# Bioprospecting for entomopathogenic fungi against a foliar citrus pest

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# Abstract

Historically, pest management was highly dependent on the use of chemical insecticides for the control of agriculturally important pests. However, more recently, key export markets have imposed stringent chemical residue restrictions for citrus export. This deterring factor for chemical use has been coupled with the fact that these economically important pests are experiencing insecticidal resistance. As a result, the use of entomopathogenic fungi (EPF) has been explored as a complementary control option in integrated pest management (IPM) regimes. Previous research in South Africa identified several strains of *Beauveria bassiana* and *Metarhizium* spp. (including isolate *M. pinghaense* FCM Ar 23 B3). Laboratory bioassays evaluating the virulence of these isolates against major pests such as the false codling moth (FCM) (*Thaumatotibia leucotreta*, Lepidoptera: Tortricidae), citrus thrips (*Scirtothrips aurantii*, Thysanoptera: Thripidae), and citrus mealybugs (*Planococcus citri*, Hemiptera: Pseudococcidae) highlighted the potential of these EPF. While field trials targeting FCM with soil-applied treatments yielded encouraging results, foliar applications aimed at controlling citrus thrips and mealybugs showed limited success. These findings highlighted the need to assess the biological traits of the recovered isolates. Varying temperature ranges and humidity levels were found to not hinder the isolates' efficacy in the field. Conidial inactivation induced by ultraviolet (UV) radiation however, was. As these strains were recovered from the soil environment, it stood to reason that EPF isolates recovered from the foliar environment may be more suited for foliar application. Thus, bioprospecting for isolates from the aboveground environment was initiated and was the focal point of this thesis. Following the isolation and identification, the pathogenic ability and virulence, as well as the UV tolerance of these novel strains were established. Of the isolates recovered from the aboveground environment

and identified using morphological and molecular techniques, four were *B. bassiana* (Px LM 4, Ha LM 11, Ha LM 12, Coe 18), one *M. anisopliae* (Hu LM 14), one *Fusarium oxysporum* (Pc HV 9), and one *Geotrichum candidum* yeast (Ha LM 2). The majority were isolated from insect cadavers, but one (Coe 18) was isolated as a foliar endophyte from an organically managed citrus farm in the Eastern Cape. Using standard protocols and conidial doses, the virulence of the recovered isolates was established against a common foliar pest of citrus, citrus mealybug. Isolate FCM Ar 23 B3 was included as a comparative control in this study as the virulence against citrus mealybug has previously been established. The initial screening of the isolates ranged between 15 and 90 % mortality. Isolates Px LM 4 and FCM Ar 23 B3 both induced an average mortality of 90 %. Isolates Ha LM 11, Ha LM 12, Hu LM 14, and Coe 18 caused mortalities greater than 60 % and were further investigated under dose-response assays. Of the six isolates measured for $LC_{50}$, FCM Ar 23 B3 was the most virulent ($5.25 \times 10^5$ conidia/ml), followed by Px LM 4 ($1.09 \times 10^6$ conidia/ml) and Hu LM 14 ($1.32 \times 10^6$ conidia/ml). The UV susceptibility to simulated sunlight of the six most virulent isolates was investigated. Whilst UV radiation certainly delayed the conidial germination of all the isolates, all the strains isolated from the aboveground environment demonstrated significant initial tolerance to UV radiation compared to the most virulent *M. pinghaense* FCM Ar 23 B3, which was recovered from the soil environment. Even though the *B. bassiana* Coe 18, which was recovered as an endophytic EPF, was not the most virulent, it stood out with strong initial UV tolerance and sustained a relatively high germination rate over time, establishing it as the most UV-tolerant isolate. Although formulation for development as a microbial biocontrol programme should not be overlooked for these isolates, the initial UV and sustained tolerance demonstrated by these aboveground isolates warrants further investigation under field conditions.

