	Synergistic
	Acephate + B27	76.0 ± 7.48	55.72	7.381	Synergistic
	Acephate + Velifer®	68.0 ± 9.17	53.56	3.893	Synergistic
	Indoxacarb + ECS1	58.0 ± 14.28	48.20	1.993	Additive
	Indoxacarb + B27	60.0 ± 8.94	42.60	7.107	Synergistic
	Indoxacarb + Velifer®	38.0 ± 9.17	39.80	0.081	Additive
	Acephate	46.0 ± 8.72	–	–	–
	Indoxacarb	30.0 ± 8.37	–	–	–
	ECS1	26.0 ± 11.66	–	–	–
	B27	18.0 ± 5.83	–	–	–
	Velifer®	14.0 ± 7.48	–	–	–
12 days	Acephate + ECS1	80.0 ± 7.7	78.16	0.043	Additive
	Acephate + B27	80.0 ± 6.3	76.08	0.202	Additive
	Acephate + Velifer®	70.0 ± 9.5	55.28	3.920	Synergistic
	Indoxacarb + ECS1	80.0 ± 4.5	79.00	0.013	Additive
	Indoxacarb + B27	78.0 ± 4.9	77.00	0.013	Additive
	Indoxacarb + Velifer®	66.0 ± 6.8	57.00	1.421	Additive
	Acephate	48.0 ± 6.6	–	–	–
	Indoxacarb	50.0 ± 7.7	–	–	–
	ECS1	58.0 ± 14.3	–	–	–
	B27	54.0 ± 14	–	–	–
	Velifer®	14.0 ± 5.1	–	–	–

[a]Observed mortality at 12 days post-application was corrected with Abbott's formula (8% mortality was observed in the control treatment) whereas mortality at 6 days post-application was not corrected as no control weevil mortality occurred.

this, Anderson and Roberts (1983) found that EC formulations of selected insecticides (e.g., permethrin, piperonyl butoxide) showed negative impacts on six strains of *B. bassiana*. The formulation additives for these insecticides included toluene and xylene-type aromatic solvents, which were toxic to the EPF. Our previous *in vitro* study examining the effects of beta-cyfluthrin and methidathion in EC and non-EC formulations also showed that beta-cyfluthrin at 12.5 mg AI/L and methidathion at 250 mg AI/L without EC formulation additives were compatible with *M. anisopliae* strain QS155 and *B. bassiana* strain B50 but when EC formulations were tested at the same active ingredient concentrations they were toxic to both fungal species (Khun et al., 2021a).

Studies have suggested that solar UV radiation has a significant effect on the viability of EPF conidia (Alves et al., 1998; Moore et al., 1993). Without protective formulations, EPF are poorly active against targeted insects under field conditions (Kaiser et al., 2020). Studies have shown that oil-based formulations can provide protection from unsuitable weather conditions (de Oliveira et al., 2018) and result in a greater impact on insect pests (Kaiser et al., 2020; Luz & Batagin, 2005). In this study, vegetable oil, canola oil (Codacide®) and olive oil were able to protect the conidia of both fungal species from solar UV radiation under glasshouse conditions. Notably, the germination of both fungal species in canola oil (10% v/v) after exposure to solar UV radiation was significantly lower than in vegetable oil (10% v/v) and this was in contrast to the results of Alves et al. (1998) who found that Codacide® was a good conidial protectant from solar UV radiation. In this study, the germination of fungi in vegetable oil formulations only occurred after 48 h of incubation. This is in agreement with the findings of Moore et al. (1993) who hypothesized that even though fungal conidia were protected by the oil, they were still damaged to some extent by UV radiation and as a consequence, conidia may be diverting resources to repair cellular damage, delaying the germination process. This delay did not have any significant impact on fungal virulence (Moore et al., 1993), and this is also supported by our results. In addition, oil formulations may prevent desiccation of conidia, prolong fungal infectivity, weaken the insect cuticle and facilitate the adherence of conidia (Ibrahim et al., 1999; Prior et al., 1988), leading to improved overall efficacy (Brito et al., 2008; Kaiser et al., 2020).

In this study acephate performed better and faster than indoxacarb in both the laboratory and glasshouse. Unlike indoxacarb, acephate has an LC_{95} value lower than its FFC, and it is also a systemic broad-spectrum insecticide that causes mortality to insects both via direct contact and ingestion (Tomlin, 2006). Indoxacarb is

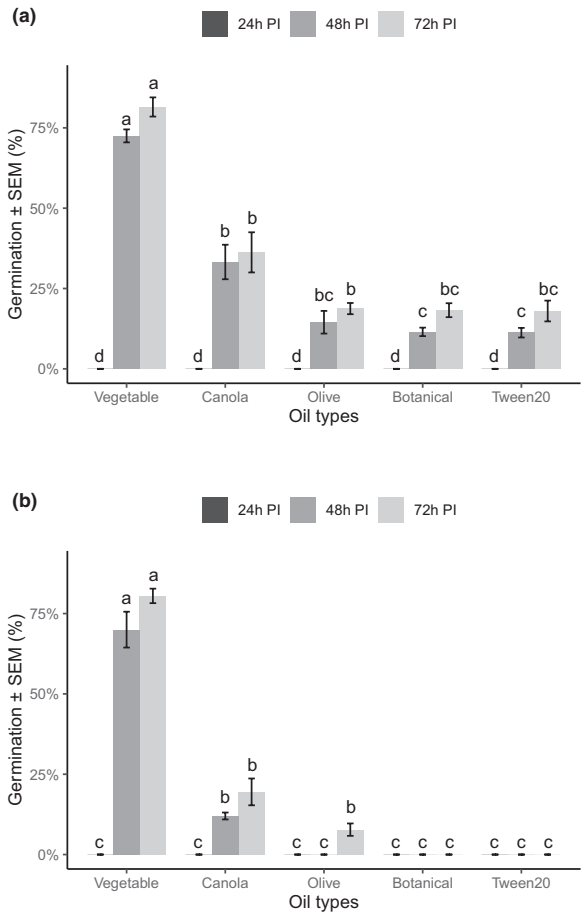


FIGURE 2 Germination (± SEM) of (a) *Beauveria bassiana* B27 and (b) *Metarhizium anisopliae* ECS1 in oil formulations after exposure to solar radiation in the glasshouse for 24 h. Columns with different letters are significantly different from each other (Wald-type statistics test, pairwise Wilcoxon rank-sum test, $p < .05$). (a) and (b) analysed separately. PI, Post-incubation

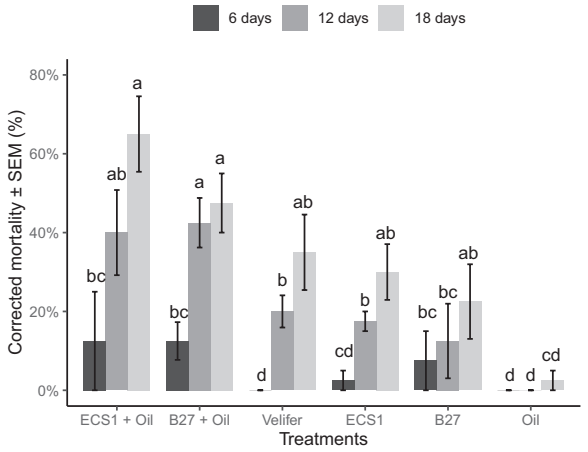


FIGURE 3 Mortality (± SEM) of *Kuschelorhynchus macadamiae* at 6 days, 12 days and 18 days post-application after treatment with EPF with or without vegetable oil. Columns with different letters are significantly different from each other (Wald-type statistics test, pairwise Wilcoxon rank-sum test, $p < .05$) (Bioassay 4)

a broad-spectrum oxadiazine insecticide that predominately causes death by ingestion (although mortality after direct contact has been observed (Wing et al., 2000)), so the response of insects to indoxacarb could be expected to be somewhat slower. The topical application of indoxacarb to several species of lepidopteran larvae was slower to take effect than feeding the larvae with food containing indoxacarb (Wing et al., 2000). This supports what we observed in the glasshouse, where the application of indoxacarb did not protect seedlings from damage by *K. macadamiae* since substantial weevil mortality occurred only after they had fed on the seedlings, whereas acephate produced a much more rapid response, preventing severe damage to the young plants.

Although these insecticides were highly effective and provided better control of *K. macadamiae* than EPF alone, some adults survived after treatment with both acephate and indoxacarb at their FFCs under laboratory conditions. This could indicate either that the current FFCs for these insecticides have simply been set below the optimum level, or it could reflect the development of resistance. The locations where we collected infested nuts were surrounded by orchards with a history of insecticide usage, and the development of resistance in response to frequent indoxacarb and acephate applications has been identified in a number of other insect species including the green peach aphid, *Myzus persicae* Sulzer (Attia & Hamilton, 1978), oblique banded leafroller, *Choristoneura rosaceana* Harris (Ahmad et al., 2002), diamondback moth, *Plutella xylostella* L. (Zhao et al., 2006) and the tarnished plant bug, *Lygus lineolaris* Palisot de Beauvois (Snodgrass et al., 2009). Our study cannot confirm or refute resistance to the tested insecticides in *K. macadamiae*, and a comparative toxicological study using weevil strains from different localities should be conducted.

The co-application of EPF with insecticides killed *K. macadamiae* more effectively than insecticides or EPF alone under both laboratory and glasshouse conditions. When insecticides at their FFCs were co-applied with EPF to *K. macadamiae*, interactions were generally additive, whereas when insecticides at 25% of FFC were combined with EPF and applied to *K. macadamiae*, more synergistic interactions were detected, including the only synergistic interaction that we detected at 12 days post-treatment. This reflects the strong and rapid response of *K. macadamiae* to the insecticides at their FFCs, leaving little scope for synergistic interactions with the slower-acting EPF (Khun, Ash, et al., 2020) to be detected in the small number of individuals surviving chemical treatment. In a similar study, Pelizza et al. (2018) reported that whilst insecticides (e.g., gamma-cyhalothrin, lambda-cyhalothrin, rynaxypyr, luphenuron, methoxyfenozide) and EPF at their FFCs effectively controlled the moth *Rachiplusia nu* Guenée, the combination of the control agents did not provide any further benefits. The synergistic interactions between insecticides and EPF could be detected only when both control agents were reduced to 50%

TABLE 6 Interaction of oil-formulated fungal entomopathogens at 10^7 conidia/ml and insecticides at FFC on *Kuschelorhynchus macadamiae* at 6 days and 12 days post-application in the glasshouse (Bioassay 5)

Days after application	Treatments	Observed mortality (%) ± SEM[a]	Expected mortality (%)	χ^2	Interaction
6 days	Acephate + ECS1 + VO	100	91.20	0.849	Additive
	Acephate + B27 + VO	100	91.20	0.849	Additive
	Acephate + Velifer®	100	90.80	0.932	Additive
	Indoxacarb + ECS1 + VO	82 ± 8.00	63.04	5.702	Synergistic
	Indoxacarb + B27 + VO	80 ± 5.48	63.04	4.563	Synergistic
	Indoxacarb + Velifer®	70 ± 7.07	61.36	1.217	Additive
	Acephate	90 ± 3.16	–	–	–
	Indoxacarb	58 ± 8.60	–	–	–
	ECS1 + VO	12 ± 4.90	–	–	–
	B27 + VO	12 ± 5.83	–	–	–
	Velifer®	8 ± 3.74	–	–	–
12 days	Acephate + ECS1 + VO	96.0 ± 2.4	97.52	0.024	Additive
	Acephate + B27 + VO	96.0 ± 2.4	97.60	0.026	Additive
	Acephate + Velifer®	96.0 ± 2.4	96.72	0.005	Additive
	Indoxacarb + ECS1 + VO	94.0 ± 2.4	95.04	0.011	Additive
	Indoxacarb + B27 + VO	94.0 ± 2.4	95.20	0.015	Additive
	Indoxacarb + Velifer®	94.0 ± 4.0	93.44	0.003	Additive
	Acephate	96.0 ± 2.4	–	–	–
	Indoxacarb	92.0 ± 5.8	–	–	–
	ECS1 + VO	38.0 ± 3.7	–	–	–
	B27 + VO	40.0 ± 3.2	–	–	–
	Velifer®	18.0 ± 2.0	–	–	–

Abbreviation: VO, vegetable oil (10% v/v).

[a]Observed mortality at 12 days post-application was corrected with Abbott's formula (4% mortality was observed in the control treatment) whereas mortality at 6 days post-application was not corrected as no control weevil mortality occurred.

of their FFCs or lower (Pelizza et al., 2018). Morales-Rodriguez and Peck (2009) also found that synergistic interactions were only apparent when imidacloprid was reduced from half to one-quarter of its FFC when combined with *B. bassiana* for controlling the white grub *Popillia japonica* Newman.

Many studies have demonstrated synergistic interactions between EPF and sublethal doses of insecticides for controlling pests of horticultural crops (Anderson & Roberts, 1983; Brito et al., 2008; Quintela & McCoy, 1997). These synergistic interactions were attributed to sublethal doses of insecticides weakening the insect cuticle by acting as a general insect stressor (Kumar et al., 2018), reducing the target pest's mobility, or disrupting the removal of fungal conidia via grooming behaviour (Brito et al., 2008; Khun et al., 2021b; Quintela & McCoy, 1997). As a consequence, insects were more vulnerable to the attachment and entry of EPF. However, exploiting the synergistic interactions identified in these studies in the field often cannot be recommended, since reducing insecticide application rates generally does not conform with mandatory registered use patterns. Sublethal insecticide rates used alone can increase selection pressure leading to the rapid development of resistant populations (Bantz et al., 2018) in both target and non-target pest species (Guedes et al., 2017). To avoid the development of resistance, some studies have suggested that EPF alone or the integration of EPF with synthetic insecticides at their full recommended rates could be used (Bahiense et al., 2006; Barbarin et al., 2017; Farenhorst et al., 2009, 2010; Farooq et al., 2018; Howard et al., 2011).

Some studies have suggested rotational applications of insecticides and EPF in the field (Bitsadze et al., 2013; Farenhorst et al., 2010; Farooq et al., 2018), and this approach may be the only viable option for integrating the two control methodologies when a particular insecticide is antagonistic to the EPF being used. If this is the case, information on the degradation rate of the insecticide and the survival of infective fungal conidia on plant surfaces can be used to ensure the timing of treatments avoids adverse effects on EPF efficacy. The use of rotational treatments, however, may lead to increased operational costs, particularly those associated with fuel and labour. In contrast, when the effects of a combined insecticide/EPF treatment are either additive or synergistic on the target pest, their simultaneous application may avoid these additional costs, providing rapid control of insecticide-susceptible individuals and an additional control pathway that may eliminate

any insecticide-resistant individuals which may otherwise lead to the development of a broader resistance problem. The choice between rotational or simultaneous insecticide/EPF treatment relies on a detailed understanding of the interactions between the component treatments, both in tank mixes and on plant surfaces, and evaluation of their respective levels of efficacy under field conditions.

In conclusion, acephate and indoxacarb in their current formulations were found to be compatible with *M. anisopliae* strain ECS1, *B. bassiana* strain B27 and Velifer® Biological Insecticide but synergistic interactions on adult weevils were rarely detected when FFCs of insecticides were used. This study provided two clear results relevant to the management of *K. macadamiae*, (a) if acephate and indoxacarb formulations are deployed in the field for controlling *K. macadamiae*, they will not have any negative impact on EPF (either naturally occurring or resulting from inundative application) and (b) acephate and indoxacarb could be combined with EPF prior to application in the field, leading to more effective control of *K. macadamiae*. This strategy could also help mitigate against the development of insecticide resistance in *K. macadamiae*.

ACKNOWLEDGEMENTS

Ian Purdue, Jarrah Coates and David Robertson are thanked for their support with field work. Craig Maddox of Wollongbar Primary Industries Institute is thanked for providing insecticide samples and Dr. Diana Leemon is thanked for providing *B. bassiana* strain B27. This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation, Australia with the Australian macadamia industry's research and development levy and contributions from the Australian Government under the project title 'The IPM program for the Australian Macadamia Industry' (project: MC16004).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors conceived the research and reviewed the manuscript. KKK conducted the experiments, analysed data and wrote the manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All raw data can be accessed at http://doi.org/10.5281/zenodo.4289403

ORCID

Kim Khuy Khun https://orcid.org/0000-0002-6937-2721
Gavin J. Ash https://orcid.org/0000-0002-6703-8295
Mark M. Stevens https://orcid.org/0000-0002-7581-0783
Ruth K. Huwer https://orcid.org/0000-0003-3435-3106
Bree A. L. Wilson https://orcid.org/0000-0003-4036-9980

REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*, 265–267. https://doi.org/10.1093/jee/18.2.265a

Ahmad, M., Hollingworth, R. M., & Wise, J. C. (2002). Broad-spectrum insecticide resistance in obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) from Michigan. *Pest Management Science*, *58*, 834–838. https://doi.org/10.1002/ps.531

Akbar, S., Freed, S., Hameed, A., Gul, H. T., Akmal, M., Malik, M. N., Naeem, M., & Khan, M. B. (2012). Compatibility of *Metarhizium anisopliae* with different insecticides and fungicides. *African Journal of Microbiology Research*, *6*, 3956–3962. https://doi.org/10.5897/ajmr12.417

Alves, R. T., Bateman, R. P., Prior, C., & Leather, S. R. (1998). Effects of simulated solar radiation on conidial germination of *Metarhizium anisopliae* in different formulations. *Crop Protection*, *17*, 675–679. https://doi.org/10.1016/s0261-2194(98)00074-x

AMS. (2019). *Yearbook 2019*. Australian Macadamia Society Ltd. https://d1bel7n84kyh0s.cloudfront.net/uploads/2019/11/2019-AUSTRALIAN-MACADAMIAS-YEARBOOK-FINAL-V1.0.pdf

Amutha, M., Gulsar Banu, J., Surulivelu, T., & Gopalakrishnan, N. (2010). Effect of commonly used insecticides on the growth of white Muscardine fungus, *Beauveria bassiana* under laboratory conditions. *Journal of Biopesticides*, *3*, 143–146.

