NUTRIENT-FORTIFIED *METARHIZIUM ANISOPLIAE* GRANULES FOR THE MANAGEMENT OF SOIL-BORNE INSECT PESTS OF SWEETPOTATO

A thesis submitted by

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ABSTRACT

Sweetpotato (*Ipomoea batatas*) is a small but important vegetable crop in Australia, with a total annual production of about 100,000 tonnes, estimated to be worth AU$100 million. Sweetpotato is susceptible to damage by many root herbivores like wireworms, nematodes, and weevils. Wireworm damage is sporadic but significant in sweetpotatoes, despite the deployment of insecticides. Inferior performance of soil insecticides and the discouraged use of hazardous insecticides has encouraged research into biological control agents such as entomopathogenic fungi (EPF). Entomopathogenic fungi like *Metarhizium anisopliae* are frequently used in integrated pest management programs and may be a suitable candidate for wireworm control. The capacity of an EPF to grow in soil may determine the outcome of wireworm control in sweetpotato fields. This study assessed the resporulation potential of nutrient-fortified fungal granules on different soil types with various levels of reduced soil microbes. The infectivity of the resulting resporulated fungal granules in soil was also assessed using larval mealworms (*Tenebrio molitor*) as a model insect under the laboratory and glasshouse conditions.

When the resporulation of fungal granules was examined in response to nutritive additives, the combination of 20% w/v compressed baker's yeast (*Saccharomyces cerevisiae*) and 20% w/v corn starch (*Zea mays*) induced the greatest fungal resporulation and this formulation was used for subsequent experimentation. Soil type had no effect on fungal resporulation from granules; however, the role of soil treatment (sterilised, pasteurised, and non-sterile soils) was significant. Fungal resporulation was significantly pronounced in sterilised soil possibly due to the reduction in the background, resource-competing soil microbes (bacteria and fungi).

Despite the variability of fungal resporulation among sterilised, pasteurised, and non-sterile soil, the overall mealworm mortalities caused by the resporulated fungal granules were not different. However, the fungal infectivity against larval mealworms was significantly higher on sterilised soil than on non-sterile soil under glasshouse conditions. The inability of fungal granules to resporulate on non-sterile soil in the glasshouse may have resulted in low mealworm mortalities.

Although soil fumigation only causes short-term suppression in the antagonistic microorganisms, this was sufficient to maximise resporulation of fungal granules. The fungal granules inoculated on fumigated soil resporulated profusely and the resulting granules killed at 100% larval mealworms after 10 days. Additionally, the fungal conidia from resporulated granules had excellent germination, which was significantly greater than those found germinating on non-sterile soil. The information and knowledge gained during this research have important implications for the biological control-based management of insect pests, especially in high-value horticulture like sweetpotato.

CERTIFICATION OF THESIS

This Thesis is the work of **Sudhan Shah,** hereby declare that this is my original work and contains no material previously published or written by another author nor material which to a substantial extent has been accepted for the award of any other degree or diploma at the University of Southern Queensland or any other educational institutions, except where due reference has been made in the text.

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

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

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

STATEMENT OF CONTRIBUTION

The following detail is the agreed share of contribution between candidate and co-authors for this thesis:

Chapter 3: Roles of nutritive additives, substrates, and soil types on the fungal resporulation of calcium alginate encapsulated *Metarhizium anisopliae*.

Sudhan Shah, Gavin Ash, Bree Wilson

Sudhan Shah (SS) contributed 60% of this chapter, consisting of the concept and experimental design, literature collection and interpretation, conducting the experiments, data collection, analysing the data, and writing the chapter. Gavin Ash (GA) and Bree Wilson (BW) contributed 40% of the chapter towards the experimental design, supervision on experimental works, and revising and editing the writing.

Chapter 4: Laboratory evaluation of larval mealworm, *Tenebrio molitor* mortality caused by *Metarhizium anisopliae* QS155 formulated in a calcium alginate granule

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SS contributed 60% towards the experimental concept and design, conducting experiments, data collection and analysis, and writing the chapter. GA and BW contributed 40% towards the experimental design, experimental supervision and guidance, data analysis and interpretation, and revising and editing the writing.

Chapter 5: Glasshouse evaluation of infectivity of soil-applied calcium alginate formulated *Metarhizium anisopliae* QS155 to mealworm larvae, *Tenebrio molitor*

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SS contributed 60% towards the experimental concept and design, conducting experiments, data collection and analysis, and writing the chapter. GA and BW contributed 40% towards the inputs on the experimental design, providing feedback

on the experimental process, data analysis, and revising and editing the chapter writing.

Chapter 6: Resporulation of fungal granules, *M. anisopliae* encapsulated into calcium alginate, on Metham® fumigated soil and the fungal infectivity to larval mealworms, *Tenebrio molitor*

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LIST OF CONFERENCES ATTENDED AND CONFERENCE PAPERS

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Chapter 3

ABBREVIATIONS

ANOVA	Analysis of Variance
APVMA	Australian Pesticides and Veterinary Medicines Authority
ASPG	Australian Sweetpotato Growers
BCA	Biological Control Agent
CAG	Calcium Alginate Granule
CAG_{Ma}	Calcium Alginate Granules with *Metarhizium* (*Meta.*)
CAG_{Ma+Cs}	Calcium alginate granule containing *Meta* and corn starch (Cs.)
CAG_{Ma+By}	Calcium alginate granule with *Meta* and baker's yeast (By.)
$CAG_{Ma+Cs+By}$	Calcium alginate granule containing *Meta*, Cs., and By.
CAG_{Cs+By}	Calcium alginate granules containing Cs. and By.
COI	Cytochrome oxidase subunit 1
CFU	Colony forming unit
DAP	Days after planting
df	degree of freedom
DSM	Defatted seed meal
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematodes
GL	Glucosinate
GPS	Global Positing System
IJ	Infective Juvenile
ITC	Isothiocynates
Mad 1	*Metarhizium* adhesion protein 1

MITC	Methylisothiocynate
NA	Nutrient Agar
OC	Organochlorine
OP	Organophosphate
PDA	Potato dextrose agar
Pers. Comm.	Personal communication
RCBD	Randomised complete block design
RH	Relative humidity
SE	Standard error
SDAY	Sabourad dextrose agar with yeast
UV	Ultraviolet

CHAPTER 1: THESIS INTRODUCTION

1.1 Introduction

The sweetpotato (*Ipomoea batatas* L. Family: Convolvulaceae) industry is worth AU$ 100 million annually in Australia, where an estimated 90% of national production is produced in Queensland (ASPG 2020). Sweetpotato occupies approximately 9 million ha of cultivated land that subsequently produces 124 million tonnes of fresh root per annum globally (Loebenstein & Thottappilly 2009). Although sweetpotato is indigenous to Central America, it is distributed globally including North America, Europe, Africa, Asia, and Pacific regions (Mukhopadhyay et al. 2011). Root herbivores, particularly root-knot nematode (*Meloidogyne javanica* and *M. incognita*), sweetpotato weevil (*Cylas formicarius*), and wireworm (unspecified species), are regarded as the most important pests for the sweetpotato industry in Australia (McCrystal 2010). Of these, wireworm, a coleopteran soil insect including both Elateridae and Tenebrionidae, is characterised as a cryptic species complex due to its subterraneous nature, multiple genera, and spatio-temporal dynamics in soil (Finney 1946; Parker 1996). Due to its cryptic nature, it is challenging to control, compared to for example the sweetpotato weevil. Wireworm generally causes damage in the form of shallow holes on the root periderm of sweetpotato but can also cause deeper holes with high pest pressure. Although this damage often does not cause direct yield loss, it does reduce the cosmetic value of the root crop, making it unmarketable (Brill 2005). Although wireworm is a sporadic pest of sweetpotato in Australia, significant losses in sweetpotato have been observed in the field when heavy infestations occur (McCrystal 2010). To avoid the potential risk of wireworm damage in sweetpotato, growers often apply insecticides to the soil before planting and foliar insecticides during the crop development to mitigate beetle oviposition on the ground (DAF 2010). However, current chemical control is unable to protect sweetpotato throughout the growing season, especially as most of the damage occurs just before harvest when chemicals cannot be used. Furthermore, many insecticides are on the verge of deregistration because they pose hazards to the environment and have implications for food safety (McCrystal 2014). For example, many companies are ceasing the supply of chlorpyrifos, and the current minor used permit expires in October 2021. Whilst

several insecticides are registered for use under minor permits, grower concern over the development of resistance and ineffectiveness has highlighted the need for alternative chemistry and management options.

In this study, we proposed the use of nutrient-fortified *Metarhizium anisopliae* granules as a prospective control agent for wireworm management in sweetpotato. The genus *Metarhizium* is a resident soil fungus and has the potential to infect and kill a large number of soil insects including wireworms (Ericsson et al. 2007; Zimmermann 2007). Besides its natural affinity with soil insects, *Metarhizium* has demonstrated a unique characteristic of being able to adapt to the belowground environment, particularly in colonising the rhizosphere and establishing endophytic relationships with the host plant especially in the roots (Parsa et al. 2013; Barelli et al. 2016). *Metarhizium* species such as *M. anisopliae*, *M. brunneum*, and *M. robertsii*, which are regarded as 'generalist' species in terms of insect host range pathogenicity, are highly abundant and persistent in soil (Castro et al. 2016). Both laboratory bioassays and semi-field studies have demonstrated that *M. brunneum* is highly virulent on wireworm (larvae) and adult (click beetle) of *Agriotes* spp. infesting potato (*Solanum tuberosum* L.) (Kabaluk et al. 2015; Brandl et al. 2016). However, results of soil insect control between laboratory bioassays and field experiments are inconsistent (Samson et al. 2006). For instance, the use of *M. anisopliae* conidial granules (about 10^{14} conidia/ha) as pre-plant broadcast along the seed potato row did not confer any significant crop protection against *A. obscurus* attack on potato tubers (Kabaluk et al. 2005; Goettel et al. 2007).

Several factors may contribute to the poor biological control efficacy of the genus *Metarhizium* for soil insects, like wireworms. For example, the transient nature of wireworms in the soil profile limits the fungal biological control performance by not allowing direct physical contact between them (Brandl et al. 2016), as direct adherence of the fungal conidia onto the insect cuticle is crucial for insect disease initiation (Zimmermann 2007) and at least 48 h of physical contact between wireworm and *Metarhizium* inocula is required for wireworms to acquire the fungal infection (Ericsson & Kabaluk 2007). The lack of persistence and infectivity of the fungal pathogen can be a major hurdle for successful soil insect biological control (Jaronski

et al. 2007). The ability of the conidia to persist in the soil is influenced by soil texture, soil moisture, soil temperature, farming practices, food substrates, and density of other soil antagonists (Jaronski et al. 2007; Zimmermann 2007). A bioassay study carried out by Goettel et al. (2007) confirmed that dry soil conditions were detrimental for *M. anisopliae* infection to wireworm (*A. obscurus*) regardless of the soil texture.

1.2 Aim

In this study, we aimed at examining the fungal resporulation in soil from the fungal granule, *Metarhizium anisopliae* encapsulated into a calcium-alginate granule with nutritive fortification and evaluating the infectivity of resulting resporulated fungal granules against wireworms in sweetpotato. Wireworms are characterised as hardy insects developing chemical resistance, thus the abundance of fungal inocula accompanied with great viability and infectivity is crucial to control the potential risk of wireworm damage on sweet potatoes. To obtain the fungal density in soil, fungal saprophytic growth in the soil is essential especially for annual crops like sweetpotato to maintain protection from wireworm infestation. Wireworms are a subterraneous and cryptic species complex that appear sporadically in sweetpotato fields in Australia constraining their collection at adequate numbers, same instars, and same species. Thus, this study used larval mealworms (*Tenebrio molitor*, Coleoptera: Tenebrionidae) as a proxy to wireworms to examine the infectivity of resporulated *M. anisopliae* from the fungal granules in soil. Larval mealworms have been extensively used as a bioassay test host to evaluate the infectivity of entomopathogenic fungi (EPF) because they are convenient to obtain and maintain in the laboratory (Batta et.al 2010, Lestari & Rao 2016), further some species of wireworms, for example false wireworms, belong to same taxonomical family (Tenebrionidae) as mealworms. A study by Bharadwaj & Stafford (2011) revealed that larval mealworms are more resistant than adult *Ixodes scapularis* to *M. brunneum* with LD50 values 4.4×10^7 and 1.7×10^5 conidia/ml, respectively. Wireworm exposed to *M. anisopliae* with the rate of 1×10^8 conidia/ml caused up to 100% mortality of larval wireworms (*Agriotes lineatus*) within three weeks (Razinger et.al 2013). From the above evidence, we can infer that mealworm can be used as a model insect for wireworms.

Since soil is a heterogeneous substrate with confounding multiple interactions, the saprophytic growth of fungal inocula in the soil is still challenging. Therefore, series of experiments were conducted to achieve the aim of the research reported in this thesis.

1.3 Outline

The literature review presented in Chapter 2 provides the background literature with respect to (1) overview of sweetpotato farming in Australia, (2) wireworm damage, (3) wireworm management practices, (4) entomopathogenic fungi, and (5) *Metarhizium anisopliae*: Distribution, mode of infection, formulation, and use of *M. anisopliae* against wireworm. This Chapter also provides the specific objectives of the research undertaken.

In Chapter 3, various experiments are detailed including (1) the effect of various nutritive additives on fungal sporulation on calcium alginate granules, (2) evaluates the resporulation efficacy of fungal granules on different substrates, and (3) investigates the effect of soil types with three levels (non-sterile soil, pasteurised soil, and sterilised soil) on fungal resporulation is reported.

In Chapter 4, experiments are presented that evaluate (1) the mortality efficacy of resporulated fungal granules against larval mealworms, (2) the effect of fungal conidia on soil for larval mealworms, and (3) the infectivity of fungal granules for larval mealworms on either on non-sterile soil or simulated-solarised soil or sterilised soil.

In Chapter 5 results from the previous experiments are extended to further investigate (1) the mortality efficacy on the fungal granules on mealworm, as either preconditioned fungal granules or non-preconditioned fungal granules, inoculated in either sterilised soil or non-sterile soil under glasshouse conditions and (2) the effect of temperature on the fungal resporulation from the fungal granules.

In Chapter 6, the last experimental chapter, experiments are presented on (1) the resporulation efficacy of fungal granules on fumigated soil and (2) the infectivity of resulting resporulated fungal granules to larval mealworms.

In Chapter 7, the summary of findings, general discussion, future research, and conclusion are presented.

CHAPTER 2: LITERATURE REVIEW

2.1 Sweetpotato

Sweetpotato, *Ipomoea batatas* L. (Family: Convolvulaceae), is the 6th most important root crop based on dry weight production in the world (Loebenstein & Thottappilly 2009). Sweetpotato occupies approximately 9 million ha of cultivated land that subsequently produces 124 million tonnes of fresh root per annum globally (Loebenstein & Thottappilly 2009). Although sweetpotato is indigenous to Central America, it is distributed globally across North America, Europe, Africa, Asia, and the Pacific Islands including Papua New Guinea (Mukhopadhyay et al. 2011). China is the largest producer of sweetpotato and accounts for approximately 75% of the global production, followed by sub-Saharan African countries, which produce 14% of the global output (FAO 2013). Sweetpotato is one of the major staple food sources in Papua New Guinea (PNG) (Bourke 2009), where 6, 57,351 tonnes of fresh weight are produced annually (FAO 2013). An estimated 100,000 tonnes of sweetpotato are produced annually in Australia, which accounts for a gross value of AU$100 million (ASPG 2020).

Sweetpotato root is a rich source of carbohydrate (80-90%) on a dry weight basis and is used as a fresh produce for human consumption, feed for livestock, and industrial products such as ethanol and starch (Woolfe 1992). Additionally, the leaves of sweetpotato are a rich source of minerals and vitamins and can also be used as a human food source (Islam 2006), while the entire vine can be fed to livestock (Scott 1992). In the Australian context, an estimated 97% of fresh weight production is supplied to domestic fresh markets as human food, 3% is utilised for factory processing and, <1% is exported as fresh supply (Hort Innovation 2018). Sweetpotato is highly susceptible to frost, but it has a unique capacity to utilise solar energy for a prolonged period, resulting in the accumulation of high dry matter content in storage roots (Hahn 1977). For this reason, in Australia, the majority of sweetpotatoes are grown in Queensland, particularly in the Bundaberg region, accounting for 76% of the national sweetpotato production (Hort Innovation 2018). Although the genetic diversity of sweetpotato is high globally (Woolfe 1992), only varieties are cultivated in Australia including Beauregard (gold flesh), Northern Star (purple skin and white flesh), WSPF

(white skin and purple flesh), and Kestle (white skin and flesh) (ASPG 2020). Among all the varieties, Beauregard, which was initially released by the Louisiana Agricultural Experiment Station in 1987, is the dominant variety across sweetpotato growing regions in Australia and the US, occupying 90% of total production in Australia due to consumer preference (Hort Innovation 2018). The sensory attributes of sweetpotato varieties were found as the main driver for consumer preferences, in which the influence of colour and textural properties were the highest (Leksrisompong et al. 2012). Varietal attributes such as root shape and size, skin and flesh colour, and shelf life have been taken into account before selecting the sweetpotato variety for field cultivation in Australia (Wolfenden 2014). As result, only a limited number of 'gold fleshed' varieties have been commercially grown in Australia, although there are a range of sweetpotato varieties currently available in Australia (Wolfenden 2014).

Like many horticultural crops, sweetpotato is attacked by large numbers of pathogens, insects, mites, and nematodes (Chalfant et al. 1990). Root herbivores cause direct damage to sweetpotato roots, making them less marketable. Therefore, soil pests including weevils (*Cylas formicarius*, *Naupactus leucoloma*), wireworms (representing both Elateridae and Tenebrionidae), and root-knot nematodes (*Meloidogyne incognita* and *M. javanica*) have been classified as high priority pests attacking sweetpotato in Australia (McCrystal 2010). *Cylas* spp. are the most destructive pest globally and in some regions of China, a yield loss of up to 80% of sweetpotato has been recorded (Hue & Low 2015). *Cylas* spp. are a heterogynous species complex, and of them, *C. formicarius* (Fabricius) is prevalent globally (Figure 2.1) but dispersed predominantly in the tropical regions (Hue & Low 2015). In this literature review, the following sections focus on the management and control of wireworms.

Figure 2.1: Sweetpotato roots infested by sweetpotato weevils (*Cylas formicarius* Fabricius)
Photo credit: Bree Wilson

2.2 Wireworms

Wireworms are the subterraneous larval stage of beetles (Order: Coleoptera) belonging to the families Tenebrionidae and Elateridae (McDonald 1995). Wireworms that are associated with the Tenebrionidae are referred to as false wireworms, while wireworms related to the Elateridae are broadly known as true wireworms (Figure 2.2) (McDonald 1995). Wireworms comprise a large range of genus and species; therefore, their lifecycles are highly variable. The time required to complete their life cycle has been attributed to several factors including climatic conditions, food availability, genetic variation, and sex (Traugott et al. 2015). Moreover, the development of wireworms in the field may be impacted by cropping practices, which is difficult to model from laboratory studies. A phenology study of the true wireworm, *Agriotes sordidus* (Illinger), showed that they complete their lifecycle in one to three years, passing through 8 to 11 larval instars (Furlan 2004). When there is sufficient food provided and adequate moisture, the temperature is the main determinant factor for the duration of the life cycle and the number of larval instars in *A. sordidus* (Furlan 2004). For instance, the spring oviposited eggs by *A. sordidus* in lower latitudes of Italy (the predominantly warmer regions) complete their lifecycle in one year, and consequently,

they emerge as an adult in next summer season, whereas two years are required for lifecycle completion in northern Italy, where temperatures are cooler (Furlan 2004). The lifecycle of *A. sordidus* and *A. ustulatus* revealed that their larvae (from 2nd instar onward) and adult overwinter during the winter period in certain regions of Italy, and the adults of both species begin oviposition in the subsequent spring season (Furlan 1996; Furlan 1998). The larvae of *A. ustulatus* can burrow as deep as 60 cm into the subsurface soil in winter to escape from the lower temperature on the soil surface, and its upward movement is highly responsive to the rising temperature of the profile soil (Furlan 1998). Due to this unique characteristic of spatio-temporal population dynamics, wireworms are broadly known as cryptic species.

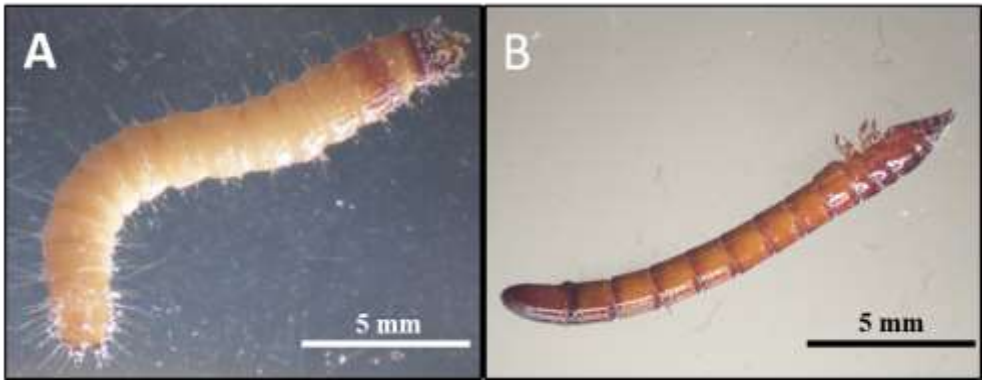


Figure 2.2: A true wireworm (for example *Agrypnus* spp.) body with 15 mm long and 3 mm wide (A) (Kimber 2015) and a false wireworm (for example *Gonocephalum* spp.) body 17 mm long and 2 mm wide (B) (Umina & McDonald 2015). Both wireworms were collected from the agricultural field of the University of Southern Queensland, Toowoomba, during the summer fallow season, while barley (*Hordeum vulgare*) was planted as a cover crop in the previous season.

A review study undertaken by Robertson (1993) explained that the false wireworm species *Gonocephalum macleayi*, *Pterohelaeus alternatus*, and *P. darlingensis*, which are common in summer grain crops in southern and central regions of Queensland, usually complete their lifecycle within one year. The adult beetles normally start to emerge from late spring to early summer (October-January) and begin to oviposit after one month after emergence (April to May), which lasts for about 20 weeks until the temperature cools down (Robertson 1993). The larvae undergo

different developmental stages throughout the autumn, winter, and spring; passing through up to 11 instars before they pupate (Robertson 1993). Soil moisture is crucial paramount for successful pupation from the fully grown larvae and rain during early spring is considered as a trigger for pupation (Robertson 1993). In the absence of rainfall, it is believed that the larval stage can be prolonged until the successive spring season (Robertson 1993).

Click beetles, the adult stage of true wireworms, are globally distributed and the number of species is estimated at over 10,000 (Traugott et al. 2015). Although the morphological characteristics of the adult click beetles allow identification at the genus level, species-level identification is not feasible through morphological characteristics (Traugott et al. 2015). In the case of the larval stage of click beetles (i.e., wireworms), the morphological characteristics of specimen identification are complicated because there is only limited knowledge of their morphology (Barsics et al. 2013). Considering these constraints, wireworms have been identified using genetic methods. In diversity studies of wireworm, the mitochondrial cytochrome c oxidase subunit I (COI) and 16S genes, which are regarded as highly conservative genes are often used (Etzler 2013). The DNA sequencing of the COI region of wireworm allows a comparison with the adult wireworm, whereas the study of the 16S gene demonstrates genetic diversity among cryptic species of wireworm, which are morphologically indistinguishable (Etzler 2013). In North America, the COI genes were utilised for the DNA barcoding of three elaterid genera (*Agriotes*, *Conoderus*, and *Melanotus*) representing 12 species (Traugott et al. 2015). Similarly, the 16S rDNA gene region was used for DNA barcoding species belonging to the genera *Aelos*, *Agriotes*, *Hypnoidus*, *Limonius*, *Melanotus*, and *Selatosomus* (Traugott et al. 2015). The DNA Barcode Reference Library in respect to adult beetles is a key determinant factor for wireworm species identification (Etzler 2013). A similar effort was recorded in Japan, in which 275 elaterid species belonging to 90 different genera including *Melanotus* and *Agrypnus* were examined using COI gene amplification and DNA barcoding (Oba et al. 2015). These molecular tools allow for rapid crop risk assessment and may lead to sustainable wireworm control in agriculture (Benefer et al. 2013).

Darkling beetles are generally referred to as the adult beetles of Tenebrionid beetles, which rank as the seventh order among the Coleopteran insects in terms of numbers of species, with at least 20,000 described species worldwide (Kergoat et al. 2014). The larval stage of darkling beetles is regarded as false wireworms because of their morphological resemblance to true wireworms, a larval stage of elaterid beetles (Wakeland 2016). Both adult and larvae of Tenebrionidae are sub-classified into different functional groups based on feeding habits and habitat, namely the cereal group, the forest group associated with rotten wood, the macro-fungi group, and the dune groups (Cho et al. 2013).

There are only a few examples of molecular studies of Tenebrionidae beetles that are solely focused on diversity and phylogenetic studies. For example, a DNA barcode library for 15 tenebrionid species including 9 genera, 7 tribes, and 4 sub-families in Korea was generated using the 658 bp partial mitochondrial cytochrome c oxidase subunit I (COI) gene (Cho et al. 2013), and phylogenetic studies were carried out in Europe across 404 taxa (250 species) using four mitochondrial gene fragments and four nuclear fragments comprising three different genes (Kergoat et al. 2014).

2.3 Crop damage by true and false wireworms

Although click beetles (true wireworms) occur naturally in forests and grasslands and are thought to be largely harmless to agriculture crops (Traugott et al. 2015), some pestiferous click beetles, (for example *Agrypnus variabilis* as a sugarcane wireworm and *Hapatesus hirtus* as a potato wireworm in Australia) do lay eggs on arable land, and their larvae inflict damage on growing crops (Horne & Horne 1991; Barsics et al. 2013). Larvae mainly feed on the crown and below-ground plant organs, which results in seedling death and subsequent yield loss (Barsics et al. 2013). Wireworm damage to tuberous crops like potato (*Solanum tuberosum*) and storage roots crops like sweetpotato is largely confined to holes (2-4 mm deep), narrow tunnels, with scarring to the periderm. This damage results in significant quality loss, rather than yield loss (Parker & Howard 2001). True wireworms (for example *Agriotes* spp.), in particular *A. obscurus*, *A. lineatus,* and *A. sputator* are the dominant species

in Europe and Northern America and cause considerable damage to potatoes, corn, strawberries, and cereal crops (Vernon & Toth 2007). *Melanouts communis* (Gyll.) is an abundant species of true wireworm in sugarcane fields in South Florida, USA (Hall & Cherry 1993). Likewise, *M. okinawensis* is an economic pest for sugarcane in Japan (Kishita et al. 2003). True wireworm *Agrypnus variabilis*, which is alternatively referred to as sugarcane wireworm in Australia, infests sugarcane fields in Australia damaging the germinating buds of billets mainly in autumn (Samson & Calder 2003) (Figure 2.2). A native click beetle species to Australia, *Hepatesus hirtus* infests potato fields in Australia, and its larva (wireworm) causes extensive feeding damage to tubers resulting in the significant economic loss (Horne & Edward 1995).

Unlike true wireworms, false wireworms have not received any significant global scrutiny. In Australia, false wireworms are reported as causing sporadic damage to several summer grain and pulse crops. For instance, the southern false wireworm *Gonocephalum* spp. and the eastern false wireworm *Pterohelaeus* spp. cause damage to summer crop seedlings particularly sorghum (*Sorghum bicolor*), sunflower (*Helianthus annus*), soybean (*Glycine max*), and mungbean (*Vigna radiata*) in central Queensland (Robertson 1993). The other two false wireworm species, the grey false wireworm *Adelium* spp. and the bronze field beetle *Isopteran* spp. attack canola (*Brassica napus*) seedlings resulting in poor seedling establishment in Western Australia, South Australia, and Victoria (Miles & McDonald 1999). Adopting cultural practices like stubble retention from the preceding crops optimises oviposition and over-summering for both the neonate larvae and adult beetles, thus the successive crop falls vulnerable to wireworm attack (Bowden et al. 2014). Generally, the damage to broadacre crops due to the false wireworms does not warrant any crop protection in Australia because the pests occur sporadically and unevenly (Bowden et al. 2014). However, the bronze field beetle (*Adelium brevicorne*) and the grey false wireworm (*Isopteran punctatissimus*) are currently regarded as emerging pests for canola in Western Australia and Victoria (Miles & McDonald 1999; Bowden et al. 2014), without typical damage to grain and pulse crops (Bowden et al. 2014).

The adults of *Gonocephalum* sp. (darkling beetles) have been recorded as an economic pest of chickpea (*Cicer arietinum*) and ground nut (*Apios Americana*) in

India and corn (*Zea mays*) in South Africa (Wadaskar & Patil 2016). In some chickpea growing areas of India, darkling beetles caused extensive damage to emerging seedlings, resulting in 60% of the cropping area needing to be re-sown (Wadaskar & Patil 2016). Similarly, the non-irrigated wheat (*Triticum aestivum*) growing regions in eastern Idaho, USA were reported to have a high abundance of *Eleodes* spp., and their larval damage to the sown wheat crop seeds caused 10-40% economic loss (Wakeland 2016). More recently, adults and larvae of this species were reported as being a major pest for grass and seedling crops such as corn, wheat, and pea (*Pisum sativum*) in Columbia, which was previously considered as a minor pest (Quiroga-Murcia et al. 2016; Quiroga-Murcia & Posada-Florez 2013).

2.4 Wireworms in sweetpotato

Soil insects such as wireworm (mainly *Conoderus* spp.), corn rootworm (*Diabrotica* spp.), and flea beetle (*Systena* spp.), which are collectively referred to as the WDS (Wireworm, *Diabrotica,* and *Systena*) complex, are regarded as economic pests for sweetpotato in the USA because their scattered shallow feeding damage results in the produce being unmarketable due to quality loss of produce (Figure 2.3) (Brill 2005). A sweetpotato farmer's field survey in North Carolina revealed an economic loss of about 25% due to the injury inflicted by the WDS complex, followed by sweetpotato flea beetle (*Chaetocnema confinis*) (18%), white grub (*Cotinis nitida*) (4.6%) and white-fringed weevil larvae (*Graphognathus* spp.*/ Naupactus leucoloma*) accounting for 3% of damage in sweetpotato fields (Brill 2005). To date, multiple species belonging to the genus *Conoderus*, for example, *C. falli*, *C. vespertinus*, *C. ampliccollis*, *C. scissus,* and *C. rudis,* are increasingly problematic for sweetpotato especially in southern regions of the USA, particularly in North Carolina, Mississippi, and California (Chalfant et al. 1990). Of these, the population of the tobacco wireworm *C. vespertinus* was found to be very abundant, representing more than 66% of the total wireworm sampled from sweetpotato plantation in Northern Carolina (Arrington et al. 2016). Historically, records of the Gulf wireworm (*C. amplicolis* Gyll.) causing injury to sweetpotato in Alabama, USA has dated back to 1936 and for its control, the dust

formulation of aldrin, a member of organochlorine insecticide, was applied during that period (Eden et al. 1956).

Figure 2.3: Typical symptoms of wireworm damage on sweetpotato found in sweetpotato fields in Bundaberg, Australia (Photo credit: Gavin Ash)

Customer demand for premium quality fresh produce in Australia drives sweetpotato farmers to produce blemish-free roots (Akers 2014). However, sweetpotato growers incur economic losses every year due to unmarketable produce caused by wireworm damage (McCrystal 2010). Although wireworm damage has been observed in different sweetpotato growing regions in Australia, specific identification of wireworm species has not yet been carried out (McCrystal 2010). There is not any record of molecular-based identification of wireworms infesting sweetpotato in Australia to date, and this necessary to develop sustainable wireworm control strategies in sweetpotato.

True wireworms, particularly representing the genera *Agrypnus, Conoderus, and Heteroderes*, are declared as pests for sugarcane (Samson & Calder 2003) and it is speculated that these wireworms have infested sweetpotato as both crops are grown in central Queensland (Bundaberg region) and northern New South Wales (NSW) (McCrystal 2014). The true wireworm, *Heteroderes* was anecdotally reported as an abundant genus in a sweetpotato field from the Cudgen region of NSW, as documented by McCrystal (2010), but additional studies to validate this raw information are not available.

2.5 Wireworm management practices

It is generally believed that the severity of wireworms as an agricultural pest came to prominence following the global deregistration of organochlorine (OC) insecticides in the 1990s, as the chemicals are highly persistent in the soil (Vernon & Van Herk 2013). Significant economic damage by wireworms was first noted in potato and cereal crops (Vernon & Van Herk 2013), and their impacts progressively moved to different root crops such as sweetpotato, beet root, carrot, and legume crops (Barsics et al. 2013).

In response to increasing wireworm damage to the sweetpotato crop, farmers have adopted the best practice control measures to protect the crop from wireworm feeding damage and to maximise an economic return from the crop (Barsics et al. 2013). Wireworm management in sweetpotato can be broadly categorised into cultural, chemical, and biological control methods.

2.5.1 Cultural methods of control

Crop rotation with non-host crops of wireworm is always a priority of sweetpotato growers. Sugarcane is susceptible to wireworm (Samson & Calder 2003) so, a crop following sugarcane could be highly vulnerable to wireworm attack. Some research has demonstrated that rotation with brassicas alleviates wireworm feeding damage to sweetpotato (Furlan et al. 2010). Brassicas like Indian mustard (*Brassica*

juncea) exhibit anti-microbial characteristics against wireworm, which contain a glucosinolate (GL) compound that produces toxic secondary metabolites such as isothiocynates, nitriles, epithornitriles, and thiocynates in the presence of water (Furlan et al. 2010). Similarly, a winter rotation with wheat *(Triticum aestivum*) can result in a low wireworm population in the succeeding crop because the wheat stubble is not optimal for wireworm survival over summer (Robertson 1993). As a result, a low wireworm population was evident in successive summer crops following wheat such as sorghum, sunflower, and mung bean (*Vigna radiata*) although the false wireworms, particularly *Pterohelaeus* spp. and *Gonocephalum* spp., are declared as major pests for sunflower, and minor pest for sorghum in Queensland (DAF 2011).

Field hygiene practices like removal of surrounding weeds to eliminate shelter for the overwintering adults and subsequent oviposition in spring is another cultural practice touted to manage wireworm populations (Parker & Howard 2001). However, there is no direct correlation found between wireworm abundance and surrounding weed coverage (Parker & Howard 2001). Stubble retention from wireworm susceptible crops such as sorghum, sunflower, and mung bean increase the likelihood of wireworm infestation to successive crops (Robertson 1993), suggesting that cultural practices like stubble removal from these crops could reduce wireworm infestation in the successive crop.

Larvae, eggs, and pupae of wireworm are subject to desiccation and predation when tillage practices like deep ploughing are carried out in spring or summer, while wireworm abundance is more prevalent in zero tillage soil compared to conventionally tilled soil (Seal et al. 1992a). Soil flooding accompanied by elevated temperature can cause wireworm mortality (Van Herk & Vernon 2006), implying that summer would be potentially a favourable time to control wireworm, but field flooding is not feasible in Australia due to the scarcity of irrigation water. A field study revealed that wireworms, for example, *Conoderus* spp., were abundant in raised beds of sweetpotato field in Georgia, USA (Seal et al. 1992a), suggesting that optimisation of soil bed depth of sweetpotato field can reduce potential wireworm severity in, particularly wireworm prone fields. Other cultural practices include mass trapping of adult males using the sex pheromone, for example, 1:1 mixture of geranyl octanoate and geranyl hexanoate

is highly attractive to *A. obscurus* (Vernon & Toth 2007) and adjusting crop planting and harvesting time to avoid wireworm infestation, for example, post-mid-July is the active feeding season for the wireworm (*Conoderus* spp.) in sweetpotato fields of the USA (Traugott et al. 2015). Planting a wheat crop as a companion crop along with potato resulted in low wireworm damage to potato (Vernon et al. 2015) and could be implemented in sweetpotato production in small, concentrated areas of infestations. Selection of resistant sweetpotato varieties, which have thick a periderm, can tolerate wireworm damage to some extent (Schalk et al. 1986). A study evaluating the resistant genotypes of sweetpotato varieties against soil insect injury in the USA revealed that the varieties 'Ruddy' and 'Charleston' have demonstrated the highest level of resistance to attack by the WDS insect complex (Jackson 2010). Although the sweetpotato variety 'Beauregard' is highly vulnerable to soil insect injury, high consumer preference has led it to be one of the dominant varieties, occupying over 90% of sweetpotato production in the USA (Jackson 2010). Furthermore, an extensive study regarding sweetpotato varietal evaluation in Australia by Wolfenden (2014) reported that the predominant sweetpotato variety in Australia 'Beauregard' is found to be more prone to soil insect damage than the other gold varieties particularly 'Bienville' and 'Evangeline' (Wolfenden 2014). However, their undesirable characteristics, especially cracking and splitting of their roots during harvest and lower yield limit their use as an alternative to Beauregard (Wolfenden 2014).

2.5.2 Chemical control

After the Second World War, organochlorine (OC) insecticides were widely utilised to control wireworms in potato and cereal crops across Europe and North America (Vernon & Van Herk 2013). Organochlorine insecticides are characterised as having significant persistence in soil, for instance, one soil application of OC insecticides, for example, aldrin and heptachlor caused mortality to wireworms (*A. obscurus*) for up to 13 years (Vernon & Van Herk 2013). With a short persistence in soil, lindane was broadly utilised as a seed treatment insecticide in wheat and corn crops, which was reported to cause a 70% reduction of the wireworm population

(*Ctenicera destructor* Brown and *Hypolithus nocturnus* Esch.) from the field (Vernon & Van Herk 2013). However, due to their extreme persistence in the soil environment and associated consequences to human health, all OC insecticides have been deregistered and banned globally (Elibariki & Maguta 2017).

Following the global ban, growers have redirected their focus towards organophosphate (OP) and carbamate insecticides, in which phorate and chlorpyrifos (both are OP insecticides), to some extent, were found to be effective against wireworm (*Agriotes* spp) in potato, but not as effective as OC insecticides like aldrin (Parker & Howard 2001; Vernon & Van Herk 2013). However, the activity of the insecticide phorate against wireworm in potatoes was found to be ineffective because of its short residual effect in the soil, while wireworms were shown to actively feed on potatoes up to the harvesting period (Vernon & Van Herk 2013). Phorate is currently registered to use against wireworms in sweetpotato across Australia except Victoria under the minor permit (permit number-PER13902) (APVMA 2021). A report presented by Horticulture Innovation Australia (2014) indicated that phorate is ineffective against wireworm control in sweetpotato and, growers prefer alternatives to phorate such as bifenthrin.