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**CHAPTER 4**

# List of Abbreviations

| | |
|---|---|
| **'** | minute(s) |
| **"** | second(s) |
| **%** | percentage |
| **®** | registered trademark |
| **™** | trademark |
| **°** | degree(s) |
| **°C** | degree(s) Celsius |
| **a.i.** | active ingredient |
| **CBS** | citrus black spot |
| **CE** | controlled environment |
| **CFU** | colony forming unit |
| **CGA** | Citrus Growers' Association |
| **CRI** | Citrus Research International |
| **E** | East |
| **EIL(s)** | economic injury level(s) |
| **EPF** | Entomopathogenic fungi |
| **EU** | European Union |
| **FCM** | false codling moth |
| **h** | hour(s) |
| **ha** | hectare |
| **IGR** | insect growth regulator |

| | |
|---|---|
| **i.e.** | *id est* (that is) |
| **IPM** | integrated pest management |
| **$LC_{50}$** | median lethal concentration |
| **MB** | methyl bromide |
| **MEA** | Malt extract agar |
| **MEAc** | Malt extract agar supplemented with 1 ml chloramphenicol (50 mg/mL) |
| **ml** | millilitre |
| **mm** | millimetre |
| **mg/ml** | milligrams per millilitre |
| **min** | minute(s) |
| **MRL(s)** | maximum residue level(s) |
| **µl** | microlitre |
| **NaCl** | sodium chloride (salt) |
| **NaOCl** | sodium hypochlorite (bleach) |
| **PPP(s)** | plant protection product(s) |
| **PPRI** | Plant Protection Research Institute |
| **RH** | relative humidity |
| **s** | second(s) |
| **S** | South |
| **SDA** | Sabouraud dextrose agar |
| **SDAc** | Sabouraud dextrose agar supplemented with 1 ml chloramphenicol (50 mg/mL) |
| **sd $H_2O$** | sterile distilled water |
| **SE** | standard error |

| | |
|---|---|
| **SIT** | sterile insect technique |
| **USA** | United States of America |
| **UV** | ultraviolet |
| **VOC(s)** | volatile organic compound(s) |
| **et al.** | *et alia* (and others) |
| **e.g.** | *exempli gratia* (for example) |
| **X** | magnification |

# CHAPTER 1: GENERAL INTRODUCTION

**Problem Statement:** The use of entomopathogenic fungi as biological control agents against insect pests is a promising alternative to chemical pesticides. However, the efficacy of these entomopathogenic fungal products when applied to the aboveground environment is limited by UV irradiation. As a result, the use of entomopathogenic fungi for the control of foliar pests in South African citrus orchards has produced unsatisfactory results. Interestingly, most of these commercial products may have been sourced from the belowground environment, where UV radiation is not a factor. To address this limitation, new fungal strains with enhanced natural UV tolerance need to be identified and further developed.

## 1.1 CITRUS IN SA

### 1.1.1 Citrus taxonomy

Genetic analysis has shown that the genus *Citrus*, family Rutaceae, comprises two subgenera: *Citrus* and *Papeda*. Furthermore, *Citrus* (edible fruit) and *Papeda* (non-edible fruit) originate from the sub-tropical and tropical regions of Southeast Asia, respectively, and subsequently spread to other continents (Nicolosi et al. 2000). Classifying *Citrus* is complicated mainly owing to its long history of cultivation, dispersal and reproductive biology. A relatively large proportion of citrus reproduces asexually by producing apomixis through nucellar embryony (Wu et al. 2021). Due to this form of reproduction, gene exchange is generally limited, resulting in reproductive isolation. However, within the genus *Citrus*, there are supposedly few true species with many subspecies due to the high frequency of hybridisation between the true species (Nicolosi et al. 2000). Swingle (1944) and Tanaka (1954) proposed the most commonly followed classification of citrus (Moore

2001). More recently, the use of DNA markers suggests that the Citron (*Citrus medica* L.), the Mandarin (*C. reticulata* Blanco), and the Pummelo (*C. maxima* Merril) are the only true species with other cultivars deemed as hybrids (Nicolosi et al. 2000).

### 1.1.2 Citrus production and export

Globally, citrus is considered the largest and most widely cultivated fruit tree crop (Mo et al. 2022). The South African citrus industry is vast, with the production areas stretching across eight of the nine provinces, covering a total of 96 277 ha of land (Citrus Growers Association (CGA) 2023). The top producing provinces are Limpopo (39 524 ha), Eastern Cape (24 508 ha), and Western Cape (19 208 ha), followed by Mpumalanga (8 127 ha) and KwaZulu-Natal (2 350 ha) (Figure 1.1)