Anderson, T. E., & Roberts, D. W. (1983). Compatibility of *Beauveria bassiana* isolates with insecticide formulations used in Colorado potato beetle (Coleoptera: Chrysomelidae) control. *Journal of Economic Entomology*, *76*, 1437–1441. https://doi.org/10.1093/jee/76.6.1437

Asi, M. R., Bashir, M. H., Afzal, M., Ashfaq, M., & Sahi, S. T. (2010). Compatibility of entomopathogenic fungi, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* with selective insecticides. *Pakistan Journal of Botany*, *42*, 4207–4214.

Attia, F., & Hamilton, J. (1978). Insecticide resistance in *Myzus persicae* in Australia. *Journal of Economic Entomology*, *71*, 851–853. https://doi.org/10.1093/jee/71.6.851

Bahiense, T. C., Fernandes, È. K. K., & Bittencourt, V. R. E. P. (2006). Compatibility of the fungus *Metarhizium anisopliae* and deltamethrin to control a resistant strain of *Boophilus microplus* tick. *Veterinary Parasitology*, *141*, 319–324. https://doi.org/10.1016/j.vetpar.2006.05.011

Bantz, A., Camon, J., Froger, J. A., Goven, D., & Raymond, V. (2018). Exposure to sublethal doses of insecticide and their effects on insects at cellular and physiological levels. *Current Opinion in Insect Science*, *30*, 73–78. https://doi.org/10.1016/j.cois.2018.09.008

Barbarin, A. M., Bellicanta, G. S., Osborne, J. A., Schal, C., & Jenkins, N. E. (2017). Susceptibility of insecticide-resistant bed bugs (*Cimex lectularius*) to infection by fungal biopesticide. *Pest Management Science*, *73*, 1568–1573. https://doi.org/10.1002/ps.4576

Bitsadze, N., Jaronski, S., Khasdan, V., Abashidze, E., Abashidze, M., Latchininsky, A., Samadashvili, D., Sokhadze, I., Rippa, M., Ishaaya, I., & Horowitz, A. R. (2013). Joint action of *Beauveria bassiana* and the insect growth regulators diflubenzuron and novaluron, on the migratory locust, *Locusta migratoria*. *Journal of Pest Science*, *86*, 293–300. https://doi.org/10.1007/s10340-012-0476-4

Bright, J. (2017a). *Macadamia seed weevil* (Kuschelorhynchus macadamiae) *life cycle and monitoring*. Wollongbar, Australia: Primefact 1586. https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/731982/Macadamia-seed-weevil-update-lifecycle_2.pdf

Bright, J. (2017b). *Sigastus weevil update. Part 1. Life cycle and monitoring keys to control*. Australian Macadamia Society Ltd. https://www.horticulture.com.au/globalassets/hort-innovation/resource-assets/mc-ipm-program-sigastus-weevil-fact-sheet.pdf

Bright, J. (2019). *Macadamia plant protection guide 2019–20*. NSW Department of Primary Industries. https://www.dpi.nsw.gov.

au/__data/assets/pdf_file/0006/529161/Macadamia-plant-protection-guide-2019.pdf

Brito, E. S., de Paula, A. R., Vieira, L. P., Dolinski, C., & Samuels, R. I. (2008). Combining vegetable oil and sub-lethal concentrations of imidacloprid with *Beauveria bassiana* and *Metarhizium anisopliae* against adult guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae). *Biocontrol Science and Technology*, *18*, 665–673. https://doi.org/10.1080/09583150802195965

de Oliveira, D. G. P., Lopes, R. B., Rezende, J. M., & Delalibera, I. (2018). Increased tolerance of *Beauveria bassiana* and *Metarhizium anisopliae* conidia to high temperature provided by oil-based formulations. *Journal of Invertebrate Pathology*, *151*, 151–157. https://doi.org/10.1016/j.jip.2017.11.012

Dolinski, C., & Lacey, L. A. (2007). Microbial control of arthropod pests of tropical tree fruits. *Neotropical Entomology*, *36*, 161–179. https://doi.org/10.1590/s1519-566x2007000200001

Farenhorst, M., Knols, B. G. J., Thomas, M. B., Howard, A. F. V., Takken, W., Rowland, M., & N'Guessan, R. (2010). Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PLoS One*, *5*, e12081. https://doi.org/10.1371/journal.pone.0012081

Farenhorst, M., Mouatcho, J. C., Kikankie, C. K., Brooke, B. D., Hunt, R. H., Thomas, M. B., Koekemoer, L. L., Knols, B. G. J., & Coetzee, M. (2009). Fungal infection counters insecticide resistance in African malaria mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 17443–17447. https://doi.org/10.1073/pnas.0908530106

Farooq, M., Steenberg, T., Højland, D. H., Freed, S., & Kristensen, M. (2018). Impact of sequential exposure of *Beauveria bassiana* and imidacloprid against susceptible and resistant strains of *Musca domestica*. *BioControl*, *63*, 707–718. https://doi.org/10.1007/s10526-018-9892-6

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (3rd ed.). Sage Publications.

Ghajar, F., Holford, P., Cother, E., & Beattie, A. (2006). Enhancing survival and subsequent infectivity of conidia of potential mycoherbistats using UV protectants. *Biocontrol Science and Technology*, *16*, 825–839. https://doi.org/10.1080/09583150600700149

Guedes, R. N. C., Walse, S. S., & Throne, J. E. (2017). Sublethal exposure, insecticide resistance, and community stress. *Current Opinion in Insect Science*, *21*, 47–53. https://doi.org/10.1016/j.cois.2017.04.010

Hlina, B. L. (2019). Ecotox: Analysis of ecotoxicology. Ver. 1.4.0.: R package. Retrieved from https://CRAN.R-project.org/package=ecotox

Howard, A. F. V., N'Guessan, R., Koenraadt, C. J. M., Asidi, A., Farenhorst, M., Akogbéto, M., Knols, B. G. J., & Takken, W. (2011). First report of the infection of insecticide-resistant malaria vector mosquitoes with an entomopathogenic fungus under field conditions. *Malaria Journal*, *10*, 8. https://doi.org/10.1186/1475-2875-10-24

Huwer, R. (2016). *Ecology and management of Sigastus weevil in macadamias*. (Final report, Project MC15010). Horticulture Innovation Australia Limited. https://www.horticulture.com.au/globalassets/laserfiche/assets/project-reports/mc15010/mc15010-final-report-514.pdf

Ibrahim, L., Butt, T. M., Beckett, A., & Clark, S. J. (1999). The germination of oil-formulated conidia of the insect pathogen, *Metarhizium anisopliae*. *Mycological Research*, *103*, 901–907. https://doi.org/10.1017/s0953756298007849

Inglis, G. D., Enkerli, J., & Goettel, M. S. (2012). Chapter 7: Laboratory techniques used for entomopathogenic fungi: Hypocreales. In L. A. Lacey (Ed.), *Manual of techniques in invertebrate pathology* (2nd ed., pp. 189–253). Elsevier Academic Press Inc.

Jennings, D., & Oberprieler, R. G. (2018). A review of the Tribe Cryptoplini (Coleoptera: Curculioninae), with revision of the Genus *Menechirus* Hartmann, 1901 and description of a new Genus associated with *Macadamia*. *Diversity*, *10*, 71. https://doi.org/10.3390/d10030071

Kaiser, D., Handschin, S., Rohr, R. P., Bacher, S., & Grabenweger, G. (2020). Co-formulation of *Beauveria bassiana* with natural substances to control pollen beetles - Synergy between fungal spores and colza oil. *Biological Control*, *140*, 104106. https://doi.org/10.1016/j.biocontrol.2019.104106

Khun, K. K., Ash, G. J., Stevens, M. M., Huwer, R. K., & Wilson, B. A. L. (2020). Response of the macadamia seed weevil *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) to *Metarhizium anisopliae* and *Beauveria bassiana* in laboratory bioassays. *Journal of Invertebrate Pathology*, *174*, 107437. https://doi.org/10.1016/j.jip.2020.107437

Khun, K. K., Ash, G. J., Stevens, M. M., Huwer, R. K., & Wilson, B. A. L. (2021a). Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia. *Pest Management Science*, *77*, 709–718. https://doi.org/10.1002/ps.6065

Khun, K. K., Ash, G. J., Stevens, M. M., Huwer, R. K., & Wilson, B. A. L. (2021b). Transmission of *Metarhizium anisopliae* and *Beauveria bassiana* to adults of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae) from infected adults and conidiated cadavers. *Scientific Reports*, *11*, 2188. https://doi.org/10.1038/s41598-021-81647-0

Khun, K. K., Wilson, B. A. L., Stevens, M. M., Huwer, R. K., & Ash, G. J. (2020). Integration of entomopathogenic fungi into IPM programs: Studies involving weevils (Coleoptera: Curculionoidea) affecting horticultural crops. *Insects*, *11*, 659. https://doi.org/10.3390/insects11100659

Kumar, V., Francis, A., Avery, P. B., McKenzie, C. L., & Osborne, L. S. (2018). Assessing compatibility of *Isaria fumosorosea* and buprofezin for mitigation of *Aleurodicus rugioperculatus* (Hemiptera: Aleyrodidae): An invasive pest in the Florida landscape. *Journal of Economic Entomology*, *111*, 1069–1079. https://doi.org/10.1093/jee/toy056

Lacey, L. A., Frutos, R., Kaya, H. K., & Vail, P. (2001). Insect pathogens as biological control agents: Do they have a future? *Biological Control*, *21*, 230–248. https://doi.org/10.1006/bcon.2001.0938

Lacey, L. A., & Shapiro-Ilan, D. I. (2008). Microbial control of insect pests in temperate orchard systems: Potential for incorporation into IPM. *Annual Review of Entomology*, *53*, 121–144. https://doi.org/10.1146/annurev.ento.53.103106.093419

Luz, C., & Batagin, I. (2005). Potential of oil-based formulations of *Beauveria bassiana* to control *Triatoma infestans*. *Mycopathologia*, *160*, 51–62. https://doi.org/10.1007/s11046-005-0210-3

Moore, D., Bridge, P. D., Higgins, P. M., Bateman, R. P., & Prior, C. (1993). Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. *Annals of Applied Biology*, *122*, 605–616. https://doi.org/10.1111/j.1744-7348.1993.tb04061.x

Morales-Rodriguez, A., & Peck, D. C. (2009). Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. *Biological Control*, *51*, 169–180. https://doi.org/10.1016/j.biocontrol.2009.06.008

Nehare, S., Ghodki, B. S., Lande, G. K., Pawade, V., & Thakare, A. S. (2010). Inheritance of resistance and cross resistance pattern in indoxacarb-resistant diamondback moth *Plutella xylostella* L. *Entomological Research*, *40*, 18–25. https://doi.org/10.1111/j.1748-5967.2009.00261.x

Noguchi, K., Gel, Y. R., Brunner, E., & Konietschke, F. (2012). nparLD: An R software package for the nonparametric analysis of longitudinal data in factorial experiments. *Journal of Statistical Software*, *50*, 23. https://doi.org/10.18637/jss.v050.i12

Ogle, D., Wheeler, P., & Dinno, A. (2019). FSA: Simple fisheries stock assessment methods. Version 0.8.25. R package. Retrieved from https://CRAN.R-project.org/package=FSA

Pachamuthu, P., & Kamble, S. T. (2000). *In vivo* study on combined toxicity of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) strain ESC-1 with sublethal doses of chlorpyrifos, propetamphos,

and cyfluthrin against German cockroach (Dictyoptera: Blattellidae). *Journal of Economic Entomology*, *93*, 60–70. https://doi.org/10.1603/0022-0493-93.1.60

Pelizza, S. A., Schalamuk, S., Simón, M. R., Stenglein, S. A., Pacheco-Marino, S. G., & Scorsetti, A. C. (2018). Compatibility of chemical insecticides and entomopathogenic fungi for control of soybean defoliating pest, *Rachiplusia nu*. *Revista Argentina de Microbiologia*, *50*, 189–201. https://doi.org/10.1016/j.ram.2017.06.002

Pires, L. M., Marques, E. J., de Oliveira, J. V., & Alves, S. B. (2010). Selection of isolates of entomopathogenic fungi for controlling *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and their compatibility with insecticides used in tomato crop. *Neotropical Entomology*, *39*, 977–984. https://doi.org/10.1590/S1519-566X2010000600020

Prior, C., Jollands, P., & Le Patourel, G. (1988). Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) to the cocoa weevil pest *Pantorhytes plutus* (Coleoptera: Curculionidae). *Journal of Invertebrate Pathology*, *52*, 66–72. https://doi.org/10.1016/0022-2011(88)90103-6

QDAF. (2019). *Macadamia industry benchmark report: 2009 to 2018 seasons. (Project MC18002)*. Queensland Department of Agriculture and Fisheries. Retrieved from https://www.publications.qld.gov.au/dataset/18517168-df7c-41d9-bf92-50ff4ccfb6ac/resource/76587ac2-fb21-4483-bc61-1a5088d02712/fs_download/macadamia-industry-benchmark-report-2009-18.pdf

Quintela, E. D., & McCoy, C. W. (1997). Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environmental Entomology*, *26*, 1173–1182. https://doi.org/10.1093/ee/26.5.1173

R Core Team. (2018). *R: A language and environment for statistical computing*. R Foundation for statistical computing. https://www.r-project.org

Rachappa, V., Lingappa, S., & Patil, R. K. (2007). Effect of agrochemicals on growth and sporulation of *Metarhizium anisopliae* (Metschnikoff) Sorokin. *Karnataka Journal of Agricultural Sciences*, *20*, 410–413.

RStudio Team. (2018). *RStudio: Integrated development for R*. RStudio, Inc. https://rstudio.com

Saito, T. (1984). Effect of pesticides on conidial germination and hyphal growth of the entomopathogenic fungus *Beauveria bassiana*. *Japanese Journal of Applied Entomology and Zoology*, *28*, 87–89. https://doi.org/10.1303/jjaez.28.87

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*, 591–611. https://doi.org/10.1093/biomet/52.3-4.591

Snodgrass, G. L., Gore, J., Abee, C. A., & Jackson, R. (2009). Acephate resistance in populations of the tarnished plant bug (Heteroptera: Miridae) from the Mississippi river delta. *Journal of Economic Entomology*, *102*, 699–707. https://doi.org/10.1603/029.102.0231

Tomlin, C. D. S. (2006). *The pesticide manual: A world compendium* (14th ed.). British Crop Protection Council.

Treverrow, N. (1987). Latania scale (*Hemiberlesia lataniae*). Paper presented at the Second Australian Macadamia Research Workshop. T. Trochoulias & I. Skinner Eds., 82–86.

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer.

Wing, K. D., Sacher, M., Kagaya, Y., Tsurubuchi, Y., Mulderig, L., Connair, M., & Schnee, M. (2000). Bioactivation and mode of action of the oxadiazine indoxacarb in insects. *Crop Protection*, *19*, 537–545. https://doi.org/10.1016/S0261-2194(00)00070-3

Zhao, J.-Z., Collins, H. L., Li, Y.-X., Mau, R. F. L., Thompson, G. D., Hertlein, M., Andaloro, J. T., Boykin, R., & Shelton, A. M. (2006). Monitoring of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad, indoxacarb, and emamectin benzoate. *Journal of Economic Entomology*, *99*, 176–181. https://doi.org/10.1093/jee/99.1.176

How to cite this article: Khun KK, Ash GJ, Stevens MM, Huwer RK, Wilson BAL. Interactions of fungal entomopathogens with synthetic insecticides for the control of *Kuschelorhynchus macadamiae* (Coleoptera: Curculionidae). *J Appl Entomol*. 2021;00:1–14. https://doi.org/10.1111/jen.12879

Chapter 7: Summary of findings, general discussion, future research and conclusion

7.1. Summary of findings

The chief aim of this study was to select potential biopesticides and integrate them with registered synthetic pesticides in order to maximise the success of *K. macadamiae* management and minimise any unintentional impacts of synthetic pesticides on biopesticide performance. The findings presented in Chapter 3-6 are summarised in this chapter.

7.1.1. Summary of Chapter 3

The overall objective of the experiments in Chapter 3 was to identify, evaluate and compare the pathogenicity of *M. anisopliae* and *B. bassiana* strains on *K. macadamiae*. Six experiments were performed to investigate this objective and the results showed that:

1. Molecular characterisation based on the 5' region of elongation factor-1 alpha (EFT1) and the B locus nuclear intergenic region (Bloc) of all strains of *Metarhizium* spp. and *Beauveria* spp. confirmed that they belonged to *Metarhizium anisopliae* and *Beauveria bassiana,* respectively.
2. The germination of all strains of *M. anisopliae* and *B. bassiana* was not significantly different and was always above 86% when the fungi were incubated at 25˚C for 18 h (Appendix B).
3. At 25˚C, the mycelial growth rates of all strains of *M. anisopliae* were not different from each other (4.58 – 5.05 mm/day), whereas B50 was significantly different to other *B. bassiana* strains (3.61 mm/day versus 2.96 – 3.1 mm/day) (Appendix B).
4. Of all the strains of *M. anisopliae,* application of ECS1 at 1 x 10^7 conidia/mL resulted in the highest mortality of *K. madadamiae* adults (97.5%). At the same concentration, *B. bassiana* strain B27 was the most effective, also inducing high mortality to adults (92.5%). The median lethal time (LT_{50}) for both strains was around 5 days.

5. The number of external conidia on each cadaver did not differ significantly between fungal strains. On average, the number of conidia on cadavers killed by *M. anisopliae* and *B. bassiana* were around 1.12 x 10^8 conidia/cadaver and 1.2 x 10^8 conidia/cadaver, respectively.
6. The median lethal concentration (LC_{50}) of *M. anisopliae* strain ECS1 was 1.48 x 10^5 conidia/mL with a 95% confidence interval of 6.88 x 10^4 to 2.89 x 10^5 conidia/mL. For *B. bassiana* strain B27, the LC_{50} was 1.65 x 10^5 conidia/mL with a 95% confidence interval of 7.22 x 10^4 to 3.36 x 10^5 conidia/mL. The LC_{95} values for ECS1 and B27 were 2.49 x 10^7 conidia/mL and 4.64 x 10^7 conidia/mL, respectively.