As a result of their high toxicity to humans and the environment, some of the insecticides from the OP and carbamate group are being withdrawn from use (Barsics et al. 2013). Consequently, insecticides from the pyrethroid, the neonicotinoids, and the phenyl pyrazoles were subjected to global scrutiny (Vernon & Van Herk 2013). Bifenthrin, an insecticide that is related to the pyrethroid group was found to be effective against wireworms, but its mechanism was that of a repellent rather than being mortality inducing (Van Herk & Vernon 2013). Similarly, neonicotinoid insecticides such as thiamethoxam and clothianidin function systemically in plants and are principally employed as a seed treatment for wheat and potato prior to planting (Vernon et al. 2013). However, a field study in Canada conducted by Vernon et al. (2013) reported that the use of thiamethoxam as a seed treatment in wheat seed did not reduce the wireworm population (*Agriotes obscurus*) from the subsequent wheat crop field. The application of thiamethoxam caused wireworms to become moribund, but

shortly after the wireworms made a full recovery and wireworm damage was severe in the successive crop during the summer period (Vernon et al. 2013).

Unlike bifenthrin and thiamethoxam, fipronil, a member of the phenyl pyrazole group, causes direct mortality to wireworms (Vernon et al. 2013). The in-furrow application of fipronil during the planting season is widely adopted to produce blemish-free potatoes (Vernon & Van Herk 2013). A field study showed that when fipronil was applied to wheat seed at rates 10 times lower than that was formerly used for lindane resulted in significant improvement of crop stand and yield due to the high mortalities of neonate and resident wireworms (Vernon & Van Herk 2013).

According to the Australian Pesticides and Veterinary Medicines Authority (APVMA), sweetpotato is classified as a minor crop. Australian sweetpotato growers only have access to a limited range of pesticides for crop protection. Based on this information, the incorporation of soil insecticides before planting is universally adopted across the sweetpotato growing regions in Australia as a pre-emptive measure against potential wireworm damage. The commonly used soil insecticides in Australian sweetpotato are chlorpyrifos (OP), phorate (OP), bifenthrin (synthetic pyrethroid), and fipronil (phenyl pyrazole). Initially, these insecticides are sprayed on the soil surface and immediately incorporated using a rotary hoe to a depth of 10-20 cm (McCrystal 2010). Of these insecticides, bifenthrin is the most efficacious and shows the longest period of protection, up to 140 days after planting (DAP). However, when bifenthrin was sprayed on the soil surface and not incorporated prior to planting, control was poor, resulting in 96.7% of sweetpotato storage roots with wireworm feeding injury at 139 DAP McCrystal (2010). The commercial harvest of sweetpotato occurs from 140 to 260 DAP (Akers 2014), thus, the short residual effect of these soil insecticides cannot assure the protection against wireworm infestation especially when severe wireworm feeding injury can occur as little as two weeks before the commercial harvest of sweetpotato (Akers 2014).

Wireworm management in the sweetpotato crop has historically consisted of two approaches: application of soil insecticides before planting, and foliar spraying (mainly bifenthrin and chlorpyrifos) during crop development (DAF 2014). The foliar

spraying over an established crop is intended to control migrating beetles before they oviposit and to kill a surface lying neonate wireworm, while the purpose of soil insecticide application before planting is to kill resident wireworm (DAF 2014). However, soil-applied insecticides tend to repel wireworms from the root zone (McCrystal 2010). Despite these approaches to wireworm control in sweetpotato crops, severe wireworm injury to sweetpotato has persisted. Because of this continued damage, chemigation was introduced, where an insecticide can be applied into the sweetpotato root zone via a drip irrigation system (Arrington et al. 2016).

Adopting new classes of insecticides such as neonicotinoid and anthranilic diamides through drip irrigation to manage wireworm is growing in the USA because chlorpyrifos, a widely used soil insecticide, may face deregistration in the future (Arrington et al. 2016). Laboratory bioassays using topical applications of various insecticides on wireworm (*A. obscurus*) showed that fipronil caused significant mortality when compared to other chemicals such as OP (diazinon, chlorpyrifos), pyrethroid (tefluthrin), thiamethoxam, clothianidin, and acetamiprid (neonicotinoid), OC (lindane), and spinosyn (spinosad) (Van Herk et al. 2008). Subsequently, a chemigation trial conducted by Akers (2014) in Cudgen, NSW reported that multiple applications of fipronil in the root zone of an established sweetpotato crop resulted in an almost complete absence of wireworm damage, while 18% feeding damage was observed in the control treatment without insecticide. Drip irrigation allows for multiple applications of specific chemicals in an established crop. However, the short persistence of these insecticides in the soil is a major limiting factor especially for sweetpotato, which has a field life of up to 260 days in Australia (Akers 2014). However, the assessment of environmental impact and food safety issues must be equally addressed to ensure that chemigation is a safe process. Therefore, fipronil, the highly efficacious insecticide used in the chemigation trial, is registered only for soil application before planting, but not via drip irrigation over the established crop (APVMA 2021).

Sodium N-methyldithiocarbamate is the active constituent of metham sodium, which converts to methylisothiocynate (MITC) upon contact with moist soil (Di Primo et al. 2003). The MITC normally imposes toxicity against soil fungi, insects, and plants

depending on MITC persistence in soil while soil temperature, soil types and soil microorganisms determine the fate of MITC in soil (Sederholm et.al 2018). Total population of soil bacteria and fungi was found to have been substantially reduced following soil fumigation with metham sodium (Li et.al 2017). The abundance of heterotrophic bacteria is suppressed persistently, while actinomycetes and gram-positive bacteria recovered quickly along with plant growth (Macalady et.al 1998). A study by Meszka et.al (2011) indicated that fungal communities in soil are more susceptible than bacterial populations to metham sodium application. Such state of microbial suppression in soil following metham sodium fumigation can allow the nutrient fortified fungal inocula to establish and resporulate in soil as soil saprophytic microbes are a major hindrance to the nutrient fortified soil inocula to be established in soil (Lestan et.al 1996). Metham sodium is also applied in sweetpotato production, primarily in bedding root nurseries as a soil fumigant against a wide range of soil insect pests, plant pathogenic diseases and nematodes, and weeds (APVMA 2021). Thus, the strategic application of fungal granules application on the post-fumigated soil with metham sodium can enhance the resporulation efficacy of fungal granules and subsequently improve the soil insect control as well.

Controlling soil pests using metham sodium is not a sustainable practice as it poses various risks to soil health, ground water, and environment. Therefore, other alternatives for soil disinfectation are sought out such as soil solarisation and bio-fumigation using biocidal plants. A study by Camprubi et.al (2007) indicated that solarisation is more effective in soil disinfectation than metham sodium in terms of the disease severity reduction in strawberry. Moreover, beneficial microbes such as mycorrhizal fungi are adversely impacted by solarisation. Soil solarisation conducted for up to 59 days during summer can raise soil temperature to 53 ºC and 34 ºC on 5 and 30 cm deep from the soil surface respectively, resulting in significant control of soil borne plant pathogens in cucumber at 5 cm deep and even pathogens like *Pratylenchus penetrans* at 30 cm deep (Pinkerton et.al 20017). Since soil solarisation acts as a broad-spectrum disinfectant, solarised soil can support the fungal resporulation.

2.5.3 Biological control

To date, wireworm control in sweetpotato has relied heavily on chemical insecticides in Australia. Despite this, sweetpotatoes sustain continuous wireworm feeding injury, even with the application of various soil and foliar insecticides. The poor efficiency of insecticides applied against wireworm has been attributed to the insect's hardy exocuticle, subterraneous life stages, and cryptic nature (Chalfant et al. 1990). Further, no evidence of direct wireworm mortality was witnessed in potato fields when one of these newly developed soil insecticides, such as pyrethroids, neonicotinoids, and phenyl pyrazole (fipronil) was applied in the field (Parker & Howard 2001).

Instead, different behavioural responses of wireworms in terms of repellence, morbidity, reverse intoxication, and sub-lethal effects were pronounced. Moreover, soil insecticides incorporated before the sweetpotato planting usually fail to maintain their control efficacy throughout the cropping period, as wireworm damage in sweetpotato is severe as late as just before the harvest (McCrystal 2014). The future of insecticides for wireworm control is also uncertain due to the gradual withdrawal of insecticides globally, for example, the deregistration of phorate in Canada in 2015 due to its detrimental effect on humans and the environment (Vernon & Van Herk 2013; Vernon et al. 2015). Due to these reasons, wireworm control based on biological approaches has received renewed attention, leading to the development of several research teams to investigate alternative methods of wireworm control in various crops (Van Herk & Vernon 2011). Biological methods are often formulated based on pest ecological niche (Shah & Pell 2003). Therefore, an understanding of biological and ecological perspectives of an individual pest is the foundation of biological methods. The following section summarises parasitoids and predators, plant-derived chemicals, entomopathogenic nematodes, and entomopathogenic fungi as options for wireworm biological control. Entomopathogenic fungi, with specific reference to the use of *Metarhizium* spp. and *Beauveria bassiana*, are further reviewed in greater detail.

2.5.3.1 Parasitoids and predators

There are numerous biocontrol agents (BCA) which occur naturally in agroecosystems and perennial grasslands that predate and parasitise wireworms (Kergunteuil et al. 2016). For instance, many wireworm species fall prey to different natural predators such as the common brown earwig (*Labidura truncate*) and the adults of carabids and staphylinids in field conditions (Traugott et al. 2015). Similarly, a variety of parasitoids, particularly proctotrupids (*Paracodrus* spp.), bethylids (*Pristocera* spp.), and ichneumonids (*Anomalon* spp.), can establish parasitic relationships with certain wireworms such as *Agriotes*, *Aeolus*, *Agrypnus*, and *Conoderus* naturally (Traugott et al. 2015). However, their contribution to natural wireworm control is insignificant (Traugott et al. 2015). To date, there has remained a significant lack of research directions towards the commercial utilization of these BCAs for wireworm control.

2.5.3.2 Plant-derived chemicals

A variety of plant species are characterised as producing various anti-herbivore properties that act as antifeedants, deterrents, and are sometimes lethal to wireworms. The cultural practice of the green manuring of brassica crops, for example, *Brassica napus* and *B. juncea* has been found to control soil insects like wireworm (Morra & Kirkegaard 2002). In principle, the biocidal chemicals, for example, glucosinolate (GL) compounds contained by these brassica crops are converted into secondary metabolites such as isothiocyanates (ITC) when they are hydrolysed (Barsics et al. 2013). These secondary metabolites, especially ITC, are characterised as having biofumigation potential against soil pests (Morra & Kirkegaard 2002). In practice, the incorporation of foliage of the brassica crop (*B. juncea*) was ineffective for the control of wireworms (*A. sordidus* Illiger, *A. brevis* Candeze, and *A. ustulatus* schäller) in field conditions due to the low GL content and short persistence of the ICT metabolite, resulting in repellence of the wireworm rather than mortality (Furlan et al. 2010). In contrast, the application of defatted seed meal (DSM) of the brassica crop, *B. carrinata* ($\geq$ 1.1g DSM l^{-1} = about 160 µmoles GL l^{-1}), which constitutes relatively high GL

compounds with longer stability in soil, resulted in complete mortalities of wireworms (*Agriotes* spp) in both pot bioassays and natural field conditions; demonstrating a promising concept for wireworm biocontrol (Furlan et al. 2010).

The botanic-induced chemical like cinnamaldehyde, a plant-derived extract, has also been reported as an anti-feeding component against wireworm damage on mother potato tubers when they were drenched at a rate of 150 g cinnamaldehyde as an active ingredient per tonne of tuber (Barsics et al. 2013). Similarly, a repellence effect elicited by neem extract (*Azadirachta indica*) is the main tool of defence mechanism against wireworms in susceptible crops like potatoes (Barsics et al. 2013). Tefluthrin, a plant-based organic insecticide, is used to treat wheat seeds before planting resulting in significant improvement in crop establishment and incremental increase in crop yield (Van Herk & Vernon 2007). Tefluthrin insecticide imposes a repulsion and short-term control against wireworm, resulting in no observed damage in the immediate crop (Van Herk & Vernon 2007). These control measures can be used as a companion with the other control measures, but a full level of crop protection is unlikely to be obtained from botanical-based insecticides.

2.5.3.3 Entomopathogenic nematodes (EPN)

A study by Morton and Garcia-del-Pino (2017) examined the use of various EPNs (Entomopathogenic nematodes) to control wireworm (*A. obscurus*) by applying 100 infective juveniles (IJs)/cm to potato in the laboratory and glasshouse. They demonstrated that one EPN, *Steinernema carpocapsae* (Weiser) strain B14 caused significant wireworm mortalities, 75.6% and 48.3% in laboratory and potato field conditions, respectively. In another study, *S. carpocapsae* was found to be ineffective against *Conoderus vespertinus* (tobacco wireworm) in sweetpotato crops of the USA, when it was applied via drip irrigation at the rate of 5 billion IJs/ha (Arrington et al. 2016). In principle, drip irrigation systems would be an ideal system for nematode delivery to cause infection against insect pests because the available free moisture in the soil is considered optimal for nematodes to initiate infection (Arrington et al. 2016). While Arrington et al. (2016) did not supply an explanation regarding the poor

efficiency of *S. carpocapsae* for tobacco wireworm control, they hypothesised that the residual effect of soil incorporated insecticide, chlorpyrifos may have resulted in detrimental effect to *S. carpocapsae*, which were applied through the drip irrigation.

2.6 Entomopathogenic fungi: *Metarhizium anisopliae*

The entomopathogenic fungi *Metarhizium anisopliae and Beauveria bassiana* have been studied extensively and intensively against an extensive range of insects and arthropods (Zimmermann 2007; Zimmermann 2007a). Both entomopathogenic fungi have demonstrated insecticidal activity against a broad range of insect hosts and are thereby known as generalists. Studies have shown that the host range of *B. bassiana* is even wider than *M. anisopliae* (Zimmermann 2007a). The liquid formulation of *B. bassiana* (Naturalis-L®, *B. bassiana* strain ATCC 74040) has been registered for wireworm (mainly *Agriotes* species) in potatoes in Italy (Vernon & Van Herk 2013). Similarly, the conidia of *B. bassiana* harvested from solid substrate caused substantial control of aerial insects like grasshoppers, whiteflies, thrips, aphids, and other insects. This information aided in the registration of *B. bassiana*, traded as Mycotrol®, for those insects in North America (Shah & Pell 2003). *Beauveria bassiana* is characterised as having a multifunctional lifestyle because it shows a unique characteristic of being able to colonise plant parts asymptomatically (McKinnon et al. 2016), allowing for protection against insects, whereby the colonised fungal mass in plant parts reduced the herbivore attack, as well as being a plant growth promoter (McKinnon et al. 2016).

Metarhizium spp. have received widespread attention especially for soil insect management (Zimmermann 1986). *Metarhizium* spp. are highly adaptive and have been noted to persist in the soil environment for 12 months, even in the absence of a host insect (Zimmermann 2007). Previous studies have shown that *Metarhizium* spp. can survive in soil either in the form of mycosis development on an insect host or as a resting spore (Zimmermann 2007). Moreover, recent reports have demonstrated that *Metarhizium* spp. can survive in the plant rhizosphere and can colonise the plant endophytically (Parsa et al. 2013; Greenfield et al. 2016). First described by Metschnikoff in 1879, the genus *Metarhizium* is now one of the major sources of

mycoinsecticides globally, in which 33.9% of mycoinsecticide products are based on *Metarhizium* (De Faria & Wraight 2007). Originally found on the larvae of the wheat cockchafer (*Anisoplia austriaca*) and then on the sugarbeet weevil (*Cleonus punctiventris*) near Odessa (Ukraine), *M. anisopliae* was commonly referred to as a green muscardine fungus (Figure 2.4) (Roberts & Leger 2004). After its first detection, *M. anisopliae* had been intensively employed to control several damaging insects such as *Clenus punctiverntris*, *Oryctes rhinoceros* (rhinoceros beetle), *Bombax mori* (silkworm), *Ostrinia nubilasis* (European corn borer) (Zimmermann 2007). At present, 204 different insect species within seven insect orders have been recorded as being naturally infected by *M. anisopliae* while coleopteran insects, in particular soil-inhabiting insects such as scarab and elaterid insects, are more susceptible to soil entomopathogens such as *Metarhizium* and *Beauveria* (Zimmermann 2007). Because of its ease of mass production in the laboratory, and lethal efficacy to several insects, *M. anisopliae*-based mycoinsecticides are dominant in the global market. Previously, there were three *Metarhizium*-based mycoinsecticides available in Australia (BioCane®, Chafer Guard®, and Green Guard®), however, only Green Guard® is currently registered and available for purchase (APVMA 2021). *Metarhizium anisopliae*-based Biocane® and Chafer Guard® granules were formulated for the biocontrol of the greyback canegrub *Dermolepida albohirtum* Waterhouse (Coleoptera: scarabaeidae) in sugarcane, and the redheaded cockchafer *Adoryphorus couloni* Burmeister (Scarabaeidae) in turf and pasture crops, respectively (Milner 2000; De Faria & Wraight 2007). The mycoinsecticide Green Guard®, containing *M. acridum*, has been registered for controlling the Australian plague locust *Chortoicetes terminifera* (Walker) and the wingless grasshopper *Phaulacridium vittatum* (Sjöstedt) (De Faria & Wraight 2007; APVMA 2021).

Figure 2.4: A 21-day old fungal colony of *Metarhizium anisopliae* strain QS155 grown on Sabouraud dextrose agar with yeast extract (SDAY).

2.6.1 Distribution

The genus *Metarhizium* is ubiquitous and is distributed from the arctic to the tropics (Zimmermann 2007). *Metarhizium* spp. occur naturally in diverse habitats such as field crop soil, orchard soil, natural forest soil, coniferous soil, meadow soil, grassland soil, pastureland soil, and termite mounds (Milner et al. 1998; Zimmermann 2007). However, the frequency, abundance, and distribution pattern of *Metarhizium* spp. are variable and influenced by biotic and abiotic factors (Zimmermann 2007a). A field study of the distribution of *M. anisopliae* in different soil habitats in Switzerland demonstrated that the abundance of *M. anisopliae* was found to be the highest in meadow land, followed by crop land soil while its natural occurrence in forest soil was insignificant (Rodrigues et al. 2005). The high abundance of insects in natural habitats (meadows) is believed to have a positive correlation with *Metarhizium* abundance, but a lower insect host population in arable land was observed, possibly due to frequent insecticide application, could explain the low density of *Metarhizium* observed (Rodrigues et al. 2005). However, other studies have demonstrated that the natural occurrence of *M. anisopliae* has a strong correlation to cultivated habitats (Quesada-Moraga et al. 2007). While the natural occurrence of *B. bassiana* is unaffected by habitat types, its abundance is connected to undisturbed soils such as orchard soils or meadows (Bidochka et al. 1998; Sun et al. 2008).

Among the several species of the *Metarhizium anisopliae* complex, two species; *M. robertsii* and *M. brunneum*, which are referred to as generalist in terms of insect pathogenesis, are found as the most predominant and stable species in the soil. For example, both *M. robertsii* and *M. brunneum* occupy the highest density among the other *Metarhizium* spp. population in strawberry fields in the tropical region of Brazil and Denmark (temperate climate), respectively (Castro et al. 2016). Similarly, a study by Kepler et al. (2015) revealed that the abundance of fungal colonies (*M. anisopliae*) was recorded in conventionally tilled soil compared to those in a zero-tilled field. Research investigating *Metarhizium* population diversity has shown that *M. robertsii* tends to have a high affinity with warmer regions in both cultivated and non-cultivated areas (Rocha et al. 2013). The above claim has been substantiated with evidence of recovery of high fungal persistence in strawberry fields in tropical regions of Brazil (Castro et al. 2016). In contrast, *M. brunneum* is prevalent in temperate regions, exemplified by the greatest density of *M. brunneum* (86.3%) detected in the agricultural fields of Denmark, followed by *M. robertsii* (11.3%) (Steinwender et al. 2014).

In contrast, *M. acridum* and *M. album* are considered specialists due to their restricted pathogenicity against insects, while *M. guizhouense* and *M. majus* show an intermediate range of insect virulence (Sbaraini et al. 2016). The geographical distribution of *M. majus* shows an abundance in tropical and sub-tropical regions (Zimmermann 2007). Likewise, *M. acridum*, a highly UV tolerant pathogen, has a limited pathogenic range, largely specific to orthopteran insects such as desert locust (*Schistocerca gregaria*) and multiple grasshoppers. For that reason, *M. acridum* was commercialised as a mycopesticide and trades as 'Green Muscle,' in Africa for the control locusts and grasshoppers (Shah & Pell 2003).

The abundance, distribution, persistence, and pathogenesis against insects are impacted by several abiotic and biotic factors, in which abiotic factors, particularly ambient temperature, humidity, and solar radiation, are considered as determinant factors (Zimmermann 2007). *Metarhizium* spp. are known as mesophilic fungi because the temperature range between 15 $^{\circ}$C to 35 $^{\circ}$C, is considered optimal for their growth (Zimmermann 2007). However, there are few consistent results to validate the

interactions between the soil insect pathogen's origin and its heat-resistance capacity (Alston et al. 2005).

The spore germination, vegetative growth, and insect pathogenesis of *Metarhizium* spp. are moisture-dependent processes (Lazzarini et al. 2006) so, maintaining a high humidity environment is of paramount importance to ensure the success of biological control. The highest germination is attainable when there is 100% RH (Milner 1997), but in some species, for example, *M. acridum* is quite effective at germinating at lower humidity of 92.5% RH (Zimmermann 2007). The conidial germination of *M. anisopliae* (isolate FI25 and FI610), grown in liquid culture at various levels of humidity, exhibited 58% germination in 100% RH followed by 19% in 99% RH and 0% germination at lower RH (below 99% RH) for 12 hours (Milner 1997).

UV radiation, particularly UV-A and UV-B, is a significant constraint to the success of a biopesticide, which is targeted at soil and plant-surface dwelling pest insects (Zimmermann 2007a). Reduced persistence of conidia is one of the direct consequences of UV radiation in insect biological control (Jaronski 2010). In one study, the conidial germination of *M. anisopliae* and *B. bassiana* was reduced from 94% to 52% and from 96% to 54% respectively, following five minutes of exposure to artificial UV radiation (Falvo et al. 2016). Formulation, to maintain the integrity of conidia is discussed later in the literature review.

2.6.2 Mode of infection by Metarhizium

Insect pathogenic fungi are characterised by a unique mode of insect pathogenesis that infects the insect through the cuticle (Figure 2.5) (Zimmermann 2007). When conidia of *Metarhizium* are deposited on an insect body, they attach to the cuticle because of hydrophobic interaction between a conidia surface protein and the outer lipid layer on the insect cuticle (Fang et al. 2007). Under favourable environmental conditions (temperature range 20 °C to 35 °C and >95 % RH), conidia germination occurs within 20 hours of attachment, followed by germ tube development (Zimmermann 2007). A progressive adhesion of conidia onto the insect cuticle has

been implicated to the *Metarhizium* adhesion protein 1 (Mad 1) gene in *Metarhizium* spp. (Broetto et al. 2010). The germ tube developed from the conidia differentiates into a special cuticle penetrating peg or 'appressoria,' which mechanically invades the insect cuticle, aided by proteases, chitinases, and lipases secreted by the fungus to hydrolyse proteins, chitins, and lipids respectively, which are major components of insect cuticle (Charnley 1989). Although the fungal infection via insect cuticle is a broadly accepted idea, the advent of modern new generation sequencing makes feasible to explore other possible routes of fungal infection into host insects, for example oral ingestion. The orally ingested *M. anisopliae* grows in the insect gut and further colonises in the haemocoel that eventually leads to the insect mortality (adult *Sitophilus granarius*) (Batta 2018). But the existing microbiome in the insect gut is a hinderance for the fungal germination and further growth (Batt et.al 2013). A chemical, for example boric acid, coapplied with *M. anisopliae* hastens the fungal penetration into the insect gut and supports further growth by altering the gut microbiome (Yang et.al 2021). With better understanding of insect routes that the fungus exploits to enter the insect can support the scientists to design the appropriate fungal formulation to achieve the best possible pest control outcomes.

The fungus eventually penetrates the insect body and rapidly disperses throughout the haemocoel, producing yeast-like structures called 'blastospores' (Charnley 1989). The intense nutrient acquisition occurs from the insect body due to the dense host colonisation by the fungus that eventually leads to insect mortality (Shah & Pell 2003). During the fungal colonisation inside a host body, some *Metarhizium* spp. also induce several secondary metabolites (toxins), particularly destruxins that also facilitate insect mortality (Kershaw et al. 1999; Rios-Moreno et al. 2016). Following insect death, the saprophytic fungus growth occurs out of the cadaver and produces masses of conidia in a humid environment (Zimmermann 2007).

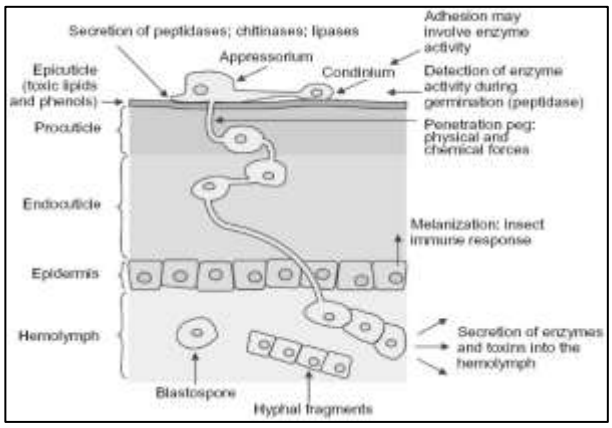


Figure 2.5: A typical insect fungal pathogenesis in insect body Source: (Charley 1989)

2.6.3 Fungal propagules, formulation, and mode of application

Aerial conidia, microconidia, blastospores, and microsclerotia are the vegetative spores produced by *Metarhizium* that can be utilised for application to both foliar and soil insects (Jaronski & Jackson 2012). Among them, the use of aerial conidia dominates biopesticide formulations for both foliar and soil insects (Jaronski & Jackson 2012). The mass production of aerial conidia is economic when compared to the other propagules because they are produced via a solid substrate fermentation, while microsclerotia, blastospores, and microconidia require a liquid fermentation to be produced (Jackson et al. 2010). Microsclerotia, a compact mass of fungal mycelia grown in liquid culture, in which carbon ratio is higher than nitrogen, demonstrates high persistence and desiccant tolerance in soil (Jackson et al. 2010). Moreover, microsclerotia produce conidia through sporogenic germination after rehydration, which can cause significant mycosis against soil-dwelling insects (Jackson et al. 2010). Unformulated aerial conidia are rarely applied to the soil to control soil insects (Batta 2003). Instead, fungal propagules are formulated with varied materials to enhance and stabilise conidia during the production process, storage, application, efficacy, and persistence (Jaronski & Jackson 2012). Conidia of hypocrealean fungi such as *Metarhizium*, *Beauveria*, and *Isaria* show hydrophobic characteristics and are therefore immiscible in water (Zimmermann 2007a). The addition of surfactants can improve their miscibility in water and adherence on the plant leaf surface (Jackson et al. 2010) and the addition of oil dispersants to formulations can enhance the efficacy

of *M. anisopliae* for aerial insects like whitefly and red spider mites, in comparison to formulations without oil (Batta 2003). UV radiation from sunlight is a major limiting factor for the application of *Metarhizium* in liquid formulations (for foliar insects) (Jaronski 2010), therefore, correct, and effective formulations are crucial for efficacy. The use of vegetable oil as an adjuvant in biopesticide formulations protects from UV radiation (Moore et al. 1993). The use of canola oil in combination with the commercial sunscreen (Everysun™) induced the conidial survivorship of *M. anisopliae* to 40% from UV radiation when the conidia were exposed to the artificial UV radiation for 5 hours, which was significantly higher than that of canola oil and aqueous suspension individually (Hedimbi et al. 2008). Consequently, the conidia suspended in canola oil and guarded by Everysun™ confers 91-94% mortality to the larvae of the red-legged tick *Rhipicephalus evertsi* in laboratory bioassays, indicating that the formulation is compatible to conidia for further growth and development (Hedimbi et al. 2008).

Biological control strategies for soil insects are different from aerial insects because the fungal propagules applied in soil are unlikely to be always in direct contact with soil insects (Jaronski 2007). Therefore, the persistence of conidia in the soil is of paramount importance until insects physically contact the fungal inocula in the soil. When *M. anisopliae* was applied inundatively for wireworm biological control in Canada, it took 40 weeks before the insect intercepted the fungal propagule (Kabaluk et al. 2007). As a result, the driving principle behind the formulation of soil-applied biopesticides is to extend conidial virulence until insects come into proximity and to maximise the time of contact timing between conidia and insects so that insects receive enough conidia to be fatal (Jaronski 2010). Previously, either drenching with an aqueous suspension or broadcasting conidial dust were the major types of biopesticide applications (De Faria & Wraight 2007). A review by Jackson et al. (2010) described that fungal spore density with at least 10^5-10^6 CFU cm^{-3} or g^{-1} soil is required to achieve a satisfactory level of wireworm control or soil insects like the greyback canegrub (Milner et al. 2002).

2.6.4 The use of Metarhizium spp. to control wireworms

The use of *Metarhizium* species to control wireworms in natural field conditions is difficult because wireworms show vertical dynamics in the soil (up to 60 cm deep from the soil surface), and feed actively mainly in spring and autumn; only 20% of its life span is spent actively feeding (Furlan 1998). Conventionally, *Metarhizium* has been applied either as conidial broadcast or a soil drench with an aqueous solution or solid matrix for the control of subterranean insects (Jaronski 2010); however, these applications are inefficient and ineffective against wireworm due to the wireworm' cryptic behaviour (Finney 1946; Parker & Howard 2001), and the lack of fungal persistence and abundance once they were applied in soil (Inglis et al. 2001).

Several factors may contribute to the poor biological control efficacy of the genus *Metarhizium* for soil insects like wireworms. For example, the transient nature of wireworms in the soil profile limits the fungal biological control performance by not allowing direct physical contact between them (Brandl et al. 2016), while direct adherence of the fungal conidia onto the insect cuticle is crucial for insect disease initiation (Zimmermann 2007); at least 48 h of physical contact between wireworm and *Metarhizium* inocula is required for wireworms to acquire the fungal infection (Ericsson & Kabaluk 2007). Currently, there is no research that reports the exact contact period required for *M. anisopliae* to successfully infect mealworms. However, we can make a comparison between mealworms and wireworms in terms of their *in vitro* mortalities in response to *Metarhizium.* Wireworms (*Agriotes lineatus*) exposed to conidial suspensions (1×10^8 conidia/ml) of *M. anisopliae* succumbed with 100% mortality within three weeks (Razinger et.al 2013), while the LD_{50} value for mealworms with *M. brunneum* was shown to be 4.4×10^7 conidia/ml (Bhardwaj et.al 2011). From this, we can infer that mealworm can be used as a model insect for wireworms. Furthermore, some host insects, for example adult *Ixodes scapularis*, are more susceptible to *M. brunneum* than larval mealworms. This indicates that larval mealworms are suitable as susceptible hosts to entomopathogenic fungi (Krams et.al 2013). The lack of persistence and infectivity of the fungal pathogen can be a major hurdle for successful soil insect biological control (Jaronski et al. 2007). The ability of the conidia to persist in the soil is influenced by soil texture, soil moisture, soil

temperature, farming practices, food substrates, and density of other soil antagonists (Jaronski et al. 2007; Zimmermann 2007). A study carried out by Goettel et. al (2007) indicated that dry soil conditions are detrimental for *M. anisopliae* to cause wireworm infection (*A. obscurus*) and further light soil texture like sandy loam is also found to be unfavourable for the fungal persistence (Jaronski et al. 2007). Therefore, continuous improvement on fungal formulation is of paramount importance to enhance the fungal persistence and abundance in soil which are the basic criteria for successful wireworm control.

Thus, the objective of the research presented in this thesis was to optimise the fungal formulation by combining the fungus and nutrients and further evaluate the resporulation and infectivity efficacy of those fungal inocula in soil.

CHAPTER 3: ROLES OF NUTRITIVE ADDITIVES, SUBSTRATES, AND SOIL TYPES ON THE RESPORULATION OF CALCIUM ALGINATE ENCAPSULATED *METARHIZIUM ANISOPLIAE*

Abstract

Ecological adaptation is one of the desirable attributes of EPF candidates. Fungal saprophytic growth in the soil can ascertain fungal ecological fitness. But the fungus requires exogenous nutritive additives for its saprophytic growth. However, there is still a dearth of knowledge regarding the types and concentrations of nutritive additives that are required to drive the fungal resporulation at the optimum. Thus, the resporulation response of *Metarhizium anisopliae* was assessed in conjunction with nutritive additives such as compressed baker's yeast (*Saccharomyces cerevisiae*) or corn starch (*Zea mays*) or the combination thereof by encapsulating the fungal propagules and nutritive additives into calcium-alginate granule under laboratory condition. The results showed that the fungus supported with the combination of baker's yeast (20% w/v) and corn starch (20% w/v) has been shown the optimal resporulation compared to the fungus with the individual nutritive additives. As a result, the fungus with the mixed nutritive additives, referred to as fungal granules henceforth, were further examined for their resporulation over various substrates, soil types, and soil levels under laboratory conditions. The results showed that four different soil types consisting of variable physical and chemical properties did not impact the fungal resporulation, but different soil levels such as sterilised, pasteurised, and non-sterile soils significantly influenced the fungal resporulation as the highest resporulation was found on sterilised soil, whereas the lowest resporulation was obtained on non-sterile soil. The poor resporulation from fungal granules on non-sterile soil has been attributed to the competition fungistasis exerted by soil microbes. This study provides insights into the integration of fungal granule application with the existing farming disinfestation practices to enhance the resporulation potentiality of fungal granules.

3.1 Introduction

The genus, *Metarhizium* is one of the most widely studied of the entomopathogenic fungi (EPF), particularly concerning the evaluation of its biocontrol efficacy for an extensive range of insect pests and acarids (Inglis et al. 2001; Aw & Hue 2017). Some species of *Metarhizium* are highly adapted to the soil environment and can colonise the plant's rhizosphere and roots (Liu et al. 2016). By the virtue of these attributes, EPF particularly *Metarhizium* species have been studied for the control of a vast array of root herbivores, such as cabbage root fly (*Delia floralis*) (Razinger et al. 2014), pupal fruit fly (*Ceratitis* spp.) (Ekesi et al. 2005), white grubs (*Polyphylla fullo*) (Samson et al. 2006), sweetpotato weevils (*Cylas formicarius*) (Dotaona et al. 2015), corn rootworms (*Diabrotica undecimpunctata*), wireworms (*Agriotes obscurus*) (Ericsson et al. 2007) and grape phylloxera (*Daktulosphaira vitifoliae*) (Kirchmair et al. 2007). Although *Metarhizium* species have shown to have high virulence against several soil insects during *in vitro* studies (Ericsson & Kabaluk 2007), the formulation of the fungal inoculum has proven to be problematic and be unreliable in crops for soil insect control (Brandl et al. 2016). Non-conducive soil environments and antagonistic interaction with naturally occurring soil microbes may impede the establishment of the fungal inoculum preventing the fungal proliferation and persistence in the agroecosystem, leading to poor control (Jaronski 2010). Moreover, unlike foliar insects, direct targeting of soil insects *in situ* is not feasible due to their cryptic nature (Vernon et al. 2015). It is, therefore, crucial to design an appropriate formulation that can complement the fungal inoculum to maintain its virulence and abundance in soils until soil insects encounter the inoculum (Gasic & Tanovic 2013). Fungal re-sporulation in the soil can also enhance the persistence and abundance of the EPF in the soil leading to improved soil insect control (Jackson et al. 2010).

A range of fungal propagules, such as aerial conidia, blastospores, microconidia, microsclerotia, and mycelia, can be utilised for insect biocontrol (Jaronski & Jackson 2012). Aerial conidia of *Metarhizium* have been predominantly utilised as a propagule for insect biocontrol (Jaronski & Jackson 2012). Practically, the direct application of pure conidia into fields as a means of insect control is not likely to be feasible because

the germination of conidia degrades rapidly (Jaronski 2007). Conidia can be presented in various formulations: wettable powders, water-dispersible granules, granules, technical concentrates, and oil emulsions to protect the fungus from the adverse environment during the storage and post-application in the field, for ease of handling at the application and to reduce the health risk to workers during handling (De Faria & Wraight 2007; Zimmermann 2007; Gasic & Tanovic 2013). The first two formulations, which are normally an aqueous suspension following water dilution, are not considered as an effective formulation for soil application as uniform dispersal in the soil is difficult (Chandler & Davidson 2005). Although 'wettable powder' is a common formulation of *M. anisopliae* or *Beauveria bassiana* used by several biopesticide companies (De Faria & Wraight 2007), myceliated granules, referred to as 'technical concentrate', has been used in several research studies for subterraneous insects (De Faria & Wraight 2007). The formulation is mostly prepared by embedding conidia and/or hyphae of *M. anisopliae* onto a solid substrate, for example, corn, millet, barley, or rice grains (Mayerhofer et al. 2017). For the control of grape phylloxera, the myceliated granule of *M. anisopliae* (GranMet®) was found to be effective in the vineyard, allowing the fungus to extensively proliferate for one year (Kirchmair et al. 2007). However, in another study, it was found that the GranMet® could not reduce the sugarbeet damage caused by sugarbeet root maggot (*Tetanops mycopaeformis*) (Jaronski et al. 2007). The infestation of sugarbeet root maggot in sugarbeet fields was, however, significantly reduced to below the threshold level when the sugarbeet cultivation was followed with rye as a cover crop coupled with the application of myceliated granule of *M.* anisopliae (Majumdar et.al 2005). This study alluded to the fact that the fungus requires sufficient time to establish and colonise in the field. Alternatively, to expedite fungal sporulation, the concept of additional nutrients added to the granules to support fungal growth has been proposed by Gerding-Gonzalez et al. (2007), who formulated the conidia of *B. bassiana* with chitin and wheat bran as the additional nutrient sources, encapsulating the ingredients in a calcium alginate granule. The additional nutrients in the granule improved the fungal sporulation to three times the initial conidia number after 21 days of incubation. When these fungal inocula are applied in fields, they resporulated in the soil before host crops were infested by soil insects (Vemmer & Patel 2013; Przyklenk et al. 2017). Several

nutritive additives such as molasses, lactic acid, polyethylene glycol, rice, wheat and sorghum grain, as well as skim milk have been shown to elicit positive responses to the growth and sporulation of entomopathogenic fungi (Dasgupta et.al 2016; Balakrishnan et.al 2011). For calcium alginate encapsulation, additives only with suitable mass density, shape and size are fit for purpose. For example, skim milk alone does not fit for the granular formulation because of its low density.