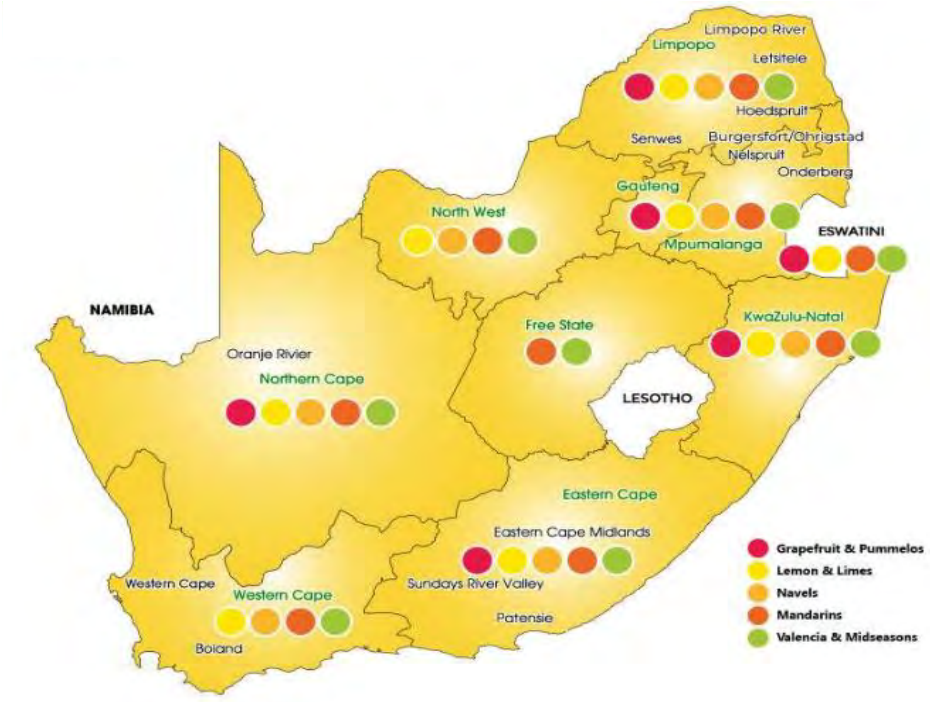


**Figure 1.1:** Different citrus cultivar production regions of South Africa (CGA 2023).

The differences in climatic conditions across South Africa influence production regions (Figure 1.1). For example, relatively colder areas, such as the Eastern and Western Cape, prioritise the production of lemons, Navel Oranges and mandarin types. The warmer climatic regions, such as Mpumalanga, Limpopo and KwaZulu-Natal, are better suited for Valencia oranges and grapefruit. Most export-quality fruits are grown south of 17°S, as colder nights are needed to naturally induce the expected colour change necessary for the commercial appearance and value (Grout & Moore 2015).

The South African citrus industry is the second largest citrus exporter, after Spain (CGA 2023). The major export destinations are Europe, the Middle East and South East Asia, constituting 33%, 19%, and 13% of the total export market, respectively. The majority of citrus produced in South Africa is destined for export (69%), whilst a relatively small portion is processed (25%) and sold locally (6%) (CGA 2023). The main citrus products exported are Valencias (32%), mandarins (20%), lemons (20%), Navels (17%), and grapefruits (11%). However, the South African citrus industry is threatened by an array of pests.

### 1.1.3 Citrus pests: a general overview

Globally, citrus is a host to a multitude of pests. The different climatic conditions across the production regions in South Africa make it an exceptional host to a wide range of citrus pests (Urquhart 1999). As previously mentioned, the climatic conditions across South Africa characterise different cultivar production regions. Similarly, the occurrence and abundance of citrus pests are directly influenced by climatic conditions and thus, specific pests may only attain pest status during certain times of the year when environmental conditions are favourable for proliferation, i.e., exceed economic injury levels (EILs) (Smith & Peña 2002, Grout & Moore

2015). Additionally, some pests may only be problematic for specific cultivars. For example, even though the false codling moth (FCM), *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), is known to infest most citrus types, the Navel orange cultivar (Osbeck var Navel) is most prone to damage as opposed to other citrus types with Lemons (cultivar Eureka) regarded as a non-host (Love et al. 2014, Stotter et al. 2014, Moore et al. 2015).

Citrus pests can be categorised into key groups: major pests, disease vectors, phytosanitary (or quarantine) pests, cosmetic pests and minor pests. Each of these categories can include both major and minor pests depending on the magnitude and frequency of attack and the specific targets of citrus plants: the branches, twigs, leaves, flowers, and fruit (Smith & Peña 2002).