7.1.2. Summary of Chapter 4

The overall objective of the experiments in Chapter 4 was to evaluate the potential of conidia transmission from fungus-infected adults and conidiated cadavers to fungus-free/healthy adults. Four experiments with two EPF investigated the mortality of healthy adults driven by the proportion of the fungal infected adults and conidiated cadavers; and the results revealed that:

1. The mortality of healthy adults was 37.5% and 46.25% at 12 days after *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 infected adults were introduced into a confined environment (500 mL plastic container) at the ratio 1:1. When the ratio of fungal infected adults decreased in the container, the mortality of healthy adults also decreased significantly. The mortality of healthy adults was 8.13% and 18.75% when *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 infected adults were introduced at the ratio 1:20 under the same conditions for 12 days.
2. In the same confined environment (500 mL plastic container), the mortality of adults caused by B27 and ECS1 conidiated cadavers at a ratio 1:1 was 95% and 100%, respectively, after 9 days of observation. At the ratio 1:20 cadavers to adults, the mortality of initially uninfected adults remained greater than 80% for both fungal species after 9 days of observation.
3. In the insect cages the mortality of adults exposed to B27 and ECS1 conidiated cadavers at the ratio 1:1 was 40% and 25%, respectively, after 9 days of observation, or 77.5% and 50%, respectively, after 18 days of observation. The mortality of adults decreased when the ratio of the fungal

conidiated cadavers decreased. The mortality of adults decreased to 25% and 6.25% when B27 and ECS1 conidiated cadavers were introduced at the ratio 1:20 in the insect cages for 18 days.

4. The scanning electron microscopy images provided insight into the behaviour of adults towards conidiated cadavers inside the insect cage. Adults did not avoid conidiated cadavers and the hairs of their tarsal pad were frequently covered in conidia. The conidia attached on the tarsal pad were presumably transferred to the rostrum and compound eyes as a result of grooming, which was an observed behaviour.

7.1.3. Summary of Chapter 5

The overall objective of the experiments in Chapter 5 was to evaluate the compatibility of registered synthetic pesticides used in macadamia production with *M. anisopliae* and *B. bassiana.* Four *in vitro* experiments were performed to examine this objective and the results are summarised below:

1. Most strains of *M. anisopliae* and *B. bassiana* responded similarly to the insecticide spinetoram at 50% and 100% of its full field concentration (FFC).
2. At their FFCs the formulated insecticides trichlorfon, acephate and indoxacarb were compatible with *M. anisopliae* whereas *B. bassiana* showed compatibility with five formulated insecticides; trichlorfon, acephate, indoxacarb, sulfoxaflor and spinetoram. However, methidathion, diazinon and beta-cyfluthrin were toxic to both fungal species. Both fungicides, carbendazim and pyraclostrobin, were very toxic to both fungal species.
3. Acetone at 2% (v/v) was toxic to both fungal species, but when the concentration of acetone was around 1% (v/v), it was toxic to *M. anisopliae* only. At a concentration of 0.5% (v/v), acetone was compatible with both fungal species.
4. At their FFCs, the laboratory-grade beta-cyfluthrin was compatible with both fungal species and laboratory-grade methidathion was compatible with *B. bassiana* only. Laboratory-grade diazinon, carbendazim and pyraclostrobin at their FFCs were toxic to very toxic to both fungal species.

7.1.4. Summary of Chapter 6

The overall objective of the experiments in Chapter 6 was to evaluate the synergistic interactions of the registered insecticides and EPF on *K. macadamiae.* Seven experiments were performed either in the laboratory or glasshouse to investigate this objective and the results validated that:

1. The median lethal concentrations (LC_{50}) of acephate and indoxacarb were 20.2% and 10.9% of their full field concentrations (FFCs), respectively, whereas their LC_{95} values were 97.1% and 148% of their FFCs, respectively. The LT_{50} of acephate and indoxacarb at their FFCs were 0.9 days and 1.6 days with 95% confidence intervals of 0.5 – 1.3 days and 1.1 – 2 days, respectively.
2. Neither of these insecticides had any negative effects on the germination or daily mycelial growth of EPF (*M. anisopliae* strain ECS1, *B. bassiana* strain B27 and a commercial biopesticide (Velifer®) based on *B. bassiana* strain PPRI 5339) when they were mixed to simulate a spray tank mix. The germination of all the fungi was greater than 86% whereas the mycelial growth of ECS1, B27 and Velifer® were 4.6 – 4.8 mm/day, 3.4 mm/day and 3.1 – 3.3 mm/day, respectively.
3. In the laboratory, the combination of EPF (ECS1 at 1 x 10^7 conidia/mL, B27 at 1 x 10^7 conidia/mL, Velifer® at FFC) with insecticides (acephate, indoxacarb) at their FFCs produced only additive effects in their overall activity against *K. macadamiae.*
4. Under laboratory conditions the combination of EPF at lower concentrations (reduced by 75% compared to the previous experiment) with insecticides (acephate, indoxacarb) at 25% of their FFCs produced both additive and synergistic effects against *K. macadamiae.*
5. Among the 5 oil types used as a solar UV protectant for the fungal conidia, vegetable oil at 10% (v/v) provided the best protection from solar UV radiation.
6. In the glasshouse, ECS1 and B27 in a vegetable oil formulation (10% v/v) induced higher mortality of adult *K. macadamiae* than ECS1 and B27 without vegetable oil, or that achieved with the commercial biopesticide (Velifer®).

7. In the glasshouse, the combination of EPF (ECS1 at 1 x 10^7 conidia/mL, B27 at 1 x 10^7 conidia/mL, Velifer® at FFC) with insecticides (acephate, indoxacarb) at their FFCs produced both synergistic and additive effects on *K. macadamiae.*

7.2. General discussion

Kuschelorhynchus macadamiae has been a significant pest on macadamia in the Northern Rivers for over a decade, causing nutlets to drop (Lee, 2014) and resulting in significant yield loss (Huwer, 2016). Over the past 5 years, the management of this weevil has moved from multiple applications of acephate (Bright, 2016) to a maximum of two applications of indoxacarb per season, combined with the collection and destruction of the fallen nutlets that contain developing larvae (Bright, 2020).

Prior to this study, Maddox *et al.* (2015) demonstrated the success of *K. macadamiae* management with EPF in laboratory experiments. Spraying adults with 2 mL suspensions of *M. anisopliae* and *B. bassiana* was highly effective, giving 100% control of adults. However, mortality in the control treatment was also high (around 70%), voiding these results. Dipping insects in a fungal suspension is commonly used in screening studies, for example dipping sweetpotato weevil, *Cylas formicarius* F. (Coleoptera: Brentidae), in 10 mL of fungal suspension at 1 x 10^7 conidia/mL for 10 – 12s (Dotaona *et al.*, 2015). In this study, both of these methods were tested. Dipping the weevils (as used by Dotaona *et al.* (2015)) resulted in high mortality in the control treatment, whereas using a spray application volume of 1 mL avoided mortality in the controls. Consequently, a spray application of 1 mL was used as the standard application techniques for all laboratory bioassays in this study (Chapters 3, 4 and 6).

Before conducting systematic experiments in this study, no peer-reviewed paper had been published on the potential of EPF to control *K. macadamiae*. The presence of adults in the field is restricted to a period between September and December each year, and this short window of availability of sufficient insects to run systematic experiments was a major challenge in this research. In addition, there was no information available on laboratory rearing and breeding using artificial diets, therefore protocols for rearing *K. macadamiae* in the laboratory were developed here (Chapters 3, 4 and 6). The protocols for rearing adults, as well as the experimental procedures developed in this study have contributed new information on the methods necessary for effective research on *K. macadamiae*.

Beauveria bassiana and *M. anisopliae* are two common EPF that are isolated from agroecosystems (Shapiro-Ilan *et al.*, 2003; Korosi *et al.*, 2019) and are usually isolated from infected insects and soil with selective media and insect baits (e.g. greater wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae) or mealworm larvae, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)), respectively (Meyling and Eilenberg, 2007). They have the potential to be used and integrated with other components of IPM programs for controlling weevils affecting many horticultural crops (Chapter 2). Morphological identification is not sufficient for distinguishing between EPF species (Bischoff *et al.*, 2009) whereas molecular techniques using primers to target specific gene region, for example B22U/B822L primers for *Beauveria* (Fisher *et al.*, 2011) and EF1T/EF2T primers for *Metarhizium* (Rehner and Buckley, 2005), have been found to be reliable (Fisher *et al.*, 2011) (Chapter 3).

In most of the bioassays with EPF, mortality of adults was recorded for only 12 days post-application (Chapters 3 and 6). This limitation was due to the mortality in the control treatments that increased rapidly after 12 days, and which may have been caused by unidentified stresses associated with the experimental conditions. In the glasshouse experiment, the feeding routine of the adults was not reduced as a result of fungal infection over the 12 days of observation. The macadamia seedlings used to feed adults in the laboratory and glasshouse were killed within 12 days (10 adults/seedling, Figure 10d-e) despite the adults being infected with EPF, or the seedlings being sprayed with fungal suspensions (Chapters 4 and 6). This damage indicates that although the adults were infected with EPF, they may still cause some damage to the tree since the EPF require several days to infect and kill the adults (Chapter 3). These live fungus-infected adults could be the carriers (or donors) to deliver conidia to other adults after application, and therefore could provide additional control to that derived from the initial EPF application. This is an important aspect of using EPF to control pests. In confirming this possibility, Chapter 4 showed that adults which were infected with fungal conidia were able to transfer infective propagules to other adults. As a result, other adults were infected with and killed by the EPF. As there is currently no effective laboratory rearing and breeding method for *K. macadamiae*, it was not possible to investigate the effect of EPF on subsequent generations as a result of horizontal transmission, rather than just on the mortality of healthy adults. Studies on other weevils affecting horticultural crops suggested that conidial transmission not only impacts on the reproductive partners (horizontal

transmission), but that adult fecundity and egg survivorship can be reduced (Dembilio *et al.*, 2010; Dotaona *et al.*, 2017). For example, the horizontal transmission of fungal conidia from male adults did not just kill the female adults, but also reduced the number of eggs produced and percentage of egg viability by 65% and by 75%, respectively, for sweetpotato weevil (*C. formicarius*) (Dotaona *et al.*, 2017) and by 55% and by 49%, respectively, for red palm weevil (*Rhynchophorus ferrugineus* Olivier, Coleoptera: Curculionidae) (Dembilio *et al.*, 2010) before the females died. This suggests that the use of EPF may bring more sustainable and on-going suppression to pest populations after application.

When implementing EPF for suppressing insect pests in the field, from a sustainability perspective, it is also important to know whether the insects killed by the EPF could conidiate and become the source of inoculum for other insects in their respective environments. This outgrowth of conidiation on the insect can only be supported if the weather conditions are conducive. During the activity season of *K. macadamiae* between September and December, the mean temperature between 1991 and 2011 was < 27˚C with the humidity of 65 – 75% (BOM, 2020), conditions ideal for EPF growth and persistence in the field. The microclimate in the orchard could be far more suitable for the EPF than the data from the meteorology station (e.g. the temperature could be lower and the humidity could be higher within the orchard). These more suitable conditions for EPF to thrive could be attributed to the dense canopy and shade produced by the mature macadamia trees (Figure 2). Mature macadamia trees are generally >18 m in height with a canopy diameter of around 12 m (Hamilton *et al.*, 1983; Rosengarten, 2004). Typically the tree density is between 250 and 350 trees/ha, but 313 trees/ha is the recommended density across the industry (AMS, 2017). By simulating the field environment in the laboratory, we found that *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 are the most virulent EPF for controlling *K. macadamiae* and they are more active than the commercially available Velifer® Biological Insecticide, which is registered for protected cropping only. In addition, the number of conidia produced on the cadavers was also very high, suggesting that both strains show potential to be commercialised and potentially registered for the control of *K. macadamiae* (Chapter 3).

If the cadavers of *K. macadamiae* conidiate in the field, the results of the horizontal infection in Chapter 4 as a result of physical contact with the conidiated cadavers could be replicated. If this is the case, enhancing the field conditions to be

more inducive could encourage the persistence and infectivity of EPF. For example, manipulating tree density/canopy coverage between rows in the orchard could provide large shaded areas to protect the EPF from UV radiation and may provide a suitable microclimate (high humidity and cooler temperatures) to complement the inundative application of EPF. However, encouraging a shaded orchard with a closed canopy to enhance EPF persistence in the field comes with issues such as the discouragement of beneficial arthropods (Govender, 2015) and may encourage the activity of other pest insects like macadamia lace bug, *Ulonemia* spp. and fruitspotting bug, *A. nitida* (Govender, 2015).

Insect outbreaks including both primary and secondary pests that emerge as a result of pesticide overuse are a serious issue for modern-day agriculture (Dutcher, 2007). Generally speaking, pest outbreaks caused by pesticide overuse can be triggered by three different factors. Firstly, the pesticides which are used to control plant diseases and other pests may also be detrimental to naturally-occurring EPF or to natural enemies which keep secondary pests in check (see section 2.3 for examples). The second factor is insecticide resistance. Insecticides may suppress susceptible insects in favour of individuals with higher pesticide tolerance, leading to the development of insecticide resistant populations (Snodgrass *et al.*, 2009). For example, research to quantify acephate resistance in populations of the tarnished plant bug (*Lygus lineolaris* Palisot de Beauvois, Hemiptera: Miridae) along the Mississippi river delta showed that the use of acephate between 2001 and 2006 had reduced the number of susceptible populations of *L. lineolaris* from 4 to 1, whereas acephate resistant populations had increased from 12 to 18 (Snodgrass *et al.*, 2009). In addition, the median lethal concentration (LC_{50}) of acephate for killing *L. lineolaris* in deposit-based bioassays increased from 5.6 µg/20 mL glass vial to 16.1 µg/20 mL glass vial (Snodgrass *et al.*, 2009). Similarly, Nehare *et al.* (2010) demonstrated that repeated applications of indoxacarb for controlling diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) in the field increased the LC_{50} of indoxacarb from 18.5 mg AI/L in the first generation to 167.8 mg AI/L in the tenth generation. The third and related factor is based around dose; insecticides applied at the recommended rate degrade over time, consequently, changing the concentration from a lethal to a sublethal dose (Bantz *et al.*, 2018). The sublethal dose of insecticide further contributes to the selection of more tolerant individuals, leading to the development of resistant populations (Bantz *et al.*, 2018). In this case, the insects are able to produce higher

levels of insecticide detoxifying enzyme(s) that allow them to survive higher pesticide application rates (Bantz *et al.*, 2018). This advantage can then be passed to their progeny and further selection can occur in response to increases in chemical application rates, exacerbating the problem over time (Ayyanath *et al.*, 2013; Tang *et al.*, 2019). Along with the accumulation of insecticide resistant populations of the targeted pest, the degradation of an insecticide to a sublethal dose may also contribute indirectly to secondary pest outbreaks as the results of non-target pests becoming resistant to the insecticides (Guedes *et al.*, 2017).

Like most other horticultural industries, Australian macadamia growers rely on synthetic pesticides for pest and plant disease control and it is important to understand if these pesticides have any effects on EPF. Natural infection of EPF on *K. macadamiae* has been reported a few times in the Northern Rivers (one strain of *B. bassiana* isolated from *K. macadamiae* was used by Maddox *et al.* (2015) and two strains were used in Chapter 3) but their abundance in macadamia orchards has not been investigated. Their presence in the field was noticeable to growers and agronomists when infected adults were found on lower branches. We hypothesised that the presence and persistence of EPF in the field could be suppressed by the spray application of synthetic pesticides. Chapter 5 investigated and discussed the effect of commonly used insecticides and fungicides on EPF which could be used during the period when *K. macadamiae* is active. Not surprisingly, the fungicides carbendazim and pyraclostrobin, which are primarily used to control husk spot disease (*P. macadamiae*) caused detrimental effects to both EPF species, whereas most insecticides showed only low to moderate effects (Chapter 5). Fungicides were very toxic to both fungal species even when the concentrations were reduced by 93% of the recommended rate (Chapter 5). The results of this study suggest that the natural presence and persistence of EPF in the Northern Rivers could be being suppressed by the poorly timed application of fungicides. On zucchini (*Cucurbita pepo* L., Cucurbitales: Cucurbitaceae), Roberti *et al.* (2017) found that the application of *B. bassiana* to leaves ten days after fungicide application (e.g. boscalid + pyraclostrobin, cyprodinil + fludioxonil) was not a suitable treatment regime since the mortality of whitefly, *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae), was less than 20%, whereas without fungicide applications, the mortality of whitefly caused by *B. bassiana* was almost 100%. On soybean, the co-application of *B. bassiana* with pyraclostrobin in the field did not provide any control on kudzu bug, *Megacopta*

cribraria F. (Hemiptera: Plataspidae) (Knight *et al.*, 2017) because the fungicide completely inhibited the germination and infection of *B. bassiana* on the targeted insects.

The inundative application of EPF onto crops may not yet be suitable for commercial macadamia growers, especially when fungicides are still recommended for controlling plant diseases on the same crop. There was a suggestion that EPF should be applied during winter (June to August) and combined with the removal of fallen nutlets (which might contain overwintering weevils) when most synthetic pesticides, especially fungicides, are not deployed in the field (R.K. Huwer, pers. comm., October 29, 2020). Currently, there is no information available on the habitats of the overwintering population, so this approach may or may not be effective. In contrast, organic macadamia production seems to be an alternative avenue for the use of EPF. Since the market for organic macadamia nuts has been growing in recent years, especially in North America and Europe (Transparency, 2020), the expansion of organic farms in order to supply the growing demand for organic nuts over the next several years will provide an opportunity for EPF to be used as a holistic tool for controlling insects pests in organically managed orchards.