Originally applied in the pharmaceutical (Vidhyalakshmi et al. 2009) and food industries (Onwulata 2012), calcium alginate encapsulation, which is characterised as being bio-degradable and non-toxic to any living organism, has also been adopted in agriculture. Examples include calcium alginate encapsulation of *Rhizobium* spp. (Bashan 1998), *Trichoderma* spp. targeting plant pathogenic fungi (Maruyama et al. 2020) and *B. bassiana* targeting soil insects (Vemmer et al. 2016). The underlying approach to calcium alginate encapsulation consists of dissolving sodium alginate powder in water, incorporating microorganisms into the alginate suspension, co-encapsulating with/without the other microbial enhancers, and crosslinking the sodium alginate suspension with calcium chloride solution resulted in the calcium alginate granule (Vemmer et al. 2016). The encapsulated microorganisms have been shown to have an extended shelf-life during storage and are more suited to manual handling (Vemmer & Patel 2013). It is also believed that encapsulated organisms are better protected in soil against any biotic and abiotic stressors. Owing to its hydrophilic nature, the calcium alginate encapsulated product can readily absorb moisture that favours the fungal germination, as high-water activity is a crucial condition for conidial germination, particularly for *M. anisopliae* and *B. bassiana* (Dillon & Charnley 1990). In addition to water, an exogenous source of the nutrient helps to trigger conidial germination as conidia contain low reserves of endogenous nutrients (Dillon & Charnley 1990). In such a case, calcium alginate encapsulation provides an opportunity to co-encapsulate external food sources, such as baker's yeast (*Saccharomyces cerevisiae*) and corn starch (*Zea mays*), which aids conidial germination and subsequent fungal growth (Przyklenk et al. 2017). Moreover, the delivery of the fungal inocula is also a prime concern, particularly when targeting soil insects like wireworms that can avoid the fungal inocula due to their movement in the

soil profile (Parker & Howard 2001). For such cryptic species, the calcium alginate granule can also enhance the efficacy of fungal infection to the insect by associating the fungal inocula with insect-specific luring agents, such as a source of carbon dioxide (CO_2) as a chemical cue for wireworms (Doane et al. 1975) or sex pheromones for adult beetles (*Agriotes* spp.) (Kabaluk 2014). Live baker's yeast (*Saccharomyces cerevisiae*) has been included as a source of carbon dioxide production following the encapsulation to attract soil insects that can be co-applied and/or co-encapsulated with conidia (Schumann et al. 2013; Brandl et al. 2016). The above strategy for insect biocontrol has been generally referred to as the "Attract & Kill" strategy (Kabaluk et al. 2015).

Previous research has shown that calcium alginate granules containing food sources trigger the optimal conidiation of co-encapsulated *M. anisopliae* or *B. bassiana* when the granules are exposed to water agar and incubated at 25 $^{\circ}$C (Przyklenk et al. 2017). However, it is crucial to ascertain which food sources drive the optimal fungal resporulation under aseptic conditions and subsequently examine for them whether the fungal formulation can proliferate and persist in soil or not, as the number of infective fungal propagules in soil determines the level of soil insect control (Rath & Worledge 1995). EPF such as *B. bassiana* and *M. anisopliae* function as an amylolytic fermenter (Vemmer et al. 2016). Therefore, sources of complex carbohydrates, for example, grain or potato starch, are commonly incorporated as a food source for *M. anisopliae.* Since the target is a soil insect, the ultimate goal is to obtain the optimal fungal resporulation in soil from the fungal inocula applied in the soil. Previous studies indicated that the nutrient-fortified fungal granules are predisposed to competition and proliferation by native soil microbes when the granules are applied in soil (Lestan & Lamar 1996). This is compounded by the fact that *M. anisopliae* is a poor saprotroph (St. Leger & Wang 2020). To reduce competition for fungal resporulation, microbially depleted soil could be plausible; this would emulate soil disinfestation, which is prevalent in the sweetpotato fields in Australia, a method used to control soil-borne pests. Methods for soil disinfestation consists of soil fumigation or soil solarisation or biocidal plant extracts (Skipper & Westermann 1973; De Vera et al. 2018). Following the soil disinfestation, overall soil microbial

suppression has been reported for a transient period (Kapagianni et al. 2010). We hypothesised that soil microbial antagonism against the fungal granules can be overcome somewhat by applying the granules into pre-disinfested soil, resulting in optimum resporulation of the fungus on the granule surface.

Besides soil biological aspects, the fate of *M. anisopliae* in soil has been also linked with soil physical properties, mainly soil moisture, soil texture, pH, cation exchange capacity, and organic matter (Wraight et al. 2001). A study conducted by Vanninen et al. (2000) noted that both *M. anisopliae* and *B. bassiana* applied in the temperate region persisted longer in clay than peat soil. A study by Jabbour & Barbercheck (2009) also indicated that soil with high organic matter adversely impacts the persistence of *M. anisopliae*. However, this organic rich soil, for example peat soil, allows the conidial penetration to the deep soil profile for the topically applied conidial suspension in comparison to clay and sandy soil. In terms of soil moisture requirement for the fungus, moderate soil moisture is optimal for the survival and infection of *M. anisopliae* in soil, whereas low moisture content (less than 10% of field capacity) is not favourable for *M. anisopliae* infection, for example giving a low mortality against sugarbeet root maggots (*Tetanops mycopaeformis*) (Jaronski et.al. 2007). However, there is still a lack of knowledge regarding how the soil inoculated fungal inocula respond to the soil environment. Besides soil as a physical property, the soil harbours myriad of soil microbiota that interact in multiple levels. Thus, the efficacy of *M. anisopliae* against soil insects depends on how the fungus adapts in the soil (Garrido-Jurado et al.2011, Rath et al. 1992). Therefore, further study is still warranted to elucidate how the fungus interacts with different soil as sweetpotato production stretches over a wide range of geography in Australia.

Having an improved knowledge of the performance of formulated fungal granules and their sporulation in the soil is important before its application in the field for soil insect control. The objectives of this study were: (i) to measure the *in vitro* resporulation of calcium alginate encapsulated *M. anisopliae* in response to the co-encapsulated nutrient additives; (ii) to assess the resporulation of fungal granules in various substrates; and (iii) to evaluate the effect of soil sample on the resporulation of the fungal granules.

3.2 Materials and methods

3.2.1 Metarhizium anisopliae

Metarhizium anisopliae strain QS155 was originally isolated from sweetpotato fields at Mapuru, Northern Territory. It has been maintained in the New South Wales Department of Primary Industries Herbarium with the accession number DAR 82480 (Dotaona et al. 2015). Cultures of *M. anisopliae* were maintained on sabouraud dextrose agar amended with 1% yeast extract (SDAY) (Merck KGaA, Germany) (Figure 3.1). For the conidial production, *M. anisopliae* was grown on SDAY at 27 °C with a 12:12 h light and dark photoperiod for 21 days. The conidia were subsequently harvested using a sterile scalpel by gentle scrapping the colony and dried in a laminar flow cabinet (Esco class II BSC) for 2 h. The air-dried conidia were then stored in a sterile plastic 50 mL Falcon container and sealed with a lid at 5 °C for 7 days until the conidia were prepared for formulation.

Before all experiments, the conidia viability was assessed by inoculating 20 µL of a conidial suspension (10^6 conidia per ml) over a thin layer of SDAY medium (1.5 × 1.5 × 0.5 cm) on a glass slide, which was covered with a coverslip and placed inside a Petri dish (Ø, 9 cm) containing Whatman® filter paper moistened with sterile distilled water. The Petri dish was sealed with Parafilm® and incubated at 27 °C and 12:12 h dark and light photoperiod. Following 14 h of incubation, 200 conidia were assessed at ×400 using a compound microscope (Olympus, Model Bx53). Only samples with >98% germination were used for further experimentation.

3.2.2 Preparation of M. anisopliae granules

To prepare the calcium-alginate formulation, 2% (w/v) sodium alginate (Chem-Supply Pty Ltd. Australia) was dissolved in 0.05% sterile Tween®80 (VWR Chemicals) in water; and the resultant suspension was heated with continuous agitation for 30 min before the suspension was autoclaved at 121 °C for 6 min only, as sodium alginate is chemically denatured from overheating (Vemmer & Patel 2013). The

conidia of *M. anisopliae* QS155 were mixed into the sodium alginate, in combination with nutritive additives, either 20% w/w corn starch (Sigma-Aldrich) or 20% w/w compressed baker's yeast (Lesaffre Australia Pacific Pty Ltd) or a combination thereof. The above nutritive additives, which were autoclaved at 121 °C for 15 min, were suspended in sterile sodium alginate suspension and homogenised thoroughly using a stirrer for 10 min. Fresh conidia of *M. anisopliae* QS155 (1% w/w) were then added into the suspension and stirred using a stirring rod for 5 min. The homogenised suspension was immediately dropped into sterile 2% (w/w) calcium chloride solution ICN Biomedicals Inc. USA) using a syringe (Norm-Ject®, drain tube Ø = 4 mm, length = 10 mm). The droplets of suspension remained immersed in the calcium chloride solution for 30 min with continuous agitation for complete gelatinisation (Vemmer et al. 2016). Granules were separated from the calcium chloride solution by collecting them on a Buchner funnel. Granules were rinsed twice with sterile water before being dried for 2 h inside a laminar flow cabinet (Labec Laboratory Equipment) at room temperature (22-24 °C). For the experiments, 'the resporulation test of fungal granules in different substrates' and 'the resporulation test of fungal granules in multiple soil samples,' the fungal granules were further dried for an additional 12 h (Figure 3.1). Following the overall drying, the fungal granules resulted in 55% of moisture loss from their initial fresh weight and then granules were sealed in a 100 mL sample tube and stored at 5 °C until the experiment commenced.

3.2.3 Fungal resporulation in response to nutrient additives

A single fungal granule either of corn starch (CAG_{Ma+Cs} Ø 3.5 mm; weight 25 mg per granule; ~ 9×10^6 conidia per granule) or baker's yeast (CAG_{Ma+By}, Ø 3.5 mm; weight 29 mg per granule; ~ 9×10^6 conidia per granule) or the combination thereof ($CAG_{Ma+Cs+By}$, Ø 4 mm; weight 36.5 mg per granule; ~ 9×10^6 conidia per granule) was placed onto the centre of a Petri dish (Ø = 3 cm, depth = 1 cm) containing 3% water agar (Figure 3.1). The inoculated Petri dishes were sealed with Parafilm® and incubated (Incubator: Labec Laboratory Equipment) at 27 °C with a 12:12 h photoperiod. Conidia of *M. anisopliae* QS155 encapsulated into calcium alginate

granules without any additional nutrients, symbolised as CAG_{Ma} (Ø 3.5 mm; 20.5 mg weight per granule; ~ 9 × 10^6 conidia per granule) were included as the control (CAG_{Ma}). Ten replications were made per treatment with the individual Petri dish (an experimental unit) containing a single granule from each treatment. The experiment was arranged as a randomised complete block design (RCBD), created using the Edgar II template (http://www.edgarweb.org.uk).

At 14 days post-inoculation, the individual granules and resporulated conidia for each treatment and replicate were dislodged using a sterile scalpel and suspended in three mL of 0.05% sterile Tween® 80 in water. The subsequent conidial suspension was transferred to a McCartney tube (50 mL) and was homogenised using a vortex (Vortex-Genie® 2, Mo Bio Laboratories, INC) at maximum speed for 5 min. The number of conidia in the suspension was quantified using a hemocytometer (Neubauer improved double net ruling) at ×400 magnification using a compound microscope (Olympus, Model BX53).

Figure 3.1: Conidiated *M. anisopliae* QS155 ready for the conidial harvest (A); a mixture of conidia, corn starch, and dead baker's yeast into sodium alginate suspension before the granulation (B); immersing the granules into the calcium chloride bath (C); rinsing off the granules with distilled water (D); air-drying of granules in a laminar flow (E); and air-dried granules ready for the inoculation

3.2.4 Resporulation test of fungal granules in different substrates

Granules ($CAG_{Ma+Cs+By}$) demonstrated the maximum resporulation under aseptic conditions (section 3.2.3), subsequently these granules $CAG_{Ma+Cs+By}$ herein referred to as fungal granules, were proposed for further resporulation tests. Six treatments were established in individual Petri dishes (Ø=9 cm). The soil used in this experiment was collected from the USQ agricultural field (Latitude: -27° 36' 15.12" S, Longitude: 151° 55' 55.20" E). After collection, the samples were air-dried, homogenised in a laboratory, and immediately transferred into a cool room (10 °C). Two-thirds of the soil samples were used for pasteurisation and sterilisation, while one-third was used for non-sterile treatment. This study consisted of the following treatments:

1. Non-sterile soil (50 g)
2. Pasteurised soil (50 g of soil pasteurised at 80 °C for 24 h in an oven)
3. Sterilised soil (50 g soil-autoclaved twice at 121 °C for 60 min at 2-day intervals)
4. Perlite (1.5 g weight)
5. 3% Water agar, and
6. Filter paper (sterilised 90 mm Whatman®)

Each dish was inoculated with four fungal granules, giving six treatments altogether, namely. After the inoculation, 10 mL of sterile water was applied to all soil and perlite treatments and one mL of sterile water to the filter paper; no additional water was added onto the water agar. The Petri dishes were sealed with Parafilm® and incubated at 25 °C in the dark in a growth chamber (CONVIRON® CMP6010). Each treatment was replicated three times and all Petri dishes were arranged into the growth chamber in an RCBD during the incubation period (Figure 3.2). After 21 days, three resporulated fungal granules were removed from each Petri dish using sterile forceps. After the removal, the granular diameter was measured. Then, granules were individually inserted into a 2 mL tube containing one mL solution of citric acid (0.03 M) and sodium carbonate (0.05 M), where sterile 0.05% Tween®80 was used instead

of water, to enhance the conidial miscibility into the solution. Each tube was vortexed for 30 min to separate the conidia from the granule. Following the vortexing, granular debris remaining in the suspension was allowed to settle for one min, and then a 100 µL of suspension was pipetted out and regarded as the stock suspension. To quantify the conidial concentration, a 10 µL aliquot from the stock suspension was used for enumeration of the conidia using an improved haemocytometer (Neubauer improved double net ruling, ProSciTech Pty Ltd). For dense conidial suspensions, the suspension was further diluted (1: 10 dilution factor) before the counts. For the germination test, a 10 µL aliquot of the conidial suspension was spread over the SDAY medium poured out on a glass slide. The inoculated media on the glass slides were covered with a coverslip, inserted into a Petri dish (9 cm diameter) lined with a moist filter paper, sealed with Parafilm®, and incubated at 27 ˚C and a 12:12 h dark and light photoperiod. At 14 h post-incubation, conidial germination was determined using a × 400 compound microscope (Olympus MVX10). Samples with non-germinating conidia were incubated further and examined for germination every 2 h for 24 h. Two hundred conidia per slide were counted for the germination test. Conidia with a germ-tube twice as long as the width of conidia were considered germinated (Rangel et al. 2010). The length of germ tubes was also measured.

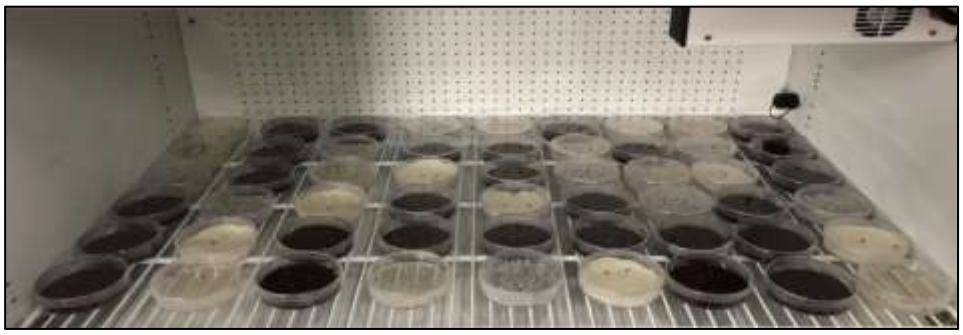


Figure 3.2: Petri dishes arranged inside a growth chamber during the incubation, on which individual Petri dish containing either non-sterile soil or pasteurised soil or sterilised soil or perlite or filter paper or water agar as a substrate and inoculating with fungal granules.

3.2.5 Effect of soil sample on resporulation of fungal granules

In this experiment, the resporulation of fungal granules was tested on four different soil samples collected from agricultural fields in Australia (Table 3.1). Each soil sample was further treated as non-sterile, pasteurised, and sterilised. Three soil samples were collected from sweetpotato fields in Bundaberg (GPS coordinate: 24°86′70″S, 152°21′4″E) Queensland, identified as ‘soil 1’; ‘soil 2’; and ‘soil 3’ (Figure 3.3). The fourth soil sample, identified as ‘soil 4’ was collected from an agricultural field of the University of Southern Queensland (USQ), Toowoomba (GPS coordinate: 27°36′33″S, 151°55′55″E) Queensland, Australia. After the soil collection, samples were immediately transported to the laboratory, where the samples were air-dried, homogenised, and graded, by passing them through a 10 mm sieve before storage at 10 °C. Analysis of soil properties from these soil samples were also conducted at the soil laboratory of the University of Southern Queensland. Equal size soil samples were pasteurised (oven-dried at 105 °C for 24 h), sterilised (autoclaved twice at 121 °C for 1 hr) or left untreated (non-sterile).

Figure 3.3: A sweetpotato field (Bundaberg, Queensland), one of the sites was selected for the soil sample collection.

For each soil type, three freshly prepared fungal granules ($CAG_{Ma+Cs+By}$) were inoculated onto a Petri dish (Ø=9 cm, 1.5 cm deep) containing 50 g of either non-sterile or sterilised or pasteurised soil (Figure 3.4). The soils were moistened with 10 mL of distilled water per Petri dish. The Petri dishes were then sealed with Parafilm® and incubated at 25 °C in the dark in a growth chamber and arranged in an RCBD. This experiment consisted of twelve treatments with three replications per treatment per Petri dish.

At 28 days post-incubation, the fungal granules from the soil surface in the Petri dishes were individually excised using a sterilised scalpel for assessment of conidia. Each individually excised granule was transferred to a Falcon tube (50 mL) containing 10 mL of sterile 0.05% Tween®80 solution and homogenised for 1 min using a vortex (Select Vortexer). Six serial dilutions (×10 dilution factor) were made from the stock suspension, and each dilution was replicated thrice. An aliquot of 100 µL of soil suspension from each dilution was spread over SDAY amended with 0.01% chloramphenicol in a Petri dish, sealed with Parafilm® and incubated (25 °C and a 12:12 h light and dark photoperiod) in a growth chamber (Conviron MP6010) for 48-72 h (Castro et al. 2016). Fungal colonies established on the medium were visualised using a stereomicroscope (Olympus S251) and the colonies counted. Hyphal and spore morphologies were considered for the fungal confirmation, as described by Humber (2012).

Table 3.1: Soil properties of each soil used in this experiment.

Soil ID	Clay %	Silt %	Sand %	pH	EC mS/m	C %	N %	Crop history
Soil 1	75*	15	10	5.7*	33	1.53	0.20	Sweetpotato
Soil 2	10	75	15	6.2	77	0.51	0.05	Sweetpotato
Soil 3	13	48	40	6.4	89	0.53	0.05	Sweetpotato
Soil 4	60	20	20	6.6	7*	3.22*	0.22	Barley

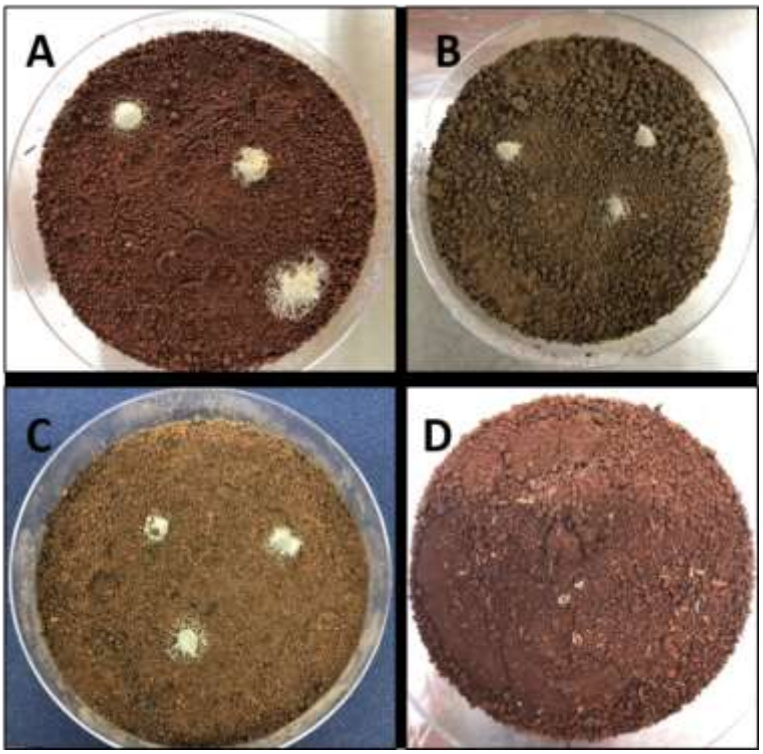


Figure 3.4: A Petri dish containing either soil 1(A) or soil 2(B) or soil 3(C) or soil 4(D) topically inoculated with three pieces of fungal granules, which resporulated during a 28-day incubation.

3.2.6 Data analysis

All statistical analyses were performed using the software IBM SPSS version 24 (SPSS, USA). All data were checked for normality and homogeneity of variance using the Shapiro-Wilk and Levene test, respectively. All datasets satisfied the criteria of normality and homogeneity of variance and were, therefore, analysed through parametric tests. Data obtained from 'Fungal resporulation in response to nutrient additives,' and 'Resporulation test of fungal granules in different substrates' were analysed by one-way analysis of variance (ANOVA) at $P \leq 0.05$ followed by a Tukey *post hoc* test for pairwise comparisons. For the experiment of 'Effect of soil samples on the resporulation of fungal granules,' the data analysis was performed using a Two-way ANOVA and pairwise comparisons were undertaken using a Tukey *post hoc* test.

3.3 Results

3.3.1 Fungal Resporulation in response to nutrient additives

The nutritive additives associated with the fungal granules significantly induced the fungal resporulation from the fungal granules ($P < 0.00$) (Figure 3.5). The combination of 20% corn starch and 20% autoclaved baker's yeast ($CAG_{Ma+Cs+By}$) significantly ($P < 0.001$) increased fungal resporulation to 1.4×10^8 ($\pm 2.07 \times 10^7$) conidia per granule, which is about 16 times higher than the initial number of encapsulated conidia (mean 8.88×10^6 conidia per granule) (Figure 3.6). However, supplementation with 20% corn starch alone (CAG_{Ma+Cs}) did not significantly increase ($P > 0.05$) the fungal resporulation, resulting in 2.4×10^7 ($\pm 5.8 \times 10^6$) conidia per granule in the comparison to the control granules (CAG_{Ma}). Conversely, fungal granules fortified with autoclaved baker's yeast (CAG_{Ma+By}) alone significantly ($P < 0.05$) increased fungal resporulation, culminating in a mean of 6.3×10^7 ($\pm 1.60 \times 10^7$) conidia per granule, compared to control or corn starch only granules. Control granules (CAG_{Ma}), containing calcium alginate encapsulation alone, without the food additives, did not demonstrate any significant ($P > 0.05$) increase in the number of fungal conidia following incubation.

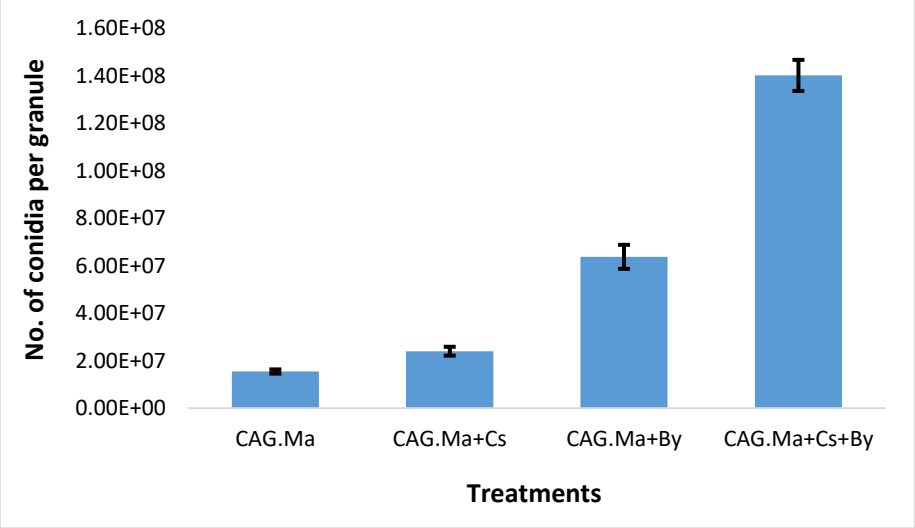


Figure 3.5: The number of conidia harvested from the fungal granules in response to food additives co-encapsulated with the fungus *(M. anisopliae*) into a calcium alginate granule (mean ±SE, number of replications = 10).

3.3.2 Resporulation test of fungal granules in different substrates

The mean number of fungal conidia produced from the fungal granules inoculated into different substrates were significantly ($P = 0.001$) different from one another (Figure 3.8). Pairwise comparisons showed that the number of fungal conidia harvested from the fungal granules placed on non-sterile soil was significantly lower than that of sterilised soil, pasteurised soil, filter paper, or water agar ($P < 0.05$), but not significantly ($P > 0.05$) different to the granules placed on perlite. The greatest number of fungal conidia arose from the fungal granules inoculated onto pasteurised soil or sterilised soil, but these values were not significantly different from each other ($P > 0.05$). No germination was observed from conidia harvested from granules inoculated onto the non-sterile soil (Figure 3.8 B). Conidia harvested from fungal granules inoculated on pasteurised soil, sterilised soil, and water agar showed the greatest conidial germination but was not significantly different from one another ($P > 0.05$). The conidial germination from the fungal granules inoculated on filter paper was greater than that of perlite, but both were significantly lower than that of sterilised soil, pasteurised soil, and water agar ($P < 0.05$). Likewise, substrate type significantly impacted the length of conidial germ tubes after their germination ($P = 0.001$). Conidia obtained from the granules inoculated onto sterilised or pasteurised soil produced significantly ($P < 0.05$) longer germ tubes than those produced on perlite, filter paper, or water agar. The diameter of the fungal colony arising from the fungal granules (inoculated in non-sterile soil, pasteurised soil, and sterilised soil) was also measured at the end of the experiment. Among those three soil types, the radial growth of fungal granules was significantly different among each another ($P = 0.001$). The greatest diameter of fungal granules was obtained on sterilised soil (14 mm ± 0.455), whereas the lowest fungal granules were found on non-sterile soil (2 mm ± 0.043).

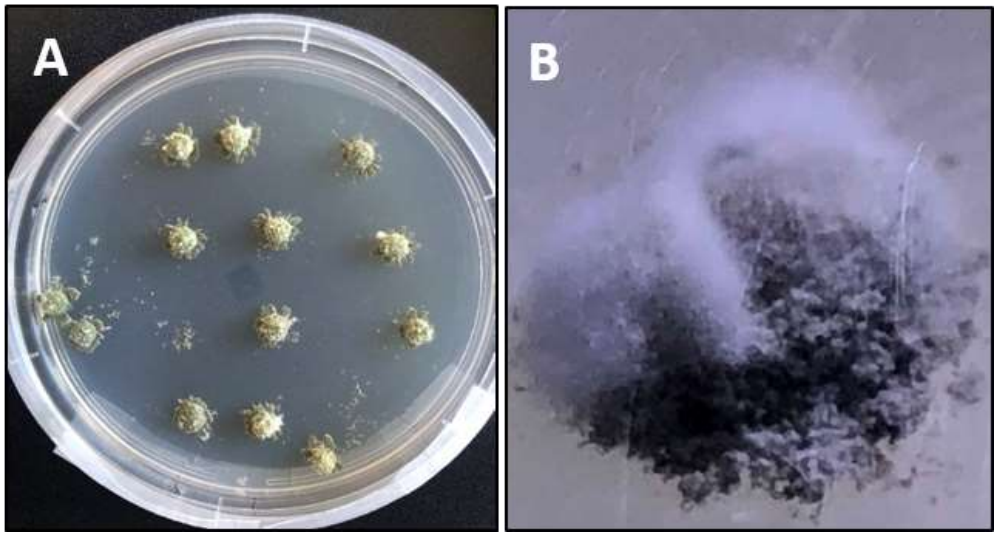


Figure 3.6: Resporulated fungal granules ($CAG_{Ma+Cs+By}$) following a two-week incubation on 3% water agar, supplemented with corn starch and autoclaved baker's yeast as food additives for *M. anisopliae* QS155 (A); and a magnified view (×40) of the single resporulated granule (B).

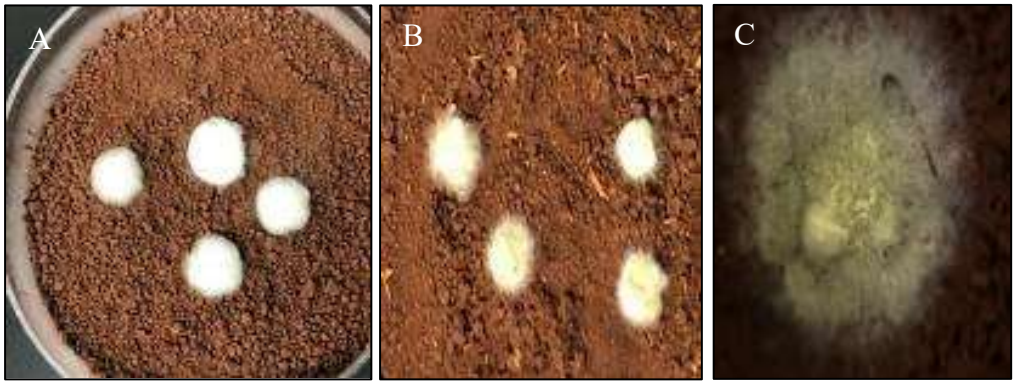


Figure 3.7: Growth of fungal granules after 7 days incubation when extensive mycelial growth appeared on the fungal granules inoculated in sterilised soil (A); mycelial growth with conidial development in pasteurised soil (B); growth of saprotroph (*Aspergillus* sp.) on non-sterile soil (C).

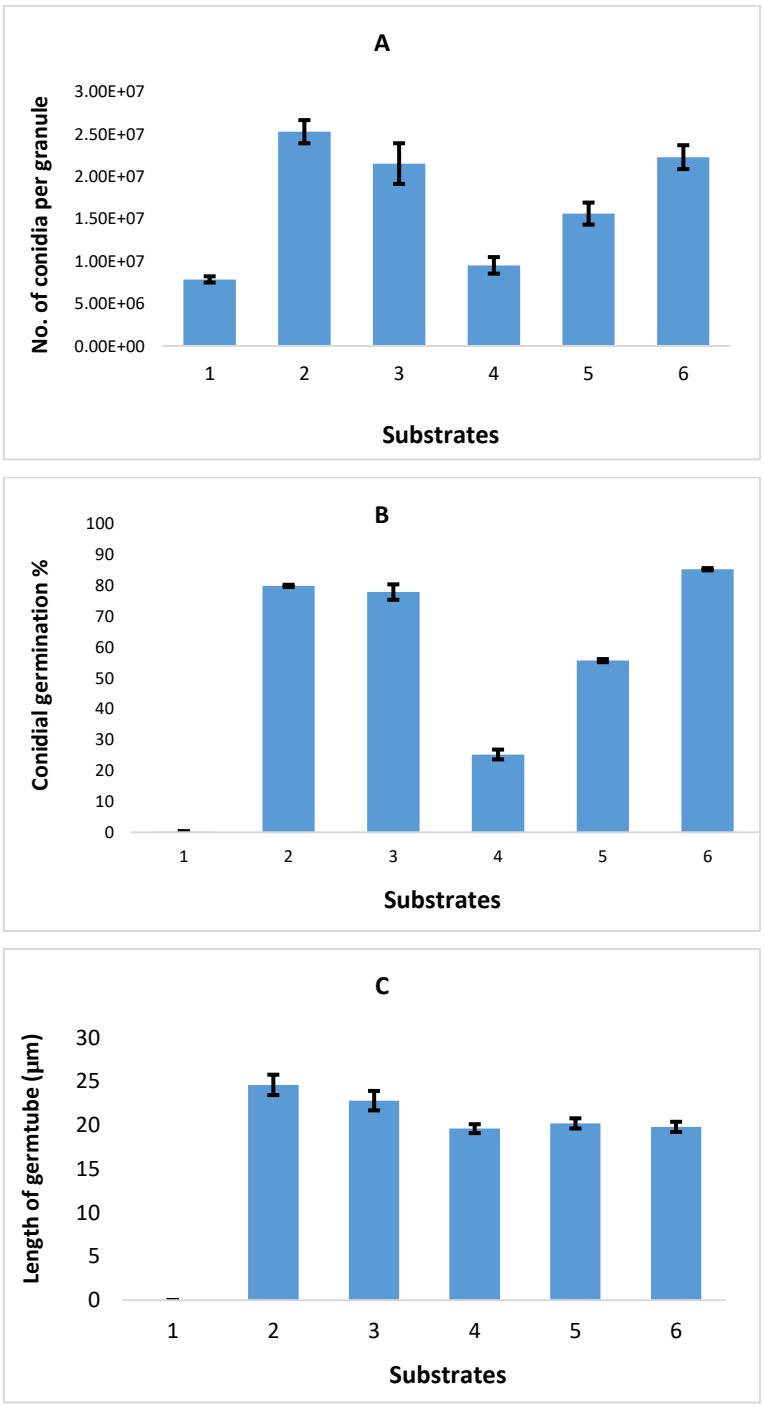


Figure 3.8: Harvest of conidia from granules at 21 days after inoculation (mean ± SE, replicates = 3, *P* = 0.05). Conidia harvested from granules from different substrates (A); conidial germination (B) and Germ tube length (C). Substrate names: non-sterile soil (1), pasteurised soil (2), sterilised soil (3), Perlite (4), Filter pater (5), and Water agar (6).

3.3.3 Effect of soil sample on resporulation of fungal granules.

The effect of using the four soil samples on fungal resporulation was not significant (P = 0.422). However, the effect of soil levels, namely non-sterile soil, pasteurised soil, and sterilised soil on fungal resporulation was significant (P = 0.000), regardless of soil types (Figure 3.9). The greatest numbers of fungal CFU were obtained from fungal granules on sterilised soil which was significantly higher than that on pasteurised and non-sterile soil (P < 0.05) (Figure 3.10), whereas the lowest fungal CFU were evident on non-sterile soil. No significant interaction was observed between soil types and soil levels (P = 0.453). A greater number of resporulated conidia was observed in sterilised soil (P = 0.001). No significant differences were observed between non-sterile soil and pasteurised soil in relation to resporulation of conidia (P = 0.652).

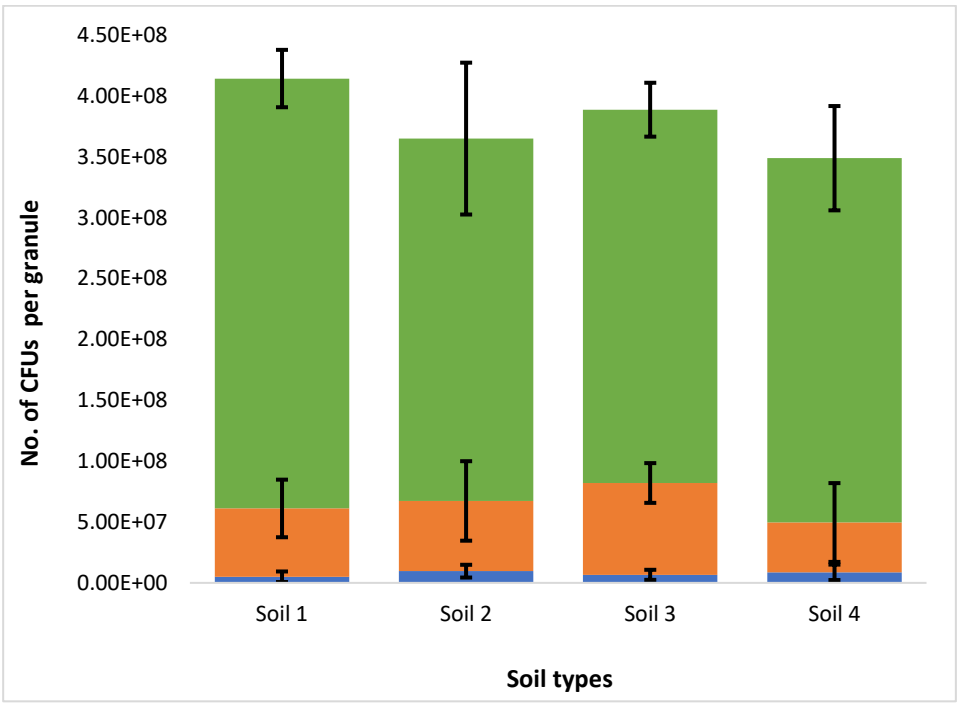


Figure 3.9: Fungal CFUs (mean ± SE, replicates = 3, P = 0.05)) were compared among four different soil samples, which were collected from various locations. Soil sample is further categorised into non-sterile (blue), pasteurised (orange), and sterilised soil (green).

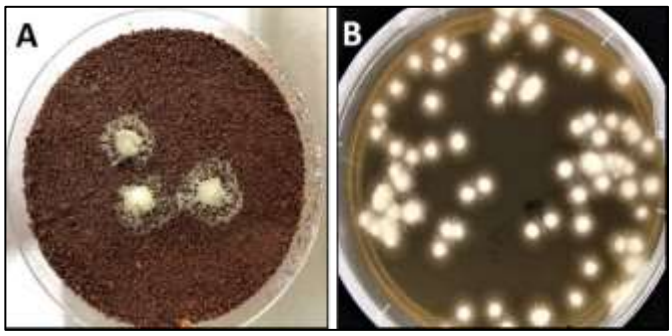


Figure 3.10: Resporulation on granules placed on sterilised soil (soil 1) at 28 days after incubation (A); and the fungal colonies on the selective media (SDAY amended with 0.01% chloramphenicol) from the individual resporulated fungal granules (B).