Major pests are defined as those most prevalent throughout the season, causing severe declines in fruit yield and tree health if considered a production pest. Each citrus region is associated with a select few (usually three or four) major pests causing the most damage, which are further classified as key pests. Not all major damage imposed by pests is induced directly, but rather indirectly through the transmission of pathogenic diseases (disease vector). For instance, the African citrus psyllid *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) is considered of economic importance as it transmits a devastating phloem-restricting intracellular bacterium that causes African greening disease, *Candidatus liberobacter africanus* (Smith & Peña 2002).

More prominently, specific key export markets impose stringent regulations against certain citrus pests. These key pests can be further classified as phytosanitary pests, where importing countries exercise zero tolerance for that pest in the commodity. A phytosanitary or quarantine pest is an insect pest deemed to have potential economic importance in regions where it is not present (Follett & Neven 2006, Moore 2021, Moore & Manrakhan 2022). FCM and three fruit fly species are classified as phytosanitary pests due to the obligate internal lifestyle of the larvae of these pests.

However, phytosanitary measures imposed by markets are more focused on FCM. Since the larvae develop and infest within the fruit, detecting contaminated commodities is extremely challenging, and if one individual is found during inspections, entire consignments are rejected by some export markets (Follett & Neven 2006, Moore 2021, Moore & Manrakhan 2022), making it imperative to optimally control these pests. Certain mealybug species are also deemed phytosanitary pests in many South African export markets (Muller & Pountney 2013).

Cosmetic pests, although they do not significantly affect yield or tree health, can cause external blemishes that reduce the fruit's market value, specifically for export markets where appearance is crucial. Such damage, while superficial, can result in economic losses due to the devaluation of cosmetically affected fruit.

Minor pests are classified as having the potential to inflict damage but are usually present in small populations or occur sporadically. Some citrus pests, such as mealybugs and scale insects, can be deemed as secondary pests which are generally successfully controlled by natural enemies. However, excessive use of broad-spectrum pesticides disrupts this natural control and results in increased populations of these secondary pests (Smith & Peña 2002, Grout & Moore 2015).

Bedford et al. (1998) listed more than 110 insect and mite citrus pests in South Africa. More recently, though, Grout & Moore (2015) listed 63 citrus pests as they purposely left out the very minor and sporadic pests. However, only 11 insect pests are considered to pose an economic threat to the South African citrus industry. These pests include red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diapsidae), citrus thrips, *Scirtothrips aurantii* Faure (Thysanoptera: Thripidae), African citrus psylla, citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), oleander mealybug, *Paracoccus burnerae* (Brain) (Hemiptera: Pseudococcidae), long-tailed mealybug, *Pseudococcus longispinus* Targioni-Tozzetti (Hemiptera: Pseudococcidae), Oriental

fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), Natal fruit fly, *Ceratitis rosa* Karsch (Diptera: Tephritidae), and most notably, FCM (Table 1.1) (Smith & Peña 2002, Grout & Moore 2015, Meyer et al. 2016, Manrakhan et al. 2018). Even though the Oriental fruit fly is known to be able to infest citrus (Theron et al. 2023), there appears to be no record of infestation in citrus orchard in South Africa.

**Table 1.1:** Common arboreal pests of citrus in South Africa and the type of damage imposed [Adapted from Grout & Moore (2015)].

| **Family** | **Common name** | **Scientific name** | **Feeding damage locality** |
|---|---|---|---|
| Cicadellidae | Citrus leafhopper | *Penthimiola bella* | Young leaves, fruit |
| | Green citrus leafhopper | *Empoasca distinguenda* | Young leaves, fruit |
| Triozidae | Citrus triozids | *Trioza erytreae* | Young leaves, vector |
| Aleyrodidae | Woolly whitefly/Citrus whitefly | *Aleurothrixus floccosus* | Young leaves, promotes sooty mould |
| Aphididae | Spirea aphid | *Aphis spiraecola* | Young leaves, twigs |
| | Black citrus aphid | *Toxoptera citricidus* | Young leaves, twigs, vector |
| Diaspididae | Red scale | *Aonidiella aurantii* | Leaves, fruit, branches |
| | Circular purple scale | *Chrysomphalus aonidum* | Leaves, fruit |
| | Citrus mussel scale | *Lepidosaphes beckii* | Leaves, fruit, twigs, branches |
| | Long mussel scale | *Lepidosaphes gloverii* | Leaves, fruit, twigs, branches |
| Coccidae | Citrus wax scale | *Ceroplastes destructor* | Leaves, twigs |
| | White wax scale | *Ceroplastes brevicauda* | Leaves, twigs |
| | Brown softs scale | *Coccus hesperidum* | Leaves, fruit |
| | Green soft scale | *Pulvinaria aethiopica* | Leaves, twigs |
| | White powdery scale | *Pseudocribrolecanium andersoni* | Leaves |