If and when attractants or pheromones for *K. macadamiae* are identified and synthesised, it may be possible to use EPF in an attract-and-infect strategy for the management of adults. Understanding the movement of adults after they emerge from infested nuts may allow EPF to be used in an alternative way. For example, once it is established how adults move into the tree (e.g. via crawling on the ground and crawling up the tree trunk), it could be possible to position an infective barrier with a high concentration of EPF around the trunk that *K. macadamiae* are forced to encounter. This could avoid the detrimental effects of fungicides and other environmental factors on the EPF. Some studies have also suggested that electrostatically charged powder could be used as an EPF carrier as it has the ability to stick to the surface of the insect exoskeleton (Baxter *et al.*, 2008; Andriessen *et al.*, 2015; Athanassiou *et al.*, 2017). This could also be a viable option to integrate with EPF in an attract-and-infect strategy in order to improve the ability of EPF conidia to transfer more easily to *K. macadamiae* and in sufficient numbers to cause mortality. Additionally, the application of EPF suspensions on the tree trunk (Shapiro-Ilan *et al.*, 2008; 2009) or banding the trunk with polyester fibre, foam, cardboard and impregnated with EPF (Dubois *et al.*, 2004;

Ugine *et al.*, 2014; Hounmalon *et al.*, 2018) are also viable options if adult *K. macadamiae* crawl on the trunk and move upwards to the higher branches.

There was an abundance of weevil infested nuts on the orchard floor of the organic farm in the Northern Rivers, where infested nuts were collected for experiments. The fallen nutlets were abundant in the first few rows next to the conventional farm (Appendix H, Figure A.11-a). There was speculation that the high nutlet drop in the organic farm could be the result of the movement of adults from a nearby conventional farm, particularly after the application of low-efficacy insecticides there. In another farm in the Northern Rivers where the weevil infested nuts were collected, there was also an abundance of weevil-induced dropped nutlets, particularly in the unsprayed areas at the back of the grower's house (Appendix H, Figure A.11-b). Indoxacarb seems to have a repellent effect on *K. macadamiae*, which has been noted in trials at the CTH in Alstonville (C. Maddox and R.K. Huwer, pers. comm., October 29, 2020). This possibility is supported by data presented in this project, which shows that indoxacarb at its current FFC may have relatively low efficacy in terms of acute effects. There was speculation that the *K. macadamiae* showed avoidance to the sprayed area in other parts of the farm and instead, moved into the unsprayed area. If this is the case, a push-pull strategy could be implemented (Cook *et al.*, 2007). The regular removal of the fallen nutlets that contain developing larvae in a small unsprayed site may provide an effective and logistically viable solutions to minimise *K. macadamiae* across individual farms.

In Chapter 6, adults that emerged specifically from these nutlets detailed above were treated with acephate and indoxacarb at their full field concentrations (FFCs) in the laboratory. After 12 days, 12.5 – 20% of adults remained alive (Chapter 6). These initial results may suggest that the current FFCs of both registered insecticides could be set below the optimum level for control, or it could reflect the development of resistance in the field. The possibility of resistance can only be assessed by obtaining a reference susceptible population from a region with no history of pesticide usage, and comparing baseline toxicology data for that population with data from populations taken from commercial orchards where there has been prolonged chemical usage. Under commercial conditions, the performance of acephate and indoxacarb could be further compromised by poor spray coverage due to the height of the trees (> 18 m) and suboptimal timing of insecticide applications. Poor spray coverage in particular

has the potential to lead to underdosing that could contribute to the development of resistant populations (R.K. Huwer, pers. comm., October 29, 2020).

The results of Chapter 6 demonstrated the potential of combinations of insecticides with EPF for the control of *K. macadamiae* in the laboratory and glasshouse. The success of the combination of EPF with both acephate and indoxacarb at their respective full doses on *K. macadamiae* suggest that insecticides might weaken the insect cuticle by acting as a general insect stressor (Kumar *et al.*, 2018), reduce the target pest's mobility, or disrupt the removal of fungal conidia due to specific grooming behaviours (Quintela and McCoy, 1997; Brito *et al.*, 2008). After being exposed to insecticides, it is likely that insects were more vulnerable to the attachment of conidia and subsequent infection by EPF. Although the combination of insecticides with EPF was more effective than insecticides or EPF alone, it was clear that EPF had received the benefit of the insecticides' activities on *K. macadamiae* when they were co-applied. The mortality of adults caused by EPF was 45 – 75% and 18 – 40% in the laboratory and glasshouse, whereas the addition of insecticides to the EPF treatments lifted these values to total mortalities of 80 – 92.5% and 94 – 96% respectively. Encouragingly, other studies have supported that the combination of insecticides with EPF may provide a suitable solution for controlling pesticide resistant populations of the cattle tick *Boophilus microplus* Canestrini (Ixodida: Ixodidae) and the mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) (Bahiense *et al.*, 2006; Farenhorst *et al.*, 2010). The most important component of this co-application strategy is the role that EPF could play in providing additional control of the small number of surviving insects post-application of insecticide.

7.3. Future research

The findings in this thesis have provided fundamental information regarding the feasibility of using EPF alone or with registered insecticides for controlling *K. macadamiae*. These findings also provide more opportunities for further research:

- Performing a cost-benefit analysis to determine the feasibility of using EPF or EPF co-applied with insecticides for the management of *K. macadamiae.*
- Trials examining the performance and efficacy of formulated EPF should be conducted on conventional and organic farms.
- Potential resistance to conventional insecticides should be examined by comparing a susceptible reference strain of weevils with no history of

chemical exposure to populations taken from conventionally managed orchards.

- Field trials examining the synergistic effects of EPF and registered insecticides on *K. macadamiae* will potentially demonstrate their efficacy in controlling pest populations and contribute to sustainable crop protection.
- Investigations on the suitable interval between fungicide and EPF applications may allow EPF to be more effectively integrated into the insect pest and plant disease management program in macadamias.
- Some growers use mulch and compost in their orchards to improve soil nutrition and to protect the soil from erosion. It may be useful to investigate the effects of incorporating EPF with the compost or mulch to cover fallen, weevil infested nuts. Observations of adult weevil emergence, subsequent fungal infection and yield (or % fallen nutlets) will be the parameters to measure the effectiveness of this strategy.
- Understanding weevil biology and their behaviour after emerging from the infested nuts in the field, and particularly how adults move into the macadamia trees after emerging from infested nuts, may allow us to develop a more suitable method of EPF utilisation. In addition, identifying the overwintering sites may allow EPF to be deployed in the field for controlling overwintering populations.
- Studying the presence and abundance of EPF in the field may provide a better understanding of how to develop a conservation program to better exploit naturally-occurring EPF for suppressing pest populations, possibly in conjunction with the inundative applications of formulated EPFs.

7.4. Conclusion

The results from this study are the first to provide an alternative option to control *K. macadamiae*, either with EPF alone or in combination with registered insecticides. Of the many strains tested, *B. bassiana* strain B27 and *M. anisopliae* strain ECS1 were shown to be the most promising EPF for the control of *K. macadamiae,* and they offered a superior level of control compared to the South African *B. bassiana* product (Velifer® Biological Insecticide) in laboratory and glasshouse bioassays. These two strains were able to trigger horizontal transmission

and infection to other adults, providing additional evidence that EPF can be used for sustainable control of *K. macadamiae* in the field beyond just killing adult weevils directly from a spray application. It is highly unlikely that synthetic pesticides for controlling pests and plant diseases will disappear from the industry any time soon, although understanding their effects on EPF could provide a possibility to integrate them appropriately. This study confirmed that insecticides such as acephate, indoxacarb and trichlorfon are compatible with both fungal species as they are currently formulated, whereas sulfoxaflor and spinetoram were compatible with *B. bassiana* only. Other insecticides such as beta-cyfluthrin, methidathion and diazinon were toxic to both fungal species and the fungicides were extremely toxic. In order for the synthetic pesticides and EPF to be integrated in an IPDM program further research is required to ascertain the appropriate intervals between spraying pesticides and EPF. If the pathways to use EPF and fungicides are different (i.e. fungicides are sprayed, but the EPF are protected and possibly combined with attractants), there are still possibilities for both options to be integrated in the same orchard simultaneously. Growers are concerned that insecticide resistance is developing in the field and this is a long-term issue that needs further investigation. Growers need to be mindful regarding their choice of pesticides and follow strict pesticide rotations using chemicals with different modes of action. The results from the laboratory and glasshouse studies detailed here introduce a solution to minimise insecticide resistance by integrating acephate and indoxacarb at their full field concentration with EPF (B27 and ECS1). Overall, this thesis has demonstrated that EPF show good potential as biological control agents or biopesticides that can be integrated into existing practices to effectively manage *K. macadamiae*.

References

Acheampong, M. A., Coombes, C. A., Moore, S. D., Hill, M. P., 2020. Temperature tolerance and humidity requirements of select entomopathogenic fungal isolates for future use in citrus IPM programmes. *Journal of Invertebrate Pathology* 174, 107436. https://doi.org/10.1016/j.jip.2020.107436

AgriFutures Australia. 2017. Macadamia. Retrieved from https://www.agrifutures.com.au/farm-diversity/macadamia/ (Accessed on 28 July, 2018)

Akinsanmi, O. A., Miles, A. K., Drenth, A., 2008. Alternative fungicides for controlling husk spot caused by *Pseudocercospora macadamiae* in macadamia. *Australasian Plant Pathology* 37, 141-147. https://doi.org/10.1071/ap08001

Alves, R. T., Bateman, R. P., Prior, C., Leather, S. R., 1998. Effects of simulated solar radiation on conidial germination of *Metarhizium anisopliae* in different formulations. *Crop Protection* 17, 675-679. https://doi.org/10.1016/s0261-2194(98)00074-x

AMS. 2017. The Australian Macadamia Industry: Information for new and potential growers and investers. Australian Macadamia Society Ltd, Lismore, Australia. Retrieved from https://app-ausmacademia-au-syd.s3.ap-southeast-2.amazonaws.com/page/1516112185_new-potential-investors.pdf (Accessed on 12 September, 2020)

AMS. 2019. Yearbook 2019. Australian Macadamia Society Ltd, Lismore, Australia. Retrieved from https://d1bel7n84kyh0s.cloudfront.net/uploads/2019/11/2019-AUSTRALIAN-MACADAMIAS-YEARBOOK-FINAL-V1.0.pdf (Accessed on 26 February, 2020)

Andriessen, R., Snetselaar, J., Suer, R. A., Osinga, A. J., Deschietere, J., Lyimo, I. N., Mnyone, L. L., Brooke, B. D., Ranson, H., Knols, B. G. J., Farenhorst, M., 2015. Electrostatic coating enhances bioavailability of insecticides and breaks pyrethroid resistance in mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America* 112, 12081-12086. https://doi.org/10.1073/pnas.1510801112

ANIC. 2019. Growing for success: Australian's Tree Nut Industry 2019. Australian Nut Industry Council, Wangaratta, Australia. Retrieved from https://nutindustry.

org.au/wp-content/uploads/2019/05/Growing_Success_2019_Email_LoRes.pdf (Accessed on 16 December, 2019)

APVMA. 2015. Permit to allow minor use of an agvet chemical product for the control of sigastus weevil in macadamia (Permit 81463). Australian Pesticides and Veterinary Medicines Authority, Canberra, Australia. Retrieved from http://permits.apvma.gov.au/PER81463.PDF (Accessed on 5 July, 2017)

APVMA. 2018. Permit to allow minor use of an agvet chemical product for the control of seed weevil in macadamia (Permit PER86827). Australian Pesticides and Veterinary Medicines Authority, Canberra, Australia. Retrieved from http://permits.apvma.gov.au/PER86827.PDF (Accessed on 5 July, 2019)

Arthurs, S., Thomas, M. B., 2001. Effects of temperature and relative humidity on sporulation of *Metarhizium anisopliae* var. *acridum* in mycosed cadavers of *Schistocerca gregaria*. *Journal of Invertebrate Pathology* 78, 59-65. https://doi.org/10.1006/jipa.2001.5050

Athanassiou, C. G., Rumbos, C. I., Sakka, M., Potin, O., Storm, C., Dillon, A. B., 2017. Delivering *Beauveria bassiana* with electrostatic powder for the control of stored-product beetles. *Pest Management Science* 73, 1725-1736. https://doi.org/10.1002/ps.4522

Ayyanath, M. M., Cutler, G. C., Scott-Dupree, C. D., Sibley, P. K., 2013. Transgenerational shifts in reproduction hormesis in green peach aphid exposed to low concentrations of imidacloprid. *PloS One* 8, e74532. https://doi.org/10.1371/journal.pone.0074532

Bahiense, T. C., Fernandes, È. K. K., Bittencourt, V. R. E. P., 2006. Compatibility of the fungus *Metarhizium anisopliae* and deltamethrin to control a resistant strain of *Boophilus microplus* tick. *Veterinary Parasitology* 141, 319-324. https://doi.org/10.1016/j.vetpar.2006.05.011

Bantz, A., Camon, J., Froger, J. A., Goven, D., Raymond, V., 2018. Exposure to sublethal doses of insecticide and their effects on insects at cellular and physiological levels. *Current Opinion in Insect Science* 30, 73-78. https://doi.org/10.1016/j.cois.2018.09.008

Baxter, I. H., Howard, N., Armsworth, C. G., Barton, L. E. E., Jackson, C., 2008. The potential of two electrostatic powders as the basis for an auto dissemination control method of *Plodia interpunctella* (Hubner). *Journal of Stored Products Research* 44, 152-161. https://doi.org/10.1016/j.jspr.2007.08.004

Bischoff, J. F., Rehner, S. A., Humber, R. A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101, 512-530. https://doi.org/10.3852/07-202

Biswas, T., Joop, G., Rafaluk-Mohr, C., 2018. Cross-resistance: a consequence of bipartite host-parasite coevolution. *Insects* 9, 28. https://doi.org/10.3390/insects9010028

BOM. 2020. Monthly climate statistics: Period 1991-2020 at Alstonville Tropical Fruit Research Station. *Bureau of Meteorology, Australian Government.* Retrieved from http://www.bom.gov.au/jsp/ncc/cdio/cvg/av?p_stn_num=058131&p_prim_element_index=0&p_comp_element_index=0&redraw=null&p_display_type=statistics_summary&normals_years=1991-2020&tablesizebutt=normal (Accessed on 8 September, 2020)

Bouchard, P., Smith, A. B., Douglas, H., Gimmel, M. L., Brunke, A. J., Kanda, K., 2017. Biodiversity of Coleoptera. In Foottit, R. G., Adler, P. H. (Eds.), *Insect Biodiversity: Science and Society* (2nd ed., Vol. I), pp. 337-417. John Wiley & Sons Ltd, West Sussex, UK.

Boucias, D. G., Pendland, J. C., 1998. Chapter 1: Insect-pathogen relationships. In Boucias, D. G., Pendland, J. C. (Eds.), *Principles of Insect Pathology*, pp. 1-30. Springer, New York city, NY.

Boucias, D. G., Zhou, Y. H., Huang, S. S., Keyhani, N. O., 2018. Microbiota in insect fungal pathology. *Applied Microbiology and Biotechnology* 102, 5873-5888. https://doi.org/10.1007/s00253-018-9089-z

Braga, G. U. L., Flint, S. D., Messias, C. L., Anderson, A. J., Roberts, D. W., 2001a. Effect of UV-B on conidia and germlings of the entomopathogenic hyphomycete *Metarhizium anisopliae*. *Mycological Research* 105, 874-882. https://doi.org/10.1017/S0953756201004270

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J., Roberts, D. W., 2001b. Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. *Photochemistry and Photobiology* 74, 734-739. https://doi.org/10.1562/0031-8655(2001)074<0734:bsuaur>2.0.co;2

Bright, J., 2016. Macadamia plant protection guide 2016–17. NSW Department of Primary Industries, Wollongbar, Australia. Retrieved from http://www.dpi.nsw.

gov.au/__data/assets/pdf_file/0006/529161/Macadamia-plant-protection-guide.pdf (Accessed on 12 September, 2019)

Bright, J., 2017a. Macadamia seed weevil (*Kuschelorhynchus macadamiae*) life cycle and monitoring. Primefact 1586, Wollongbar, Australia. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/731982/Macadamia-seed-weevil-update-lifecycle_2.pdf (Accessed on 15 August, 2017)

Bright, J., 2017b. Macadamia seed weevil (*Kuschelorhynchus macadamiae*) orchard management. Primefact 1585, Wollongbar, Australia. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0008/731987/Macadamia-seed-weevil-update-orchard-management_2.pdf (Accessed on 15 August 2017)

Bright, J., 2017c. *Sigastus* weevil update. Part 1. Life cycle and monitoring keys to control. Australian Macadamia Society Ltd, Lismore, Australia. Retrieved from https://www.horticulture.com.au/globalassets/hort-innovation/resource-assets/mc-ipm-program-sigastus-weevil-fact-sheet.pdf (Accessed on 4 August, 2017)

Bright, J., 2019. Macadamia plant protection guide 2019-20. NSW Department of Primary Industries, Wollongbar, Australia. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/529161/Macadamia-plant-protection-guide-2019.pdf (Accessed on 16 December, 2019)

Bright, J., 2020. Macadamia plant protection guide 2020-21. NSW Department of Primary Industries, Wollongbar, Australia. Retrieved from https://www.dpi.nsw.gov.au/agriculture/horticulture/nuts/growing-guides/macadamia-protection-guide (Accessed on 08 July, 2020)

Brito, E. S., de Paula, A. R., Vieira, L. P., Dolinski, C., Samuels, R. I., 2008. Combining vegetable oil and sub-lethal concentrations of imidacloprid with *Beauveria bassiana* and *Metarhizium anisopliae* against adult guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae). *Biocontrol Science and Technology* 18, 665-673. https://doi.org/10.1080/09583150802195965

Butt, T. M., Greenfield, B. P. J., Greig, C., Maffeis, T. G. G., Taylor, J. W. D., Piasecka, J., Dudley, E., Abdulla, A., Dubovskiy, I. M., Garrido-Jurado, I., Quesada-Moraga, E., Penny, M. W., Eastwood, D. C., 2013. *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. *PloS One* 8, e81686. https://doi.org/10.1371/journal.pone.0081686

Charnley, A. K., Collins, S. A., 2007. Chapter 10: Entomopathogenic fungi and their role in pest control In Kubicek, C. P., Druzhinina, I. S. (Eds.), *Environmental and Microbial Relationships IV* (2 ed.), pp. 159-187. Springer, Berlin, Germany.