3.4 Discussion

Co-encapsulation of nutrient sources with *M. anisopliae* is important for prolific fungal sporulation. Calcium alginate does not have any detrimental effects on *M. anisopliae*, in terms of vegetative growth, conidiation, and conidial viability (Rodrigues et al. 2017). However, calcium-alginate alone as an encapsulating polymer does not lead to any substantial fungal sporulation without additional nutrients. Furthermore, aerial conidia of *M. anisopliae* are unlikely to optimally germinate without the supply of externally supplied nutrients because of their low nutrient reserve within the conidia (Dillon & Charnley 1990). In this study, the sporulation of encapsulated *M. anisopliae* in response to autoclaved baker's yeast and corn starch co-encapsulated in sodium alginate, individually or in combination, was examined. The results here showed that the combination of autoclaved baker's yeast (20% w/v) and corn starch (20% w/v) as a nutrient for the fungus yielded the greatest sporulation on the surface of calcium alginate granules, compared to when the autoclave baker's yeast or corn starch was individually encapsulated with *M. anisopliae.* A study Przyklenk et al. (2017) showed that the combination of corn starch and baker's yeast optimised the fungal resporulation. Corn starch as a carbon (C) source and autoclaved baker's yeast as a nitrogen (N) source has been shown to maximise the sporulation of encapsulated *M. anisopliae*; as the fungal mycelium growth has been linked to corn starch as a carbon source, whereas baker's yeast as a nitrogen (N) source stimulates the fungal conidiation (Jaronski & Jackson 2012). However, corn starch (20% w/v)

alone as food for encapsulated *M. anisopliae* has been implicated in producing hyphal overgrowth leading to limited resporulation. A study conducted by Gerding-Gonzalez et al. (2007) showed that *B. bassiana* granules fortified with > 2 % chitin (a carbon source) alone stimulated the fungal mycelial growth but the addition of rice bran (2 % w/v) into chitin (2% w/v) significantly transformed the fungal mycelia into the conidiation (Gerding-Gonzalez et al. 2007), confirming the nitrogen source as a conidiation trigger for *B. bassiana*. Although the carbon source has not directly contributed to the fungal conidiation, the addition of carbon source, particularly corn starch has a role to prevent the fungal granules from desiccation-derived mortalities during storage (Pereira & Roberts 1991), while maintenance of fungal viability after the fungal granule desiccation and then storage is a challenge without the desiccation-protectant like corn starch. Generally, the fungal granules have been undergone through drying process as the incidence of saprophytic attack is higher on the non-dried fungal granules. Particularly nitrogen sources, for example, baker's yeast or rice bran, have been implicated in inducing the saprophytic growth on the fungal granules (Behle & Jackson 2014), whereas corn starch cannot be readily utilised by saprotrophs because the microbes need the starch degrading enzyme like amylase to digest corn starch, reducing the likelihood of saprophytic incidence on the fungal granules. This study confirmed that the addition of 20 % corn starch in combination with 20 % autoclaved baker's yeast triggers the optimal fungal resporulation from the fungal granules under laboratory conditions.

Effective soil insect control has been linked to the fungal abundance and persistence of the infective fungal colonies in soil (Ekesi et al. 2005). Fungal colonisation in crop fields can ensure the high fungal density with infectivity which eventually confers the protection of a host plant against soil insect infestation (Mayerhofer et al. 2015). To encourage the rapid growth in soil, the fungal inoculum can be supplemented with nutrient additives (Knudsen et al. 1991). In respect to the fungal multiplication from the nutrient-supplemented fungal granule, our study showed that the number of fungal CFU recovered from sterilised soil statistically outnumbered those from pasteurised or non-sterile soils. It may be implied that the potential suppression of soil microorganisms during the soil sterilisation enabled the

encapsulated *M. anisopliae* to utilise the co-encapsulated foods in sterilised soil, culminating in vigorous mycelial growth and subsequent sporulation (Jaronski 2010). Non-sterile soil naturally contains a diverse range of soil microbes such as bacteria, archaea, and fungi and these soil microbes inextricably interact with each other to maintain the balance status of the ecosystem. Saprotrophic fungi, particularly *Aspergillus*, *Penicillium, Fusarium, Trichoderma,* and *Mucor* are natural decomposers of plant and animal debris (Lestan & Lamar 1996; Aislabie et al. 2013). For this reason, they quickly colonise co-encapsulated food sources leading to competitive fungistasis (Jaronski 2007). *M. anisopliae* is considered a weaker saprotroph than the other soil saprophytes, which likely outcompete the encapsulated *M. anisopliae* for food (Zimmermann 2007). Moreover, these saprotrophs release several types of enzymes and metabolites which induce the antibiosis against nearby soil microbes, for example, *Penicillium urticae* imposes an antibiotic fungistasis to other microbes by releasing the toxin patulin as a metabolite (Jaronski 2010). Based on this information, the lack of germination from the conidia extracted from the fungal granules inoculated on non-sterile soil may be attributed to the antibiosis imposed by the presence of growth of contaminating saprotrophs on the granules. Bacterial growth was also observed on the granules applied to non-sterile soil. *In vitro* studies showed that volatile and non-volatile metabolites produced by bacteria, such as *Bacillus*, *Pseudomonas*, and *Streptomyces* can be detrimental to the viability of the externally applied EPF (Jaronski 2007). Interestingly, actinomycetes, for example, *Streptomyces* spp. can produce a broad-spectrum antibiotic against other soil microbes (Aislabie et al. 2013). Some studies elucidated that the phenomena of soil mineralisation, for instance, the conversion of organic nitrogen into ammonia (NH_4), the increased bioavailability of soil elements e.g., manganese (Mn), and alteration of soil pH occurring during soil sterilisation may favour soil microbes in the exponential growth phase when they are released into sterilised soil (Kitur & Frye 1983). However, since the fungal granules that we used in our experiment already contained the nutrient additives to support its growth in soil, the fungus *M. anisopliae* does not rely on soil nutrients alone for its growth, implying that inherent soil nutrients might not have a significant impact on the *Metarhizium* growth. On the contrary, we observed the rapid and extensive growth of saprotrophic fungi over the food granules (control granules) when food granules

were placed onto sterilised soil, whereas such extensive growth was not seen in non-sterile soil. Regarding the impact of edaphic factors on *M. anisopliae* growth, there is still ambiguity. A study by Rath et al. (1992) presented that soil physical properties such as soil texture, pH, electrical conductivity, and cation exchange capacity do not induce any significant effect on the growth and development of EPF. Our study is also in agreement with the latter claim because no variability in terms of fungal colonies was evident among four different soil types, despite the contrasting soil physical properties.

The combination of corn starch and baker's yeast as nutritive additives significantly induced the fungal resporulation when these nutritive additives were coencapsulated with the conidia of *M. anisopliae* into calcium alginate. This fungal granule demonstrated its potentiality to be resporulated on diverse substrates maintaining significant conidial viability. This study marked that sterilised soil optimally favoured fungal resporulation. The greatest fungal resporulation found on sterilised soil has been attributed to the reduced soil microbes from soil because of soil sterilisation. This study implied that the efficacy of fungal granules can be enhanced either by the conjunction of the fungal granules with the reduced microbial soil, for example, disinfested soil, or by further optimising the fungal granules to stimulate the fungal growth on field soil.

The above study was attempted to evaluate only the resporulation aspect of fungal granules on different substrates including soil, on where the resporulation of *M. anisopliae* was significantly variable in response to various substrates. However, understanding the infectivity of those resporulated fungal granules against insect hosts is also crucial as our prime objective is to control wireworm insects. Thus, the following study is going to evaluate the infectivity of resporulated fungal granules against insect hosts on both soil and soilless substrates, while various soil levels such as sterilised, pasteurised, and non-sterile soil induced various levels of fungal resporulation.

CHAPTER 4: LABORATORY EVALUATION OF LARVAL MEALWORM (*TENEBRIO MOLITOR*), MORTALITY CAUSED BY *METARHIZIUM ANISOPLIAE* QS155 FORMULATED IN CALCIUM ALGINATE GRANULES

Abstract

Infectivity of entomopathogenic fungi as potential biocontrol candidates needs thorough evaluation before deployment in the field. However, there is a significant paucity of knowledge regarding the infectivity of resporulated fungal granules (for example EPF formulated in calcium-alginate) once applied in the field and how they remain infective against host insects. In this chapter, the infectivity of resporulated fungal granules was assessed under laboratory conditions using larval mealworms, *Tenebrio molitor* as a model insect. Due to their subterraneous and cryptic behaviour, a population of wireworms sufficient for experimentation was not available. We used resporulated fungal granules to challenge mealworms, which resulted in up to 100% larval mealworms within 14 days of insect exposure on the resulting resporulated fungal granules. We investigated the effect of soil treatment on insect mortality but found that the soil treatments: sterilised, simulated-solarised, and non-sterile soil did not induce any significant difference in mealworm mortality. Non-discriminatory results of mealworm mortality on three different soil levels with fungal resporulation have been attributed to the larval mealworms as a susceptible host to the insect pathogen. Rapid death after exposure to the resporulated fungal granules may also be able to effectively kill wireworms in sweetpotato, provided they come into contact with the propagules.

4.1 Introduction

The fungal genus *Metarhizium* contains several species that are considered potential candidates of entomopathogenic fungi for insect control (Shah & Pell 2003). Some *Metarhizium* species, particularly *M. anisopliae, M. brunneum,* and *M. robertsii*, are generalist pathogens as they attack a broad range of arthropods including aerial insects (Zimmermann 2007). Because of their ecological plasticity, these generalist fungal pathogens of insects can also colonise the plant rhizosphere and further establish endophytic relationships with plants, especially in the roots (Greenfield et al. 2016; Vega 2018). The plant-fungi endophytic interactions may protect the host plant from herbivores' attacks (Razinger et al. 2020) and further facilitate nutrient exchange between the fungus and the plant (Behie & Bidochka 2014). These attributes have contributed to these fungi being extensively studied as biocontrol agents for a broad range of insect pests (Inglis et al. 2001). For many insects, especially for soil insects, entomopathogenic bacteria or virus-based insecticides are not effective as insects must ingest these organisms to be effective (Copping & Menn 2000), whereas external contact to insect cuticle is sufficient for EPF to initiate the infection (Aw & Hue 2017). These fungi can grow saprophytically on nutrient substrates and reproduce asexually.

In the work presented here, we aimed to increase the growth of *M. anisopliae* in the soil, especially in proximity to crop roots, so as to enhance protection from root-feeding herbivores such as wireworms. To achieve the fungal multiplication in soil, the selection of appropriate fungal formulation is always of paramount importance (Gasic & Tanovic 2013). According to the study by Ekesi et al. (2005), soil treated with granular-formulated *M. anisopliae* (pumice/maize granule) maintained fungal persistence and infectivity against soil-dwelling insects (pupariating fruit flies, *Ceratitis* spp.) for a prolonged period, but liquid formulations failed to perform as well. Secondly, they noted that granular formulations are convenient for manual handling during storage, transportation, and delivery into the fields.

From the previous Chapter (section 3.3.1) encapsulation of conidia of *M. anisopliae* in calcium alginate granules supplemented with corn starch and autoclaved

baker's yeast as nutritive additives ($CAG_{Ma+Cs+By}$) lead to maximum resporulation. Moreover, these fungal granules may not require as many initial fungal conidia as required in conventional granules, as the nutrients may induce secondary sporulation on the granule surface. It is proposed that the fungus can better compete for the co-encapsulated nutrient additives when the activity of soil microbes, especially saprotrophs, is reduced. For the infection, soil insects must have physical contact with the fungal granules. The physical contact between soil insects and fungal inocula may be affected by the ability of some insects to detect the fungal inoculum and migrate into non-contaminated areas to prevent the infection (Ericsson & Kabaluk 2007). Moreover, certain insect behaviour reduces the fungal infection, for example, mutual grooming among termites (*Coptotermes formosanus* Shiraki) dislodges the fungal inoculum from the insect body (Yanagawa et al. 2008) or thermoregulation modulated by the Migratory Grasshopper (*Melanoplus sanguinipes*) to elevate the body temperature decimates the microbial growth (Dakhel et al. 2019). Thus, it is plausible to know that how soil insects interact with the resporulated fungal granules.

The application rate of fungal (*M. anisopliae*) granules is generally recommended at $>10^6$ spores cm^{-3} soil to achieve effective control against wireworms (Kabaluk et al. 2007). However, the conventional recommendation rate may be reduced using encapsulated granules as it is anticipated that the fungus may produce secondary resporulation in the soil. A study by Przyklenk et al. (2017) mentioned that the encapsulation of *M. brunneum* conidia at 0.01% can multiply the fungal resporulation up to 1000 times, referred to as the microfermentation, when the conidia were fortified with the nutritive additives. These resporulated conidia produced on fungal granules are considered a primary source of infection for any soil insects which encounter the resporulated fungal granules. The success of encapsulated fungal granules for soil insect control relies on the resporulation efficacy of fungal granules once they are applied to the soil. However, there has been a significant paucity of knowledge about the infectivity of soil resporulated fungal granules against soil insects. Thus, this study emphasised evaluating the infectivity of fungal granules following their application on soil against insect hosts.

Our previous chapter (section 3.2.4) concerning resporulation of fungal granules as affected by treatments to reduce levels of other soil microbes found the highest resporulation on granules in sterilised soil, lower resporulation in pasteurised soil, and lowest in non-sterile soil. However, there is a significant lack of knowledge about how the variable fungal resporulation among sterilised soil, pasteurised soil, and non-sterile soil translates into soil insect mortality. The practice of soil disinfestation is common in horticultural crops in Australia, including sweetpotato, using methods such as fumigation or soil solarisation to suppress soil-borne pathogens, insects, and weeds (Henderson & Dennien 2018). Soil fumigation is practically feasible for many commercial growers, whereas non-chemical options, for example, soil solarisation, are available for organic growers. A variety of soil disinfestation carried out in fields has been simulated with sterilised soil, simulated solarised soil, and non-sterile soil in the study. Based on the variable fungal resporulation from the fungal granules among three different soil levels, we postulate the fungal granules inoculated on sterilised soil could cause the greatest level of insect mortality, moderate mortality in simulated solarised soil, and lowest mortality in non-sterile soil. According to our laboratory evaluation (section 3.3.2), fungal granules inoculated on non-sterile soil neither significantly resporulated, nor preserved the germination of original conidia that were encapsulated into the fungal granules. Thus, it is warranted to understand whether these non-resporulated fungal granules contribute to insect mortality or not.

Our previous research has shown that substantial larval mealworms introduced into sterilised soil with food granules (control) were recovered with the notable mortality caused by *Metarhizium* organisms (section 4.2.4). A similar scenario was shown in a study conducted by Van Herk et al. (2016) who reported the field-collected wireworms started to die when they were reared in sterilised soil. They implied that the enzootic pathogen *M. brunneum* caused the mortality of the wireworms when they were reared in sterilised soil (Kabaluk et al. 2017). This study further explains that soil insects, for example, wireworms, harbour a multitude of symbiotic microbes that may confer protection against infection by naturally occurring pathogens like *M. brunneum*, whereas sterilised soil, potentially devoid of soil microbes, triggers the expression of

enzootic pathogen i.e., *M. brunneum*, which remains asymptomatic for host insect when the insects live in fields (Kabaluk et al. 2017).

The work in Chapter 3 (3.3.2) demonstrated that the resporulation pattern on fungal granules differed among sterilised, pasteurised, and non-sterile soils. Here, we are going to include the simulated sterilised soil because soil solarisation is a type of soil disinfectants that is usually adopted by organic sweetpotato growers in Australia (Bree Wilson pers. comm.). The idea of simulated solarised soil has been adapted from the study by Stapleton et al. (2000) based on heat exposure to soil. Once the fungal granules were incorporated into the soil, they imbibed the moisture from the soil, initiating conidial germination in the granules. But the patterns of mycelial development and subsequent conidiation were found to be variable and were dependent on the different soil types and levels.

The objectives of this study were: (i) to assess the efficacy of resporulated fungal granules against larval mealworms; (ii) to evaluate the effect of different fungal granule rates on soil for larval mealworm mortality; and (iii) to examine the infectivity of soil-applied fungal granules for larval mealworm mortality.

4.2 Materials and methods

4.2.1 Treatments

Metarhizium anisopliae QS155 was encapsulated in calcium alginate granules supplemented with 20% corn starch (w/v) and 20% autoclaved baker's yeast (w/v), ("fungal granules" or $CAG_{Ma+Cs+By}$). Calcium alginate granules containing the same volume of corn starch and autoclaved baker's yeast, but without *Metarhizium anisopliae* ("food granules' or CAG_{Cs+By}) were used as the control (Figure 4.1 B).

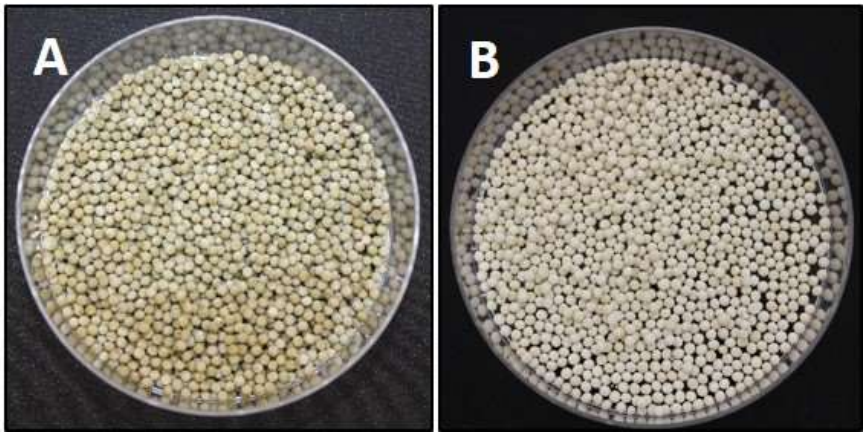


Figure 4.1: Fungal granules, $CAG_{Ma+Cs+By}$ used as a treatment for mealworm mortality (A); and food granules, CAG_{Cs+By} used as a control for mealworm mortality (B).

4.2.2 Insects

The target insect for this study was wireworms (Coleoptera: Elateridae and Tenebrionidae), which are subterraneous insects causing feeding damage to the underground plant parts of various crops including sweetpotato. Wireworms are a sporadic insect pest on sweetpotatoes, although wireworm infestation is impacted by the season, soil moisture, soil temperature, and vegetation cover and is commonly referred to as a cryptic insect. Because of these factors, wireworms do not always respond to the grain-based baits, which are placed 5-10 cm deep in the soil. The indifferent response of wireworms to the grain baits was also evident in our trial conducted in a sweetpotato field, Gatton, QLD in October 2016 (Figure 4.2). But a non-response to the bait on fields does not signify those wireworms are absent in the soil. The grain baits consisted of a mixture of pre-soaked corn and wheat seeds in a mesh bag (50 g seeds per bag) and the individual bags were buried at a depth of 5-10 cm in a sweetpotato field (Beauregard, 35 days after planting).

In addition, wireworms exist as a cryptic species complex in fields that challenge the wireworm collection as a cohort and homogenous species. Therefore, the establishment of a wireworm colony was not possible. For these reasons, larval mealworms (*Tenebrio molitor*, Coleoptera: Tenebrionidae) were used as a model

insect in this study to evaluate the infectivity of fungal granules. Both false wireworms and larval mealworms belong to the same family, Tenebrionidae, and making them a good model insect in our study. In addition, these larval mealworms are regularly available, easy to rear, and maintainable in the laboratory. The larval mealworms used in our study were supplied from Bio Supplies (https://biosupplies.net.au), Yagoona, NSW (Figure 4.3). The mealworms were reared in the laboratory of the University of Southern Queensland, Toowoomba at room temperature (20-22 ˚C) in a diurnal light regime and were supplied with wheat germ and sweetpotato roots as a food source.

Figure 4.2: A sweetpotato farm in Lockyer Valley, Gatton, Queensland where the presence of larval wireworms was assessed by incorporating corn baits in field soil in October 2016. A mesh bag containing pre-soaked grains of corn and wheat was used as a seed bait for wireworm isolation.

Figure 4.3: Larval mealworms used as a model insect to assess the mortality efficacy of fungal granules

4.2.3 Mortality Efficacy of fungal granules

In a previous experiment, we found that the granules containing corn starch and baker's yeast produced the highest number of conidia after resporulation and therefore this formulation ($CAG_{Ma+Cs+By}$) was used in all experiments to challenge larval mealworms (*T. molitor*). For the bioassay, ten resporulated fungal granules were inoculated into a Petri dish (Ø=9 cm) containing a mixed-sex cohort of 10 larval mealworms (mean body length 2.65 cm; body weight 0.1 g; and 8 abdominal rings) (Figure 4.4 A). A piece of diced carrot (35 g) was also suppled as food for the insect in each Petri dish. The Petri dishes were then sealed with Parafilm® and incubated at 25 °C, 75% RH, and a 12:12 h dark and light photoperiod in a growth chamber (Conviron MP6010). After 24 h of incubation, the Parafilm® was removed, and the original lids of the Petri dishes were replaced with perforated ones to facilitate aeration. Calcium alginate granules containing corn starch and baker's yeast, without the fungal conidia (CAG_{Cs+By}) were used as the control (Figure 4.4 B). To obtain the resporulated fungal granules, the fresh fungal granules were inoculated into a Petri dish containing

3% water agar and incubated (27 ˚C, and a 12:12 h dark and light photoperiod) for 14 days to encourage sporulation. Similarly, the food granules were also pre-treated under the same conditions as fungal granules before inoculation. There were eight replicates per treatment per Petri dish, and the Petri dishes were arranged in an RCBD in an incubator (Conviron MP6010). The experiment was repeated twice over time.

Every 24 h post-inoculation, the mortality of mealworms was assessed and this continued for 14 days. During these observations, dead mealworms were removed using sterile tweezers and immediately transferred into a moist chamber to encourage mycosis following the surface-sterilisation of the cadavers, using the method described by Lacey and Brooks (1997).

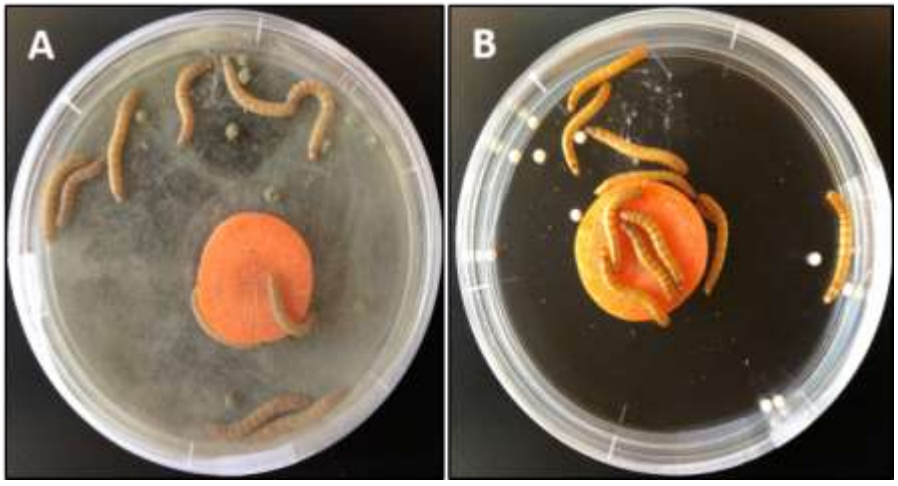


Figure 4.4: An experimental unit consisted of a Petri dish containing 10 larval mealworms inoculated with either of 10 pieces of resporulated fungal granules (A) or the same number of pre-incubated food granules (B) regarded as the control.

4.2.4 Effect of different conidia concentration on mealworms

The experimental design was factorial set-up (3×2×3), with three different soil treatments (non-sterile soil, simulated solarised soil, and sterilised soil), two granule treatments (fungal granules and food granules), and three concentrations of conidia:

low (2×10^6 conidia g^{-1} soil), medium (3×10^7 conidia g^{-1} soil), or high (4×10^{10} conidia g^{-1} soil). For the experimental set-up, individual plastic containers (volume 500 mL) were filled with 150 g of either non-sterile soil (collected from the USQ agriculture field), simulated solarised soil (constant heat treatment at 45 °C for 14 d for the USQ field-collected soil), or sterilised soil (oven-dried USQ field-collected soil at 105 °C for 3 days). For this experiment, all granules were dried for 14 h in a laminar flow following their preparation. This prevented the initial growth of soil saprotrophs on the granules when they were first inoculated in the soil. The mixture of soil and granules was homogenised by inverting the containers twenty times and then moistening the soil with 15 mL of distilled water. The containers were secured with a lid and then placed in a growth chamber (Conviron MP6010) at 25 °C, 60% RH in the dark) to encourage fungal sporulation. Calcium alginate granules containing corn starch and autoclaved baker's yeast (CAG_{Cs+By}), without the fungal conidia, were included as the control (denoted as the food granules). Initial moisture levels in non-sterile soil, simulated-solarised soil, and sterilised soil were not equal, and therefore, soil moisture levels among these soils were adjusted to the same level using sterile water. The incubated granules are herein referred to as pre-treated (control granules) or resporulated fungal granules

After 28 days, a cohort of 30 larval mealworms (mean body length 2.65 cm; body weight 0.1 g; and 8 abdominal rings) was released into each container containing soil resporulated fungal granules or pre-treated food granules (control). Fifteen mL of distilled water was applied to the soil in each container. Additionally, 1 g of autoclaved and air-dried corn seeds was also supplied as food for the mealworms. After the insect release, containers were sealed with a perforated lid to facilitate aeration and re-incubated at 22 °C, 80% RH in the dark. Each treatment was replicated three times, and all treatments were arranged in a randomised complete block design during the incubation.

After 14 days following the insect release, insect mortality was examined, in which dead mealworms were removed using sterilised forceps and recorded. Dead

mealworms were separated into mycosed and non-mycosed cadavers. For the non-mycosed cadavers, dead insects were surface-sterilised, rinsed with distilled water twice, placed over a moist filter paper, and incubated (25 ˚C and a 12:12 light and dark photoperiod) to encourage conidiation.

4.2.5 Examining unexpected Metarhizium sporulation in control treatments

Despite careful handling of the fungal granules around the food granules and the use of aseptic technique during the experimental set-up, mealworm mortality was found in sterilised soil inoculated with food granules (control) in the previous experiment (section 4.3.2). Most of the dead mealworms were recovered as a conidiated cadaver in the experimental pot during the assessment. Other non-conidiated cadavers produced the conidiation when they were placed in a moist chamber. Based on the morphological features, the suspected pathogen causing mealworm mortality was expected to the genus *Metarhizium*. Few *Metarhizium*-like fungi appeared in mealworms reared in non-sterile soil, but the conidial colour and shape were different from what is observed in isolate QS155 used for all experimentation described in this research. We hypothesised that the *Metarhizium* found in sterilised soil was QS155, but the *Metarhizium* observed in non-sterile soil was possibly a different indigenous USQ soil strain.

The initial assumption in relation to the *Metarhizium* infection of mealworms in sterilised soil with food granules was that there was either contamination of the food granules from the fungal granules during the preparation or cross-contamination occurred between fungal treated soil and food treated soil in the growth chamber during the experimental period. To confirm the contamination of food granules, the food granules (6 granules) sourced from the same lot of granules used for the infectivity assessment experiment were placed in Petri dishes (Ø 9 cm) containing SDAY, sealed with Parafilm®, and incubated at 25 ˚C for 14 days. Six replications (Petri dishes) were used.

To rule out whether the cross-contamination occurred in the growth chamber during the experimental period, a scenario of "enforced cross-contamination" was created, in which four plastic containers each containing sterilised soil (50 g) with 10 larval mealworms (mean body length 2.65 cm; body weight 0.1 g; and 8 abdominal rings) were intermixed with three Petri dishes with the fully sporulated culture of QS155 *M. anisopliae* and a conidiated mealworm cadaver in each dish. When no sign of insect infection was experienced over 7 days, 0.25 g of fungal conidia was spread over the top of perforated lids so that mealworms could acquire the fungal inoculum.

4.2.6 Infectivity assessment of fungal granules in soil

The experiment was set up as a factorial (2×3), with two treatments (fungal granules and food granules) and three soil treatments (non-sterile soil, simulated solarised soil, and sterilised soil). Before the insect release, either fungal granules ($CAG_{Ma+Cs+By}$) as the treatment or food granules (CAG_{Cs+By}) as the control were added to the soil (Figure 4.5). All soil samples were collected from the USQ agriculture field, Toowoomba. For the simulated solarised soil, soil samples were heated at 45 $^{\circ}$C for 14 d in an oven (Steridium). The soil was sterilised heating at 105 $^{\circ}$C for 72 h using an oven (Steridium). Transparent plastic containers (500 mL) were filled with 150 g of either a non-sterile, simulated solarised, or sterilised soil, and then inoculated with the fungal granules at the rate equivalent to 3.8×10^6 conidia g^{-1} soil; individual fungal granules contained an average of 10^7 conidia.

After 28 days post-incubation (Figure 4.5 B), a cohort of 30 mealworm larvae (mean body length 2.65 cm; body weight 0.1 g; and 8 abdominal rings) were released into granule-inoculated soils of individual containers. The containers were then inverted thrice. All containers containing soil were moistened with an additional 15 mL of sterile, distilled water per container. For mealworm food, corn was autoclaved and air-dried for 2 h in a laminar flow, then 1 g of corn was placed into a separate dish within the container to minimise contact with soil (minimising contaminating fungal growth that consumed the corn) (Figure 4.5 C). The containers were then sealed with

a perforated lid to facilitate the aeration for mealworms, and all containers were re-incubated (22 ˚C, 80% RH and in the dark) in the growth chamber.

Assessments of mortality began at 7 days after insect exposure (DAIE) and continued every day. Following the mortality assessment on 20 DAIE, an additional 15 mL of sterile water was added to each soil container to compensate for the moisture loss during the incubation period. Dead mealworms recovered at the assessments were immediately surface sterilised and incubated by following the method as described in the 'mortality efficacy of fungal granules' (section 2.2.3).

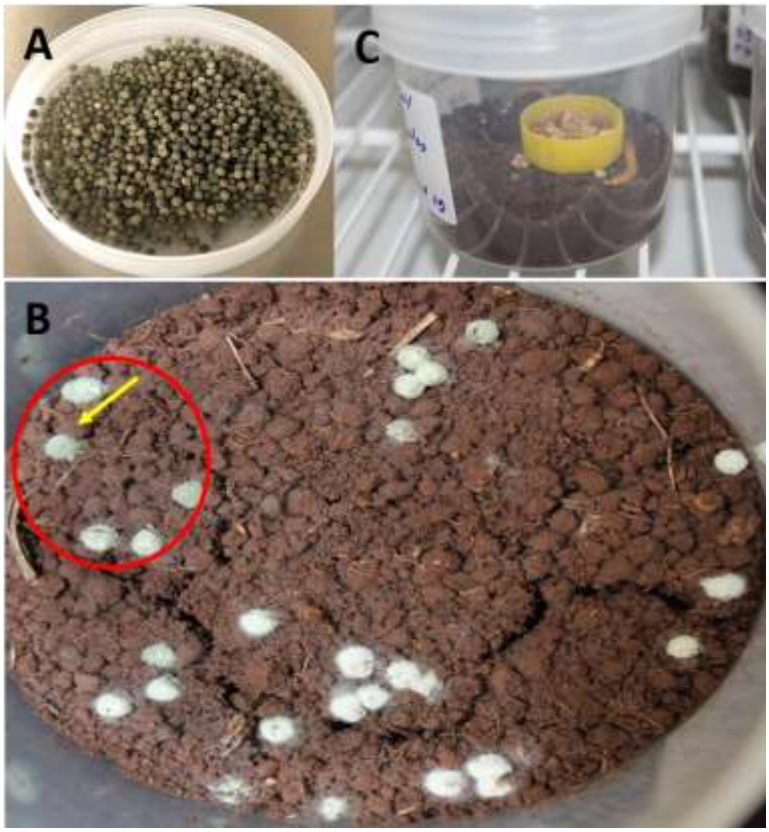


Figure 4.5: Fungal granules (mean 2.5 mm diameter, ~10^7 conidia per granule, and air-dried until 61% moisture loss from their original weight) prepared before the inoculation (A); resporulated fungal granules, along with saprotroph growth on the granules (yellow arrow), appeared on non-sterile soil following 28 days incubation (B); and larval mealworms released on soil with resporulated fungal granules and provided disinfested wheat seeds in a yellow container as food for the mealworms (C).

4.2.7 Data analysis

All statistical analyses were performed using the software IBM SPSS version 24 (SPSS, USA). All data were checked for normality and homogeneity of variance using the Shapiro-Wilk and Levene test, respectively before performing the analysis. For the experiment on pathogenicity, the mortality data were converted to percentages and then further corrected using Abbot's formula (Abbott 1925). Repeated data were pooled, and significant differences were determined using a T-test analysis. For dose-response, the mortality data were converted to a percentage. The percentage data were analysed using a two way-ANOVA to determine the factorial interaction, followed by a *post hoc* comparison (Tukey test) for an individual level comparison. There was not any mortality effect of food granules, regardless of soil level. Therefore, the effect of fungal granules in three different soil levels was analysed using an ANOVA analysis ($P \leq 0.05$), followed by a Tukey *post hoc* test to make a pairwise comparison.

4.3 Results

4.3.1 Mortality efficacy of fungal granules

There was no significant difference ($P > 0.05$) between the repeated data over time so the data from both experiments were pooled. The resporulated fungal granules caused significantly greater mortality at each assessment time than the control ($P < 0.001$). The onset of mealworm mortality started at three days post-incubation (10 % (± 9.53) mortality) and the mortality was 52 % (± 5.2) on the 5th-day post-incubation. Mortality of mealworms continued to be rapid where 76 % (± 9) mortality was observed 9 days post-incubation reaching to 88 % (± 7) mortality at 14 days post-incubation. Significant ($P < 0.001$) differences were observed in the mealworm mortality across all assessment dates. More than 95% of the dead mealworms recovered during the experiment produced profuse mycosis when they were placed into a moist chamber, confirming their mortality was caused by the fungus applied (Figure 4.6).

Figure 4.6: Cadaver of a mealworm larva infected by *M. anisopliae* QS155 during the mortality efficacy of the fungal granules ($CAG_{Ma+Cs+By}$) against larval mealworms. Sporulation on the cuticle appeared following the placement of the cadaver in a chamber with high humidity.

4.3.2 Effect of different conidia concentration on mealworms

Fungal granules inoculated in non-sterile soil, simulated pasteurised soil, or sterilised soil caused significantly ($P < 0.05$) greater mealworm mortalities than that caused by food granules (control) in all soil treatments. No significant difference ($P = 0.693$) in relation to mealworm mortality was found among three soil treatments (non-sterile, simulated pasteurised, and sterilised soil) with fungal inoculation. Similarly, the concentration of conidia in the soil did not result in any significant difference ($P = 0.493$) in mealworm mortality. No significant ($P = 0.911$) interaction between soil levels and conidial concentrations was found. From the food granules (control) treated on sterilised soil, 8% (±5) larval mealworms had *Metarhizium*-like infection on dead mealworms.

4.3.3 Examining unexpected Metarhizium sporulation in control treatments

There was no evidence of fungal contamination in fungal-treated soil and food-treated soil. Moreover, no sign of mealworm infection was observed, despite an 'enforced contamination' event that was applied to larval mealworms maintained in sterilised soil.

4.3.4 Infectivity assessment of granules in soils

Unsurprisingly, there was a significant ($P < 0.05$) effect of using fungal granules over food granules on mealworm mortality. At 14 days after the insects were released, the following mortality was recorded: 52% (±21) in non-sterile soil, 8% (±7) in simulated solarised soil, and 13% (±5) for sterilised soil, all of which were significantly ($P < 0.05$) different from one another (Figure 4.7 A, B, & C). Significant ($P < 0.05$) mortality was also observed 20 days after insects were released: 79% (± 21) mortality for non-sterile soil, at 32% (±18) for simulated solarised soil, and 27% ± 17 for sterilised soil (Figure 4.7 D, E & F). The mortality of insects in non-sterile soil was significantly ($P < 0.05$) higher than that observed in solarised and sterilised soils. Twenty-five days after insect release, the mealworm mortalities on solarised and sterilised soils were 78% (±17) and 67% (±24) respectively, which were not significantly ($P < 0.05$) different from the mortality on non-sterile soil i.e., 92% (± 8). At 30 days after the insects were released, mealworm mortalities were 96% (± 5), 91% (±14), and 84% (±17) in non-sterile soil, simulated-solarised soil, and sterilised soil respectively, and these were not significantly different from one another.

Dead mealworms recovered during the assessments that were not found with mycosis had > 95% of the cadavers had fungal outgrowth after they were placed in a moist chamber (Figure 4.7 G, H & I). Out of all the dead mealworms found in *M. anisopliae*-granule treated soils, 75% of the cadavers remained as larvae, 13% were pupae and 2% were adult beetles. At 30 days after insects were released, only two adult mealworms were recorded, although they presented as deformed adults, possibly as a

result of poor nutrition. A total of 12 larval mealworms were observed to be moribund and displayed little movement. Across all the replicates, five live pupae were also found. In contrast, the original larval mealworms released on soil with food granules (control) had predominantly metamorphosed into adults (75%), and 10% of them had emerged into pupae and 5% remained as larvae. Pupae remained highly vulnerable to cannibalism by adult mealworms. Dead larval or pupal mealworms (~5%) were also randomly recovered in the control-treated soil, particularly in sterilised soil; and the cadavers later developed into *Metarhizium*-like conidiation following their placement in a moist chamber.