| | | | |
|---|---|---|---|
| Pseudococcidae | Citrus mealybug | *Planococcus citri* | Leaves, fruit, twigs |
| | Oleander mealybug | *Paracoccus burnerae* | Leaves, fruit, twigs |
| | Karoo thorn mealybug | *Nipaecoccus viridis* | Leaves, fruit, twigs |
| | Long-tailed mealybug | *Pseudococcus longispinus* | Leaves, fruit, twigs |
| Thripidae | Citrus thrips | *Scirtothrips aurantii* | Leaves, fruit |
| Tephritidae | Natal fruit fly | *Ceratitis rosa* | Fruit |
| | Mediterranean fruit fly | *Ceratitis capitata* | Fruit |
| | Asian fruit fly | *Bactrocera dorsalis* | Fruit |
| Tortricidae | False codling moth | *Thaumatotibia leucotreta* | Fruit |
| | Citrus leaf roller | *Choristoneura occidentalis* | Leaves, fruit |
| Gracillariidae | Citrus leaf miner | *Phyllocnistis citrella* | Leaves, twigs |
| Praydidae | Citrus flower moth | *Prays citri* | Fruit, flowers |
| Pyralidae | Carob moth | *Ectomyelois ceratoniae* | Fruit |
| Geometridae | Citrus looper | *Ascotis reciprocaria* | Leaves, fruit |
| Noctuidae | Fruit-piercing moth | *Serrodes partita* | Fruit |
| | African bollworm | *Helicoverpa armigera* | Leaves, fruit, flowers |
| Papilionidae | Citrus swallowtail | *Papilio demodocus* | Leaves |

### 1.1.4 Control of citrus pests: a general overview

Citrus pests can be controlled in various ways. Historically, synthetic insecticides were heavily relied upon for the control of these pests. These chemical insecticides included pyrethroids and organophosphates. Pyrethroids and organophosphates were later discovered to be toxic to the environment, animals and humans. This extensive chemical usage caused certain pests that were previously not of major concern to become economically important due to subsequent outbreaks (Bedford et al. 1998, Urquhart 1999). For instance, the proliferation of red scale was due to the reduction of natural enemies as a result of chemical spraying (Bedford et al. 1998). Resurgences of some mealybug populations were also due to the disruption of their natural enemy complex (Franco et al. 2004). In the Eastern Cape Province, the use of the fungicide, mancozeb, has

increased due to European regulations for citrus black spot, *Phyllosticta citricarpa,* as a quarantine organism. Consequently, an increase in populations of citrus thrips have been recorded as this chemical has shown to be responsible for the decline in populations of the phytoseiid mites, *Euseius addoensis*, (McMurtry) (Acari: Phytoseiidae), which is an important thrips predator (Grout et al. 1997, Grout 2015). Excessive chemical usage also resulted in the development of insecticidal resistance in many pest species, commonly seen in red scale to organophosphates (Urquhart 1999, Grafton-Cardwell et al. 2001) and FCM to triflumuron (Hofmeyr & Pringle 1998). Recently, low levels of resistance to organophosphates have also been observed in citrus mealybug in India (Mruthunjayaswamy et al. 2019). In addition to these negative aspects of chemical insecticides, export markets (e.g., U.S.A and Europe) impose stringent maximum residue levels (MRLs) for plant protection products (PPPs). As a result, control strategies shifted toward employing more environmentally sustainable control measures and thus, integrated pest management (IPM) emerged. Urquhart (1999) discussed IPM and the citrus industry and Barzman et al. (2015) provided a practical framework of principles of IPM. To achieve control of pests, IPM integrates various control measures such as cultural control, chemical control and biological control, with the most importance placed on biological control.