Chouvenc, T., Su, N. Y., Robert, A., 2009. Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. *Journal of Invertebrate Pathology* 101, 130-136. https://doi.org/10.1016/j.jip.2009.04.005

Cook, S. M., Khan, Z. R., Pickett, J. A., 2007. The use of push-pull strategies in integrated pest management. *Annual Review of Entomology* 52, 375-400. https://doi.org/10.1146/annurev.ento.52.110405.091407

da Silva, R. A., Quintela, E. D., Mascarin, G. M., Pedrini, N., Lião, L. M., Ferri, P. H., 2015. Unveiling chemical defense in the rice stalk stink bug against the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 127, 93-100. https://doi.org/10.1016/j.jip.2015.03.009

de Faria, M. R., Wraight, S. P., 2007. Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* 43, 237-256. https://doi.org/10.1016/j.biocontrol.2007.08.001

de Oliveira, D. G. P., Lopes, R. B., Rezende, J. M., Delalibera, I., 2018. Increased tolerance of *Beauveria bassiana* and *Metarhizium anisopliae* conidia to high temperature provided by oil-based formulations. *Journal of Invertebrate Pathology* 151, 151-157. https://doi.org/10.1016/j.jip.2017.11.012

Dembilio, Ó., Quesada-Moraga, E., Santiago-Álvarez, C., Jacas, J. A., 2010. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil, *Rhynchophorus ferrugineus*. *Journal of Invertebrate Pathology* 104, 214-221. https://doi.org/10.1016/j.jip.2010.04.006

Devi, K. U., Sridevi, V., Mohan, C. M., Padmavathi, J., 2005. Effect of high temperature and water stress on *in vitro* germination and growth in isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin. *Journal of Invertebrate Pathology* 88, 181-189. https://doi.org/10.1016/j.jip.2005.02.001

Dillon, R. J., Charnley, A. K., 1986. Inhibition of *Metarhizium anisopliae* by the gut bacterial flora of the desert locust, *Schistocerca gregaria*: Evidence for an

antifungal toxin. *Journal of Invertebrate Pathology* 47, 350-360. https://doi.org/10.1016/0022-2011(86)90106-0

Dimbi, S., Maniania, N. K., Lux, S. A., Mueke, J. M., 2004. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *Biocontrol* 49, 83-94. https://doi.org/10.1023/b:bico.0000009397.84153.79

Dolinski, C., Lacey, L. A., 2007. Microbial control of arthropod pests of tropical tree fruits. *Neotropical Entomology* 36, 161-179. https://doi.org/10.1590/s1519-566x2007000200001

Dossey, A. T., 2010. Insects and their chemical weaponry: New potential for drug discovery. *Natural Product Reports* 27, 1737-1757. https://doi.org/10.1039/C005319H

Dotaona, R., Wilson, B. A. L., Stevens, M. M., Holloway, J., Ash, G. J., 2015. Screening of tropical isolates of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) for virulence to the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *International Journal of Tropical Insect Science* 35, 153-163. https://doi.org/10.1017/s1742758415000211

Dotaona, R., Wilson, B. A. L., Stevens, M. M., Holloway, J., Ash, G. J., 2017. Chronic effects and horizontal transmission of *Metarhizium anisopliae* strain QS155 infection in the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Biological Control* 114, 24-29. https://doi.org/10.1016/j.biocontrol.2017.07.008

Dubois, T., Hajek, A. E., Hu, J. F., Li, Z. H., 2004. Evaluating the efficiency of entomopathogenic fungi against the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae), by using cages in the field. *Environmental Entomology* 33, 62-74. https://doi.org/10.1603/0046-225x-33.1.62

Dutcher, J. D., 2007. A review of resurgence and replacement causing pest outbreaks in IPM. In Ciancio, A., Mukerji, K. G. (Eds.), *General concepts in integrated pest and disease management*, pp. 27-43. Springer, Dordrecht, the Netherlands.

Ekesi, S., Maniania, N. K., Ampong-Nyarko, K., 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology* 9, 177-185. https://doi.org/10.1080/09583159929767

European Commission. 2008. Encouraging innovation in biopesticide development. Retrieved from https://ec.europa.eu/environment/integration/research/newsalert/pdf/134na5_en.pdf (Accessed on 19 December, 2017)

Falvo, M. L., Pereira, R. A., Rodrigues, J., Lastra, C. C. L., Garcia, J. J., Fernandes, E. K. K., Luz, C., 2016. UV-B radiation reduces *in vitro* germination of *Metarhizium anisopliae* s.l. but does not affect virulence in fungus-treated *Aedes aegypti* adults and development on dead mosquitoes. *Journal of Applied Microbiology* 121, 1710-1717. https://doi.org/10.1111/jam.13309

Farenhorst, M., Knols, B. G. J., Thomas, M. B., Howard, A. F. V., Takken, W., Rowland, M., N'Guessan, R., 2010. Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PloS One* 5, e12081. https://doi.org/10.1371/journal.pone.0012081

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L. A., Lomer, C. J., Rougier, M., 1996. Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia* 135, 171-181. https://doi.org/10.1007/bf00632339

Fay, H. A. C., De Faveri, S. G., Storey, R. I., Watson, J., 2001. *Sigastus* weevil - an emerging pest of macadamias in north Queensland. Proceedings of the 6th Workshop for Tropical Agricultural Entomology, Darwin, Australia, 11-15 May 1998.

Fernandes, È. K. K., Rangel, D. E. N., Moraes, Á. M. L., Bittencourt, V. R. E. P., Roberts, D. W., 2007. Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. *Journal of Invertebrate Pathology* 96, 237-243. https://doi.org/10.1016/j.jip.2007.05.007

Fernández-Bravo, M., Flores-León, A., Calero-López, S., Gutiérrez-Sánchez, F., Valverde-Garcia, P., Quesada-Moraga, E., 2017. UV-B radiation-related effects on conidial inactivation and virulence against *Ceratitis capitata* (Wiedemann) (Diptera; Tephritidae) of phylloplane and soil *Metarhizium* sp strains. *Journal of Invertebrate Pathology* 148, 142-151. https://doi.org/10.1016/j.jip.2017.06.012

Fisher, J. J., Rehner, S. A., Bruck, D. J., 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology* 106, 289-295. https://doi.org/10.1016/j.jip.2010.11.001

Fourie, P. C., Basson, D. S., 1990. Sugar content of almond, pecan, and macadamia nuts. *Journal of Agricultural and Food Chemistry* 38, 101-104. https://doi.org/10.1021/jf00091a020

Govender, A. W., 2015. *Australian fruitspotting bugs, Amblypelta nitida Stål and A. lutescens lutescens Distant (Hemiptera: Coreidae), and the potential for their biologically based management in macadamia orchards.* (PhD thesis). The University of Queensland, Queensland, Australia.

Gross, J., Podsiadlowski, L., Hilker, M., 2002. Antimicrobial activity of exocrine glandular secretion of *Chrysomela* larvae. *Journal of Chemical Ecology* 28, 317-331. https://doi.org/10.1023/a:1017934124650

Guedes, R. N. C., Walse, S. S., Throne, J. E., 2017. Sublethal exposure, insecticide resistance, and community stress. *Current Opinion in Insect Science* 21, 47-53. https://doi.org/10.1016/j.cois.2017.04.010

Hajek, A. E., St. Leger, R. J., 1994. Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* 39, 293-322. https://doi.org/10.1146/annurev.en.39.010194.001453

Hallsworth, J. E., Magan, N., 1999. Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology* 74, 261-266. https://doi.org/10.1006/jipa.1999.4883

Hamilton, R. A., Ito, P. J., Chia, C. L., 1983. Macadamia: Hawaii's dessert nut. University of Hawaii, Honolulu, HI. Retrieved from https://scholarspace.manoa.hawaii.edu/bitstream/10125/53678/CtahrpsCoopExtCirc4851985.pdf (Accessed on 24 July, 2017)

Haraprasad, N., Niranjana, S. R., Prakash, H. S., Shetty, H. S., Wahab, S., 2001. *Beauveria bassiana* - a potential mycopesticide for the efficient control of coffee berry borer, *Hypothenemus hampei* (Ferrari) in India. *Biocontrol Science and Technology* 11, 251-260. https://doi.org/10.1080/09583150120035675

Hedimbi, M., Kaaya, G. P., Singh, S., Chimwamurombe, P. M., Gindin, G., Glazer, I., Samish, M., 2008. Protection of *Metarhizium anisopliae* conidia from ultra-violet radiation and their pathogenicity to *Rhipicephalus evertsi evertsi* ticks. *Experimental and Applied Acarology* 46, 149-156. https://doi.org/10.1007/s10493-008-9186-2

Hely, P. C., Pasfield, G., Gellatley, J. G., 1982. *Insect pests of fruit and vegetables in NSW*. Inkata Press, Melbourne, Australia.

Hong, T. D., Edgington, S., Ellis, R. H., de Muro, M. A., Moore, D., 2005. Saturated salt solutions for humidity control and the survival of dry powder and oil formulations of *Beauveria bassiana* conidia. *Journal of Invertebrate Pathology* 89, 136-143. https://doi.org/10.1016/j.jip.2005.03.007

Hounmalon, G. Y. A., Maniania, N. K., Niassy, S., Fellous, S., Kreiter, S., Deletre, E., Fiaboe, K. K. M., Martin, T., 2018. Performance of *Metarhizium anisopliae*-treated foam in combination with *Phytoseiulus longipes* Evans against *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae). *Pest Management Science* 74, 2835-2841. https://doi.org/10.1002/ps.5073

Huwer, R., 2016. Ecology and management of *Sigastus* weevil in macadamias. (Final report, Project MC15010). Horticulture Innovation Australia Limited, Sydney, Australia. Retrieved from https://www.horticulture.com.au/globalassets/laser fiche/assets/project-reports/mc15010/mc15010-final-report-514.pdf (Accessed on 23 March, 2019)

HVP. 1999. About Hidden Valley Varieties. Retrieved from http://www.hvp-macadamias.com/ (Accessed on 2 October, 2017)

INDFC. 2009. Statistical review. Macadamias. *The Cracker, 48(3),* 60. International nut and dried fruit council, Reus, Spain.

INDFC. 2010. Statistical review. Macadamias. *The Cracker, 51(3),* 58. International nut and dried fruit council, Reus, Spain.

INDFC. 2011. Statistical review. Macadamias. *The Cracker, 54(3),* 56. International nut and dried fruit council, Reus, Spain.

INDFC. 2012. Statistical review. Macadamias. *The Cracker, 57(3),* 54. International nut and dried fruit council, Reus, Spain.

INDFC. 2013. Statistical review. Macadamias. *The Cracker, 60(3),* 56. International nut and dried fruit council,, Reus, Spain.

INDFC. 2014. Statistical review. Macadamias. *The Cracker, 63(3),* 58. International nut and dried fruit council, Reus, Spain.

INDFC. 2015. Statistical review. Macadamias. *The Cracker, 66(3),* 38. International nut and dried fruit council, Reus, Spain.

INDFC. 2016. Statistical review. Macadamias. *NUTFRUIT, 69(3),* 72. International nut and dried fruit council, Reus, Spain.

INDFC. 2017. Statistical review. Macadamias. *NUTFRUIT, 72(3),* 74. International nut and dried fruit council, Reus, Spain.

INDFC. 2018. Statistical review. Macadamias. *NUTFRUIT, 75(3),* 70. International nut and dried fruit council, Reus, Spain.

INDFC. 2019. Statistical review. Macadamias. *NUTFRUIT, 78(3),* 70. International nut and dried fruit council, Reus, Spain.

INDFC. 2020. Statistical yearbook 2019/20. International nut and dried fruit council, Reus, Spain. Retrieved from https://www.nutfruit.org/industry/technical-resources?category=statistical-yearbooks (Accessed on 9 October, 2020)

Inglis, G. D., Enkerli, J., Goettel, M. S., 2012. Chapter 7: Laboratory techniques used for entomopathogenic fungi: Hypocreales. In Lacey, L. A. (Ed.), *Manual of techniques in invertebrate pathology* (2 ed.), pp. 189-253. Elsevier Academic Press Inc, San Diego, CA.

Jaronski, S. T., 2014. Chapter 11: Mass production of entomopathogenic fungi: state of the art. In Morales-Ramos, J. A., Rojas, M. G., Shapiro-Ilan, D. I. (Eds.), *Mass production of beneficial organisms: invertebrates and entomopathogens*, pp. 357-413. Elsevier Academic Press Inc, San Diego, CA.

Jennings, D., Oberprieler, R. G., 2018. A review of the Tribe Cryptoplini (Coleoptera: Curculioninae), with revision of the Genus *Menechirus* Hartmann, 1901 and description of a new Genus associated with *Macadamia*. *Diversity* 10, 71. https://doi.org/10.3390/d10030071

Kabaluk, T., Li-Leger, E., Nam, S., 2017. *Metarhizium brunneum* – An enzootic wireworm disease and evidence for its suppression by bacterial symbionts. *Journal of Invertebrate Pathology* 150, 82-87. https://doi.org/10.1016/j.jip. 2017.09.012

Kaiser, D., Bacher, S., Mène-Saffrané, L., Grabenweger, G., 2019. Efficiency of natural substances to protect *Beauveria bassiana* conidia from UV radiation. *Pest Management Science* 75, 556-563. https://doi.org/10.1002/ps.5209

Kaiser, D., Handschin, S., Rohr, R. P., Bacher, S., Grabenweger, G., 2020. Co-formulation of *Beauveria bassiana* with natural substances to control pollen beetles - Synergy between fungal spores and colza oil. *Biological Control* 140, 104106. https://doi.org/10.1016/j.biocontrol.2019.104106

Kawate, M. K., Tarutani, C. M., 2004. Pest Management Strategic Plan for Macadamia Nut Production in Hawai ‘i. Retrieved from https://ipmdata.ipm

centers.org/documents/pmsps/HIMacadamia_Nut%202006.pdf (Accessed on 28 July, 2017)

Klieber, J., Reineke, A., 2016. The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *Journal of Applied Entomology* 140, 580-589. https://doi.org/10.1111/jen.12287

Klingen, I., Westrum, K., Meyling, N. V., 2015. Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres. *Biological Control* 81, 1-7. https://doi.org/10.1016/j.biocontrol.2014.10.006

Knight, I. A., Roberts, P. M., Gardner, W. A., Oliver, K. M., Jacobson, A., Toews, M. D., 2017. Effect of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) and fungicide applications on *Megacopta cribraria* (Hemiptera: Plataspidae) in soybean. *Crop Forage & Turfgrass Management* 3, 8. https://doi.org/10.2134/cftm2017.05.0034

Koppenhofer, A. M., 2007. Synergy with microorganisms. In Pimentel, D. (Eds.), *Encyclopedia of pest management* (Vol. II), pp. 658-660. CRC Press, Boca Raton, FL.

Korosi, G. A., Wilson, B. A. L., Powell, K. S., Ash, G. J., Reineke, A., Savocchia, S., 2019. Occurrence and diversity of entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp.) in Australian vineyard soils. *Journal of Invertebrate Pathology* 164, 69-77. https://doi.org/10.1016/j.jip.2019.05.002

Kumar, V., Francis, A., Avery, P. B., McKenzie, C. L., Osborne, L. S., 2018. Assessing compatibility of *lsaria fumosorosea* and buprofezin for mitigation of *Aleurodicus rugioperculatus* (Hemiptera: Aleyrodidae): an invasive pest in the Florida landscape. *Journal of Economic Entomology* 111, 1069-1079. https://doi.org/10.1093/jee/toy056

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., Goettel, M. S., 2015. Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology* 132, 1-41. https://doi.org/10.1016/j.jip.2015.07.009

Lacey, L. A., Shapiro-Ilan, D. I., 2008. Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. *Annual Review of Entomology* 53, 121-144. https://doi.org/10.1146/annurev.ento.53.103106.093419

Lazzarini, G. M. J., Rocha, L. F. N., Luz, C., 2006. Impact of moisture on *in vitro* germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. *Mycological Research* 110, 485-492. https://doi.org/10.1016/j.mycres.2005.12.001

Lee, S., 2014. *Sigastus* weevil. The Nutshell - MPC's newsletter for macadamia growers, Lismore, Australia. Retrieved from https://mpcmacs.com.au/media/Nutshell-May-2014.pdf (Accessed on 16 August, 2017)

Leland, J. E., Behle, R. W., 2005. Coating *Beauveria bassiana* with lignin for protection from solar radiation and effects on pathogenicity to *Lygus lineolaris* (Heteroptera: Miridae). *Biocontrol Science and Technology* 15, 309-320. https://doi.org/10.1080/09583150400016936

Maddox, C., Huwer, R., Liang, W., Bright, J., Leemon, D. M., 2015. Ecology and management of *Sigastus* weevil in macadamias - a pilot study. (Project MC15010). Horticulture Innovation Australia Limited, Sydney, Australia. (Accessed on 12 October, 2017)

Maguire, L. S., O'Sullivan, S. M., Galvin, K., O'Connor, T. P., O'Brien, N. M., 2004. Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. *International Journal of Food Sciences and Nutrition* 55, 171-178. https://doi.org/10.1080/09637480410001725175

Mannino, M. C., Huarte-Bonnet, C., Davyt-Colo, B., Pedrini, N., 2019. Is the insect cuticle the only entry gate for fungal infection? insights into alternative modes of action of entomopathogenic fungi. *Journal of Fungi* 5, 33. https://doi.org/10.3390/jof5020033

McCammon, S. A., Rath, A. C., 1994. Separation of *Metarhizium anisopliae* strains by temperature dependent germination rates. *Mycological Research* 98, 1253-1257. https://doi.org/10.1016/s0953-7562(09)80295-5

Meyling, N. V., Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control* 43, 145-155. https://doi.org/10.1016/j.biocontrol.2007.07.007

Milner, R. J., Lozano, L. B., Driver, F., Hunter, D., 2003. A comparative study of two Mexican isolates with an Australian isolate of *Metarhizium anisopliae* var. *acridum* - strain characterisation, temperature profile and virulence for wingless

grasshopper, *Phaulacridium vittatum*. *Biocontrol* 48, 335-348. https://doi.org/10.1023/a:1023630319127

Milner, R. J., Staples, J. A., Lutton, G. G., 1997. The effect of humidity on germination and infection of termites by the hyphomycete, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 69, 64-69. https://doi.org/10.1006/jipa.1996.4636

Mishra, S., Kumar, P., Malik, A., 2015. Effect of temperature and humidity on pathogenicity of native *Beauveria bassiana* isolate against *Musca domestica* L. *Journal of Parasitic Diseases* 39, 697-704. https://doi.org/10.1007/s12639-013-0408-0

Mochi, D. A., Monteiro, A. C., Pietro, R., Corrêa, M. A., Barbosa, J. C., 2017. Compatibility of *Metarhizium anisopliae* with liposoluble photoprotectants and protective effect evaluation against solar radiation. *Bioscience Journal* 33, 1028-1037. https://doi.org/10.14393/BJ-v33n4a2017-34080

Mohammadbeigi, A., Port, G., 2013. Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* against *Uvarovistia zebra* (Orthoptera: Tettigoniidae) via contact and ingestion. *International Journal of Agriculture and Crop Sciences* 5, 138-146.