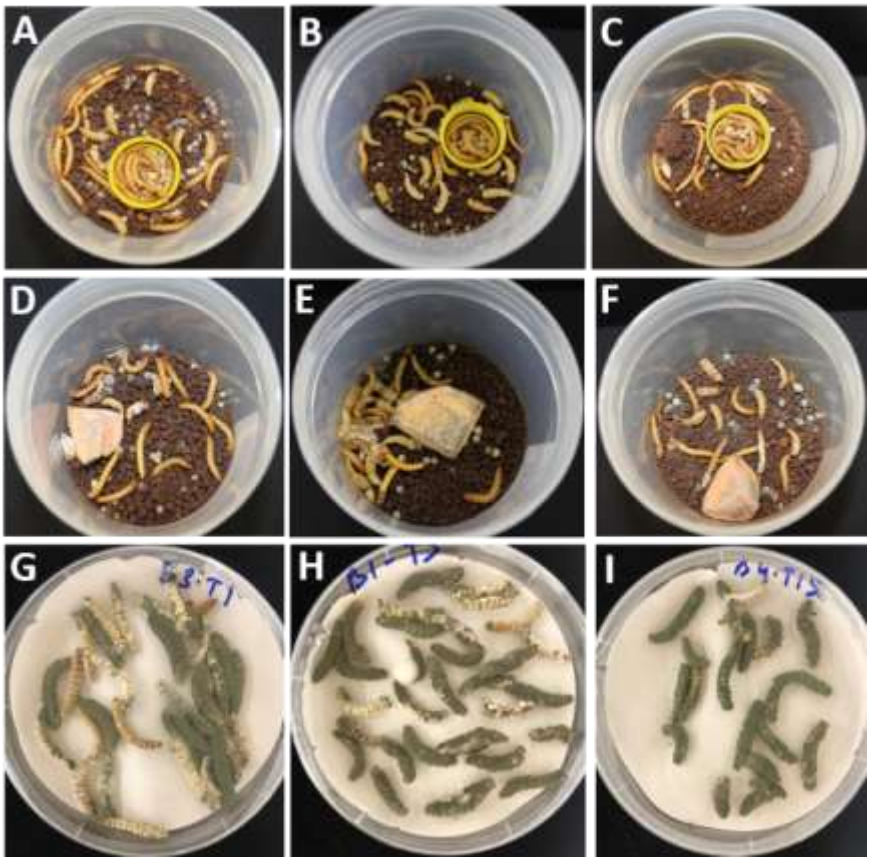


Figure 4.7: Dead mealworms after 14 days in non-sterile soil (A) or simulated solarised soil (B) or sterilised soil (C) with resporulated fungal granules; dead mealworms after 20 days on non-sterile soil (D) or simulated solarised soil (E) or sterilised soil (F) with resporulated fungal granules; and conidiated cadavers following incubation from non-sterile soil (G) or simulated solarised soil (H) or sterilised soil (I) with resporulated fungal granules

4.4 Discussion

Assessing the efficacy of fungal conidia produced from the granules is always necessary before their application for insect control. In this study, resporulated granules ($CAG_{Ma+Cs+By}$) used against larval mealworms resulted in about 90% mortality within 14 days of inoculation, confirming that the resporulated granules are highly pathogenic. Our observations of the rapid death of mealworms when exposed to *M. anisopliae* and the usefulness of them to bait EPF from the soil in low concentrations confirm that larval mealworms are quite susceptible to infection (Castro et al. 2016). It was noted that the mortality of larval mealworms was initiated as early as three days after inoculation that agrees with Lestari and Rao (2017), which reported that most larval mealworms treated with a fungal suspension (larvae were dipped) (*Metarhizium* spp. and *Beauveria* spp.) succumbed only five days after inoculation. Similarly, newly formed conidia of *M. brunneum*, from resporulated fungal granules caused 81% mealworm mortality within 14 days (Przyklenk et al. 2017). Early-onset of mealworm mortality, as observed in our study, could be attributed to the virulence of the conidia resporulated from the nutrient-rich fungal granule. Our previous study noted that fungal granules with corn starch and baker's yeast as food for the fungus can produce 100 times more conidia than that from the initial encapsulated conidia. Therefore, the early onset of mealworm mortality could also be due to the inundative fungal inoculum arising from the resporulated fungal granules. Research shows that fungal conidia originating from nutrient-rich culture, particularly nourished with nitrogen and carbon sources, tend to have more virulence than from nutrient-stressed environments (Dillon & Charnley 1990). It has been reported that conidia arising from nutrient-depleted fungal granules failed to cause as many insects' mortality as occurred in the nutrient-enriched granules (Moslim et al. 2009; Przyklenk et al. 2017).

Not surprisingly, dense fungal colonies in soil lead to effective control of soil insects. In our study, resporulated fungal granules grown on soil caused up to 96% mealworm mortality in a laboratory assay after 30 days. Mealworm mortality observed

in this experiment cannot be directly extrapolated to wireworm mortality, due to genus and species differences; for example only 60% mortality was noted after an eight-week period when wireworms were immersed in a fungal suspension (*M. brunneum*) then introduced into sterile soil in the laboratory (Razinger et al. 2020). Wireworm tolerance against some EPF has been linked with their symbiotic relationship with bacteria (for example the bacterial symbiont *Rickettsia insecticola* defends pea aphid (*Acrythosiphon pisum*) against entomopathogenic fungus *Pandora neoaphidis* (Su et al. 2013), which may protect the wireworm from the insect pathogen, for example, *M. brunneum* (Kabaluk et al. 2017). Bacterial symbiosis is common in soil insects, for example, wireworms, but such relationships might not be established in mealworms because they are usually reared in an artificial substrate, for example, wheat germ. We noted substantial mealworm mortality in non-sterile soil first at 14 days and then 20 days after insect release compared to that observed in the simulated/sterilised soils. We hypothesised the rapid and high mortality in non-sterile soil could be attributed to the prompt sporulation that occurred on the fungal granules after only a small amount of mycelial growth was produced. However, this is in stark contrast to what was observed in the sterilised soil, where excessive EPF mycelial growth occurred on the granules with little resporulation at the initial phase. In general, thermal treatment alters the soil chemistry, for example converting nitrogen into extractable ammonium (NH^+_4) in soil which favours the growth of soil microbes (Kitur & Frye 1983). Mycelial over-growth from fungal granules that occurred in sterilised soil could be attributed to the removal of fungistasis that allows the fungus to unilaterally utilise the co-encapsulated food additives and increased availability of soil nutrients following soil sterilisation favouring the fungal growth. Yet, substantial elimination of these microbes during the simulated solarisation might not occur (Wakelin et al. 1999) and consequently, both mycelial growth and subsequent resporulation were low in the fungal granules in simulated solarised soil. Soil solarisation is one soil disinfestation method, which can be used in soil to control the soil-borne pathogens before planting (Pasche et al. 2014). High concentrations of some soil microbes, for example, *Penicillium urticae*, in soil have been shown to produce substantial fungistasis against

externally applied EPF, which further imposes inhibition of the fungal growth by producing a water-soluble inhibitor (Inglis et al. 2001). Potentially, fungistasis in non-sterile soil could stimulate the fungus to conidiate on granules by reducing the length of the mycelial phase, which in turn could potentially drive the high mealworm mortality shortly after the insect release. This could be analogous to nutrient-stress conditions prevailing on substrates, which are considered as an impetus to shift from a hyphal stage to conidiation (Jaronski & Jackson 2012). Normally, the prolonged mycelial phase of EPF has been linked with the excess availability of nutrients, whereas nutrient stress leads to conidiation (Zimmermann 2007). The dense mycelium of fungal granules in sterilised soil potentially delayed the sporulation process that resulted in low mortality at 14 to 20 days after insect release.

A sharp increase in mealworm mortality observed at 25 and 30 days after the insect release in simulated solarised and sterilised soil could be attributed to the newly formed conidia. Low soil moisture usually constrains the growth and development of *Metarhizium* (Raid & Cherry 1992). Moreover, low humidity (< 90 % RH) also restricts fungal infectivity (Arthurs & Thomas 2001). Thus, soil remoistened with additional water on the 20^{th} day might have triggered the resporulation of the granules and better conditions for infection of the host, resulting in high mortality. As a result, overall mortality during the 30-day incubation was not significantly different among non-sterile, simulated solarised, and sterilised soil. However, resporulation patterns of fungal granules in non-sterile soil were found to be inconsistent with the previous experiments. Numerous interactions, including both biotic and abiotic factors, may explain these seemingly confounding results. The result from the previous study (Chapter 3) indicated that the fungal resporulation on non-sterile soil was significantly less than that on sterilised soil. However, the result of mealworm mortalities as found in this experiment did not correspond the previous finding of fungal resporulation. The high rate of mealworm mortality in non-sterile soil has been attributed to the conducive climatic conditions in the laboratory especially temperature and relative humidity, despite the limited fungal resporulation that occurred in non-sterile soil. Under the laboratory conditions, the fungus can resporulate to some extent from the fungal

granules that caused up to 100% mortality, but both resporulation and virulence may not be possible on glasshouse and field conditions due to the unfavourable abiotic conditions.

These results here showed that the low dose of conidia within granules resulted in the same level of mealworm mortality as the high dose did. The food-fortified fungal granules had the potential to reproduce a myriad of new conidia, which could lead to substantial mealworm mortality, despite the initial low dose of fungal granules applied. Secondly, mealworms are highly susceptible hosts to soil insect pathogens, which may lead to their death even in the presence of few infective propagules. Some larval mealworms quickly became melanised and such melanisation could occur because of an insect immune reaction against insect pathogens (Krams et al. 2013), on which host haemocytes are triggered to aggregate and encapsulate around the pathogen cell. However, the process of cellular immunity-mediated defence could result in the fitness cost, especially for susceptible hosts like mealworms, by reducing the insect life span (Clark et al. 2010; Krams et al. 2013).

We observed some *Metarhizium*-like sporulation on the larval mealworms reared on food granules (control) applied to sterilised soil, despite no evidence of cross-contamination between fungal granules and food granules. When food granules (control) were applied to sterilised soil, saprophytes such as *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* rapidly colonised the food granules, likely due to the lack of competing microbes in the soil. Whereas the aforementioned fungi were not visibly seen in sterilised soil with fungal granule inoculation because of the presence of *M. anisopliae* as an encapsulated organism in the fungal granules. Saprophytes such as *Aspergillus*, *Penicillium*, and *Fusarium* have been also linked with insect pathogenesis (Teetor-Barsch & Roberts 1983; Foley et al. 2014). Unlike entomopathogenic fungi, saprophytes are not well suited to insect pathogenicity, given a lack of appressorium structures and insect cuticle degrading enzymes, especially chitinase, lipase, and protease (St. Leger & Wang 2020). But these fungi can enter insects via ingestion, and induce an adverse impact on insect survival, as exemplified by immunosuppression

(Foley et al. 2014). The evidence for *Metarhizium*-like infection that appeared in larval mealworms in sterilised soil could be the result of interaction between saprophytic induced immune-suppression and an enzootic pathogen of mealworms, for example, *Metarhizium*. A study by Kabaluk et al. (2017) stated that an enzootic pathogen can turn pathogenic from asymptomatic status when microbial diversity around the host insect is depleted. However, our test showed that no mealworm mortality occurred when mealworms were reared in visually saprotrophic-free sterilised soil, hinting that soil saprotrophs might contribute to the mealworm mortality. But, according to an *in vitro* study, the saprophytic and entomopathogenic fungi interact antagonistically (Guven & Caltili 2016).

Significant mealworm mortality observed on non-sterile soil or simulated-solarised soil or sterilised soil with fungal inoculation within a short period has been attributed to the conducive arrangements in the laboratory, especially temperature and relative humidity, which could contribute to the optimal fungal granule resporulation making *M. anisopliae* more competitive against the soil microbes. However, such favourable conditions do not naturally exist on fields that potentially restrict the fungal growth, while soil microbes relatively show their great thermal plasticity making them more competitive than EPF in terms of nutritive food utilisation (Crowther & Bradford 2013). In context to our target insect pest wireworms, the significant level of fungal resporulation is necessary because wireworm is characterised with tough cuticle which is further strengthened by the microbial symbiosis defending the host insect against entomopathogens (Kabaluk et al. 2017). However, the climate conditions where sweetpotato is grown in Australia are likely to favour the persistence and resporulation of applied biopesticides, especially as targeted irrigation along the rows keeps the soil moist in times of no rainfall. These conditions are likely to maintain fungal resporulation from the fungal granules, especially near the crop root zone, which will potentially reduce the feeding damage in sweetpotato (host crop) roots. However, we know that indigenous soil microbes and their fungistasis activity can inhibit EPF resporulation and we also know that the greater fungal resporulation was found on sterilised or pasteurised soil (chapter 3, section 3.2.3) was possible linked to the

reduced soil microbes. A practice of soil disinfestation, for example, soil fumigation is used by some commercial sweetpotato growers in Australia to control soil-borne pests and pathogens. If used, fumigation is applied to the soil when the soil for bedding roots is prepared and has been used occasionally to manage soil fungi like scurf, which causes substantial cosmetic damage to storage roots. Apart from controlling soil-borne pathogens and pests, soil fumigation caused a broad range of microbial suppression, which could encourage and enhance the growth of biological control agents, for example, mycopathogenic fungus *Trichoderma harzianum* or entomopathogenic fungus *M. anisopliae* resulting in a pest-suppressive soil (Mazzola 2007).

Apart from being an excellent infect pathogen, *M. anisopliae* has also been shown to be rhizosphere competent and a root coloniser (Hu & Leger 2002), making it capable of conferring a small degree of crop protection to pest insects (Vega 2018). Soil insects including wireworms are reported to exhibit aversion towards the active colonies of *M. anisopliae* in soil (Villani et al. 1994; Ericsson & Kabaluk 2007). For long-term wireworm management, growers could focus on increasing fungal persistence and abundance in the field. To increase the fungal persistence in the field, rotation with a cover crop that can form a symbiotic relationship may be important to enhance the establishment of *M. anisopliae* in the soil (Boetel et al. 2012), whereas periodic application of fungal granules could help to maintain the presence of fungal propagules in the soil (Mayerhofer et al. 2015). Ensuring soil health is crucial to maintain long-term establishment of *Metarhizium* in soil that leads to sustainable wireworm management, for which rotation, cover crops, and increasing organic matter in soil are the basic cultural practices required. The delivery of fungal granules around sweetpotato roots is convenient using a band application method due to its granular form. Tape or drip irrigation is commonly used in sweetpotato production, the fungal application via drip irrigation could be a worthwhile study to be conducted in future but ensuring the efficacy of inundation of fungal propagules within rootzone via wetted perimeter is essential before conducting an experiment in field conditions.

The formulation used in the current study especially compressed baker's yeast was found to unwantedly encourage the growth of saprophytic microorganisms in the soil. Future studies could benefit from the use of nutrients other than those used here or investigating more sophisticated forms of encapsulating in layers to prevent the direct exposure of nutrients to competing microbes or by replacing the saprophytic-growth prone substrate with one that the saprophytic fungi do not use or by preconditioning the EPF fungal granules to allow mycelial growth before their application in soil. Preconditioned fungal granules can competitively utilise the encapsulated nutritive so that the chance of saprophytic growth in the food substrate could be abated (Lestan & Lamar 1996), while fungal mycelia are more resistant to the soil fungistasis than conidial mediated inoculum. Alternatively, the use of microsclerotia as a propagule of fungal granules can be an area to be explored as microsclerotia is reported to show resistance to fungistasis in the soil (Behle et al. 2013).

Our previous laboratory-based studies (chapters 3 and 4) clearly showed that the soil-applied fungal granules significantly resporulated and the resulting fungal resporulation was able to kill significant larval mealworms when these insects were exposed to soil with the fungal resporulation. To validate the efficacy of fungal granules, further examination of fungal granules under the glasshouse condition in the presence of a host plant is essential to conduction. Therefore, the following study consisted of evaluating the morality efficacy of fungal granules against larval mealworms in the glasshouse.

CHAPTER 5: INFECTIVITY OF SOIL-APPLIED FORMULATED *METARHIZIUM ANISOPLIAE* TO *TENEBRIO MOLITOR* IN THE GLASSHOUSE

Abstract

The performance of *M. anisopliae* as a biological control agent is heavily influenced by both abiotic and biotic factors and congruency usually lacks in the results between laboratory and field studies. Soil temperature and moisture are the primary abiotic factors impacting the fungal growth, infectivity, and persistence in soil. For the biotic factors, the indigenous soil microbiota is the most influential factor determining the fungal survival and overall biocontrol efficacy for soil insects, while host plants have a significant role in shaping the structure of the soil microbial community. A glasshouse experiment was performed to examine the infectivity study of resporulated fungal granules on mealworms. The results demonstrated that the resporulated fungal granules caused 60% mortality after 30 days. The fungal granules applied on sterilised soil caused significantly greater mealworm mortality than those applied to non-sterile soil. Results from this study suggest that *M. anisopliae* granules could play a key role in a management program for pests like wireworm in sweetpotato.

5.1 Introduction

Sweetpotato (*Ipomoea batatas* L.) is one of the major root-vegetable crops in Australia, generating AU$ 100 million annually (ASPG 2020) with 90% of the national production concentrated in Queensland, particularly in Bundaberg (ABS 2013). An estimated 96% of total production (storage roots) is supplied to the domestic fresh market (Australian Horticulture Statistics Handbook 2015). Being a root crop, sweetpotato is predisposed to attack from root herbivores, which generally leads to unmarketable produce. Root herbivores such as sweetpotato weevil (*Cylas formicarius*), root-knot nematodes (*Meloidogyne javanica* and *M. incognita*), and wireworms (both Elateridae and Tenebrionidae family) have been identified as important pests of sweetpotato crop in Australia, causing considerable damage to the storage roots, and consequently, leading to market rejection (McCrystal 2010). Moreover, these pests also pose the threat of damage in global sweetpotato production (Seal et al. 1992b; Mukhopadhyay et al. 2011; Pasche et al. 2014; Hue & Low 2015; Arrington et al. 2016).

Wireworms, the larval stage of coleopteran beetles belonging to Elateridae and Tenebrionidae, are ubiquitous, polyphagous insects attacking a diverse range of crops such as sugarcane, cereals, potato, and sweetpotato (Seal et al.1997; Parker & Howard 2001; Samson & Calder 2003). In Australia, both Elateridae (true wireworms) and Tenebrionidae (false wireworms) are also pests for sweetpotato, with the damage typically characterised as shallow and scattered holes on the root periderm (McCrystal 2010). In the United States, the Elaterid wireworm, *Conoderus* spp., has been recognised as one of the most devastating wireworms for sweetpotatoes, especially in the southern states (Brill 2005).

Wireworm damage on sweetpotato storage roots causes economic losses to the growers, especially in developed countries like Australia, either the produce is rejected (with moderate to severe damage) or downgraded from premium (with minor damage). As a result, the growers in Australia often apply soil insecticides, for example, Talstar® (Bifenthrin) or Regent® (Fipronil), to control wireworms (APVMA 2021);

however, crop losses of up to 21% are still observed. Wireworm activity is typically prevalent in the weeks leading up to harvest and the efficacy of soil insecticides generally does not persist in soil throughout the cropping period (McCrystal 2014). Thus, treating a field with soil insecticide before planting for wireworm control is not often effective. Additionally, the tough exoskeleton of wireworm, coupled with its ability to evade the chemical-treated zones by moving deeper into the soil profile, limits the effectiveness of the insecticides (Parker 1996). A 2014 study showed that the intermittent application of chemical insecticide (Regent®) via drip irrigation in sweetpotato root zones until the late stage of crop growth completely protected the crop against the wireworm infestation (McCrystal 2014), but the food safety of the chemical-treated produce for human consumption is still questionable. Ongoing economic loss for sweetpotato growers in Australia from continuous pest pressure highlights the need for an alternative to the current pest-control practices (McCrystal 2014). The adoption of alternative methods to chemical control, for example, biological control using entomopathogenic fungi, has been proposed as a promising alternative that could be incorporated into an integrated pest management program (IPM).

Entomopathogenic fungi (EPF) are soil-resident organisms that naturally infect soil insects (Zimmermann 2007). For example, *Metarhizium brunneum* has been isolated from wireworm (Kabaluk 2017). Four genera of EPF: *Metarhizium*, *Beauveria*, *Isaria*, and *Lecanicillium*, have been developed as biopesticides for insect control (De Faria & Wraight 2007). *Metarhizium* is one of the most studied, displaying pathogenicity against more than 200 insects, particularly to coleopteran insects (Pilz et al. 2011). Moreover, some species of *Metarhizium* are root endophytes and even colonise the rhizosphere (Krell et al. 2018; Vega 2018). These may endophytes play a role in crop protection by deterring attacks from root herbivores (Parsa et_al. 2013). Therefore, there has been a longstanding endeavour to exploit the *Metarhizium* species as a biological control agent for soil insect control (Roberts & Leger 2004). In conventional practice, fungal propagules have been inundatively applied to soils to control soil-dwelling insects, but fungal viability sharply declines once it is applied to

soil (Gasic & Tanovic 2013). In some cases, the fungus takes substantial time for its establishment and further colonisation following its application in soil, which limits the efficacy of the fungus to protect crops when they experience high pest pressure, especially annual field crops like sweetpotato (Pilz et al. 2011). Alternatively, EPF colonisation in fields can be expedited by adopting a modified formulation, in which the fungal propagules can be combined with the food sources to benefit the EPF. Lack of appropriate food resources in the vicinity of the fungal propagules in soils is one of the constraints that potentially limits fungal colonisation in soil (Jackson et al. 2010; Jaronski 2010). As a result, the notion of co-application of fungal inocula combined with exogenous food sources has been proposed. For example, this approach has been used for *M. anisopliae* conidiated on rice grain (Kabaluk 2014), on rice bran (Moslim et al. 2009), and millet grain (Rath et al. 1995). The co-application of fungal inocula with food additives may be further improved using a calcium alginate polymer (Humbert et al. 2017).

Fungal granules like calcium alginate encapsulated *M. anisopliae* with food substrates have been formulated for soil insect control, for example in wireworms (Lackey et al. 1993; Vemmer & Patel 2013). In principle, these fungal granules have the potential to produce rapid secondary sporulation in soil due to the supply of food additives required for fungal growth (Przyklenk et al. 2017). Despite this potential, the application of encapsulated *M. brunneum* in calcium alginate with food supplements did not reduce wireworm damage in a potato field (Brandl et al. 2016) or a glasshouse experiment (Mayerhofer et al. 2017), implying that the fungal granules might not have resporulated to the highest capacity in the soils. Food substrates associated with the EPF granules are usually targeted by soil microbes, particularly by saprotrophs when the EPF granules are applied in the field (Lestan & Lamar 1996). In general, the soil is a microbial pool, harbouring up to 10^8 to10^9 bacteria per gram of soil (Jaronski 2010). Opportunistic fungal saprotrophs, such as *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium*, and *Rhizopus* directly colonise the food substrates of the EPF granules because the conidia of *M. anisopliae* germinate relatively slowly, taking 18 to 24 h in optimal conditions (Dillon & Charnley 1990). Furthermore, *M. anisopliae* conidia

extracted from saprotroph-contaminated fungal granules fail to germinate even in optimal conditions (personal observation), implying that the conidia might have been debilitated due to antifungal compounds or other chemicals released by the saprotrophic fungi.

Fungistasis is common in the soil but may be mitigated to some extent, by the process of soil sterilisation (Jackson et al. 2010). A practice of soil disinfestation, for example, soil fumigation, has occasionally been used to control the soil-borne pests in the sweetpotato fields of Australia and is more commonly used in nursery bed preparation (McCrystal 2014). Thus, it is possible that the resporulation assessment of fungal granules in the sterilised soil, detailed in the work presented here, could be likened to the disinfested fields. However, until recently, there has not been any evidence showing the efficacy of controlling insects with calcium alginate encapsulated *M. anisopliae* fortified with food substrates inoculated in sterilised soil when sweetpotato is planted as a host plant.

Alternatively, preconditioning EPF by allowing them to resporulate before their application into the soil may enhance their ability to combat fungistasis, providing a competitive advantage against native soil organisms (Lestan & Lamar 1996). For this reason, we hypothesise that the actively growing fungi (resporulated granules), before their inoculation, can overcome the soil microbial resistance when fungal inocula are applied into the soil because they are already primed for rapid and ongoing germination.

To better understand the resporulation and infectivity of fungal granules on soil with sweetpotato as a host plant, the objectives of this study consist of evaluating the infectivity of either the preconditioned fungal granules or non-preconditioned fungal granules inoculated on either sterilised or non-sterile soil under the glasshouse condition and assessing the effect of temperature on the fungal resporulation from the fungal granules under laboratory conditions.

5.2 Materials and methods

5.2.1 Glasshouse pot experiment

5.2.1.1 Treatments

Metarhizium anisopliae strain QS155, formulated with calcium alginate encapsulation was used as the inoculum in all experiments. The fungal formulation was prepared by encapsulating the freshly harvested conidia (1% w/w) into calcium alginate granules, supplemented with corn starch (20% w/w) and autoclaved baker's yeast (20% w/w) referred to as the fungal granule ($CAG_{Ma+Cs+By}$) henceforth (Figure 5.1**)**. After the fungal granules were prepared, they immediately underwent 11 h of drying in a laminar flow cabinet (Labec Laboratory Equipment) at room temperature, resulting in 73% moisture loss from the fresh weight. This ensured that the granules were less susceptible to contamination by opportunistic saprotrophs during storage. The dried granules were packed into a sterile plastic container and stored at 5 $^{\circ}$C until used. The average dry weight of fungal granules was calculated as 14 mg per granule containing a mean of 10^7 conidia per granule. A process for determining the conidia numbers contained by the granule included the homogenisation of the fungal granule in a solution of citric acid (0.03 M) and calcium bicarbonate (0.05 M) using a vortex for 5 min, and then the enumeration of conidia in the suspension using an improved haemocytometer (Neubauer improved double net ruling), viewed at ×400 using a compound microscope (Olympus BX53).

For the glasshouse experiment, the fungal granules were used at two distinct levels: one with preconditioning of the granules before inoculation, and the other without preconditioning (Figure 5.1). For preconditioned (myceliated) granules, the fungal granules were incubated at 25 $^{\circ}$C for 24 h by placing them on 3% water agar to encourage fungal germination and growth prior to the inoculation into the soil, whereas fungal granules which were directly applied into the soil without any previous incubation, were referred to as non-preconditioned fungal granules.

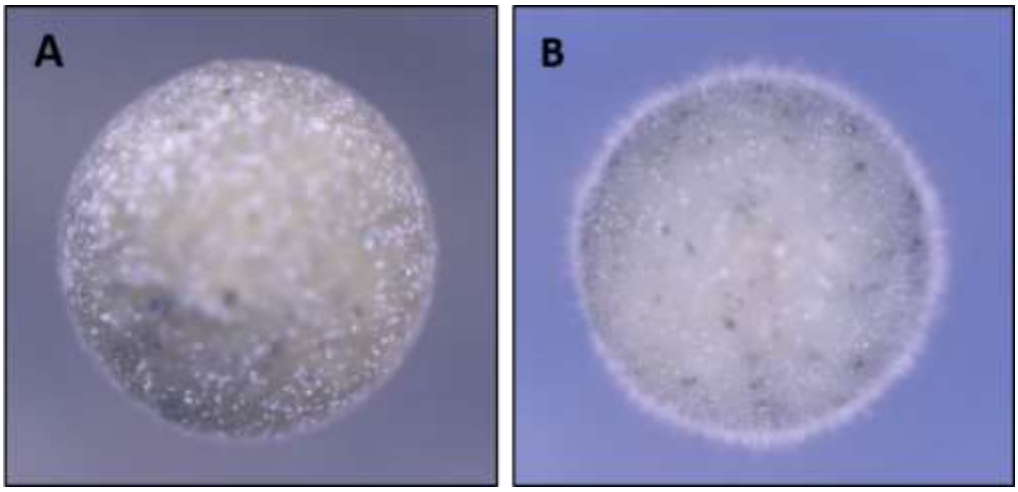


Figure 5.1: A fungal granule ($CAG_{Ma+Cs+By}$) with 3.5 mm diameter and 14 mg weight prepared for the glasshouse experiment and thermal regime experiment, referred to as a non-preconditioned granule (A); and myceliated fungal granule appeared after a 24 h incubation of fungal granules, referred to as preconditioned granules in the glasshouse experiment (B).

5.2.1.2 Soil sampling

Soil samples (soil texture: clay, pH= 6.6, EC= 0.069 dS/m, carbon %= 3.2257 and nitrogen %= 0.226) were collected from the agricultural field of University of Southern Queensland (USQ), Toowoomba (Lat. 27˚36` 33``S, Long. 151° 55` 55`` E) that had been previously cropped with barley. Following sampling, soil samples were immediately transported to the soil processing laboratory of USQ, where the soil samples were air-dried for 24 h, homogenised, and passed through a sieve strainer (10 mm) to remove plant debris and larger particles. Following soil processing, the soil sample was immediately stored in a cool room (10 ˚C) until used.

For the process of soil sterilisation, soil samples were placed inside an oven drier (Steridium Ovens, http://www.eurotherm.com) at 105 ˚C for 3 days. The oven-dried soil is denoted as ‘sterilised soil’ henceforth, while soil samples used in the glasshouse pot experiment without oven sterilisation were regarded as ‘non-sterile soil’ containing 20% (w/w) moisture content.

5.2.1.3 Glasshouse experiment

The experiment was conducted in a glasshouse (Agriculture Science and Engineering Precinct, USQ) at 18 °C- 30 °C and 60% relative humidity from May until August 2019 (Figure 5.2 A). A plastic pot (d= 25 cm, h= 23.5 cm) containing 6 kg of either non-sterile or sterilised soil was inoculated with preconditioned fungal granules or non-preconditioned fungal granules at the rate of 2 g of granules (Ø = 3.5 mm) per pot, equivalent to 10^5 conidia cm^{-3} of soil. Each of the four treatments was replicated six times resulting in 24 pots. For the controls, a calcium alginate granule containing 20% corn starch and 20% autoclaved baker's yeast without *M. anisopliae,* termed the 'food granule' (CAG_{Cs+By}) and preconditioned food granules (incubated at 25 °C for 24 h for food granules) were included. In summary, this experiment comprised eight treatments including: **(i)** Non-sterile soil plus non-preconditioned fungal granule; **(ii)** Non-sterile soil with non-preconditioned food granule (control); **(iii)** Sterilised soil with non-preconditioned fungal granule; **(iv)** Sterilised soil treated with non-preconditioned food granule (control); **(v)** Non-sterile soil with preconditioned fungal granule; **(vi)** Non-sterile soil with preconditioned food granule (control); **(vii)** Sterile soil with preconditioned fungal granule; and **(viii)** Sterile soil with preconditioned food granules (control). All pots were arranged in a randomised complete block design in the glasshouse.

Each pot was firstly filled two-thirds with soil, then a pre-sprouted Bellevue sweetpotato root was planted horizontally (mean weight 600g, mean diameter 8 cm), and the root with the remaining one-third of the soil, which was premixed with fungal granules at the rate noted above. After planting, soil in each pot was irrigated with 200 mL of tap water, while the disparity of moisture content lying between sterilised soil (0% moisture content w/w) and non-sterile soil (20% moisture content w/w) was equalised by adding watering the pots to weight, which was allowed to soak overnight prior to the inoculation.

On the 30^{th} day after the inoculation of granules, a cohort of 100 larval mealworms (mean body length 2.65 cm; body weight 0.1 g; and 8 abdominal rings)

was added to each pot. The soil surface was then covered with 2 cm pre-sterilised (105 °C for 24 h) sugarcane mulch as mealworms prefer shade. During the experimental period, sweetpotato shoots longer than 20 cm high were pruned twice, on the 20th day and the 40th day after planting to avoid the vines touching one another.

5.2.1.4 Pot harvest and data collection

On the 30th day after the insect release into the inoculated soil, the glasshouse experiment was terminated by harvesting the experimental pots. Pot harvesting was carried out by removing the shoots first, and then the roots were separated from the soil. Mealworms were recovered from the soil and their mortality status was assessed (Figure 5.2). The number of dead or live mealworms recovered from the individual pots were recorded, with the life stage of mealworms recovered from the soil recorded i.e., larva, pupa, or adult. The health status of live mealworms was further assessed classifying them into vigorous vs moribund. Similarly, the dead insects (cadavers) were categorised into mycosed and non-mycosed based on conspicuous fungal outgrowth over the cadaver (Figure 5.2). Non-mycosed cadavers were placed over a moist chamber and incubated at 25 °C to stimulate the fungal conidiation on the cadavers.

Live mealworms that were recovered after the harvest were further examined to confirm whether infection could be forced in a smaller area of soil. Collected insects were placed into a 50 ml plastic container containing 40 g of soil sampled from individual pots (Figure 5.2). After the insects were transferred, the soil in the container was moistened with 5 mL of tap water and sealed with a perforated lid. The soil in the container was inverted 5 times and half a spoonful of corn seeds (autoclave sterilised) was added as food for the insects. The containers were incubated in the same glasshouse (18 °C-30 °C and 70% RH) and after 18 days, mealworm mortality was assessed (Figure 5.2). Non-conidiated cadavers were transferred to a Petri dish lined with a moist filter paper and incubated at 25 °C (photoperiod 12:12 h day and night) for 7 days to encourage resporulation from the cadavers.

The sweetpotato storage roots were assessed for feeding damage caused by mealworms (Figure 5.2). Roots with any number of feeding holes were rated as damaged and roots without holes were rated as non-damaged roots.

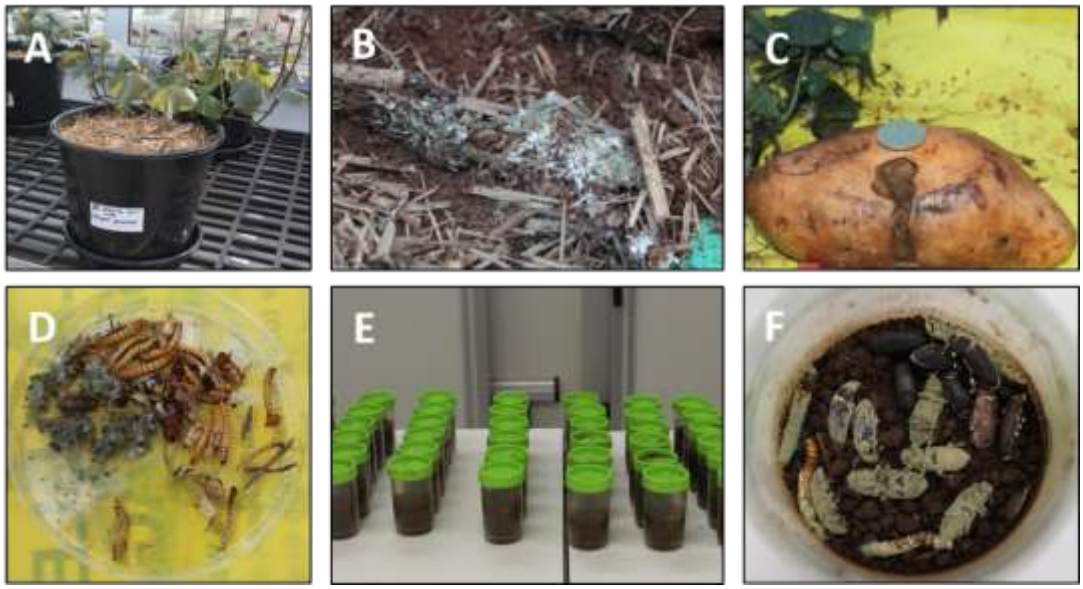


Figure 5.2: An experimental unit (pot with a sweetpotato plant) in the glasshouse (A); dense conidiation of *M. anisopliae* QS155 developed on a mycosed mealworm and surrounding mulch during the glasshouse experiment (B); a sweetpotato root with mealworm feeding damage (C); mealworm cadavers collected during the experiment (D); live mealworms recovered after the pot harvest further evaluated the latent infection of *M. anisopliae* QS155 in the laboratory (E); and *M. anisopliae* QS155 growth on mealworm cadavers after 18 days of laboratory observation were live insects were placed back into a sub-sample of experimental soil (F)

5.2.2 Effect temperature on sporulation of M. anisopliae

Fungal granules ($CAG_{Ma+Cs+By}$) were tested for their ability to resporulate in contrasting ambient temperatures. For this experiment, a single freshly prepared fungal granule was inoculated into a Petri dish (Ø, 3 cm) containing 3% water agar. The Petri dishes were sealed with Parafilm® and then incubated at three different temperature regimes: 25 ˚C, 35 ˚C or 45 ˚C in the dark. Each temperature regime was considered as the treatment giving three treatments altogether. There were six replicates per

treatment and the entire experiment was repeated thrice, rotating through the different incubators.

On the18th day after incubation, the granules in each Petri dish were inundated with 5 mL of sterile 0.05% Tween®80, all the conidia were scraped off using a sterilised spatula and transferred into a sterile 1.5 ml tube. The fungal suspension was homogenised using a vortex and the conidial concentration from fungal suspension was determined using a haemocytometer. The viability of conidia remaining in the suspension was also assessed, for which 200 µL of suspension was spread over a glass slide containing SDAY. The glass slides with the inoculated fungal suspension were sealed inside a Petri dish lined with a moist filter paper and then incubated at 25 $^{\circ}$C during a 12:12 h day and light photoperiod. At 12 h or 14 h or 16 h or 20 h after the incubation, the germination of conidia and the length of germ tubes were assessed at ×400 magnification using a compound microscope (Olympus MVX10).

5.2.3 Data analysis

Mealworm mortality data obtained from the glasshouse was converted into a percentage, transformed using a log10 transformation to achieve normal distribution, and analysed using Univariate Analysis of Variance (SPSS version 24). Multiple pairwise comparisons were performed using the *post hoc* test (Tukey). The Correlation between the surviving mealworms and sweetpotato root damage was tested by using Pearson's correlation test.

For the temperature experiment, the repeated experiment was pooled after confirmation of no significant variation between data sets and analysed with an ANOVA. All statistical analyses were carried out by using the SPSS software (IBM SPSS Statistics 24).

5.3 Results

5.3.1 Glasshouse pot experiment

Thirty days after the mealworms were added to the pots the experiment was terminated. Exposure of mealworms to the preconditioned fungal granules in sterilised soil and non-sterile soil resulted in 60.82% (±15.62) and 17.96% (±7.58) mealworm mortality, respectively. Mealworms exposed to the non-preconditioned fungal granules inoculated in sterilised soil and non-sterile soil resulted in 42.18% (±14.60) and 13.63% (±10.65) respectively ($P = 0.001$). Likewise, the main effect of fungal granules (non-preconditioned and preconditioned) was also significantly different ($P = 0.036$). However, no significant ($P = 0.178$) interaction was found between two factors i.e., fungal granule type and soil treatment. Fungal-derived mealworm mortalities were significantly greater in sterilised soil than in non-sterile soil, irrespective of the types of fungal granules i.e., preconditioned or non-preconditioned fungal granules ($P < 0.05$). No significant difference ($P = 0.556$) in terms of mealworm mortality was observed between preconditioned and non-preconditioned fungal granules inoculated in the non-sterile soil, whereas a significant ($P = 0.018$) difference was found between the preconditioned and non-preconditioned fungal granules inoculated in sterilised soil (Figure 5.4).

Mealworms introduced on sterilised soil with preconditioned food granules and non-preconditioned food granules during the glasshouse study succumbed to the fungal-derived mortalities at 3% (±4.45), and 4% (±5.5) respectively, which were significantly lower than those mortalities on soil with fungal treatments ($P < 0.05$), but preconditioned and non-preconditioned food granules on non-sterile soil did not cause any fungal related mealworm mortalities.