The aim of IPM is to suppress and manage pest populations below EILs, not to eliminate entire populations. IPM is considered a holistic plan of action, placing prominence on a systems approach. It essentially involves using a variety of control measures aimed at managing pest populations, while protecting non-target organisms in an environmentally responsible manner. It is important to highlight that monitoring is essential in all aspects of IPM, as it ultimately allows for improved pest control. This improved control is achieved, as monitoring allows for the determination of exact pest levels and the use of economic thresholds (Smith & Peña 2002).

Citrus Research International (CRI) develops comprehensive IPM programmes for the control of phytosanitary and key insect pests. For example, CRI has developed an extensive and highly effective control programme for the phytosanitary pest, FCM. This control programme encompasses cultural control (orchard sanitation), behavioural control (using mating disruption), sterile insect technique (SIT), and biological control methods (e.g., using the egg parasitoid, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae), and the newly, commercially available entomopathogenic NPV baculovirus-based product, MultiMax® (River Bioscience, South Africa). These integrated control measures have demonstrated a reduction of up to 97% in FCM infestations (Moore & Kirkman 2008, Moore & Hattingh 2012, Hofmeyr et al. 2016, Malan et al. 2018, Moore 2021).

Although IPM has proved very effective for some insects, other pests may be more challenging. For example, citrus thrips and mealybug are extremely difficult to control chemically, due to the limited abundance of chemicals registered for their control, but also the cryptic ovipositional site of citrus thrips and the wax protective covering of the mealybug (in addition to their multivoltine existence) (Muller & Pountney 2013, Moore & Hattingh 2022). As a result, citrus thrips and mealybug control rely heavily on the natural and augmented populations of their predators and parasitoids. There are at least three important biological control agents that are commercially available in South Africa for the control of citrus mealybug, i.e., the predatory beetle, *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), and parasitic wasps, *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae) and *Anagyrus vladimiri* (Triapitsyn) (Hymenoptera: Encyrtidae) (Moore & Hattingh 2004, Muller & Pountney 2013). The predatory mites, *Euseius addoensis* (Van der Merwe & Ryke) (Acari: Phytoseiidae) and *E. citri*

(Van der Merwe & Ryke) (Acari: Phytoseiidae), play an important role in reducing citrus thrips damage, when present in high numbers (Grout et al. 1997, Grout 2015).

Populations of their natural enemies are, however, under threat due to the use of chemical insecticides, subsequently leading to sporadic secondary outbreaks of these two pests. Insecticidal resistance in both these key pests has been recorded (Grout 2015). It is, therefore, vitally important to search for alternative control measures for citrus thrips and mealybug, which are substantially less harmful to the natural enemies of these two key arboreal citrus pests.

Microbes, such as bacteria, nematodes, viruses, and fungi, have been employed for the control of insect pests and plant pathogens of citrus. Most of these microbial biocontrol agents have been shown to be greatly successful and IPM-compatible (Lacey et al. 2001, Lacey & Shapiro-Ilan 2003). Amongst the entomopathogenic fungi (EPF), two fungal species, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) are being incorporated into control programmes globally. EPF for the control of arboreal citrus pests is the focal point of this research.

## 1.2 ENTOMOPATHOGENIC FUNGI

### 1.2.1 Classification

An entomopathogen is described as a disease-causing organism, such as fungi, bacteria, viruses, and nematodes, which mainly kills or disables insects and is known to infect other arthropods (Hajek & van Frankenhuyzen 2017). Of the total 1.5 – 5.1 million fungal species described, only 750 species from approximately 90 different genera are considered to be entomopathogenic (Mora et al. 2018, Bamisile et al. 2021a). Entomopathogenic fungal species mainly belong to the

Entomophthoromycota and Ascomycota phyla, which occur most frequently in nature (Litwin et al. 2020). Fungi from Entomophthoromycota are not of major interest in terms of mycoinsecticides largely owing to their highly specialised biology, making large-scale production complicated (Singh et al. 2017). Within the Ascomycota division, the majority of agriculturally important EPF genera, such as *Metarhizium, Beauveria, Paecilomyces, Isaria,* and *Akanthomyces* (previously known as *Lecanicillium*), fall within the order Hypocreales (Litwin et al. 2020).