Mola, F. L., Afkari, R., 2012. Effects of different vegetable oils formulations on temperature tolerance and storage duration of *Beauveria bassiana* conidia. *African Journal of Microbiology Research* 6, 4707-4711. https://doi.org/10.5897/AJMR11.1372

Moore, D., Bridge, P. D., Higgins, P. M., Bateman, R. P., Prior, C., 1993. Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. *Annals of Applied Biology* 122, 605-616. https://doi.org/10.1111/j.1744-7348.1993.tb04061.x

Nehare, S., Ghodki, B. S., Lande, G. K., Pawade, V., Thakare, A. S., 2010. Inheritance of resistance and cross resistance pattern in indoxacarb-resistant diamondback moth *Plutella xylostella* L. *Entomological Research* 40, 18-25. https://doi.org/10.1111/j.1748-5967.2009.00261.x

Nichols, J., 2017. Macadamia crop losses to extreme weather this year mask a cracking future. *ABC News.* Retrieved from http://www.abc.net.au/news/rural/2017-08-04/macadamia-crop-losses-this-year-masks-a-cracking-future/8773656 (Accessed on 28 July, 2017)

NZ TCA. 2017. Macadamia. Retrieved from http://www.treecrops.org.nz/crops/nut/macadamia/ (Accessed on 3 October, 2017)

O'Hare, P., Stephenson, R., Quinlan, K., Vock, N., 2004. *Macadamia grower's handbook*. Department of Primary Industries and Fisheries, Nambour, Australia.

Oberprieler, R. G., Marvaldi, A. E., Anderson, R. S., 2007. Weevils, weevils, weevils everywhere. *Zootaxa* 1668, 491-520. https://doi.org/10.11646/zootaxa.1668.1.24

Olson, S., 2015. An analysis of the biopesticide market now and where it is going. *Outlooks on Pest Management* 26, 203-206. https://doi.org/10.1564/v26_oct_04

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., Anthony, S., 2009a. *Macadamia integrifolia*. Retrieved from http://www.worldagroforestry.org/treedb/AFTPDFS/Macadamia_integrifolia.PDF (Accessed on 28 July, 2017)

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., Anthony, S., 2009b. *Macadamia tetraphylla*. Retrieved from http://www.worldagroforestry.org/treedb/AFTPDFS/Macadamia_tetraphylla.PDF (Accessed on 28 July, 2017)

Parisi, A. V., Wong, J. C. F., 1997. The erythemal ultraviolet exposure for humans in greenhouses. *Physics in Medicine & Biology* 42, 2331–2339.

Pedrini, N., Ortiz-Urquiza, A., Huarte-Bonnet, C., Fan, Y. H., Juárez, P., Keyhani, N. O., 2015. Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. *Proceedings of the National Academy of Sciences of the United States of America* 112, E3651-E3660. https://doi.org/10.1073/pnas.1504552112

QDAF. 2016. Macadamia industry benchmark report: 2009 to 2015 seasons. (Project MC15005). Queensland Department of Agriculture and Fisheries, Brisbane, Australia. Retrieved from http://horticulture.com.au/wp-content/uploads/2016/12/macadamia-industry-benchmark-report2009-2015.pdf (Accessed on 12 February, 2018)

QDAF. 2019. Macadamia industry benchmark report: 2009 to 2018 seasons. (Project MC18002). Queensland Department of Agriculture and Fisheries, Brisbane, Australia. Retrieved from https://www.publications.qld.gov.au/dataset/18517168-df7c-41d9-bf92-50ff4ccfb6ac/resource/76587ac2-fb21-4483-bc61-1a5088d02712/fs_download/macadamia-industry-benchmark-report-2009-18.pdf (Accessed on 26 February, 2020)

Quesada-Moraga, E., Martin-Carballo, I., Garrido-Jurado, I., Santiago-Álvarez, C., 2008. Horizontal transmission of *Metarhizium anisopliae* among laboratory populations of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). *Biological Control* 47, 115-124. https://doi.org/10.1016/j.biocontrol.2008.07.002

Quinlan, K., Wilk, P., 2005. Macadamia culture in NSW. Primefact 5, Wollongbar, Australia. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/75740/Macadamia-culture-in-NSW-Primefact-5---final.pdf (Accessed on 6 July, 2017)

Quintela, E. D., McCoy, C. W., 1997. Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environmental Entomology* 26, 1173-1182. https://doi.org/10.1093/ee/26.5.1173

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J., Roberts, D. W., 2005. Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *Journal of Invertebrate Pathology* 88, 116-125. https://doi.org/10.1016/j.jip.2004.11.007

Rath, A. C., Anderson, G. C., Worledge, D., Koen, T. B., 1995. The effect of low temperatures on the virulence of *Metarhizium anisopliae* (DAT F-001) to the subterranean scarab, *Adoryphorus couloni*. *Journal of Invertebrate Pathology* 65, 186-192. https://doi.org/10.1006/jipa.1995.1027

Rehner, S. A., Buckley, E., 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97, 84-98. https://doi.org/10.1080/15572536.2006.11832842

Roberti, R., Righini, H., Masetti, A., Maini, S., 2017. Compatibility of *Beauveria bassiana* with fungicides *in vitro* and on zucchini plants infested with *Trialeurodes vaporariorum*. *Biological Control* 113, 39-44. https://doi.org/10.1016/j.biocontrol.2017.06.006

Roberts, D. W., St. Leger, R. J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. In Laskin, A. I., Bennet, J. W., Gadd, G. M. (Eds.), *Advances in Applied Microbiology* (Vol. 54), pp. 1-70. Elsevier Academic Press Inc, Cambridge, MA.

Rosengarten, F. J., 2004. Chapter 7: Macadamia nuts. In *The book of edible nuts*, pp. 117-144. Dover publication Inc, Mineola, NY.

Samson, R. A., Evans, H. C., Latgé, J. P., 1988. *Atlas of entomopathogenic fungi*. Springer, Berlin, Germany.

Schabel, H. G., 1976. Oral infection of *Hylobius pales* by *Metarrhizium anisopliae*. *Journal of Invertebrate Pathology* 27, 377-383. https://doi.org/10.1016/0022-2011(76)90100-2

Shapiro-Ilan, D. I., Cottrell, T. E., Gardner, W. A., Behle, R. W., Ree, B., Harris, M. K., 2009. Efficacy of entomopathogenic fungi in suppressing pecan weevil, *Curculio caryae* (Coleoptera: Curculionidae), in commercial pecan orchards. *Southwestern Entomologist* 34, 111-120. https://doi.org/10.3958/059.034.0201

Shapiro-Ilan, D. I., Gardner, W. A., Cottrell, T. E., Behle, R. W., Wood, B. W., 2008. Comparison of application methods for suppressing the pecan weevil (Coleoptera: Curculionidae) with *Beauveria bassiana* under field conditions. *Environmental Entomology* 37, 162-171. https://doi.org/10.1093/ee/37.1.162

Shapiro-Ilan, D. I., Gardner, W. A., Fuxa, J. R., Wood, B. W., Nguyen, K. B., Adams, B. J., Humber, R. A., Hall, M. J., 2003. Survey of entomopathogenic nematodes and fungi endemic to pecan orchards of the Southeastern United States and their virulence to the pecan weevil (Coleoptera: Curculionidae). *Environmental Entomology* 32, 187-195. https://doi.org/10.1603/0046-225x-32.1.187

Shipp, J. L., Zhang, Y., Hunt, D. W. A., Ferguson, G., 2003. Influence of humidity and greenhouse microclimate on the efficacy of *Beauveria bassiana* (Balsamo) for control of greenhouse arthropod pests. *Environmental Entomology* 32, 1154-1163. https://doi.org/10.1603/0046-225x-32.5.1154

Snodgrass, G. L., Gore, J., Abee, C. A., Jackson, R., 2009. Acephate resistance in populations of the tarnished plant bug (Heteroptera: Miridae) from the Mississippi river delta. *Journal of Economic Entomology* 102, 699-707. https://doi.org/10.1603/029.102.0231

Tang, Q. L., Ma, K. S., Chi, H., Hou, Y. M., Gao, X. W., 2019. Transgenerational hormetic effects of sublethal dose of flupyradifurone on the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *PloS One* 14, e0208058. https://doi.org/10.1371/journal.pone.0208058

Thaochan, N., Benarlee, R., Shekhar Prabhakar, C., Hu, Q., 2020. Impact of temperature and relative humidity on effectiveness of *Metarhizium guizhouense* PSUM02 against longkong bark eating caterpillar *Cossus chloratus* Swinhoe

under laboratory and field conditions. *Journal of Asia-Pacific Entomology* 23, 285-290. https://doi.org/10.1016/j.aspen.2020.01.006

Transparency. 2020. Macadamia market - global industry analysis, size, trends, growth, and forecast 2020 – 2030. Retrieved from https://www.transparencymarketresearch.com/macadamia-market.html (Accessed on 12 November, 2020)

Tumuhaise, V., Ekesi, S., Maniania, N. K., Tonnang, H. E. Z., Tanga, C. M., Ndegwa, P. N., Irungu, L. W., Srinivasan, R., Mohamed, S. A., 2018. Temperature-dependent growth and virulence, and mass production potential of two candidate isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin for managing *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) on cowpea. *African Entomology* 26, 73-83. https://doi.org/10.4001/003.026.0073

Ugine, T. A., Peters, K. E., Gardescu, S., Hajek, A. E., 2014. The effect of time postexposure and sex on the horizontal transmission of *Metarhizium brunneum* conidia between Asian longhorned beetle (Coleoptera: Cerambycidae) mates. *Environmental Entomology* 43, 1552-1560. https://doi.org/10.1603/EN14116

US EPA. 2016. What are biopesticides? Retrieved from https://www.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides (Accessed on 19 December, 2017)

Vandenberg, J. D., Ramos, M., Altre, J. A., 1998. Dose-response and age- and temperature-related susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae). *Environmental Entomology* 27, 1017-1021. https://doi.org/10.1093/ee/27.4.1017

Vock, N., 1999. *Macadamia variety identifier*. Department of Primary Industries, Nambour, Australia.

Vock, N., 2003. *Field guide: macadamia problem solver & bug identifier*. Department of Primary Industries, Brisbane, Australia.

Wall, M. M., Gentry, T. S., 2007. Carbohydrate composition and color development during drying and roasting of macadamia nuts (*Macadamia integrifolia*). *Lwt-Food Science and Technology* 40, 587-593. https://doi.org/10.1016/j.lwt.2006.03.015

Wei, G., Lai, Y., Wang, G., Chen, H., Li, F., Wang, S., 2017. Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality.

Proceedings of the National Academy of Sciences of the United States of America 114, 5994-5999. https://doi.org/10.1073/pnas.1703546114

Wilkinson, J., 2005. *Nut grower's guide: the complete handbook for producers and hobbyists*. Landlinks Press, Collingwood, Australia.

Wilson, W. M., Ibarra, J. E., Oropeza, A., Hernández, M. A., Toledo-Hernández, R. A., Toledo, J., 2017. Infection of *Anastrepha ludens* (Diptera: Tephritidae) adults during emergence from soil treated with *Beauveria bassiana* under various texture, humidity, and temperature conditions. *Florida Entomologist* 100, 503-508. https://doi.org/10.1653/024.100.0302

Xiao, G., Ying, S. H., Zheng, P., Wang, Z. L., Zhang, S., Xie, X. Q., Shang, Y., St. Leger, R. J., Zhao, G. P., Wang, C., Feng, M. G., 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific Reports* 2, 483. https://doi.org/10.1038/srep00483

Yao, S. L., Ying, S. H., Feng, M. G., Hatting, J. L., 2010. *In vitro* and *in vivo* responses of fungal biocontrol agents to gradient doses of UV-B and UV-A irradiation. *Biocontrol* 55, 413-422. https://doi.org/10.1007/s10526-009-9265-2

Zhang, F., Huang, Y. H., Liu, S. Z., Zhang, L., Li, B. T., Zhao, X. X., Fu, Y., Liu, J. J., Zhang, X. X., 2013. *Pseudomonas reactans*, a bacterial strain isolated from the intestinal flora of *Blattella germanica* with anti-*Beauveria bassiana* activity. *Environmental Entomology* 42, 453-459. https://doi.org/10.1603/en12347

Zhang, F., Sun, X. X., Zhang, X. C., Zhang, S., Lu, J., Xia, Y. M., Huang, Y. H., Wang, X. J., 2018. The interactions between gut microbiota and entomopathogenic fungi: a potential approach for biological control of *Blattella germanica* (L.). *Pest Management Science* 74, 438-447. https://doi.org/10.1002/ps.4726

Zimmermann, G., 2007a. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology* 17, 553-596. https://doi.org/10.1080/09583150701309006

Zimmermann, G., 2007b. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology* 17, 879-920. https://doi.org/10.1080/09583150701593963

Appendix A

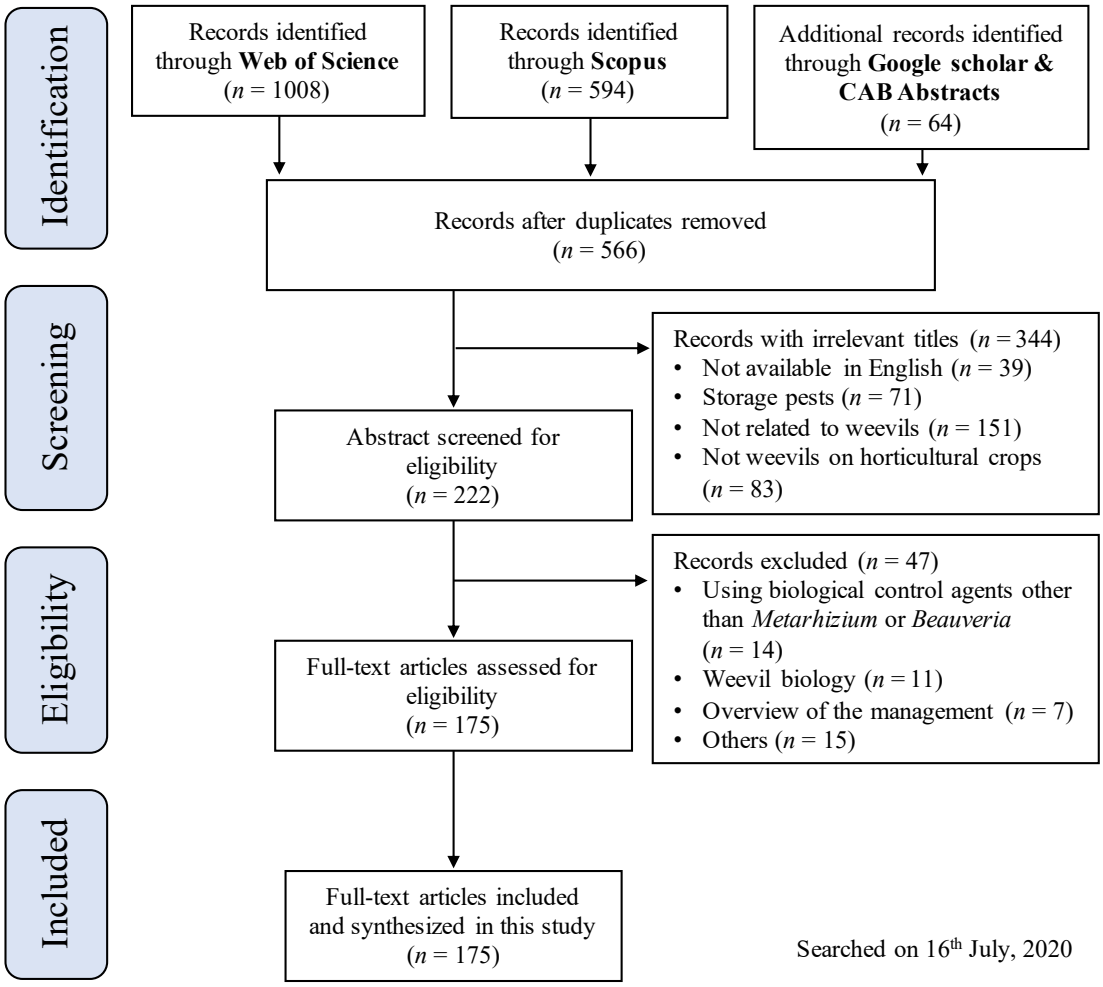


Figure A.1: Flow diagram illustrating the selection process for publications included in the review paper (Chapter 2, section 2.3)