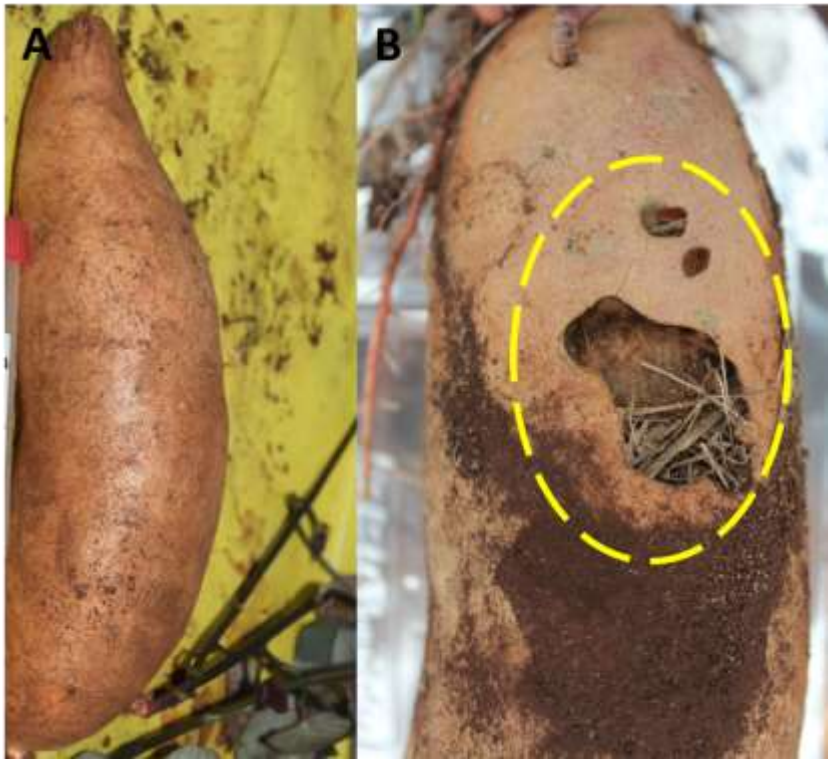


Figure 5.3: Non-damaged mother sweetpotato roots at 30 days of mealworms exposure on sterilised soil with preconditioned food granules (control treatment) (A), and mealworm damaged sweetpotato mother roots (inside the yellow circle) at the end of 30 days mealworm introduction on sterilised soil with preconditioned fungal granules (B).

No positive correlation was observed between the damaged sweetpotato roots and the number of live mealworms recovered (at $P > 0.05$) (Figure 5.3). Following the glasshouse experiment, the recovered live mealworms were further assessed to check for latent infection by the fungus. After exposing live mealworms to experimental soil in confined containers, mortality was reassessed. Mealworms added to fungal granule-treated soil had significantly greater mortality than those exposed to food granule-treated soil ($P < 0.05$) (Figure 5.4 B). Both preconditioned and non-preconditioned fungal granules inoculated in sterilised soil resulted in 81% (±8.1), and 81% (±3.8) mealworm mortality respectively, while mealworm mortalities at 58 % (±8.4) and 59% (±11.34) were achieved non-preconditioned fungal granules and preconditioned fungal granules inoculated on non-sterile soil respectively (Figure 5.4 B). The mealworm

mortality observed in sterilised soil inoculated with fungal granules was significantly ($P < 0.05$) greater than that observed in non-sterile soil, irrespective fungal granules type.

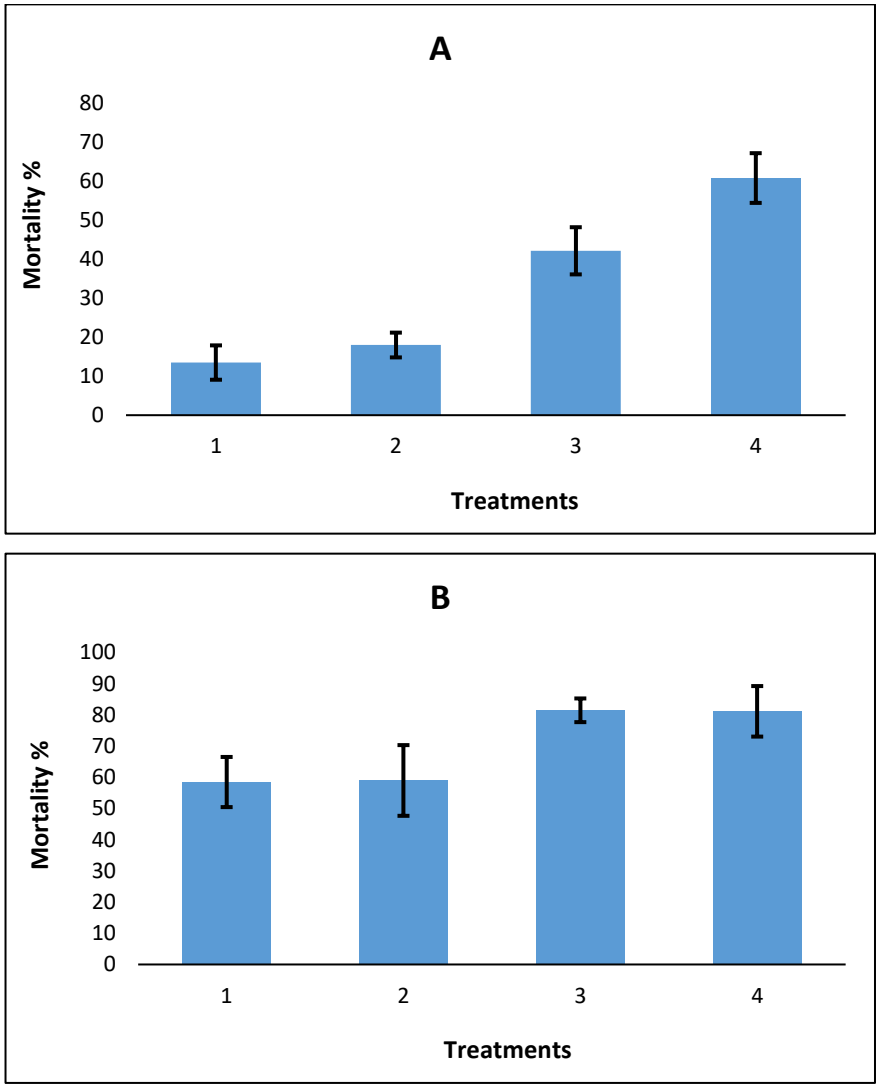


Figure 5.4: Mealworm mortality (mean ± SE, replicates = 6, $P = 0.05$) shown by non-preconditioned fungal granules on non-sterile soil (1), preconditioned fungal granules on non-sterile soil (2), non-preconditioned fungal granules on sterilised soil (3), and preconditioned fungal granules on sterilised soil (4) during the glasshouse conditions (A), and post-glasshouse bioassay in laboratory conditions (B).

5.3.2 Effect temperature on sporulation of M. anisopliae

Fungal granules incubated at 25 °C ($3.36 \times 10^7 \pm 5.2 \times 10^6$ conidia per granule), 35 °C ($2.75 \times 10^6 \pm 1.19 \times 10^6$ conidia per granule), and 45 °C ($7.08 \times 10^5 \pm 4.3 \times 10^5$ conidia per granule) produced a significantly different number of fungal conidia ($P < 0.05$). The fungal conidia at 25 °C were significantly greater than those at 45 °C ($P < 0.05$), whereas no significant ($P > 0.05$) difference was observed between 35 °C and 45 °C in terms of conidia numbers. The conidia harvested from the fungal granules maintained at 45 °C failed to germinate, whereas conidia obtained from fungal granules maintained at 35 °C reached 64% conidial germination with a mean 6 µm long germ tube within a 14 h of incubation. Conidial germination was >98% with a mean of 11 µm germ tube length occurred from the fungal granules maintaining at 25 °C. In terms of the physical appearance of fungal granules following the incubation, fungal granules at 25 °C were extensively conidiated, while myceliated fungal granules were found at 35 °C. For the granules incubated at 45 °C, no sign of fungal growth was visible over the fungal granule (Figure 5.5).

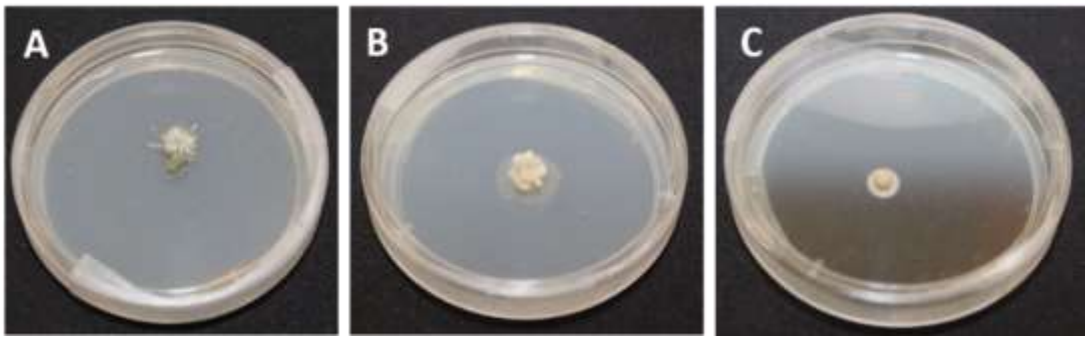


Figure 5.5: Conidiation on a fungal granule ($CAG_{Ma+Cs+By}$) after 18 days incubation at 25 °C (A); myceliated fungal granule ($CAG_{Ma+Cs+By}$) after 18 days incubation at 35 °C (B); and degraded fungal granules ($CAG_{Ma+Cs+By}$) because of incubation at 45 °C (C).

5.4 Discussion

Our glasshouse study showed that both preconditioned and non-preconditioned fungal granules on non-sterile soil caused 17.96% ±7.58 and 13.63% ±10.65 mealworm mortalities, respectively. Based on this evidence, it is implied that the fungal inocula applied on non-sterile soil are likely to be inhibited by soil microbes. A study conducted by Rogge et al. (2017) demonstrated that the fungal granules (calcium alginate encapsulated *M. brunneum* with autoclaved baker's yeast as food for the fungus) applied in soil could not enhance their density in soil, nor safeguard potato tubers against wireworm damage. Field soil containing a multitude of native microbes has been usually blamed for obstructing the fungal sporulation in soil (Garbeva et al. 2011). Fungistasis seems quite possible especially in the food-fortified fungal granules (Bonanomi et al. 2013). In our observation, when the fungal granules were applied in non-sterile soil, opportunistic saprotrophs living in the soil, such as *Penicillium*, *Aspergillus*, *Mucor*, *Rhizopus*, and *Trichoderma*, started to rapidly grow over the fungal granules by exploiting the co-encapsulated food substrates (Inglis et al. 2012). When saprotroph growth occurs over the fungal granules, the encapsulated *M. anisopliae* is also subject to the viability impairment potentially due to the antibiotic effect of saprotrophs (Lingg & Donaldson 1981). A follow-up viability test of fungal conidia, which were extracted from saprotroph-grown fungal granules, failed to revive in the selective media of *M. anisopliae*. The second plausible reason is that a temperature spike (up to 49 $^{\circ}$C for at least 7 days intermittently) that occurred during the fungal sporulation period impaired the fungal growth and development. Insect mortality is dose-dependent (Ansari et al. 2011) and the fungal density of at least 10^6 conidia g^{-1} soil is required for wireworm infection by *M. anisopliae* in the field (Kabaluk et al. 2007). In the work presented here, the fungal granules applied on non-sterile soil produced, by far, less fungal density than that needed to result in mealworm to cause the mortality on non-sterile soil. Moreover, the relatively low overall rate of death in this experiment irrespective of treatment (soil) suggests that the concentration of conidia was insufficient, or that the mealworms effectively avoided soil containing EPF, opting to shelter safely under the provided sugarcane mulch instead. The

recovery of some EPF infected mealworm cadavers on sterilised soil with food granules (control treatment) has been attributed to cross-contamination inside the glasshouse, likely due to the fan moving air and subsequent airborne conidia of QS155 during temperature regulation.

Both preconditioned and non-preconditioned fungal granules showed 60.82% ±15.62 and 42.18% ± 14.60 mealworm mortalities on sterilised soil. The greatest mealworm mortality shown in this study has been linked to the reduced fungistasis on sterilised soil, as soil sterilisation, a widely adopted method to remove the fungistasis could allow the fungal granules to resporulate into the fullest capacity by exploiting the food substrates. A study conducted by Susurluk (2007) confirmed that the mortality efficacy of entomopathogenic nematodes (*Heterorhabditis bacteriophora* or *Steinernema feltiae*) against larval mealworms (*T. molitor*) was significantly higher in sterilised soil than in non-sterile soil. The findings of this experiment align with the previous findings that showed the significant mealworm mortality found in sterilised soil inoculated with the fungal granules, whereas low mealworm mortality was obtained in non-sterile soil. Some studies suggest that an actively growing microorganism before its inoculation into the soil can resist the antagonism of indigenous soil microbes, to some extent (Lestan & Lamar 1996). Our study agrees with this because the preconditioned granules profusely resporulated in both sterilised and non-sterile soil, subsequently causing more mealworm mortality than that resulting from non-preconditioned granules. A study by Mayerhofer et al. (2017) highlighted that *M. brunneum* conidiated on autoclaved barley kernels did not allow the growth of soil saprotrophs because the fungus has already occupied the nutrient available in the barley kernels. In our case, the preconditioned granules were incubated for 24 h before the inoculation, providing a longer period of activation to achieve the profuse fungal growth over the granules. In a laboratory test, the fungal resporulation from the fungal granules was significantly lower in non-sterile soil than in sterile soil. Poor fungal resporulation in non-sterile soil has been attributed to the presence of background soil microbes in soil. To make the fungal inocula competitive with existing soil microbes, the EPF granules were preconditioned prior to their application into soil

which demonstrated comparatively better results than non-preconditioned inocula. Regarding the relevance of sterile and unsterile to the real world, sterile soil can be somewhat comparable to disinfected soil in crop fields, however soil disinfection up to the level of sterile soil is infeasible in field.

Moreover, homogeneous infection is unlikely in the soil environment, as opposed to the enforced inoculation conducted in a laboratory (Bruck et al. 2005), because insects might evade the infectious fungal propagules in soil (Jaronski 2010). Mycosed cadavers were found in the preconditioned inoculated soil which could contribute to long-term insect control by providing an additional source of inoculum, however, such consideration was beyond the scope of our study. Despite the inoculation of fungal granules in soil, the planted storage roots still experience some feeding damage by larval mealworms, probably because the root was challenged to 100 individuals and entomopathogen-based insecticides alone cannot prevent damage with such high insect pressure (Mayerhofer et al. 2015). We aimed at targeting the control of soil insects like wireworm in sweetpotato. Wireworm naturally tends to be attracted to the host roots by following the CO_2 gradient. In our observation, mealworms tend to live at the soil surface or sub-surface underneath the mulch we provided, whereas mealworms feeding in deep soil profiles were found with notable mortality due to optimal soil moisture in deep soil. Following the laboratory bioassay, live mealworms that were recovered at the time of pot harvest succumbed to fungal-derived death, confirming that mealworms that recovered at the end of the experiment carried asymptomatic infections. Some studies also revealed that fungal asymptomatic infection in insect hosts has been linked to adverse impacts of egg-laying, hatchability, longevity, and feeding efficacy of host insects (Quesada-Moraga et al. 2004; Jarrahi & Safavi 2016).

This glasshouse study has indicated that the existing level of fungal resporulation on non-sterile soil from the fungal granules may not be able to safeguard sweetpotato against wireworm infestation due to the failure of optimal resporulation from the fungal granules. However, since the results with high mealworm mortalities appeared

in sterilised soil, the efficacy of fungal granules can be enhanced by integrating the inoculation of fungal granules with a soil disinfecting method, for example, soil fumigation in very high pest populations, or with existing registered insecticides of sweetpotato, for example, Talstar® or Regent® (AVPMA 2020). *Metarhizium anisopliae* has shown compatibility with various insecticides acting synergistically against insect control, for example, *M. anisopliae* and spinosad (insecticide derived from actinomycete toxin) giving a synergistic effect for wireworm control (Erricsson et al. 2007), and similarly, the farm registered insecticides for macadamia in Australia such as certain concentrations of Lancer® (acephate) and Avatar® (indoxacarb) was synergistic with *M. anisopliae* against macadamia seed weevil (*Kuschelorhynchus macadamiae* Jennings and Oberprieler) under both laboratory and glasshouse conditions (Khun et al. 2021). Thus, future studies could be oriented towards the compatibility study between the registered sweetpotato insecticides and *M. anisopliae*. Moreover, the use of the microsclerotia or blastoconidia of *M. anisopliae* as a propagule for the fungal granules is also an area to be explored in the future, while microsclerotia is unaffected by fungistasis, and the germination of blastoconidia is faster than conidia. Additionally, the sporulation of fungal granules can be further assessed in soils pre-treated with fumigants because microbial suppression in soil resulted from soil fumigation could be conducive for the fungal resporulation. Optimising the preconditioning of fungal granules before their application into the soil can be also included in future studies.

Our laboratory study confirmed that the temperature regime at 25 ˚C not only stimulated the greatest fungal resporulation but also favoured the conidial viability by showing >98% conidial germination. This evidence again reassured the fact that the genus *Metarhizium* is characterised as a mesophilic organism showing its thermal preference between 10 ˚C and 40 ˚C (Arthurs & Thomas 2001). *Metarhizium* shows intra-species variability in relation to thermal preference, which is usually dictated by their geographical origin (Vidal et al. 1997), although the germination and subsequent growth and development of most *Metarhizium* species are impaired when exposed to a temperature of 40 ˚C (Rangel et al. 2010). *Metarhizium* species isolated from high

latitudes were found to have a lower optimal temperature, while species collected from low latitudes, for example in Thailand (15^{o} N) and Australia (19^{o} S), can tolerate higher temperatures (45 $^{\circ}$C) (Alston et al. 2005). Sweetpotato cultivation is concentrated in the sub-tropical and tropical regions of Australia, and specifically in Bundaberg Queensland, where the soil temperature exceeds 40 $^{\circ}$C for several days during summer (Henderson & Dennien 2018). The entomopathogenic fungus, *M. anisopliae* is a global pathogen of an extensive range of insects, naturally occurring in a diverse range of soils. Among various soil habitats, the affinity of *M. anisopliae* has been found with agricultural fields, while characteristics like ecological fitness are among the most desirable attributes of entomopathogenic fungi for soil insect control.

In our study, *M. anisopliae* strain QS155, originally isolated from the tropical Northern Territory, was, therefore, included in our study as a potential control agent against wireworm insects in sweetpotato fields. Sweetpotato production occurs in the subtropical and tropical regions of Australia (ASPG 2020), where the soil temperature can exceed 40 $^{\circ}$C for several weeks continuously during the summer (Henderson & Dennien 2018). Temperature and humidity are considered as the determining factors for insect pathogenesis (Rath et al. 1995). Understanding the establishment of EPFs by taking the peak temperature into account is crucial before their application in the field. Thus, the resporulation of calcium alginate formulated *M. anisopliae* QS155 (fungal granules) was tested at three different temperatures. It was found that the fungal granules placed at a constant 45 $^{\circ}$C failed to grow during the 18-days of heat exposure. Failure of fungal growth has been attributed to the prolonged dry heat exposure, leading to the damage of conidial DNA (Setlow 2006). Those conidia from the resultant fungal granule entirely lost their viability, as shown by a follow-up germination test. However, the granules placed at 35 $^{\circ}$C were densely myceliated without notable conidiation in comparison to the granules incubated at 25 $^{\circ}$C, indicating that extreme heat is more counterproductive for the process of conidiation than the mycelial growth. In contrast to exposure to constant hot temperatures, if fungal colonies are alternated with moderate temperatures to elevated temperatures, they can withstand the elevated temperatures without losing their viability (Rangel et

al. 2010), which explained how the heat-exposed (42 °C) *M. acridum* regained its mycelial growth and sporulation when the temperature was lowered to 28 °C. Similarly, fungal granules applied to sweetpotato fields are anticipated to resporulate during the cooler period of the day (especially at night), although the day temperature is too high for fungal growth. During the glasshouse experiment, the applied fungal granules (*M. anisopliae* QS155) experienced intermittent temperature peaks of up to 49 °C for 7 d due to the failure of the climate controller although it had been only 20 days since the inoculation of fungal granules in pot soil. However, *M. anisopliae* was still able to cause mealworm mortality during the experiment, and likely had time to recover and cause infection when live insects at the end of the experiment were exposed to, and were killed by propagules in the soil in a confined container. Our experiment explained that the resporulation efficacy of fungal granules was impaired at a constant 35 °C and completely devastated at a constant 45 °C during the 18-days incubation. Despite the temperature peak amid fungal growth, the results of fungal infectivity shown at the end of the experiment were attributed to the temperature fluctuation, dipping to below 30 °C at night in combination with the presence of the sweetpotato plant with mother storage root, buffering capacity of soil and mulch cover of the soil. However, the extreme temperature certainly curtailed the fungal growth to the limited sporulation, preventing the fungus from its full potential sporulation. Critical temperature alone is not responsible for causing fungal mortality, but the critical temperature combined with the exposure period determines the fate of fungal survival.

The glasshouse study indicated that the fungal granules applied on sterilised soil caused the greatest mealworm mortality and the fungal granules following their preconditioning further enhanced their infectivity. The evidence of greatest mealworm mortality on sterilised soil with preconditioned fungal granules has been attributed to the occurrence of dense fungal resporulation on sterilised soil from the preconditioned fungal granules. The laboratory study on the resporulation of fungal granules at different temperatures showed that the temperature regime at 25 °C stimulated the optima fungal resporulation. Soil temperature above 35 °C could be counterproductive

for the fungal sporulation, but fungus tends to survive in such conditions without losing its viability in fields due to the diurnal thermal fluctuation, for example, low temperature at night. These studies implied that the efficacy of fungal resporulation on the sweetpotato fields can be enhanced in conjunction with disinfested soil, for example, fumigated soil. Thus, the following study consisted of examining the resporulation and infectivity of fungal granules applied on fumigated soil as soil fumigation is one of the commonly adopted methods of soil disinfestation in sweetpotato cultivation.

CHAPTER 6: RESPORULATION OF *METARHIZIUM ANISOPLIAE* ENCAPSULATED GRANULES ON METHAM® FUMIGATED SOIL AND MORTALITY OF *TENEBRIO MOLITOR*

Abstract

Competitive fungistasis caused by soil saprophytic microbes has been implicated in hindering the resporulation from nutrient-fortified *Metarhizium*-encapsulated granules. However, microbe depleted soil, due to soil sterilisation, has been shown to improve the fungal resporulation that subsequently led to high insect mortality. The previous results spurred an additional experiment to understand how disinfested soil impacts *Metarhizium* resporulation and infectivity of fungal granules. Soil fumigation is used by some farmers as a pre-plant treatment to manage soilborne pests, diseases, and weeds. In Australian sweetpotato production, beds prepared to receive roots for generating shoots (nursery beds) are often treated with a fumigant like Metham® in Australia. Because the effects of Metham® are transient, we wanted to examine the usefulness of applying *Metarhizium* to the soil to continue protecting the plants against insect attack following fumigation. Resporulation from *Metarhizium* encapsulated granules was examined in Metham®-fumigated soil and then the infectivity of the resulting resporulated fungal granules was evaluated in the laboratory. In the fumigated soil, the resporulation of *Metarhizium* from the prepared granules led to 100% insect mortality at 9 days post-insect exposure; both fungal resporulation and insect mortality on the three different soils did not differ significantly. The conidial germination of the resporulated granules on fumigated soil was > 80%, which was significantly higher than those on pasteurised soil or field soil. The results from this study show that the fungal granules can be applied in sweetpotato fields in conjunction with soil fumigation, but further field studies are required to assess the ongoing role of *Metarhizium* encapsulated granules in insect control in fumigated soil.

6.1 Introduction

Root crops like sweetpotato are vulnerable to various soilborne pathogens and soil insect pests (Chalfant et al. 1990; Johnson & Gurr 2016). In Australia, root herbivores particularly plant-parasitic nematodes (*Meloidogyne javanica* and *M. incognita*), wireworms (both Elateridae and Tenebrionidae), and sweetpotato weevils (*Cylas formicarius*) can cause substantial damage to sweetpotato roots (McCrystal 2010; Dotaona et al. 2015; Stirling et al. 2020). This damage often leads to economic losses for growers as the produce is either rejected at the packing shed or is sold as a non-premium product. Chemical pesticides are the major tools for crop protection in Australia (Horticulture Innovation Australia 2014). However, their use against root herbivores such as wireworms in sweetpotato does not always lead to reduced insect populations. Specifically, wireworms intensify their feeding damage up until the crop is harvested (preventing the use of chemicals with harmful residues), while the efficacy of pre-planting applied soil insecticides is depleted over time. Therefore, the use of entomopathogenic fungi has been proposed as an additional tool to manage wireworms (and other pests) in an integrated pest management program for sweetpotato.

Entomopathogenic fungi like *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea*, have been frequently detected in soil from a diverse range of habitats and show great diversity due to their worldwide distribution (Medo & Cagan 2011). Several studies have noted that the natural occurrence of *B. bassiana* is predominantly found in natural habitats, for example, forests, while the prevalence of *M. anisopliae* is primarily concentrated in cultivated soils, for example, crop fields (Quesada-Moraga et al. 2007; Medo & Cagan 2011). Considering the association of *M. anisopliae* in agriculture fields, irrespective of agriculture practices such as tillage and farm inputs (Jabbour & Barbercheck 2009; Castro et al. 2016; Uzman et al. 2019), many studies have evaluated its ability to manage soil insect pests (Rath et al. 1995; Ekesi et al. 2005; Kabaluk et al. 2007). Despite this, *M. anisopliae*-based management of insects is not reliable in field conditions (Jaronski et al. 2007; Putnoky-Csicso et al. 2020), even though substantial insect mortality by *M. anisopliae* has been observed

under laboratory conditions (Ansari et al. 2009; Razinger et al. 2013). For this reason, fungal adaptation, especially in agroecosystems has been viewed as a crucial attribute that needs to be evaluated for any EPF candidates before their application into the field. Some *Metarhizium* species, for example, *M. anisopliae, M. brunneum,* and *M. robertsii* demonstrate their strong association in soil environment in terms of rhizospheres colonisation (Hu & Leger 2002; Razinger et al. 2020) and further endophytic establishment into plant roots, for example, cassava (Greenfield et al. 2016), and tomato (Krell et al. 2018). Fungal-plant interaction as endophytism or rhizosphere competence can confer crop protection against root herbivores (Razinger et al. 2020) and even the suppression of root herbivores by altering the gene expression of the host plant (Ahmad et al. 2020). Not surprisingly, the degree of crop protection conferred by EPF against soil insects relies on the density of fungal inoculum. A study by Mayerhofer et al. (2015) showed that only a repetitive application of inoculum (*B. brongniartii* Sacc.) could achieve the required EPF density in the soil to cause mortality to the European cockchafer (*Melolontha* spp.). Fungal inocula targeting the control of soil insects have been formulated as colonised grains such as barley, millet, rice and maize (Ekesi et al. 2005; Ekesi et al. 2011; Mayerhofer et al. 2019). These grains nutritionally support the growth of EPF propagules in soil, providing crop protection against soil insects (Schwarzenbach et al. 2009; Erler & Ates 2015). The presence of insect and plant hosts in the soil can further improve the EPF growth and persistence in soil, but EPF saprophytic growth is important to maintain propagules at high enough levels to result in insect infection and rhizosphere colonisation (Kessler et al. 2004). However, fungistasis initiated by indigenous soil microbes has been often implicated in hindering the growth of the target EPF in soil (Bonanomi et al. 2013). Fungistasis in the soil can be manipulated by reducing the microbes living in soil, for example, soil sterilisation, which resulted in substantial fungal growth from the applied fungal inocula leading to significantly high insect mortality in comparison to non-sterile soil (Chapter 4). The practice of soil disinfection has been universally adopted to particularly control soil-borne pathogens in many crops. Methods for soil disinfestation can include both solarisation and fumigation, these methods have been

implicated in temporarily suppressing a broad range of soil microbes, especially spore-forming fungi (Stapleton et al. 2000; Wang et al. 2014).

The practice of soil fumigation with methyl bromide (CH_3Br) was once prevalent across the world as it was highly effective nematicide, insecticide, fungicide and herbicide (De Vera et al. 2018). But its detrimental effect of depletion of the ozone layer in the atmosphere caused this chemical to be phased out worldwide (De Vera et al. 2018). The global ban of methyl bromide stimulated the search for alternative fumigation options including non-chemical methods such as solarisation, soil steaming, electromagnetic radiation and biofumigation (Casu et al. 2018; Morra et al. 2018; Tseng et al. 2018). The practice of biofumigation applied alone or in combination with solarisation, is broadly practised in fields by incorporating the parts of glucosinate-containing plants, particularly brassica crops (for example *Brassica napus*) into the soil (Omirou et al. 2011; Kruger et al. 2013). Myrosinage, an endogenous enzyme found in brassica plants (e.g., *B. napus*), hydrolyses glucosinolates converting them to isothiocyanates (ITCs), which possess biocidal properties (Borek et al. 1998).

Metham sodium (MS) is a soil fumigant based on sodium N-methyldithiocarbamate, which converts to methylisothiocynate (MITC) upon contact with moist soil (Di Primo et al. 2003). Methylisothiocynate is toxic to soil biota and has been widely used in agriculture as a suppressor of weed seeds (for example *Portulaca oleracea* and *polygonum arsenastrum*) (Klose et al. 2008), plant-parasitic nematodes (for example *Meloidogyne incognita* in cucumber) (Thies et al. 2005) and plant pathogenic fungi (for example *Verticillium dahlia*) (Fravel 1996), and soil insects (for example soil-dwelling whitefringed beetle *Graphognathus leucoloma*) (Matthiessen et al. 1996). The mixed success of soil fumigation with metham sodium against phytopathogenic bacteria have been reported, for example, no efficacy against *Pseudomonas allicola* was observed in onion (Kritzman & Ben-Yephet 1988), to significant control of *Erwinia carotovora* in summer squash in a field following metham fumigation (Bu et al. 2014). Due to its control efficacy for a broad range of

pests, metham sodium has been applied extensively to manage the pests of multiple crops including sweetpotato nursery beds in Australia, making it an alternative option to methyl bromide, since its ban (Warton & Matthiessen 2000; Li et al. 2017). In addition to the principal role of metham fumigant as an inhibitor of soil-borne pathogens, its application in the soil causes off-target effects to multiple soil microbes including bacteria and fungi. A study by Li et al. (2017) found that the abundance and diversity of soil fungal and bacterial community were significantly reduced in metham sodium fumigated soil, including the beneficial mycorrhizal fungi (Davis et al. 1996). However, MITC rapidly degrades in the soil, especially in soil with repeated fumigation (Di Primo et al. 2003); thus, some soil microbes, particularly actinobacteria and proteobacteria, which are resilient, can recolonise the soil quickly (Sederholm et al. 2018).

In our previous studies, the encapsulated *M. anisopliae* granules that were inoculated on disinfested soil effectively resporulated, resulting in high insect mortality. To the best of our knowledge, there has not been a study that has examined granule resporulation and infectivity of these granules after inoculation to metham-fumigated soil. Because metham fumigation is used in crops to control pests, including in sweetpotato production in Australia, we sought to examine the effects of fumigation on persistence, saprophytic competence, and infectivity of *M. anisopliae* in soil. *Metarhizium anisopliae* was formulated with nutrient additive fortification, referred to as fungal granules, while larval mealworms (*Tenebrio molitor*) were used as a model insect for soil insects.

6.2 Material and methods

6.2.1 Soil sampling

Soil (clay % 60; silt % 20; sand % 20; pH 6.6; EC 7 mS/m; Carbon % 3.22; Nitrogen % 0.22; barley as a winter crop) was sourced from an agricultural field of the University of Southern Queensland, Toowoomba (GPS coordinate: 27°36′33″S,

151°55′55″E) in February 2021 (monthly mean minimum and maximum temperature 16.6 and 26.6 ˚C respectively; (BOM 2021). The field was planted with cereal crops, for example, barley in winter, but left fallow during summer (Figure 6.1). The soil was sampled across the field in a diagonal transect, where 6 samples were taken over 100 m. For each sample, the top 5 cm of the topsoil was cleared, and soil was collected to a depth of 15 cm using a trowel. Soil samples were pooled (~6 kg weight) and immediately transported to the laboratory for further processing. The sample was homogenised, sieved (pore size 5 mm), and air-dried for 12 hr, before being transferred to a plastic bin (20 L) fastened with an air-tight lid and stored in a cool room (5 ˚C) until further use. This processed soil sample was referred to as field soil in the following sections.

Figure 6.1: A soil collection site at an agricultural field of the University of Southern Queensland, Toowoomba

6.2.2 Soil treatment: fumigation, pasteurisation, and field soil

In this experiment, the resporulation and infectivity potential of fungal granules was investigated on three soil treatments, fumigated, pasteurised, and field soil. To fumigate the soil, transparent plastic containers (diameter 8.5 cm; height 9 cm) were

filled with 150 g of field soil. All containers containing field soil were adjusted to 50% field capacity (16% initial moisture content) by applying 40 mL of ultra-pure Type 1 water (Milli-Q) to each container 8 h before being treated. The containers were then treated with Metham® soil fumigant (active ingredient 423 g/L Metham as a sodium salt, Nufarm Australia) at the rate of 600 mL/m^3 soil using a pipette. Following the application of the Metham®, an additional 5 mL of ultra-pure Type 1 water (Milli-Q) was added to each container using a syringe (volume 60 ^{cc}/mL; Terumo® Syringe), and the lids were sealed tightly to contain the volatile fumes. Seven days after the fumigant was applied, the lids were loosened by half a turn to allow for gas release. The process of soil fumigation was performed inside a fume hood (Lab systems, Product for Science and Life) at room temperature: 22 $^{\circ}$C and 79% RH for 19 days (Figure 6.2).

For the soil pasteurisation treatment, 2 kg of field soil (16% initial moisture content) was placed into a double-layered aluminium tray (20 cm x 15 cm x 5 cm), moistened with 400 mL of ultra-pure Type 1 water (Milli-Q), and wrapped with a double-layer of aluminium foil to prevent heat loss and cross-contamination. The soil was heated to 80 $^{\circ}$C for 40 min in an oven (Memmert GmbH + Co. KG). The pasteurised soil was dispensed into plastic containers (diameter 8.5 cm; height 9 cm) containing 150 g of pasteurised soil per container and adjusted to 50% field capacity at 8 h before being treated. Pasteurised soils were treated with 5 mL of ultra-pure Type 1 water (Milli-Q). All the procedures were carried out at the same time as the fumigated soil, but in a separate fume hood (Lab systems, Product for Science and Life) at 22 $^{\circ}$C and 79% RH to avoid cross-contamination of any volatiles. The containers were treated as described for the fumigated soil.

For the non-sterile field soil treatment, the same containers were filled with 150 g soil per container (diameter 8.5 cm, height 9 cm) and the soil moisture was adjusted to 50% field capacity 8 h before the ‘treatment’ (placement in the fumehood). The soil was treated with 5 mL of ultra-pure Type 1 water (Milli-Q) only. All procedures were carried out at the same time with the fumigated and pasteurised soil. All containers containing treated field soils were placed separately from fumigated soil into the fume

hood with the pasteurised soil during the treatment period to prevent cross-contamination. The other procedures performed were the same as for the fumigated and pasteurised soils.

Figure 6.2: Containers containing Metham®-treated soil arranged in a fume hood for 19 days before the inoculation of fungal granules

6.2.3 Colony Forming Units from soil and indigenous EPF

Prior to treating the prepared soils with fungal granules, the colony forming units (CFUs) were enumerated for each soil type, to determine the efficacy of the soil treatments on the presence of soil microbes. For the fumigated soil, CFUs were calculated from surface soil where the fumigant was applied (Fumigant 1) and from the homogenised fumigated soil after the fumigation was completed (Fumigant 2). For each soil type (field soil, pasteurised soil, or fumigated soil), 1 g of soil was suspended in a 20 mL plastic tube containing 9 mL of sterile water and was agitated for 5 min using a vortex (Select Vortexer; speed 500 RPM). A dilution series from 10^{-2} to 10^{-8} was made for each soil type. Serial dilutions of 10^{-2} to 10^{-4} were assigned to pasteurised soil and fumigated soil, whereas dilutions ranging from 10^{-4} to 10^{-8} were used for field

soil samples. For each soil dilution, 100 µL of suspension was plated out onto triplicate Petri dishes (Ø, 90 mm) containing potato dextrose agar (Merck KGaA, Germany) or onto nutrient agar (Merck KGaA, Germany). Petri dishes were sealed with Parafilm® and incubated in a controlled temperature room at 25 °C and a 12:12 day and light photoperiod.

To determine the presence of indigenous EPF from field soil, a plastic container (4 cm diameter; 8 cm height) was filled with 50 g of moist soil sourced from either field soil or pasteurised soil or fumigated soil before the fungal inoculation on them. To each container, ten larval mealworms were added, and the containers were sealed with a perforated lid and incubated in a controlled temperature room at 25 °C and a 12:12 day and light photoperiod. If any dead mealworms were found, they were surfaced-sterilised with 100% ethanol, rinsed in distilled water, and placed into a moist chamber (90 cm Petri dish lined with a moist sterile Whatman® paper). The Petri dishes were incubated as above to stimulate EPF sporulation.

6.2.4 Fungal inoculation and experimental design

After 19 days of treatment in the fumehoods, individual containers containing either fumigated, pasteurised soil, or field soil received 5 mL of sterile water before being inoculated with fungal ($CAG_{Ma+Cs+By}$) or food (CAG_{Cs+By}) granules. Prior to their application, the freshly prepared fungal granules were dried inside a sterile laminar flow cabinet (Labec Laboratory Equipment) for 14 h. This resulted in a 55% weight reduction from the initial fresh weight: each resulting granule weighed 16 mg, contained ~10^7 conidial, and was 3.5 mm in diameter (Figure 6.3 A). Freshly prepared fungal granules were placed on the soil surface at a rate of 4×10^8 conidia g^{-1} soil equivalent to 1 g (~ 60 pieces) of granules (Figure 6.4 A & B). Food granules without conidia were inoculated into the respective containers with soil as the control treatment (Figure 6.3 B). A Petri dish containing 3% water agar was inoculated with either ten fungal granules or food granules as quality control (to assess for resporulation and presence of contaminants), sealed with Parafilm® and incubated in a plant growth

chamber (CONVIRON® CMP6010) at 25 °C, 80% RH and a 12/12 h day and night photoperiod. After inoculation, the containers were loosely sealed with a lid and transferred to the same plant growth chamber, and incubated at 25 °C, 80% RH, and a 12/12 h day and night photoperiod. Six replicates were made per treatment and containers were arranged in an RCBD.