### 1.2.2 General biology

EPF belonging to the order Hypocreales have a broad host range, largely owing to their opportunistic ability. This is demonstrated by the fact that *B. bassiana* has been reported to infect over 700 insect species from an array of different orders (Elkhateeb et al. 2021). Furthermore, Elkhateeb et al. (2021) provides an extensive table with various examples of insect pest species successfully controlled by *B. bassiana* and *M. anisopliae.* The hemibiotrophic pathogenic fungi initially demonstrate biotrophy, depleting the host of its nutrients within the hemocoel, followed by a saprophytic phase where the fungi colonise the rest of the cadaver (Roy et al. 2006). Entomopathogenic fungal species and strains all demonstrate different biotic and abiotic needs; however, they all follow a similar infection process (Roy et al. 2006).

Unlike most entomopathogenic microbes, EPF infect insects through penetration of the host's cuticle (Mascarin & Jaronski 2016). The infection process of these microbes in insects is well documented and follows a six-stage process: adhesion of the spores; germination; penetration through the integument or natural openings; haemolymph colonisation, whilst attempting to overcome the host immune reactions; hyphal formation and proliferation; and sporulation and new conidia outgrowth (Elkhateeb et al. 2021) (Figure 1.2).

The infection process begins with the attachment of conidia or blastospores onto the outer layer of the host's cuticle. After successful adhesion, germination and growth of the fungus across the host and through the cuticular layers are supplemented by the expression of hydrolytic enzymes, e.g., proteases, chitinases, and lipases (Ortiz-Urquiza & Keyhani 2013). During this process, the fungal pathogen penetrates the host through natural openings, direct penetration, and/or enzymatic degradation. Direct penetration involves using specialised infection structures, such as appressoria or penetration pegs, that allow the growing hyphae to breach directly into the host integument. Enzymatic degradation involves the production of enzymes that degrade the insect cuticle, subsequently forming an entry point for the fungus. Some EPF can penetrate the host through natural openings, such as the mouthparts, spiracles, and anus (Chaurasiya et al. 2021). The fungus penetrates the host until reaching the nutrient-rich haemolymph. Inside the haemocoel, the fungal cells morphologically transform from filamentous growth to single-celled, yeast-like hyphal bodies called blastospores that exploit and colonise internal tissue sequentially (Mascarin & Jaronski 2016, Mannino et al. 2019). A few days after host death and mummification of the insect cadaver, conidiophores emerge and sporulate, subsequently releasing infective conidia to successfully continue its lifecycle (Mascarin & Jaronski 2016). EPF can affect the host in multiple ways, such as reduced feeding and fecundity, as well as behavioural changes. Roy et al. (2006) explicitly documented and reviewed various examples of host-altered behaviour inflicted by EPF.

Insects have innate defence mechanisms that have presented resistance to infective microbial pathogens and can avoid infection through behavioural and/or immune defence mechanisms. Behaviours such as grooming, which can be observed in social insects, are common. This behaviour removes EPF spores from the host body and prevents infection. Other examples include decreased social contact in ants and behavioural thermoregulation, where the host seeks out higher

temperatures in the hopes of increasing its own body temperature and overcoming EPF infection. de Roode & Lefèvre (2012) reviewed the various behavioural strategies insects employ to resist pathogenic microbes.

Insects have both humoral and cellular defences for infection prevention while making death avoidable even if the infection cycle has begun. Upon attachment of the conidia and penetration of the cuticle, initial host defence mechanisms are activated (Castrillo et al. 2005). These mechanisms usually involve the encapsulation of germ tubes or upon fungal penetration into the haemocoel, phagocytic activities, nodule formation prevention, and encapsulation (Castrillo et al. 2005). Moreover, insects secrete antimicrobial peptides into the haemolymph (Elkhateeb et al. 2021). However, secondary metabolites (some of which have insecticidal properties) produced by EPF directly and indirectly disable or counteract host defence mechanisms and accelerate the infection rate of the EPF (Castrillo et al. 2005, Paschapur et al. 2021). Ultimately, through coevolution, it is an evolutionary arms race between pathogen and host, demonstrated by the adaptions that either ensure infection and efficacy or prevent infection (Roy et al. 2006, Butt et al. 2016, Ma et al. 2024).