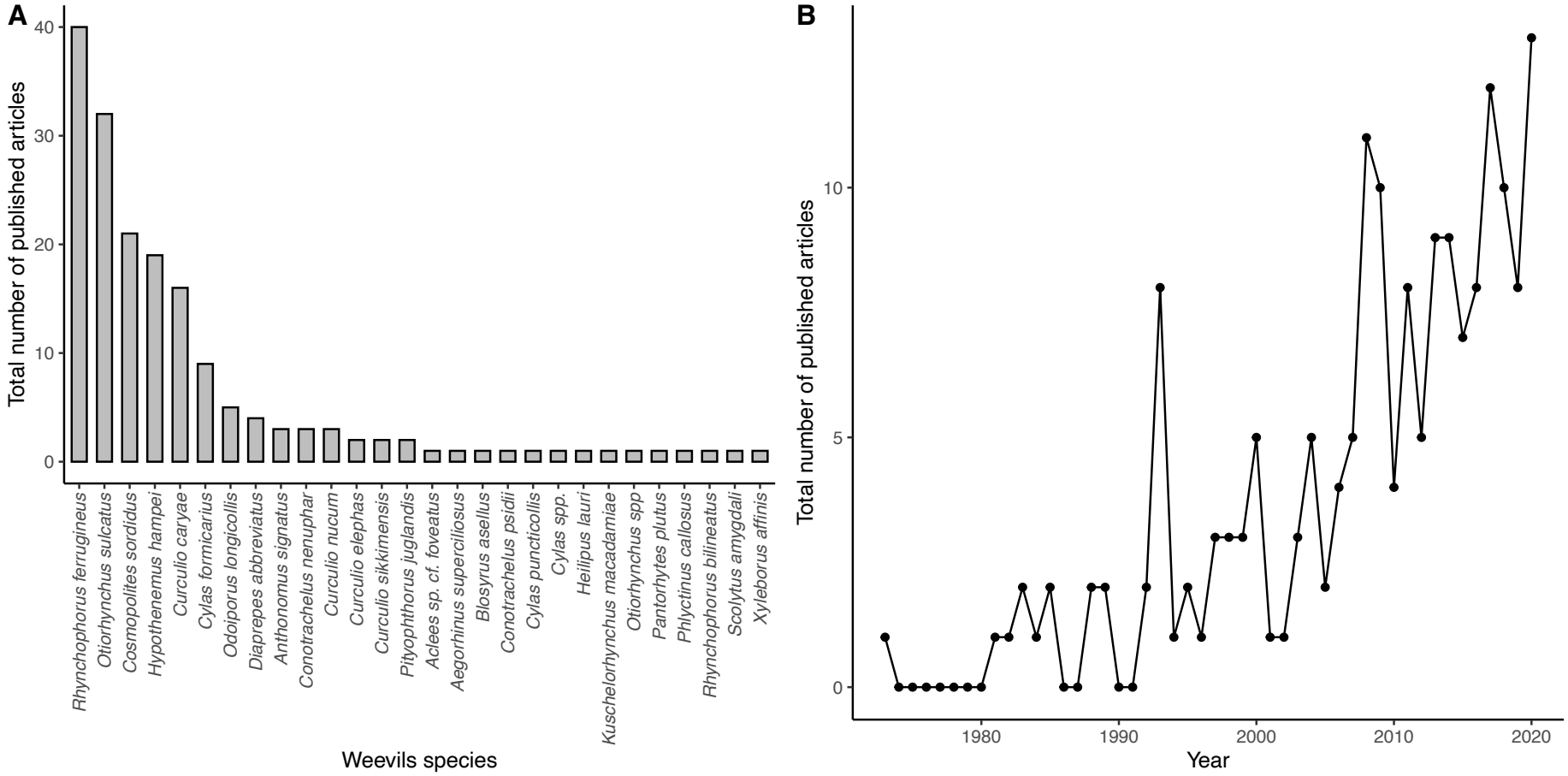


Figure A.2: (A) The number of published studies using EPF on each weevil species affecting horticultural crops and included in the review paper (Chapter 2, section 2.3), and (B) published studies using EPF for controlling weevils affecting horticultural crops and included in the review paper (Chapter 2, section 2.3) from 1973 to 2020. Last accessed on 16th July, 2020.

Appendix B

Table A.1: Germination and daily mycelial growth rate of *M. anisopliae* and *B. bassiana* isolates

Species	Isolate/ Accession	Germination (%) ± SE	Growth (mm/day) ± SE
M. anisopliae	M81/BRIP 70266	90.75 ± 1.65	4.58 ± 0.12
	B4A1/BRIP 70268	90.25 ± 1.25	4.83 ± 0.06
	ECS1/BRIP 70272	90.00 ± 1.47	4.84 ± 0.12
	ECF1/BRIP 70270	90.00 ± 1.41	4.87 ± 0.13
	DA1/BRIP 70271	88.00 ± 1.47	4.58 ± 0.18
	QS155/DAR 82480	86.75 ± 1.25	5.05 ± 0.06
B. bassiana	B50/BRIP 70276	90.50 ± 1.85	3.61 ± 0.10a
	B48/BRIP 70269	89.50 ± 1.32	3.10 ± 0.10b
	Velifer®-R	89.50 ± 1.55	2.96 ± 0.04b
	B60/BRIP 70275	87.25 ± 0.63	3.01 ± 0.11b
	B49/BRIP 70274	87.00 ± 0.82	3.03 ± 0.12b
	B27/BRIP 70267	86.75 ± 1.49	3.10 ± 0.02b
	Velifer® Biological Insecticide	-	2.97 ± 0.14b

For the growth rates of *B. bassiana* isolates, treatment means followed by different letters are significantly different (ANOVA, Tukey's HSD test, ($P < 0.05$). There were no significant differences between treatments in the other comparisons. This table is a supplement to the study in Chapter 3.

Appendix C

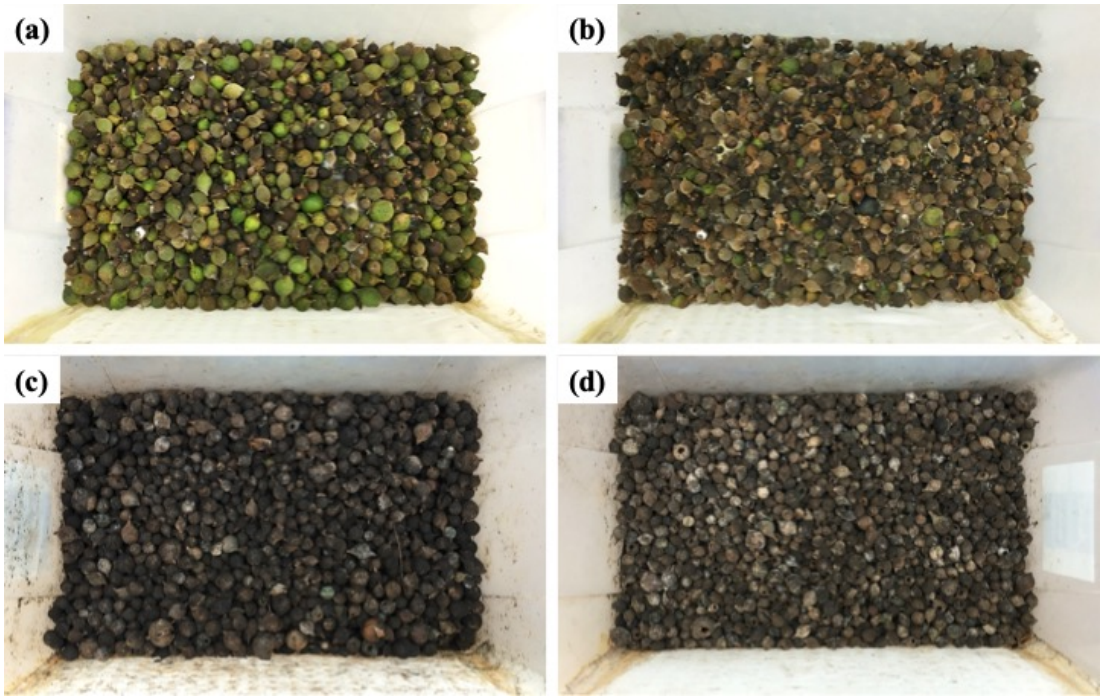


Figure A.3: Decaying progress of weevil infested nuts in 50 L containers (a) the fresh nutlets, (b) 2 weeks post collection, (c) 6 weeks post collection and (d) 8 weeks post collection. This figure supplements the methodology section in Chapters 3, 4 and 6.

Appendix D

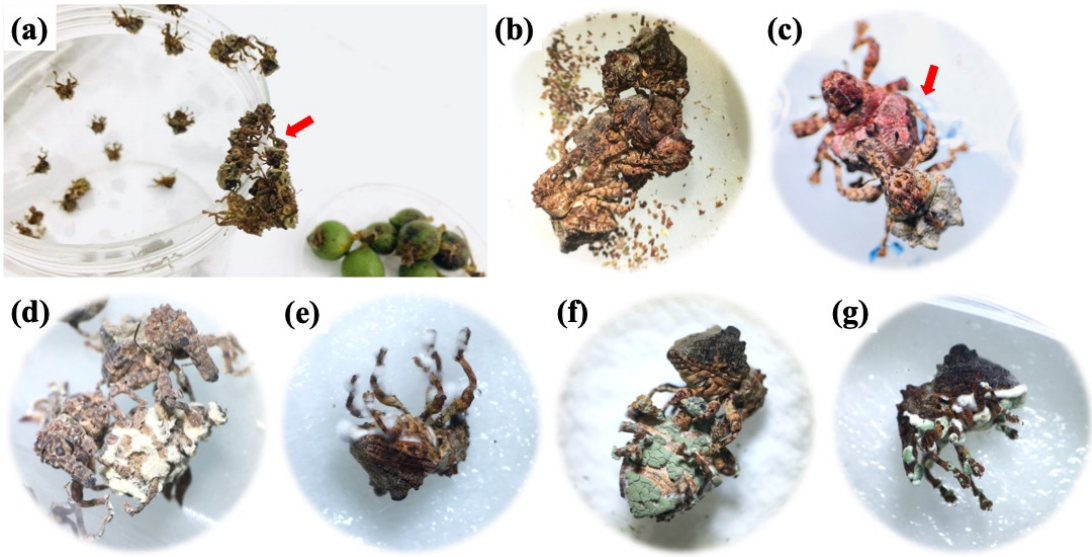


Figure A.4: (a & b) Aggregation behaviour of adults inside insect cage, (c) the permanent red pen painted adult and the non-painted adult, (d) the no-discrimination behaviour of adults toward a B27 conidiated cadaver in the confined environment (500 mL plastic container), (e) conidiation of B27 on an adult which contacted a B27 conidiated cadaver, (f) the no-discrimination behaviour of adults toward a ECS1 conidiated cadaver in the confined environment (500 mL plastic container) and (g) conidiation of ECS1 on an adult which contacted a ECS1 conidiated cadaver. This figure is supplemental to the study in Chapter 4.

Appendix E

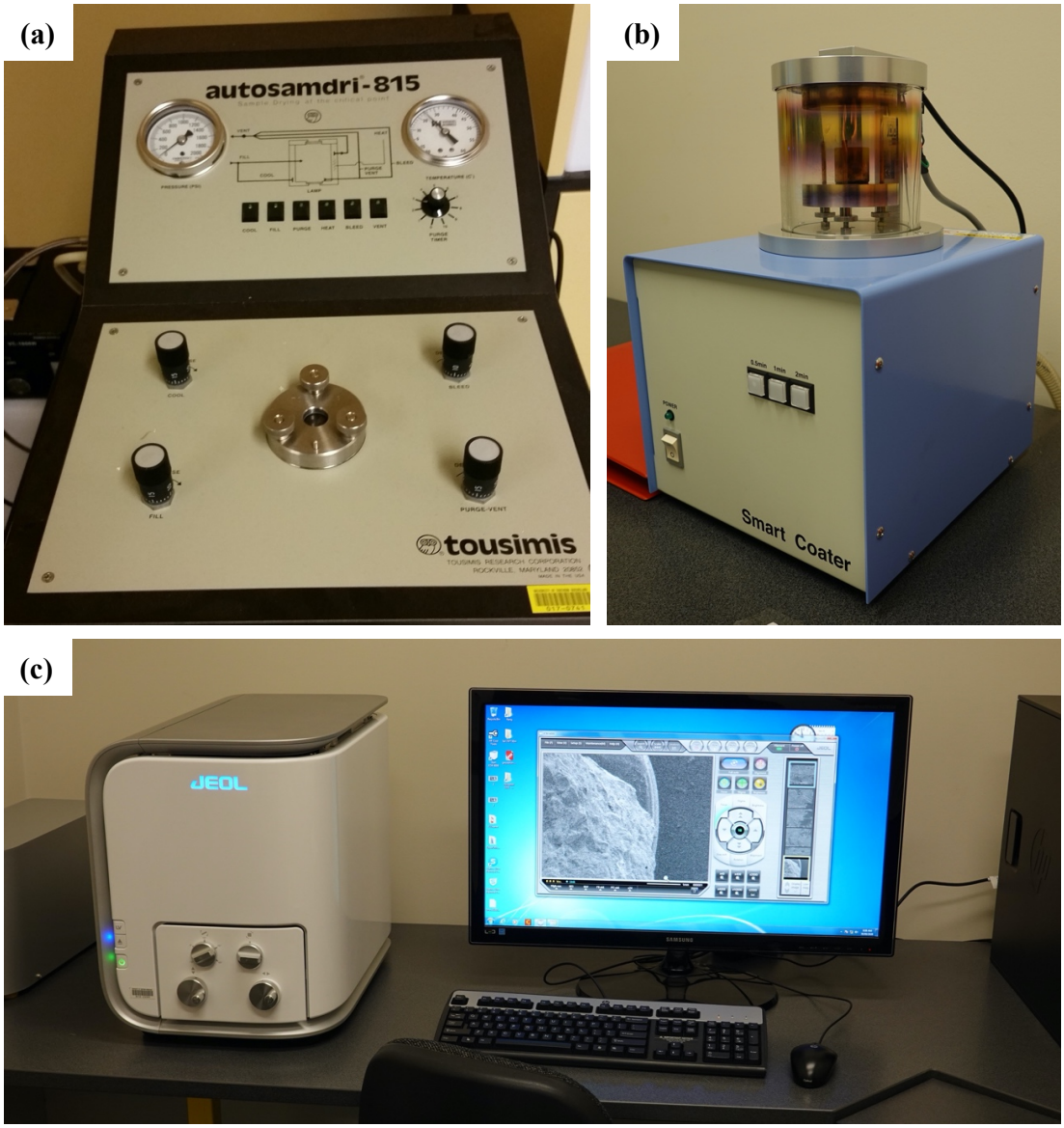


Figure A.5: (a) Autosamdri-815 series A critical point dryer, (b) gold-sputter coating machine and (c) the scanning electron microscope model Neoscope JCM-6000 connected to a computer. The use of these tools was described in the methodology section of Chapter 4.

Appendix F

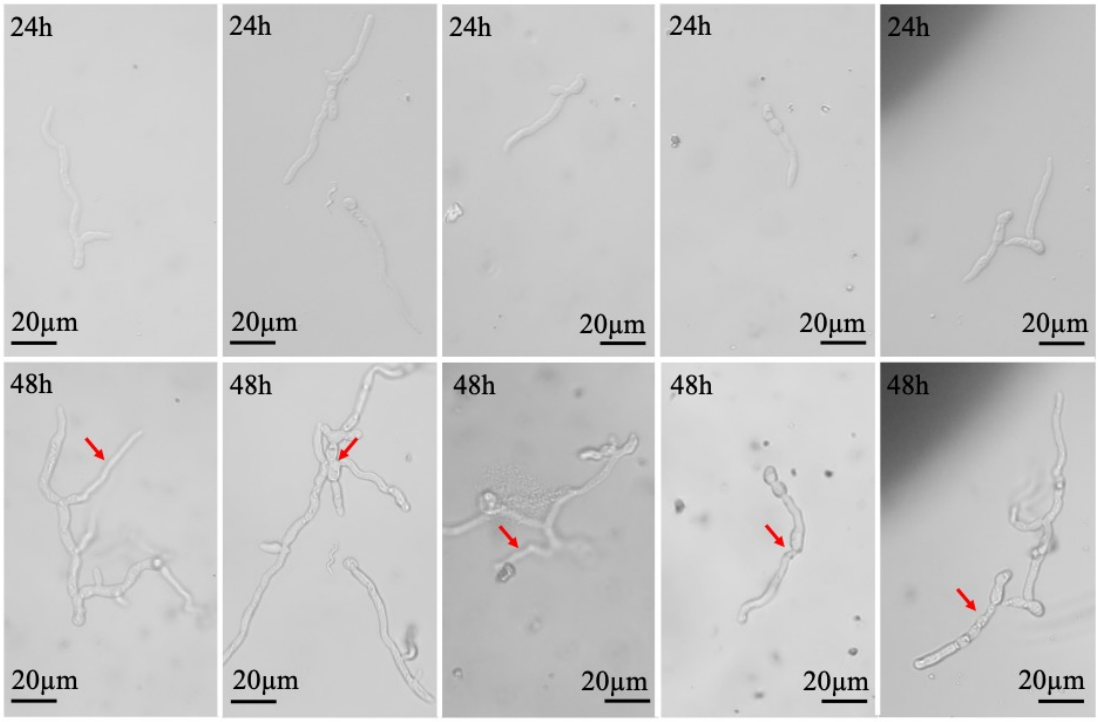


Figure A.6: Germination and germ tube growth of *M. anisopliae* strain QS155 on carbendazim media at varying concentrations after 24h and 48h of inoculation. The germ tubes appeared normal at 24h post-incubation. At 48h post-incubation, the cell-walls of the germ-tubes had burst after exposure to 100% of the FFC (red arrow), whereas at lower concentrations the conidia produced abnormal, distorted, swollen and stunted germlings (red arrows). This figure supplements the discussion in Chapter 5.