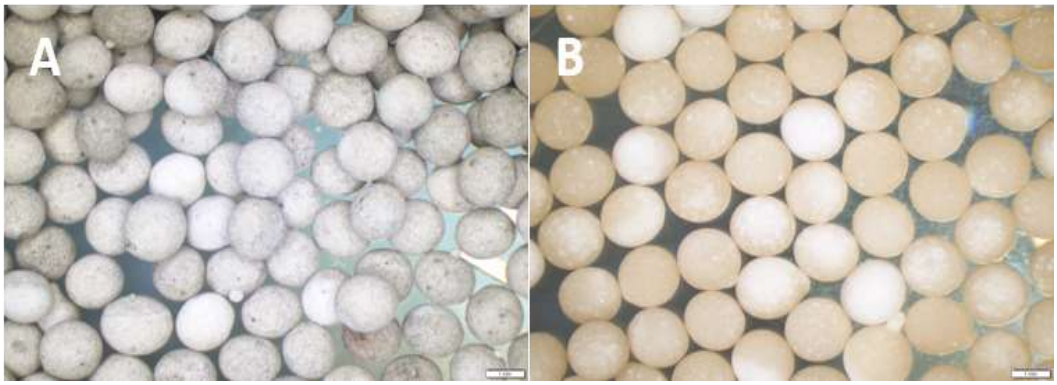


Figure 6.3: Fungal granules ($CAG_{Ma+Cs+By}$) used as a treatment (A) for the mealworm mortality, while food granules (CAG_{Cs+By}) were used as the control treatment (B)

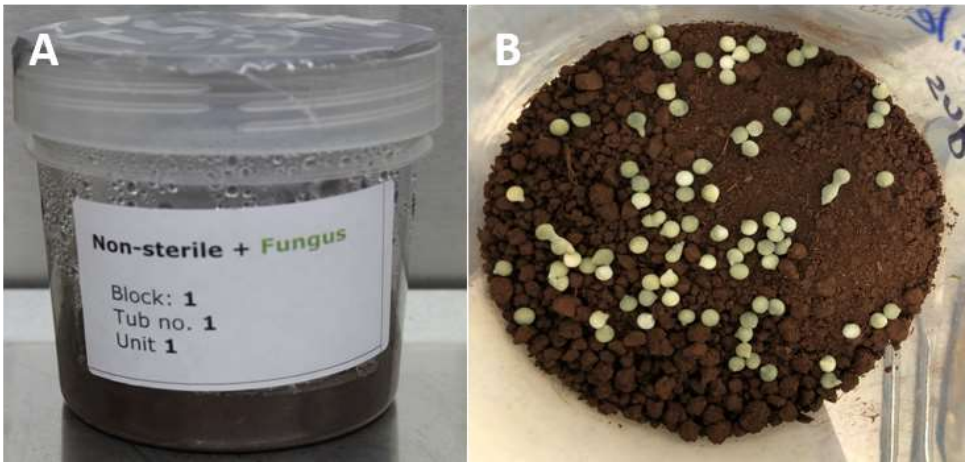


Figure 6.4: A plastic container containing 150 g of non-sterile soil (A), and view of the soil inoculated with fungal granules (B).

6.2.5 Resporulation assessment

To determine the amount of resporulation from the fungal granules at 10 days post-incubation, nine fungal granules ($CAG_{Ma+Cs+By}$) or food granules (CAG_{Cs+By}) were randomly removed from individual containers using sterile tweezers (Figure 6.5). The conidial resporulation from the fungal granules was determined by inserting an individual fungal granule into a tube (2 mL) containing 1 mL of sterile 0.05% Tween®80 solution. For the conidial resporulation test, three fungal granules were sub-sampled from the nine removed fungal granules. The tubes were agitated using a vortex (Select Vortexer; speed 1000 RPM) for 2 min to dislodge the conidia from the granule (Figure 6.5).

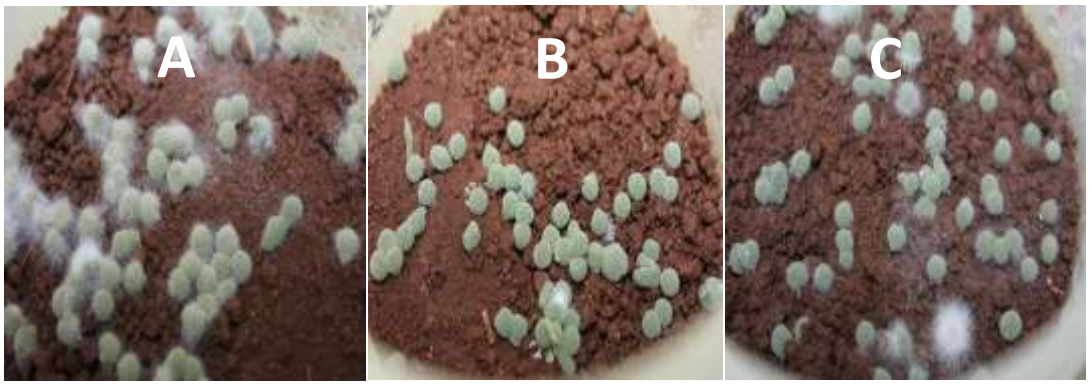


Figure 6.5: Resporulated fungal granules inoculated in non-sterile soil (A), fumigated soil (B), and pasteurised soil (C) at 10 days post-incubation at 25 °C, 80% RH, and a 12:12 day and light photoperiod

Conidia from the suspension were enumerated using a haemocytometer (Neubauer improved double net ruling, ProSciTech Pty Ltd) at ×400 magnification (Olympus, Model BX53). The conidial germination was assessed by inoculating 20 µL of each suspension onto a glass slide with a thin layer of SDAY agar (1.5 × 1.5 × 0.5 cm). A coverslip was placed on the agar and each glass slide was place in a Petri dish (Ø, 9 cm) lined with a moist Whatman® filter sealed with Parafilm® and incubated at 27 °C and a 12:12 h dark and light photoperiod. For each slide, at 12 h, 14

h, 16 h, and 24 h post-incubation, the germination of 200 conidia was assessed at ×400 magnification using a compound microscope (Olympus, Model BX53). Conidia with a germ-tube twice as long as the width of spores were considered to be germinated (Rangel et al. 2010). During the germination test, the length of the germ tube from the germinated conidia (20 conidia per replicate) was also measured.

6.2.6 Infectivity assessment

At 10 days post-incubation, the infectivity of the remaining fungal granules (calculated at ~1.43×10^7 conidia g^{-1} soil based on the resporulation of fungal granules following the incubation) was evaluated. Before mealworms were introduced to the soil, the resporulated fungal granules or food granules were thoroughly mixed into the soil by inverting the container and remoistened with 5 mL of sterile water. After this, twenty larval mealworms (mean 110 mg weight; 16 mm length and 1.6 mm wide) were added to each container (Figure 6.6 A), containers were loosely sealed with a lid, and re-incubated at 25 ˚C, 60-80% RH in the dark in the same plant growth chamber. At 24 h post-incubation, a piece of fresh sweetpotato (~30 g) was supplied as food for the mealworms, and container lids were replaced with perforated lids to facilitate the aeration inside the containers. All containers were placed back into the plant growth chamber according to the previous design.

After the mealworms were exposed to the fungal or food granules, the mortality of the larval mealworms was assessed on daily basis for 14 days. During the assessment period, any dead mealworms were removed by using sterilised tweezers. To confirm EPF-induced mortality, dead insects were immediately surface sterilised and placed into a moist chamber (a Petri dish lined with a sterile filter paper moistened with 1 mL sterile water) at 25 ˚C to stimulate sporulation. The surface sterilisation of mealworm cadavers was performed by placing the cadavers in 70% ethanol for few seconds, rinsing briefly in sterile water, dipping in 1% diluted sodium hypochlorite for 1 min, twice rinsing with sterile water, and blotting dry on a sterile filter paper.

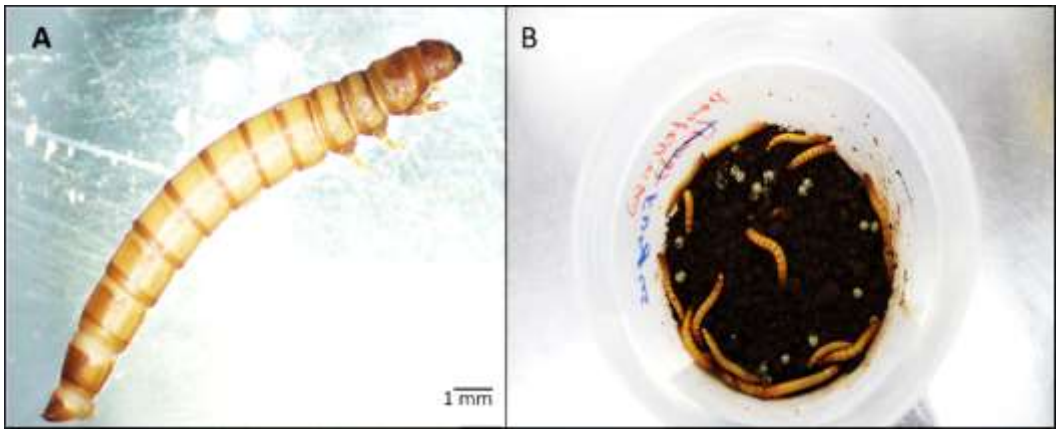


Figure 6.6: A larval mealworm before the inoculation (A); and a group of 20 larval mealworms introduced into soil containing resporulated fungal granules (B)

6.2.7 Data analysis

Data obtained from the above experiments were tested for normal distribution, and homogeneity of variance by using the Shapiro-Wilk and Levene test, respectively. Data obtained from 'colony forming units from soil and indigenous EPF' was firstly analysed through the two-way ANOVA to determine the interaction between two independent variables (soil levels and media types) in relation to the CFUs. Significant differences among field soil, pasteurised soil, and fumigated soil in terms of CFUs groups were calculated using ANOVA, and their pairwise comparisons were further performed by using the Tukey test with 95% confidence. For the 'resporulation assessment' and 'infectivity assessment,' the data were analysed using an ANOVA. For the experiment on 'infectivity assessment,' the mortality data were converted to percentages and then further corrected using Abbot's formula (Abbott 1925).

6.3 Results

6.3.1 Colony Forming Units from soil and indigenous EPF

The microbes present in field soil, fumigated soil and pasteurised soil were cultivated on nutrient agar (NA) and potato dextrose agar (PDA) media by spreading

the soil suspension derived from each soil type (Figure 6.8). A significant interaction was found between soil type (field, pasteurised, fumigated 1, and fumigated 2) and media type (PDA and NA) for CFU ($P = 0.001$). The number of CFUs from field soil, pasteurised soil, fumigated 1 soil and fumigated 2 soils were significantly different from one another, irrespective of media type ($P = 0.01$) (Figure 6.7). In terms of CFUs on NA, the CFUs from field soil were significantly greater than those on pasteurised soil and fumigated 1 soil ($P < 0.05$), but not significantly different from that observed on fumigated 2 soils ($P > 0.05$). While CFUs on field soil on PDA were significantly greater than those on fumigated 2 soils ($P < 0.05$), but it was not significantly different to pasteurised soil and fumigated 1 soil ($P > 0.05$).

One morphotype of *Metarhizium*-like fungi was isolated from field soil using the mealworm-bait method on the 14th-day post-exposure (Figure 6.9), while at this time point, there was no evidence of any EPF was found from pasteurised or fumigated soil using the mealworm-bait method. However, when examined approximately 30 days later, the same *Metarhizium*-like fungi was found across all soils (presenting as conidiated cadavers) suggesting the native EPF had recovered sufficiently from the treated soils to become infective again.

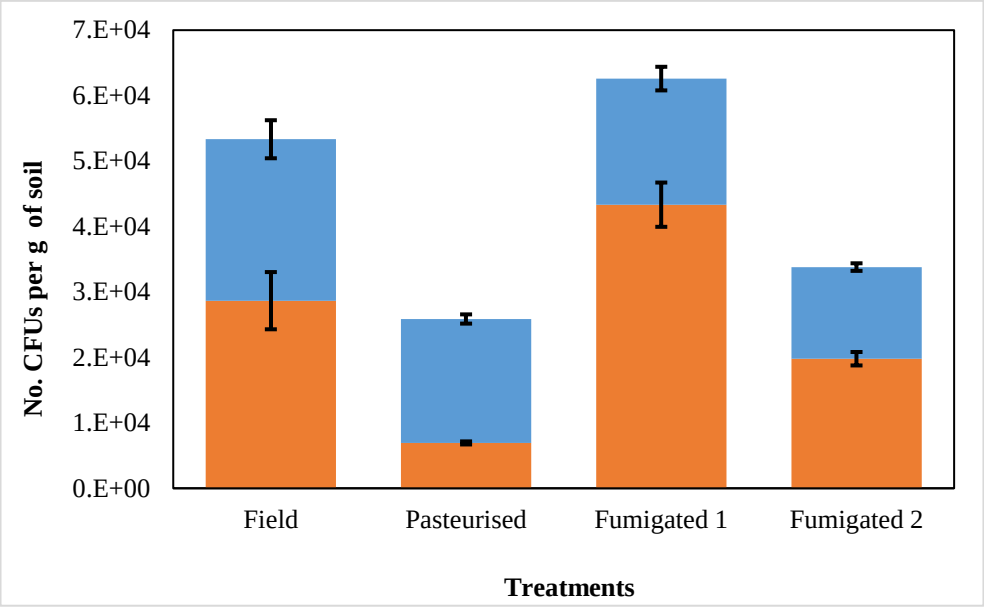


Figure 6.7: The colony forming units (CFUs) present on standard nutrient agar (NA: orange) or potato dextrose agar (PDA: blue) media soil sourced from field soil, pasteurised soil, fumigated soil with soil sampled from the top fumigant treated area (fumigated 1) and fumigated soil with soil sampled from homogenised fumigated soil (fumigated 2). All soil types produced significantly different CFUs on PDA (blue) or NA (orange) (mean ± SE, replicates = 3, $P = 0.05$).

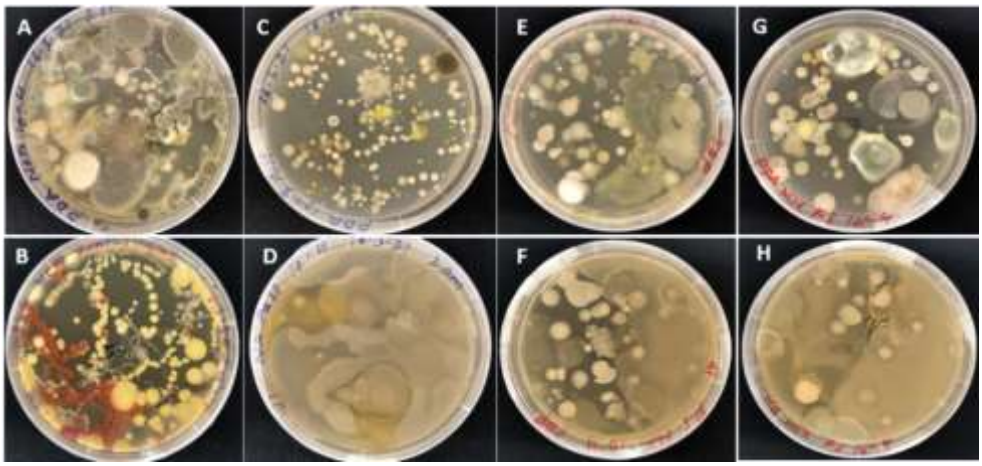


Figure 6.8: Microbial colonies appeared on nutrient agar and potato dextrose agar media from non-sterile soil (A and B), pasteurised soil (C, D); fumigated 1 soil (E, F); and fumigated 2 soils (G, H). A mixture of fungal and bacterial colonies was evident on potato dextrose agar, whereas only bacterial colonies were observed in nutrient agar, regardless of soil levels. The above pictures were captured few days after the CFU counts were completed.

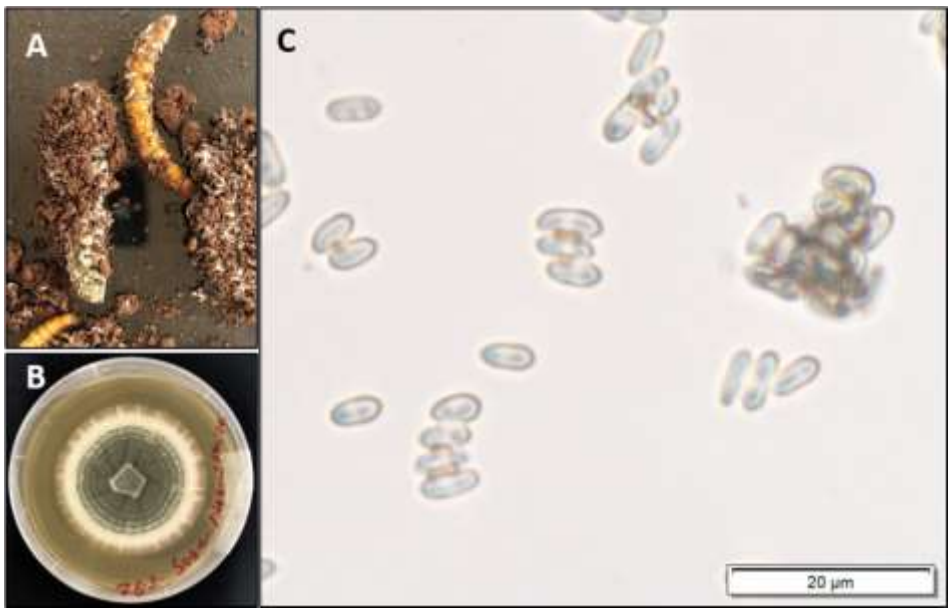


Figure 6.9: Sporulated cadavers were found in non-sterile soil samples sourced from the agricultural field at the USQ, Toowoomba (A); a fungal colony, suspected to be *Metarhizium* spp. grown from a single colony isolated from an insect cadaver (B), and the fungal conidia obtained from the fungal colony (C).

6.3.2 Resporulation assessment

Ten days after inoculation, there was substantial resporulation from the fungal granules (Figure 6.10 However, there was no significant difference in the number of conidia from resporulated granules regardless of soil treatment (P = 0.189). The resporulated conidia from the individual fungal granules on fumigated soil were calculated at 4.14×10^7 ($\pm 2.17 \times 10^6$) conidia per granule, 3.74×10^7 ($\pm 8.88 \times 10^5$) conidia per granule on nonsterile soil, and 4.28×10^7 ($\pm 2.67 \times 10^6$) conidia per granule on pasteurised soil. Conidial germination of resporulated conidia was significantly (P = 0.0001) different among the three different soils. Fungal granules inoculated in field soil had shown the highest germination at 86% (± 2), whereas the lowest germination at 63% (± 2) was recorded for the fungal granules applied on pasteurised soil. The conidia from the fungal granules applied in fumigated soil had 82% (± 6) germination.

Conidial germination of resporulated fungal granules between non-sterile soil and fumigated soil was statistically non-significant (P = 0.406), whereas the germination of conidia in pasteurised soil was significantly lower than that recorded from the field and fumigated soil (P = 0.0001) (Figure 6.11 A). Germ tube length was found significantly different among treatments (P = 0.0362). The resporulated conidia from the fumigated soil showed the greatest germ length, which was significantly greater than those on pasteurised or field soils (Figure 6.11 B). Fungal granules inoculated on 3% water agar as a quality control substantially resporulated yielding 2×10^7 conidia from a single fungal granule, whereas food granules cultured on 3% water agar did not show any changes.

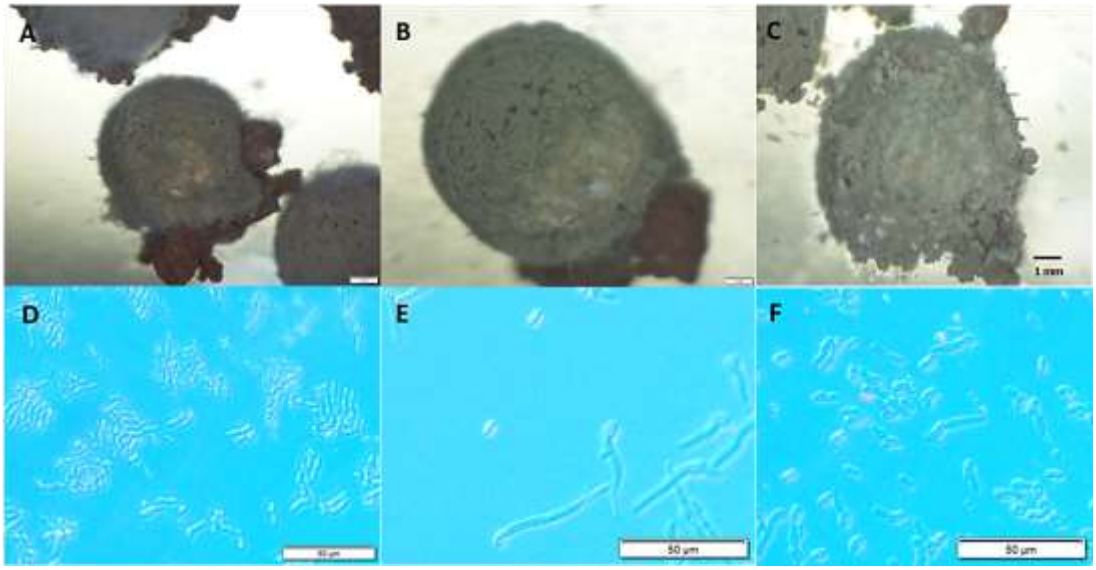
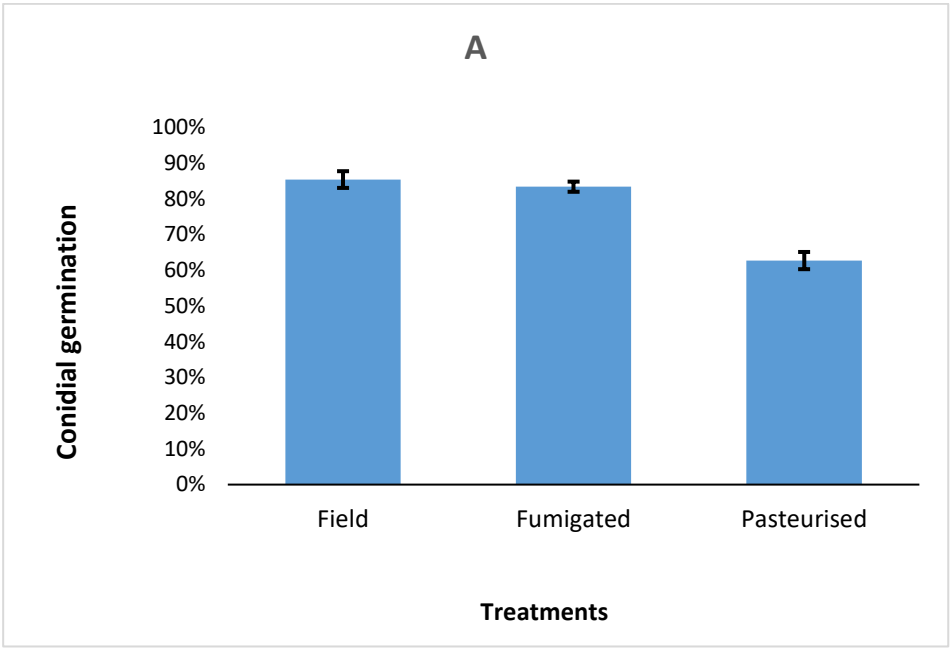


Figure 6.10: Resporulated fungal granules appeared on field soil (A), fumigated soil (B), and pasteurised soil (C) after 10 days incubation of fungal granules; and germinated conidia following a 14h incubation, which was extracted from the resporulated fungal granules grown on soil substrates (D, E, F) respectively.



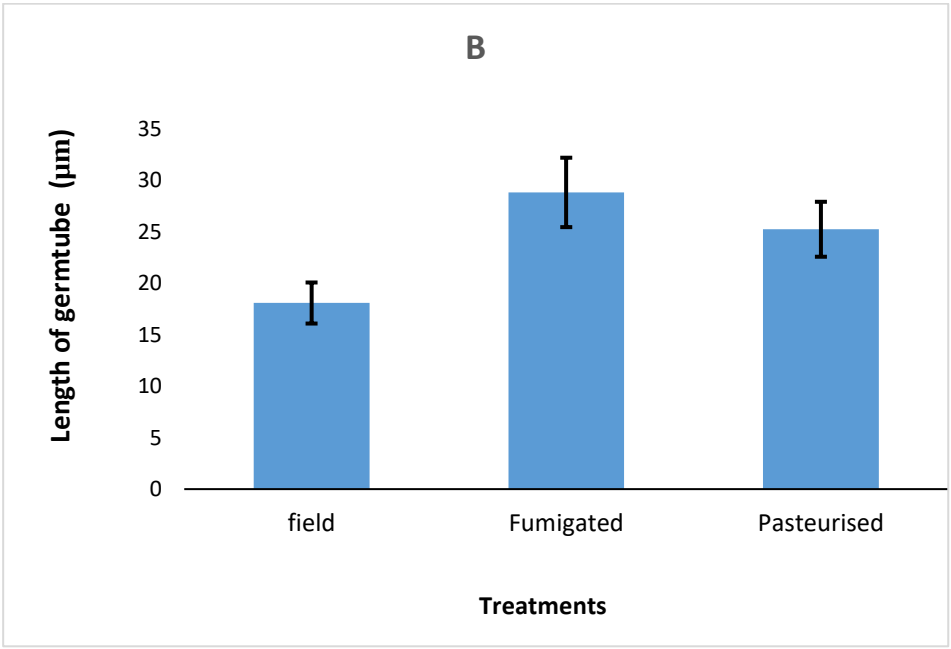


Figure 6.11: Percentage germination of extracted conidia from resporulated fungal granules applied on either field or fumigated or pasteurised soil for 10 days. Fungal granules applied on pasteurised soil had shown significant lower conidial germination than that of field and fumigated soil (Means ± SE, replicates = 6, $P = 0.05$) (A), and from the germinated conidia, the fumigated soil showed the significant greater conidial germ length than those of field and pasteurised soil (B)

6.3.3 Infectivity assessment

The assessment of mealworm mortality began four days following the insect exposure to soil with resporulated fungal granules and was observed daily. Mealworm mortality on pasteurised soil (80% ± 2) and fumigated soil (80% ± 3) with fungal granules was significantly greater than that in field soil (45% ± 7) on the 4th-day post-inoculation (P = 0.001). Mealworm mortalities among field soil, fumigated soil and pasteurised soil inoculated with fungal granules were not significantly different from one another on the 8th-day post-inoculation, where 100% ± 0 mortality was recorded from all treatments (P = 0.087) (Figure 6.12). Substantial ecdysis (>90%) from larval mealworms exposed to soil containing fungal granules was evident on the 4th-day post-inoculation, whereas there was not any mealworm mortality from the soil with food granules (control) on that assessment. Those dead larval mealworms recovered at four- and five days post-inoculation were deep brown, which led to mycosis and conidiation following the placement in a moist chamber (Figure 6.13). At mortality assessment on the 8th day post-exposure on soil, the larval mealworms introduced on pasteurised soil with food granules had 5% (±3) mortality, whereas no death was observed in field soil and fumigated soil with food granules, despite substantial saprotroph growth shown on food granules. About 50% of larval mealworms exposed to soil containing food granules pupated during the 14-day bioassay.

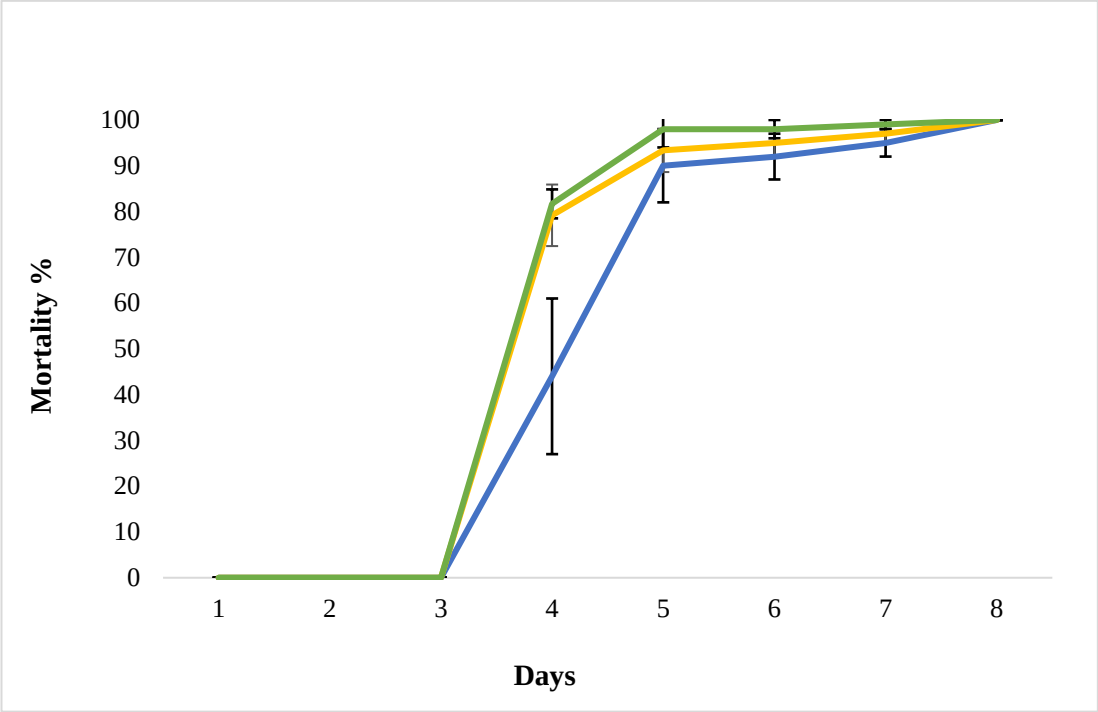


Figure 6.12: Larval mealworm mortality during the laboratory bioassay when larval mealworms were introduced into three different soils i.e., field soil (blue), fumigated soil (orange), and pasteurised soil (green) with inoculation of fungal granules. Fungal granules caused 100% mealworm mortality at 8 days post-inoculation, regardless of soil types (mean ± SE, replicates = 6, P = 0.05).

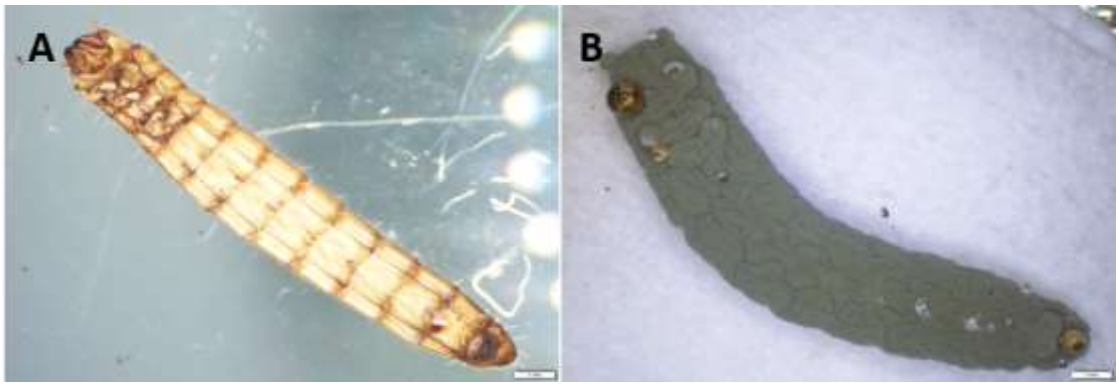


Figure 6.13: A deal larval mealworm recovered from fumigated soil containing resporulated fungal granules at 8 days post-inoculation with fungal inocula (A) which turned into a cadaver with profuse sporulation following placement in a moist chamber for 4 days (B).

6.4 Discussion

Fungal granules inoculated in soil caused 100% mortality of larval mealworms within eight days of insect exposure to soil containing resporulated fungal granules, irrespective of soil treatment. When fungal granules were introduced into the soil, they substantially resporulated, for example, 4.14×10^7 ($\pm$ 2.17×10^6) conidia per granule on fumigated soil, 3.74×10^7 ($\pm$ 8.88×10^5) conidia per granule on field soil, and 4.28×10^7 ($\pm$ 2.67×10^6) conidia per granule on pasteurised soil producing an excessive number of viable conidial, as shown by the resporulation test. The presence of this high conidial concentration with good viability resulted in the rapid death of all mealworms in our study. It is probable that the nutritional requirements of *M. anisopliae* were met in this study that allowed for prolific sporulation and saprophytic growth in soil, as shown in other studies (Wakelin et al. 1999; Zahran et al. 2008; Coombes et al. 2016). As a facultative saprophyte, *M. anisopliae* can exploit externally supplied nutrients (St. Leger & Wang 2020), therefore, the fungal propagules, such as conidia or mycelia are often fortified with nutritive additives to encourage successful saprophytic growth in soil (Rath et al. 1995; Mayerhofer et al. 2015).

In this study, the fungal granules inoculated on field soil substantially resporulated to $3.74 \times 10^7 \pm 8.88 \times 10^5$ conidia per granule. But the resporulated fungal granules did show microbial contamination based on the visual appearance compared to the granules on fumigated and pasteurised soil that could be linked to the significantly greater CFUs on field soil ($5.3 \times 10^5 \pm 1.26 \times 10^5$ CFUs per g of soil from both NA and PDA media). Despite the evidence of a multitude of microbial CFUs on field soil, this study demonstrated the food-fortified fungal granules successfully resporulated on field soil. Conducive abiotic conditions, particularly soil moisture and temperature, under the laboratory conditions and the mode of fungal inoculation on soil surface could be the possible reasons behind the successful fungal resporulation on field soil. Fungal granules on the soil surface can readily access oxygen as compared to the fungal granules inoculated on deep soil profile as the conidial germination and further growth of *M. anisopliae* significantly consumes oxygen (O_2)

(Braga et.al 1999). The slow-growing fungal granules deep soil profile are likely to be attacked by soil saprotrophs as the growth of soil saprotrophs, for example, *Mucor*, *Penicillium*, *Aspergillus*, and *Fusarium* (Lestan & Lamar 1996). Despite the evidence of microbial contaminant on the resporulated fungal granules on field soil, the resporulated fungal granules on field soil demonstrated the greatest conidial germination (86% ± 2), indicating that the resporulated fungal granules from the field showed greater germination than those resporulated fungal granules produced on microbial reduced soil such as pasteurised and fumigated soil (46% ± 3 and 82% ± 6 respectively). The variability of conidial germination from the fungal granules inoculated in differnet soil can be attributed to different microbial composition presented on soil. The results from our study may suggest that microbial communities in fumigated and pasteurised soils started to rebuild and the resulting microbial composition developed in fumigated or pasteurised soil is likely to be different to the microbial composition existing in non-sterile soil. However, mortality of the mealworms was more rapid on fumigated (82% ± 3) and pasteurised (82% ± 2) soil than on field soil (45% ± 7) after 4 days, despite the reduced conidial germination on fumigated or pasteurised soil. The infective conidia with rapid conidial attachment and their further penetration on insect hosts from the resporulated fungal granules on fumigated and pasteurised soils could be linked to the microbial reduced soil based on CFUs numbers on pasteurised ($2.59 \times 10^5 \pm 1.63 \times 10^4$ CFUs per g soil combining both NA and PDA media) and fumigated soils ($6.85 \times 10^4 \pm 8.99 \times 10^3$ CFUs per g of soil combining of PDA and NA) as compared to field soil. The result of low conidial germination from the resporulated fungal granules especially on pasteurised soil, but with the evidence of fast mealworm infection and subsequently mortality shown by resporulated fungal granules on pasteurised soil shows that there is not always congruency between the *in vitro* and *in vivo* results.

From our observations, encapsulated *M. anisopliae* showed rapid germination and good utilisation of the nutrient additives for its resporulation, surpassing the saprophytic growth stage when abiotic factors are favourable to the fungal growth, mainly soil moisture (>20% water holding capacity) and temperature (20-30 $^{\circ}$C)

(Wakelin et al. 1999). In contrast, if such conditions do not exist for the fungal growth, the soil saprotrophs usually overtake the growth of EPF, utilising the food for themselves and reducing subsequent EPF fungal resporulation. Thus, maintaining the optimal climatic conditions during the period of fungal resporulation in the soil is of crucial but is a balancing act to ensure conditions for the EPF are favoured. For this reason, an idea of preconditioning of fungal granules before the inoculation in soil has been proposed to improve the chance of EPF inocula outcompeting other soil microbes (Lestan & Lamar 1996). There have been multiple attempts to reduce fungistasis to enhance the saprophytic growth of biological control agents using labile carbon sources, for example, sugars and amino acids, while fungistasis is likely to be formidable on carbon-deprived soil (Bonanomi et al. 2013). In our study, we studied the role of soil fumigation to reduce fungistasis, so as to assess the fate of fungal granules, especially fungal resporulation in fumigated soil. We used Metham® as a fumigant that emits methyl isothiocynates (MITC) upon contact with moist soil (Matthiessen & Kirkegaard 2006). The principal purpose of soil fumigation is to control soil-borne plant parasitic fungi, nematodes, and insect pests (Borek et al. 1998; Kruger et al. 2013; Wang et al. 2014). Apart from controlling plant pathogens, soil fumigation has been linked to the suppression of a wide range of soil microbes including eukaryotes and prokaryotes (Wang et al. 2014; Li et al. 2017), but the suppressive effect is transient so that soil microbe numbers start to rebound once the chemical toxicity dissipates in the soil (Sederholm et al. 2018). Thus, we aimed to achieve optimal saprophytic growth of *M. anisopliae* from food granules in soil when fungistasis was reduced by soil fumigation. These results showed that fungal granules successfully resporulated on fumigated soil and the resulting resporulated granules in soil caused 100% larval mealworm mortality. Although the number of conidia from the resporulated fungal granules was the same among field soil, fumigated soil, and pasteurised soil; conidial qualitative parameters in terms of the physical appearance of resporulated fungal granules, conidial germination, and length of germ tube of germinated conidia was significantly different.

A recovery of EPF induced insect mortality on fumigated soil after soil treatment signified that metham fumigation can only cause a transient suppression of indigenous EPF. Similarly, a study by Camprubi et al. (2007) also noted the revival of the indigenous population of mycorrhizal fungi on fumigated soil. The application of metham as a soil fumigant does not decimate indigenous *Metarhizium* populations based on the observation from the above study, while a consortium of indigenous EPF and externally applied EPF can coexist (Mayerhofer et al. 2015). The visual appearance of resporulated fungal granules on fumigated appeared dark green and produced dry conidia without any cross-contamination, whereas resporulated fungal granules from the field soil were a lighter green and were damp conidia with visible cross-contamination (personal observation). It further suggested that metham fumigation could have stimulated the resporulation of fungal granules by suppressing the deleterious soil microbes, which antagonise *M. anisopliae* on the field soil. The germination test from resporulated fungal granules signified that field soil and fumigated soil mediated the production of resporulated fungal granules with significantly higher conidial germination than that for pasteurised soil, suggesting that the rate of microbial recovery in soil, especially fungal antagonists, is higher in pasteurised soil than fumigated soil. Furthermore, longer germ tubes of conidia from fumigated soil suggested that the growth of germinated conidia was faster in treated soils, which might cause the rapid mortality of insects once the fungus penetrates the insect cuticle.