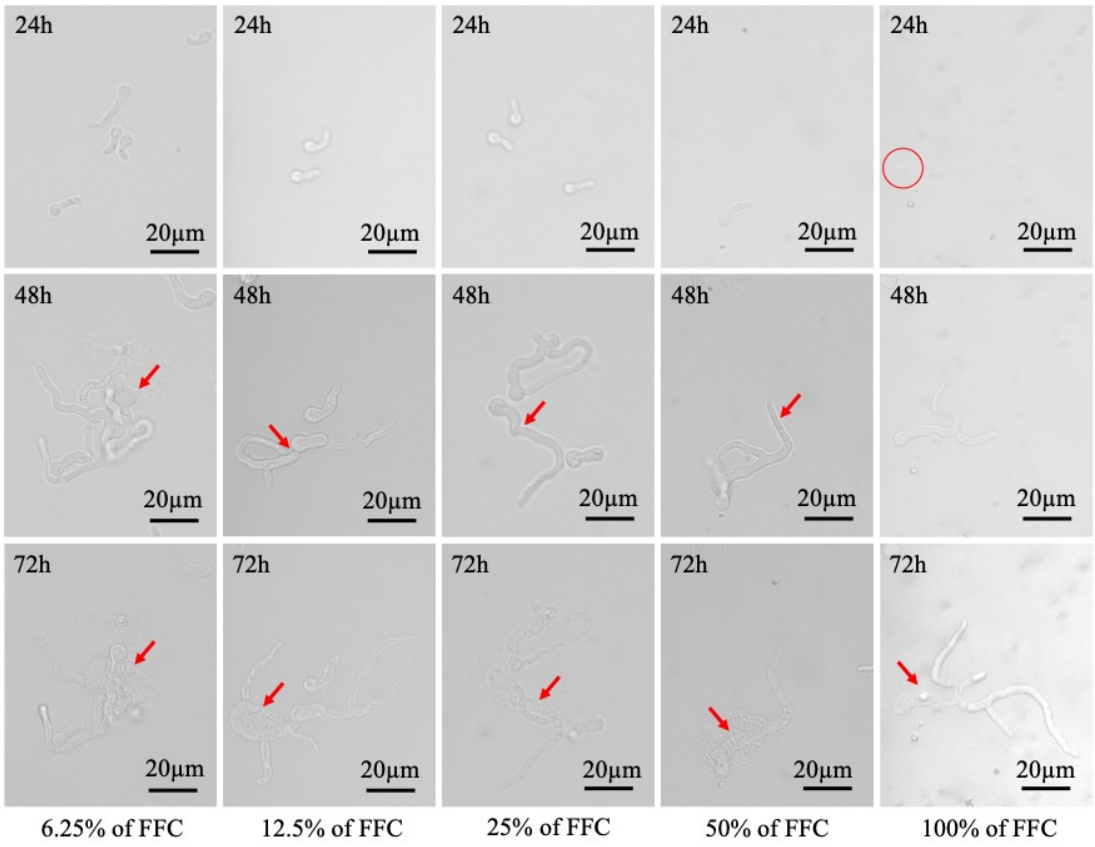


Figure A.7: Germination and subsequent germ tube growth of *B. bassiana* strain B50 on carbendazim media at varying concentrations after 24h, 48h and 72h of inoculation. The germ tubes appeared to be inhibited at 24h post-incubation, slightly elongated at 48h post-incubation with the abnormal, distorted, swollen and stunted germlings (red arrows). At 72h post-incubation, the cell-walls of the germ-tubes had burst after exposure to any concentrations of carbendazim (red arrows). This figure supplements the discussion in Chapter 5.

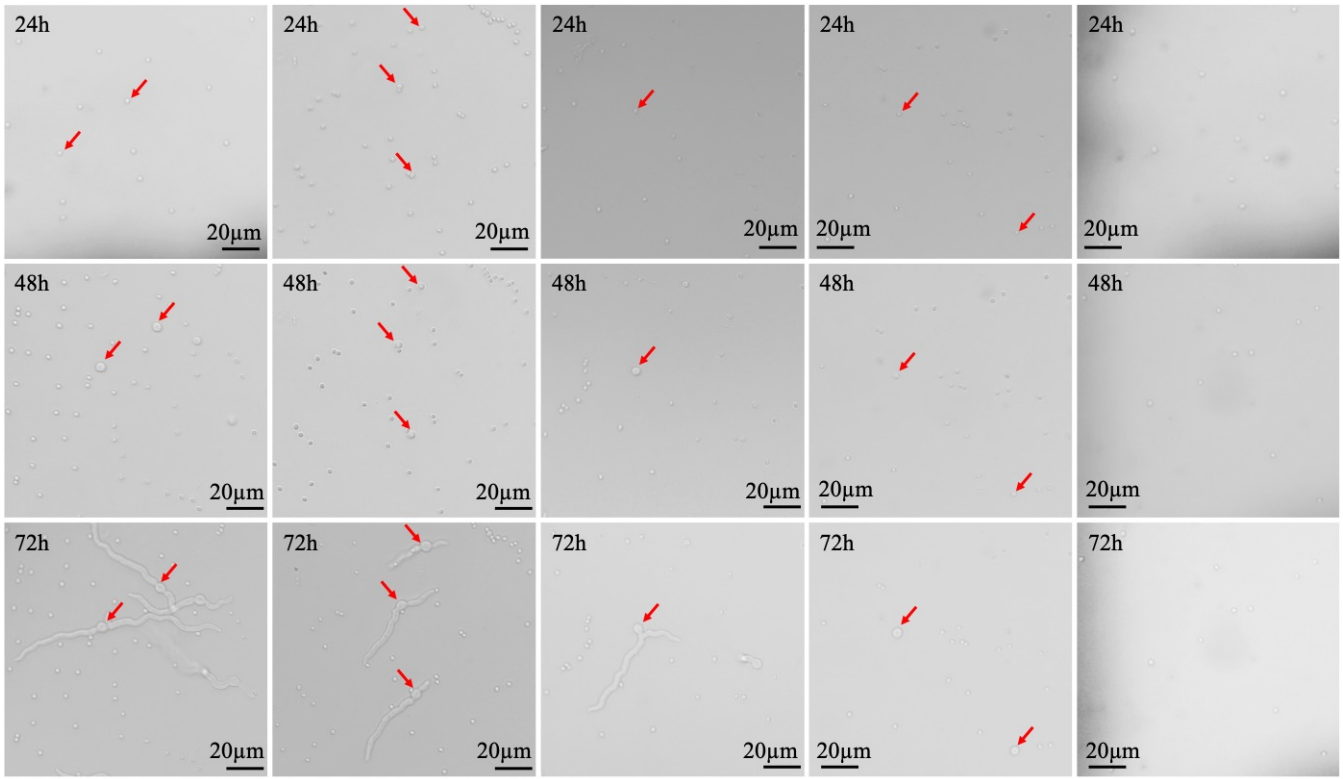


Figure A.8: Germination of *B. bassiana* strain B50 on pyraclostrobin media at different concentrations after 24h, 48h and 72h of inoculation. No germination was observed with B50 on pyraclostrobin media at different concentrations after 24h of exposure (red arrow), but after 48h, the conidia absorbed water and became swollen (red arrow), especially at below 25% of the FCC. After 72h of exposure germ-tubes were observed at concentrations below 25% of the FFC and swollen conidia were observed at 50% of the FFC (red arrow). This figure supplements the discussion in Chapter 5.

Appendix G

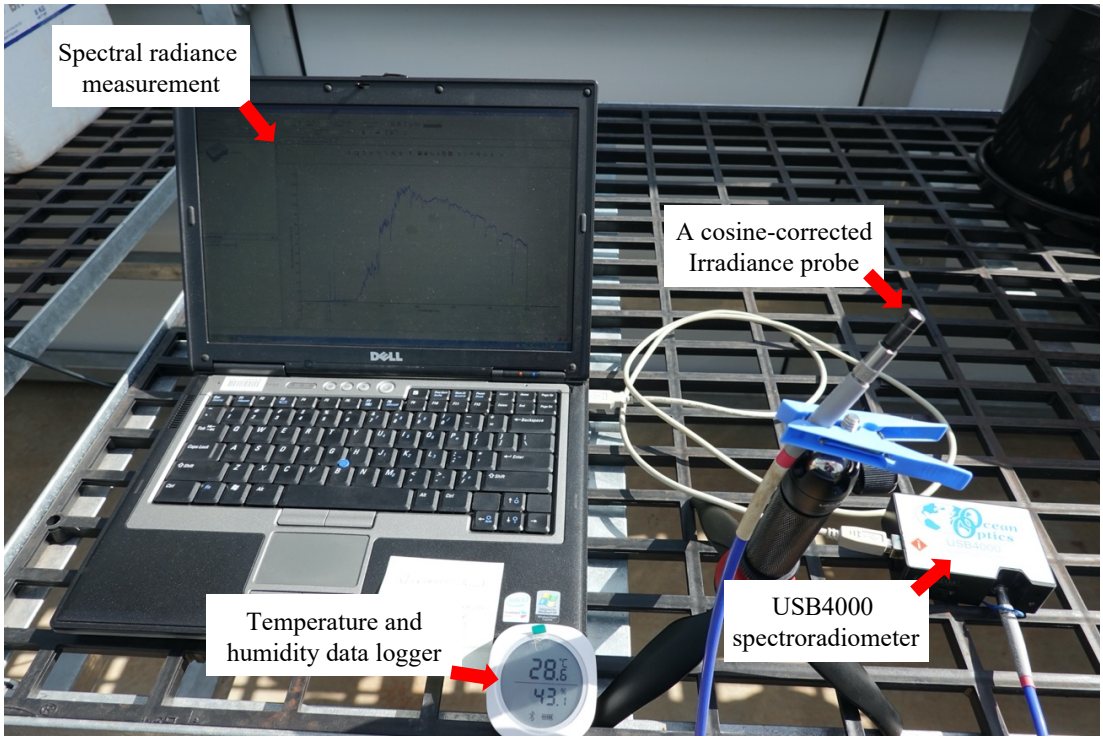


Figure A.9: The equipment for measuring solar UV radiation in the glasshouse.

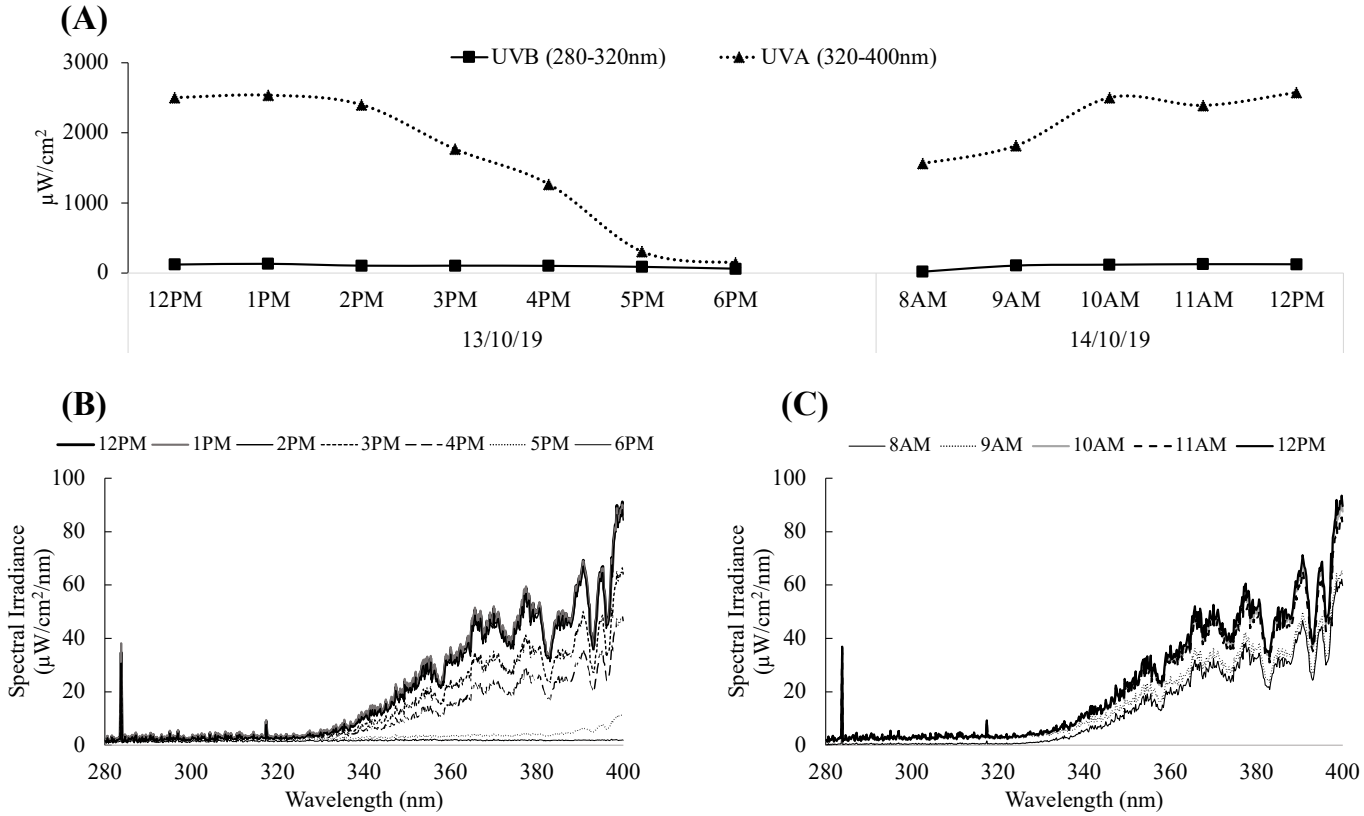


Figure A.10: Solar UV radiation in the glasshouse during the study on oils as UV protectants. (A) average solar UV radiation at each measured time, (B) solar spectra radiation over time on 13/10/2019 and (C) solar spectra radiation over time on 14/10/2019. The solar UV radiation was measured every hour in the glasshouse (27°36'29.3"S 151°55'55.1"E) from 12:00PM to 6:00PM on the 13th October 2019 and from 8:00AM to 12:00PM on the 14th October 2019 using a cosine-corrected irradiance probe (CC-3-UV, Ocean Optics, Dunedin, FL, USA) screwed onto the end of an optical fibre coupled to an USB4000 spectroradiometer (Ocean Optics). The experimental days were sunny and the temperature outside the glasshouse was around 10-25˚C.

Appendix H

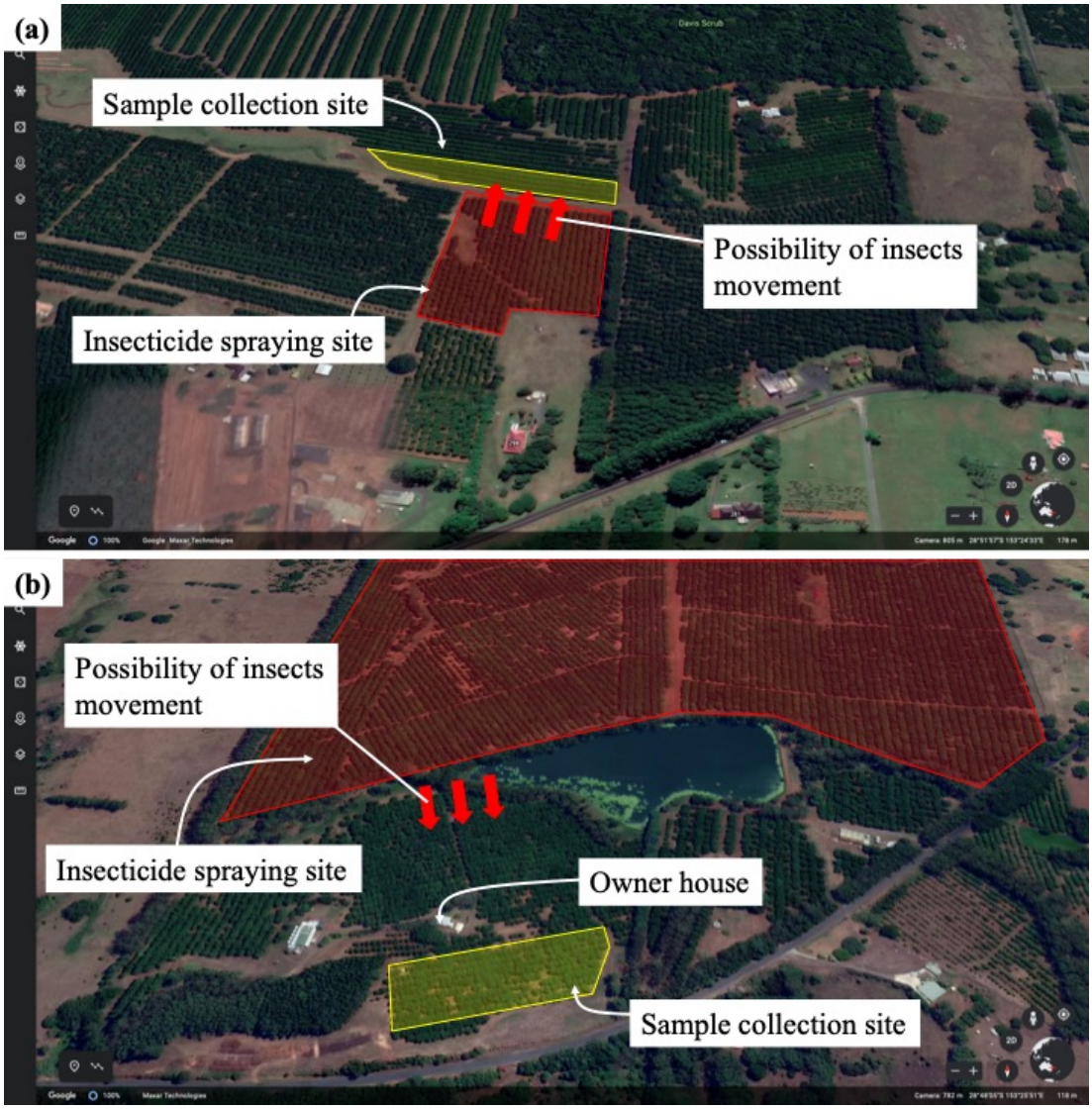


Figure A.11: Weevil infested nut collection sites: (a) at an organic farm (28°52'07"S 153°24'06"E) and (b) at a conventional farm (28°48'27"S 153°25'23"E) (Source: Google Earth). This figure supplements the discussion in Chapter 7.