Encapsulated *Metarhizium* in calcium alginate may be more suitable than the directly exposed conidial formulation. For example, in one study, the *M. anisopliae* conidia exposed to brassica produced isothiocynates failed to germinate, grow, and infect host insects (Inyang et al. 1999). The co-application of biocontrol fungi such as *Gliocladium roseum* and *Talaromyces flavus* in the form of alginate granular formulation in combination with a sub-lethal dose of metham sodium into the soil suppressed the soil-borne fungal pathogen *Verticillium dahliae* (Fravel 1996). This may imply that the combination of fungal granules and biofumigation might be possible because of the potential low deleterious effect of biofumigants against soil

microbes when applied as a sub-lethal dose. The reduced deleterious effects of bio-fumigation on soil microbes have been described in Omirou et al. (2011), who showed that the biocidal effect of biofumigation is transient and non-lethal as compared to that of metham sodium. Metham fumigation has been blamed for causing environmental degradation and adverse impact on soil health (Woodrow et al. 2014; Li et al. 2017), stimulating the adoption of biofumigation using brassica plants as an alternative (Potgieter et al. 2013). A detailed study is warranted to understand more about how the fumigation affects the indigenous EPF in soil, whether it could be a temporary or long-lasting effect.

The greater level of mealworm mortality on fumigated (80% ± 3) and pasteurised soil (80% ± 2 on pasteurised soil) than that on non-sterile soil (45% ± 7) within four days of inoculation can be attributed to the densely viable fungal propagules resulting from the resporulation of fungal granules(for example, $4.14 \times 10^7 \pm 2.17 \times 10^6$ on fumigated soil, $3.74 \times 10^7 \pm 8.88 \times 10^5$ conidia per granule on the field soil, and $4.28 \times 10^7 \pm 2.67 \times 10^6$ conidia per granule on pasteurised soil) as well as mealworms being an extremely susceptible host insect to EPF. A similar result was also reported in a study by Lestari and Rao (2017), which reported the 100% mealworm mortality on the 6^{th} day of inoculation carried out by dipping larval mealworms on the aqueous suspension (4×10^5 to 4×10^6 conidia ml^{-1}) of *Metarhizium* spp. or *Beauveria* spp. In our study, the appearance of the dark body colour of dead larval mealworms following the fungal inoculation indicated that the insect defence system was induced in the response to fungal infection by mealworms. This was also reported in a study by Hussein et al. (2012), who reported the exoskeleton of the greater wax moth (*Galleria mellonella* L.), commonly used to bait EPF from the soil, to be highly melanised when the insects were inoculated with EPF. In general, insects are equipped with both cellular and humoral immune defence systems (Krams et al. 2013). In the cellular response, haemocytes attach to pathogens and encapsulate them (encapsulation); while the humoral response functions as an accessory role to the cellular response that consists of regulated coagulation and melanisation of the hemolymph (Hancock et al. 2006) (Figure 6.14). The melanotic capsule of haemocytes breaks the supply chain of

nutrients into the pathogen and contributes to their mortality by starvation (Lavine & Strand 2002). Such immune reaction by host insects as a response to pathogen infection could be costly especially by reducing the insect life span (Krams et al. 2013). Despite the melanisation, >95% of dead cadavers produced the conidiation when the cadavers were placed in a moist chamber, suggesting that conidiation was not suppressed by the melanisation.

Figure 6.14: The darkened cuticle on a mealworm cadaver killed by *M. anisopliae* during the laboratory bioassay

Substantial ecdysis (moulting) was observed in the larval mealworms, specifically those mealworms exposed to the fungal granules. Ecdysteroids (ecdysone) hormone secreted by insects has been implicated in inducing the ecdysis (Zhu et al. 2021). The ecdysteroids hormone is also found to be activated in insects when insects are infected by insect pathogens. Generally, many moulting proteins are regulated under the ecdysteroids hormone. A melanising enzyme, called phenoloxydase (PO) is activated in moulting proteins upon infection by entomopathogens, to inhibit pathogen germination and further growth resulting in the occurrence of melanised ecdysis (Zhang et al. 2014). However, the recovery of conidiated cadavers from our experiment infers that *M. anisopliae* can escape the melanised ecdysis driven by larval mealworms, to further penetrate and invade the haemolymph, multiplying as

blastospores. Such instance of avoidance of melanised ecdysis was also evident in *B. bassiana* for the feeding larvae of silkworm (*Bombyx mori*) (Zhang et al. 2014). Some EPF, for example, *M. rileyi*, are reported with eliciting an anti-ecdysteroids enzyme to inactivate the hormone and suppress the insect's innate defence mechanism (Kiuchi et al. 2003).

Our targeted host insect is wireworm, a soil insect infesting belowground plant parts like sweetpotato roots. Generally, soil insects require longer exposure time to be infected and killed by insect pathogens, for example, soil-applied with *M. anisopliae* conidiated on millet grains at the rate of 10^7 conidia g^{-1} soil resulted in the death of 50% of the population of the subterranean scarab larvae (*Adoryphorus couloni*) after 19 days in laboratory conditions (Rath & Worledge 1995). Similarly, wireworms (*Agriotes* sp.) introduced into the soil with *M. anisopliae* as an aqueous formulation of 3.85×10^6 conidia g^{-1} air-dried soil had 50% population mortality at 50-65 days post-inoculation in the laboratory (Razinger et al. 2013). A study by Kabaluk et al. (2017) demonstrated that soil insects like wireworms establish a symbiosis with soil microbes, especially bacteria, for example, *Pantoea agglomerants* and *Pandoraea pnomenusa* which safeguard wireworms from the infection of insect pathogens like *M. brunneum*. In addition, insects were also found to maintain a host insect–actinomycete association on the insect exoskeleton, which acts as the first line of defence against insect pathogen infection (Human et al. 2017). For any insects, mortality is a function of fungal concentration and exposure time. For wireworm mortality, exposure for at least 48 h to *M. anisopliae* is estimated to be required (Ericsson & Kabaluk 2007), with a dose of at least 4×10^6 conidia cm^{-3} soil (Kabaluk et al. 2007). In this study, we exposed larval mealworms to soil containing resporulated fungal granules, where a single fungal granule produced average 4.14×10^7 conidia. Due to this high fungal resporulation, we observed rapid mealworm mortalities (up to 100% within 9 days of inoculation). Based on our observation of mealworm mortality, we hypothesised that it would be possible for wireworms to succumb to death rapidly. Results from our current study show that the soil with resporulated fungal granules resulted in 100% mealworm mortalities within eight days of inoculation inferring that these resporulated

fungal granules could also cause substantial wireworm damage, but unlike mealworms, wireworms need longer exposure time to the fungal inocula to be infected. The other factor that potentially impedes the fungal infection to wireworms is the behavioural constraint of some wireworms, which evade the fungal contaminated area to non-contaminated area (Ericsson & Kabaluk 2007). This avoidance behaviour was not observed in these experiments, in contrast, mealworms were observed to be actively feeding on the granules, and it is possible that false wireworm species infesting sweetpotato may behave similarly to mealworms.

This study showed that fungal granules applied in the field, fumigated, or pasteurised soil demonstrated substantial resporulation, despite the presence of significant differences of soil microbial numbers among these soils. But qualitative attributes of fungal granules resporulating on fumigated soil were found to be better than those in the field and pasteurised soil in terms of conidial germination, length of the germ tube, and cross-contamination by other soil saprophytic fungi. The complete mealworm mortality within eight days of exposure to resporulated fungal granules in soil showed that the resporulated fungal conidia from the granules (i.e., isolate QS155) in soil are highly pathogenic to insects. Moreover, fumigated soil (19 days after the soil fumigation) allowed the fungal granules to resporulate and subsequently, the resultant fungal granules killed the insects, confirming that fungal granules can be applied in fumigated soil to enhance the efficacy of fungal resporulation but probably only when the biofumigant toxicity receded. Future studies can be directed towards the assessment of fungal sensitivity with metham fumigant when both treatments are co-applied in the field, the impact of soil fumigation on the indigenous EPF, and fate, persistence, and infectivity of EPF in biofumigated soil.

CHAPTER 7: SUMMARY, GENERAL DISCUSSION, FUTURE RESEARCH, AND CONCLUSION

7.1 Summary of findings

This thesis presents the investigation of optimising the formulation of fungal granules and examination of their resporulation on a variety of soil types. The infectivity of the resulting soil resporulated fungal granules was also assessed against larval mealworms and this was performed under both laboratory and glasshouse conditions. Since our target was to control soil-dwelling insect pests like wireworms in sweetpotato fields, achieving effective fungal saprophytic growth in the soil is our prime focus. Formulating *M. anisopliae* into calcium alginate granules with food fortification demonstrated an enormous growth potential for this EPF in soil because of the nutritive additives it carries with the association of fungal propagules. This thesis adds insight into the knowledge pool of wireworm management by using *M. anisopliae* in sweetpotato and offers practical knowledge about the formulation of fungal granules, which fit and resporulate into diverse soil conditions, especially soil with reduced soil microbes enhancing the fungal resporulation. Fungal granules present an option to provide additional protection against insects like wireworms, contributing to the existing control strategies as a part of an integrated pest management program.

This chapter summarises the main findings with respect to the chief aims of the study and positions the work into the broader context of the biological control of wireworms using the entomopathogenic fungus *M. anisopliae*. Additionally, the chapter also outlines areas for future research and provides a conclusion.

Chapter 1 provides a brief introduction into the Australian sweetpotato industry, the extent of wireworm damage in sweetpotato, existing control measures, and future scope of biological control, while Chapter 2 reviews the current status of sweetpotato production in Australia, wireworm biological diversity, and their damage extent to the Australian crops particularly sweetpotato. It details existing control measures for

wireworm control in sweetpotato, highlighting the need for biological control in wireworm management, especially focusing on the use of entomopathogenic fungi *M. anisopliae* for wireworm control. Synthesis of the available literature on wireworm diversity, their current pest status, and future damage prospective in sweetpotato is of valuable information because this has not been done previously, while much of the information was previously inaccessible, scattered, and disparate. These two initial chapters provide a baseline of knowledge that sets the scene for the following four experimental chapters.

7.1.1 Summary of Chapter 3

The overall objective of the experiments in Chapter 3 was to examine the fungal resporulation in response to different nutritive additives, to assess the resporulation of fungal granules on different substrates, and to evaluate the effect of soil types on the growth of *M. anisopliae* in soil. The experiment identified the following findings:

1. The combination of baker's yeast (20% w/v) and corn starch (20% w/v) supported the optimal resporulation of *M. anisopliae* to 1.40×10^8 conidia per fungal granule when the freshly prepared fungal granules were placed onto 3% water agar and incubated at 25 °C for 14 days. The fungal granules containing corn starch only stimulated to the mycelial growth without stimulating conidia production ($2.4 \times 10^7 \pm 5.8 \times 10^6$), whereas fungal granules only with baker's yeast prompted the conidial development to 6.3×10^7 conidia per granule.

2. The fungal granules were able to resporulate on different substrates, but their greatest resporulation efficacy was found on sterilised and pasteurised soil with high conidial viability at >80% conidial germination, while the fungal resporulation was constrained on non-sterile soil and the resulting conidia lost their complete germinability. Fungal granules with a diameter of 15 mm were found on sterilised soil that consisted of mycelium and minimal resporulation from the fungal granules with a 3.5 mm initial diameter.

3. Different soil types characterised with variable physical and chemical properties did not affect the EPF growth, but fungal granules applied on sterilised soil induced the greatest numbers of *M. anisopliae* colonies. In comparison, fungal granules on non-sterile soil had the lowest number of *M. anisopliae* colonies indicating the poor fungal growth on non-sterile soil that has been attributed to the competition from other soil saprotrophs.

7.1.2 Summary of Chapter 4

The overall objective of the experiments in Chapter 4 was to evaluate the infectivity efficacy of fungal granules. Three different experiments were conducted on soil-less and soil substrates against larval mealworms. Moreover, the evidence of larval mealworm mortality caused by *Metarhizium*-like organisms in sterilised soil treated with control treatment (food granules) was further elucidated. These experiments presented the following findings:

1. Resporulated fungal granules, which were placed on 3% water agar and incubated at 25 $^{\circ}$C for 14 days before the inoculation to insects, resulted in mealworm mortality of 88%.

2. Three different fungal doses inoculated on soil caused substantial mealworm mortality, but the mealworm mortality did not differ significantly among the three doses and soil types.

3. The onset of larval mealworm mortality was observed at 14 days post-exposure to soil containing fungal granules. The greatest mealworm mortality was obtained in non-sterile soil (52% mortality), 8% mortality in simulated solarised soil, and 13% mortality in sterilised soil. At 30 days post insect exposure, there was no difference between treatments.

4. Fungal granule-free soil containing larval mealworms did not become infected when they were placed on the same platform consisting of the Petri dishes containing the conidia of *M. anisopliae* to understand the possible cross-contamination between

the mealworms and the conidia on Petri dishes. Nor did the enforced contamination of *M. anisopliae* conidia to mealworms in soil cause any mealworm mortality.

7.1.3 Summary of Chapter 5

The overall objectives of the experiments in Chapter 5 were to investigate the effects of preconditioned or non-preconditioned fungal granules inoculated in sterilised soil or non-sterile soil with sweetpotato as a host plant on mortality of larval mealworms in the glasshouse and to evaluate the effect of temperature on the resporulation of fungal granules. The results from the experiments showed that:

1. Fungal granules applied on sterilised soil caused up to 60% mealworm mortality, regardless of types of fungal granules i.e., preconditioned, or non-preconditioned fungal granules.

2. The fungal granules inoculated on non-sterile soil resulted in 18% mealworm mortality, which was significantly lower than that observed in sterilised soil.

3. Although preconditioned fungal granules did not induce any significant mealworm mortalities compared to non-preconditioned fungal granules, the quality of resporulated fungal granules and conidiated cadavers found in soil was better in terms of conidial density than those on non-preconditioned fungal granules. More research is warranted to better understand and improve the effects of preconditioning the fungal granules.

4. The fungal granules that were placed at 25 $^{\circ}$C and 35 $^{\circ}$C for 18 days resporulated well. However, germination was affected by temperature with only 64% of conidia able to germinate at 35 $^{\circ}$C, whereas conidial germination was recorded at 98% for the resporulated fungal granules at 25 $^{\circ}$C.

5. The fungal granules incubated at 45 $^{\circ}$C for 18 days did not exhibit any fungal growth and further lost the conidial germinability completely. The granulating

polymer, calcium alginate, was found to be degraded when it was exposed to 45 ˚C for 18 days.

7.1.4 Summary of Chapter 6

The overall objective of the experiments in Chapter 6 was to examine the resporulation efficacy of fungal granules in fumigated soil and evaluate the fungal infectivity of resulting resporulated fungal granules in soil. The experiments showed that:

1. The number of background soil microbes (CFUs) was reduced compared to field soil following fumigation (Metham®) or pasteurisation. A substantial number of fungal colonies grew on PDA plates from field soil, while PDA culture plates derived from fumigated and pasteurised soil did not have significant fungal colonies.

2. Fungal granules introduced into the field, fumigated, and pasteurised soil resporulated measuring 3.74×10^7, $4.14 \times 10^{7,}$ and 4.28×10^7 conidia per granule, respectively. Conidial germination from the resulting resporulated fungal granules over pasteurised soil was only 64% compared to that of fumigated soil at 83%, and field soil at 85%.

3. Larval mealworms introduced into the resporulated fungal granules growing over either field soil or fumigated soil or pasteurised soil were to fungal infection with 100% insect mortality at 9 days after the insect exposure to the fungal inocula. The onset of mealworm mortality was begun as early as at 4 days after the insect exposure.

7.2 General discussion

This study focuses on wireworm management on sweetpotato as it is an economically important insect pest, although a sporadic one, capable of causing severe crop damage especially before the crop harvest in Australia when the application of chemical insecticide is not possible (McCrystal 2014). Wireworm damage to

sweetpotato storage roots is characterised by shot holes scattered around the periderm that reduces the cosmetic value of sweetpotato prompting rejection from the market. There is not any exact data showing economic loss on sweetpotato caused by wireworms, but anecdotal evidence indicates that crop loss up to 30% can occur (Bree Wilson Pers. Comm.), despite the application of chemical insecticides (McCrystal 2014). Wireworm infestation to sweetpotato does not cause any yield loss, and even these infested sweetpotato roots can be still used as animal feed and industry products such as starch and ethanol. However, 96% of the sweetpotato produced in Australia is supplied to the domestic fresh market, making wireworm damage economically important. In general, there are two tiers of wireworm management practices existing in sweetpotato fields in Australia: pre-plant application of soil insecticides to kill the resident wireworm, and post-plant application of insecticides to control adult wireworms and neonate larval wireworms (DAF 2011). Despite these chemical applications, sweetpotato growers still experience wireworm feeding damage. Severity of damage caused by wireworm, especially on the harvest-ready crop, has been attributed to the short persistence of soil insecticides applied in sweetpotato fields. Decreased performance of soil insecticides and potential repercussions to the environment posed by chemical insecticides have spurred the investigation based on biological control methods for insect pest management, for example, entomopathogenic fungi (EPF).

The EPF *Metarhizium anisopliae* is a generalist insect pathogen causing infection to >200 insect species. In addition, this fungus is also characterised as rhizosphere and rhizoplane coloniser because of its adaptability in the soil environment making it a promising candidate for soilborne insect pests. In the current study, we focused on the saprophytic growth of *M. anisopliae* in soil that is likely to be of most relevance to crop protection against root herbivores. No evidence has ever revealed the interaction between the fungal saprophytic growth in soil and its further extension on rhizosphere and rhizoplane colonisation, but it is believed that they are correlated.

Ascertaining the nutritive requirements of a fungus destined for biological control is essential if fungal saprophytic growth is anticipated on the soil. Thus, this study used a fungal formulation containing fungal propagules and food additives together in a calcium-alginate granule, referred to as fungal granules. The fungal granules demonstrated their resporulation on a range of soil types (Chapter 3) and the resultant resporulated fungal granules were able to kill up to 100% larval mealworms when the insects were exposed to soil with the resporulated fungal granules under both laboratory and glasshouse conditions (Chapters 4, 5, and 6). The fungal granules were also able to resporulate on soil with sweetpotato as a host plant and then resporulated fungal granules on soil killed up to 60% of larval mealworms (Chapter 5). Those live larval, pupal, and adult mealworms recovered at the end of the glasshouse experiment eventually succumbed to *Metarhizium*-induced mortality when they were exposed to soil containing propagules. These results implied that the application of fungal granules on soil may impact the survival of wireworms, but wireworm mortality is not expected as high as the mortality of larval mealworms achieved in laboratory and glasshouse conditions (Chapters 4, 5, and 6). Possible reasons for expected low wireworm mortality are wireworm body structure and wireworm association with symbiotic bacteria which protect them from the attack of *Metarhizium* (Traugott et al. 2015; Kabaluk et al. 2017). Wireworms are generally characterised as hardy insects due to their tough exoskeleton, through which the conidia of *M. anisopliae* is required to penetrate. Wireworm behaviour to avoid the *Metarhizium* treated zone to non-treated area suggests that the presence of *Metarhizium* colonisation near the rhizosphere could deter wireworms from the roots (Ericsson & Kabaluk 2007). But our finding revealed that the feeding intensity of larval mealworms was unreduced as shown by significant root damages by larval mealworms on soil with fungal inoculation that was not significantly different from the damage that occurred on soil with food granules (control). Despite the significant mealworm mortality efficacy with *in situ* conidiated mealworms recovery from the soil with fungal inoculation, the presence of sweetpotato root damage by larval mealworm on fungal inoculated soil has been attributed to the substantial number of mealworms released on soil (100 larval

mealworms per pot). Larval mealworms were used in this study as a proxy for wireworms as wireworms are a sporadic pest of sweetpotato in Australia. Furthermore, they have a subterraneous habitat, and exist as a cryptic species complex constraining wireworm collection with adequate quantity, of the same species, and life stage (Samson & Calder 2003; McCrystal 2010; ASPG 2020). Because of these constraints, the trend of larval mealworm utilisation in other studies as a model insect for several soil insects including wireworms has increased to assess the infectivity of entomopathogenic fungi (Lestari & Rao 2017; Przyklenk et al. 2017). Wireworms and mealworms are both coleopteran insects and further both false wireworms (for example, *Gonocephalum macleayi*) and mealworms belong to the same insect family Tenebrionidae. Further, studies of wireworm control in field conditions using *M. anisopliae* is essential as research outcomes generated from laboratory and glasshouse studies may not be congruent with field conditions. Thus, further studies focusing on wireworm control using *Metarhizium anisopliae* could be a potential study in the future as wireworms are already economical soil pests for a variety of crops such as sweetpotato, sugarcane, sunflower and sorghum in Australia and further as an emerging pest in canola.

In this study, the infectivity aspect of *M. anisopliae* as shown on mealworm mortality has been extrapolated to wireworm mortalities. Because *M. anisopliae* is a generalist insect pathogen, the resporulated fungal granules have the potential to also protect against the attack from a broad range of root herbivores. Besides wireworms, sweetpotato weevil (*C. formicarius*), whitefringed weevil (*Graphognathus lecoloma*), and curl grub (*Heteronyx* spp.; *Heteronychus* spp.) are soil insect pests for sweetpotato that can cause extensive damage to the storage roots if untreated, especially on organic farms. *Metarhizium* species alone in a laboratory and the combination with the other EPF in a field caused effective control of the sweetpotato weevil (Reddy et al. 2014; Dotaona et al. 2015). Many studies have discovered the diversified role of *Metarhizium* species including being antagonistic against plant-parasitic nematodes (Ghayedi & Abdollahi 2013; Devi 2018) and pathogenic fungi (Sasan & Bidochka 2013) and have been referred to as “jack of all trades, master of many” (St. Leger & Wang 2020). The

evidence of *M. anisopliae* persistence in the rhizosphere and as an endophyte has been supported in many studies (Hu & Leger 2002; Vega 2018). Endophytic relationships can offer mutual benefit between the fungus and host plant through a nutrient exchange (Krell et al. 2018), whereas the rhizosphere colonisation renders the prospect of crop protection from root herbivores (Pava-Ripoll 2013). Plant-specific response to *M. anisopliae* colonisation on roots has been reported, thus the interaction between sweetpotato and *M. anisopliae* in relation to the root colonisation needs to be elucidated. Due to its multiple roles in the agroecosystem, *M. anisopliae* has been recognised as a multi-functional organism contributing to the plants in many ways (Vega et al. 2009).

Our study only took into account the quantitative resporulation from the externally applied fungal granules on soil and their resultant pathogenic impacts on larval mealworms. However, our study is limited to the description of the implication of these resporulated fungal granules to the environment, food consumers and agroecology. *Metarhizium* has been blamed for secreting secondary metabolites such as Destruxin A, which act as an antagonist to the other soil insects, phytopathogenic fungi and nematodes (Ravindran et al. 2014). However, the knowledge regarding these secondary metabolites being introduced into the food chain and their resultant risks to human and animal health is still lacking (Strasser et.al 2000), and an ecological risk assessment is one of the criteria that is considered as a part of biopesticide registration (Zimmermann 2007). But the secretion of secondary metabolite by EPF *in vivo* is much less than that secreted by EPF grown on nutritively rich media (Strasser et. al 2000), implying that field applied fungal granules on sweetpotato fields are unlikely to cause any food safety risk to humans.

Conventionally, the usual form of *M. anisopliae* formulation for soil insect control consists of the conidiated cereal grains, which are applied inundatively to the soil for insect control as the maintenance of high conidia density in the soil is a prerequisite for effective control (Rath et al. 1995; Ekesi et al. 2005). In this study, we used a formulation consisting of conidia and nutrient sources embedded into a calcium-

alginate granule denoted as a ‘fungal granule,’ and these granules showed the capacity to resporulate and produce copious numbers of conidia in soil. The rapid loss of conidial viability and persistence in soil are the limitations associated with the conventional fungal inocula (Samson et al. 2006). However, if soil conditions (including the availability of nutrients) are favourable, crude formulations of EPF on rice can persist in the field and cause rapid insect infection after > 6 months in sweetpotato in both Australia and Papua New Guinea (Bree Wilson Pers. Comm.). But challenges associated with conidiated rice include the health hazards present at the manual handling stage due to the dusty nature of conidia.

The unavailability of nutrient sources in the soil is one of the limiting factors for fungal growth in soil (Jaronski 2007). However, the nutrition-related limitation on *M. anisopliae* can be addressed by incorporating nutrients with the fungal propagules, as previously described by Przyklenk et al. (2017). These nutritive additives may not be readily utilisable for *M. anisopliae* in fields because the saprophytic competency of *M. anisopliae* is not as high as that of soil saprotrophs. Therefore, soil saprotrophs in soil are the major hindrance for the resporulation of *M. anisopliae*, despite the nutritive additives associated with the fungus. Thus, these fungal granules warrant additional additives which can deter the soil saprotrophic growth on granules, for example, organic antibiotics from neem (*Azadirachta indica*) or garlic (*Allium sativum*). The fungal conidia used in these granules are reported to be highly susceptible in the soil to the fungistasis. The soil resting spores of *M. anisopliae*, microsclerotia, are considered more stable on soil than the conidia (Jackson & Jaronski 2012; Behle et.al 2016)). Thus, a future study should include microsclerotia as the fungal propagules into the fungal granules. Other fungal propagules, for example, blastoconidia could be also included in the fungal granules because these blastoconidia, which are produced by *M. anisopliae* in the liquid media, have been shown more rapid germination than the conidia that could drive the fungus to utilise the nutritive additives earlier than the soil microbes and subsequently enhance the fungal saprophytic growth over the fungal granules (Wassermann et.al 2016). Optimisation of the blastoconidia formulation is needed because they are susceptible to dry conditions (Wassermann et.al 2016).

Our finding showed that the fungal granules applied on sterilised soil showed the greatest resporulation compared to non-sterile soil, indicating that reduced soil microbial populations favour resporulation from the fungal granules (Chapter 4). Fumigated soil showed some similarities to laboratory sterilisation of soil in our experiments, which can be likened to the practice of soil fumigation used during the preparation of sweetpotato plant beds by some growers in Australia. The reduced soil microbe populations resulting from soil fumigation are likely to enhance the fungal resporulation on the soil when the fungal granules were applied on fumigated soil. Thus, the resporulation and infectivity of fungal granules were evaluated on fumigated soil under laboratory conditions, and then both fungal resporulation and infectivity were optimal. But further glasshouse and/or field studies are needed to confirm the fungal resporulation enhancement induced by soil fumigated soil. The fungal granule's ability to resporulate on diverse soil types signified that the application of fungal granules is feasible across the sweetpotato fields in Australia that have contrasting soil types. Sweetpotato growers could apply fungal granules to the soil when bedding roots are laid for sprout production, to provide the roots with protection against soil insects when the protective effects of the fumigant have dissipated.

Moreover, we found that there was greater production of conidia from the granules that were on the soil surface, whereas buried fungal granules had reduced resporulation possibly because oxygen levels were lower. This has practical ramifications for field application of EPF: fungal granules could be applied as a band along the drip line, allowing them to resporulate on the surface, and building up the inoculum that potential insects could be exposed to. Or EPF as a liquid formulation could be applied through the drip tape, allowing for targeted near-surface deposition of infective propagules for insect management. Good soil moisture is crucial for subsequent fungal infection and conidiation. In the laboratory experiments in Chapter 4, extended survival of larval mealworms was observed when fungal granules were exposed to dry soil. Related results were reported in a study by Chen et. al (2014), who noted that germination and larval mealworm mortality was significantly reduced and when the fungal inocula were exposed to the moisture stress soil (<25% soil moisture).

But once the soil moisture was replenished, the fungal infectivity rapidly increased demonstrating the resilience of *M. anisopliae* to changes in abiotic factors (personal observation). Additionally, the role of fungal formulation into calcium alginate granule has been found in contributing to the fungal persistence during moisture-stress conditions (Lackey et.al 1993). Thus, it suggests that maintenance of appropriate soil moisture is essential to obtain the optimal soil resporulation and subsequent insect control in soil from the resulting fungal resporulation.

In both laboratory and glasshouse studies, the fungal granules were shown to resporulate in a broad range of soil types and the resulting resporulation caused up to 100% insect mortality. However, the feasibility of fungal resporulation from fungal granules in the field is yet to be tested. More field-based studies are required to gauge the actual resporulation capacity of fungal granules in field soil and the efficacy of these granules to manage insect pests like wireworm in sweetpotato. The challenge with pests like wireworms, however, is that they are sporadic, and finding fields with consistent pest pressure can be challenging. Field trials would need to take into consideration how various farm inputs such as fertilisers, fungicides, and insecticides interact with the fungal granules.

Entomopathogenic fungi, particularly *M. anisopliae* show great potential for controlling soil insects, for example, wireworms on sweetpotato because of their strong presence on soil environments such as saprophytic multiplication in bulk soil, predominance on rhizosphere, and endophytic relationship with host plants. A direction towards the registration of EPF-based biopesticide is essential in the future in the view of incessantly increasing pressure from soil insects on crops, despite the application of currently available control measures. Thus, *Metarhizium* based biopesticide can serve as an additional tool in the battle against pest soil-borne insects by reinforcing the existing control strategies. A continuous endeavour to generate profound understanding regarding the fungal biology and ecology on agroecosystems, and to improve the fungal formulation is needed to achieve the optimal results from the biopesticide.

7.3 Future research

The findings in this thesis have generated fundamental knowledge regarding the feasibility of *M. anisopliae*, particularly formulated it into a calcium alginate granule with nutritive fortification, as a biological control agent for controlling wireworms in sweetpotato. This study has also developed insights that could underpin future studies:

- Expanding the fungal resporulation test to a field or semi-field level provides the real picture of ecological drivers and constraints which impact the fungal resporulation in soil.
- Deciphering the endophytic status and rhizosphere competency of EPF in sweetpotato and further assessing the plant-fungus relationship in terms of root colonisation. Elucidating the correlation between the fungal resporulation in bulk soil, and rhizosphere and rhizoplane growth. Discovering the role of fungal rhizosphere and rhizoplane-induced crop protection against root herbivores like wireworm.
- Optimising the fungal formulation by incorporating the fungistasis resistant fungal propagules such as microsclerotia or mycelia, by substituting the processed nutritive substrates to industrial or agricultural by-products, for example, rice bran, based on a cost-benefit analysis, and by preconditioning the fungal granule to enhance the fungal resporulation in soil.

Boosting the fungal resporulation in soil with the conjunction of soil disinfestation such as solarisation or bio-fumigation or a combination of the treatments. Examining the method or timing of fungal inocula delivery in soil with sweetpotato to increase the efficacy of fungal granules for the management of wireworm and other soilborne pests. Exploring the compatibility of fungal granules with farm inputs such as fertilisers, insecticides, and fungicides, and cultural practices that include planting of cover crop or crop rotation or maintaining optimal soil moisture regime to encourage entomopathogenic fungal sporulation as a part of integrated pest management against wireworm.

7.4 Conclusion

The findings from this thesis are the first study to offer the feasibility of wireworm control in sweetpotato in Australia by using *M. anisopliae* as a biological control agent. For any EPF candidates like *Metarhizium*, incorporating the fungus into an appropriate formulation is important because the fungal formulation and its subsequent additives (nutrients, UV protectants, and stabilisers) contribute to the efficacy by safeguarding them from abiotic and biotic stresses. From the results from our study, we hypothesise and suggest that the *in situ* resporulation (pre-sporulation) of conidia improves sustained viability, persistence, abundance, and infectivity of *M. anisopliae* when formulated into granules. However, the feasibility of the commercial supply of such a product needs evaluation.

The *M. anisopliae* granules applied on sterilised soil had the greatest level of resporulation, indicating that a reduction in soil microbes allowed for optimal resporulation. The results of this study drove the design of the next experiment, which examined the resporulation of *M. anisopliae* granules on fumigated soil, as fumigation is a method used to destroy unwanted pests and diseases in sweetpotato plant beds. The evidence of high germination conidia from the resporulated fungal granules on fumigated soil may suggest that the resporulated conidia have high vitality that may lead to prolonged wireworm protection in sweetpotato. Our findings from the glasshouse study noted that the fungal granules inoculated on sterilised soil killed up to 60% larval mealworms (*Tenebrio molitor*), and those live mealworms recovered at the end of the glasshouse study were easily killed when the effective soil area available to the mealworm was reduced, increasing the chance of being exposed to an infective propagule. Adverse impacts on insect survival such as reduction in reproduction capability, retardation of insect development, and avoidance of plant roots have been linked to the fungal sub-lethal effects to host insects that this study could not address. Future studies may shed the light on these interesting and important aspects of insect management. The successful resporulation of fungal granules on different soil types which provide near-complete control of mealworms in both laboratory and glasshouse

assays indicates that the fungal granule on *M. anisopliae* is a promising candidate for controlling wireworms in sweetpotato and is likely to complement the existing pest management strategies used in sweetpotato.

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Appendix A

Table A.1: Initial conidia (IC), resporulated conidia (RC), and their ratio (RC/IC) obtained from the fungal granules with different levels of nutritive additives. Fungal granule with corn starch as a food additive (CAG_{Ma+Cs}) induced the lowest numbers of fungal resporulation, while the fungal conidia with the combination of corn starch and dead baker's yeast ($CAG_{Ma+Cs+By}$) as a nutritive additive gave rise to the greatest fungal resporulation.

Treatments	**Initial conidia**	**Resporulated conidia**	**Ratio RC/IC**
CAG_{Ma}-----control	9.13E+06	1.55E+07 a*	1.69
CAG_{Ma+Cs}	9.13E+06	2.40E+07 a	2.63
CAG_{Ma+By}	8.08E+06	6.38E+07 aa	7.89
$CAG_{Ma+Cs+By}$	8.88E+06	1.40E+08 ab	15.79

*Means followed by the same letter within a column stand for not significantly different according to Tukey test (P = 0.05).

Table A.2: Mean mealworm mortality (MMM) percentages recorded on 14, 20, 25, and 30 days after incubation (DAI); the fungal granules ($CAG_{Ma+Cs+By}$) inoculated in non-sterile, simulated-solarised, and sterilised soils caused the mealworm mortalities.

Treatments	**MMM % (14th DAI)**	**MMM % (20th DAI)**	**MMM % (25th DAI)**	**MMM % (30th DAI)**
Non-sterile soil	52 ± 21 ^{a*}	79 ± 21 ^{ab}	92 ± 8 ^{c}	96 ± 5 ^{d}
Simulated-solarised soil	8 ± 7 ^{b}	32 ± 18 ^{bb}	78 ± 17 ^{c}	91 ± 14 ^{d}
Sterilised soil	14 ± 5 ^{b}	27 ± 17 ^{bb}	67 ± 24 ^{c}	84 ± 17 ^{d}

*Means followed by the same letter within both column and row stands for not significantly different according to Tukey test (P =0.05)

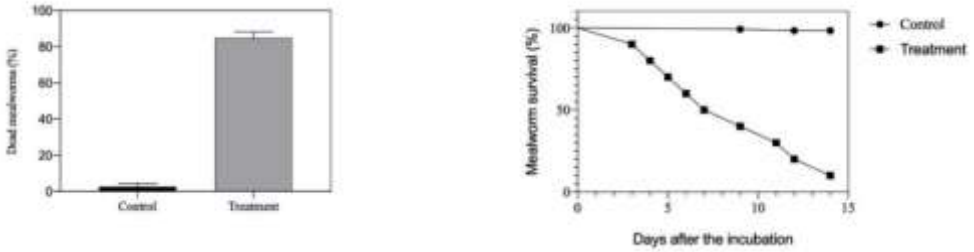

Figure A.1: Presporulated fungal granules ($CAG_{Ma+Cs+By}$) as a treatment caused a mean 90% mealworm mortalities following a two-week incubation (Left), and the survival of mealworms started to decline on the 4th-day post-incubation and constantly decreased until the second week of incubation (Right). Food granules (CAG_{Cs+} the control in this experiment caused less than 3% mealworm mortality.

Appendix B

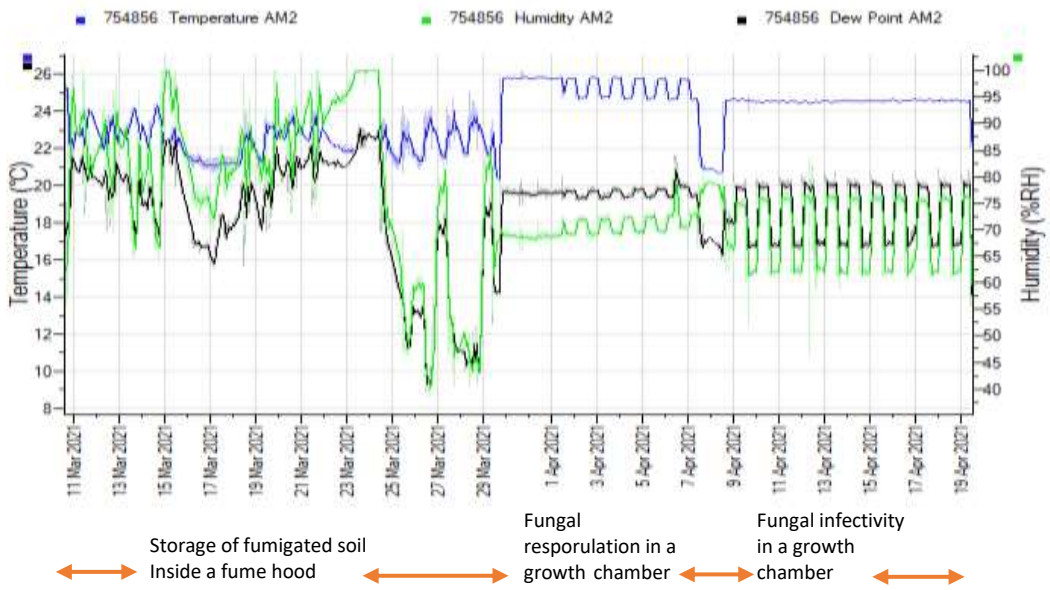

Figure A.2: Measurement of temperature and relative humidity by using a data logger (Tinytag ULTRA 2, Gemini Data Loggers Ltd) carried out inside the fume hood (Lab Systems, Product for Science and Life) during the storage of fumigated soil and inside the growth chamber (CONVIRON® CMP6010) during the fungal resporulation and infectivity (chapter 6).

Figure A.3: A glasshouse study with sweetpotatoes to examine the infectivity of fungal granules. The soil was covered with mulch (see yellow indicator) as larval mealworms require dark.