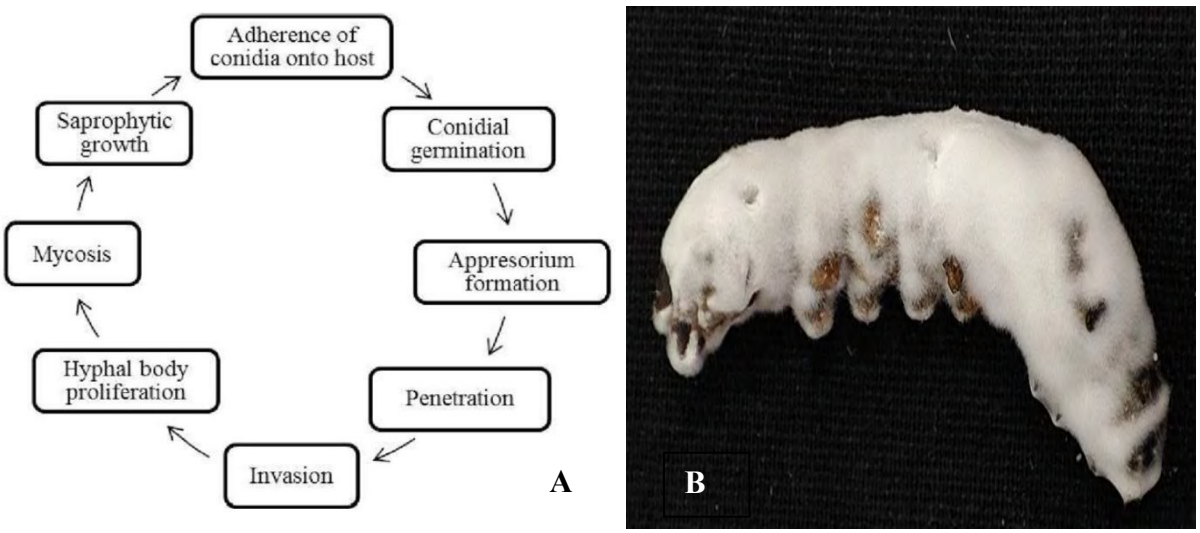


**Figure 1.2:** (A) Infection process of entomopathogenic fungi (Majumder et al. 2018), (B) *Beauveria bassiana* infected larvae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Islam et al. 2023).

### 1.2.3 Entomopathogenic fungi as biocontrol agents

Owing to their rich terrestrial diversity (Gul et al. 2014), using pathogenic fungal species to control insect pests is not a novel technique. In 1835, the first occurrence of entomopathogenic fungi was documented by an Italian entomologist. Bassi described a white muscardine fungus, later named *Beauveria bassiana* (Balsamo), killing insects (Mantzoukas et al. 2022). Shortly after, Metchnikoff suggested the use of *Metarhizium anisopliae* (Metchnikoff), the agent of green muscardine disease, for the control of insect pests (Zimmermann et al. 1995). From here, bioprospecting for fungi with insecticidal properties and using them as microbial biocontrol agents became prominent. However, the establishment of fungi in pest management was halted by the emergence of chemical pesticides (Mantzoukas et al. 2022). This is due to the fact that pesticides are cheap, readily available and easily storable products that efficiently kill pests within 24 hours, whereas fungal pathogens are

relatively costly to produce and store and may take 2 – 3 weeks to kill pests after application. Furthermore, EPF are dependent on high pest population numbers (infestations), while chemical pesticides do not rely on pest densities to be effective (Khan et al. 2012). In contrast to chemical pesticides that kill various pests, EPF are relatively species-specific and will likely need to be supplemented with other measures to achieve control (Singh et al. 2017). Regardless of these limitations, employing EPF as biological agents offers various benefits, such as being environmentally safe, generally species-specific, leaving no toxic residue on fruit nor the environment, reduced likelihood of insect resistance, and typically compatible with IPM control measures (Singh et al. 2017, Chaurasiya et al. 2021).

To date, there are a number of products registered as fungal biopesticides worldwide that are being used against several insect pests of agricultural importance (Chaurasiya et al. 2021). Based on the list of commercial EPF products available worldwide provided by Bamisile et al. (2021a), *M. anisopliae* constitutes the majority, with 54.39 %, whilst *B. bassiana*, *B. brongniartii, L. lecanii* (updated name: *A. lecanii*)*, I. farinosa,* and *I. fumosorosea* constitute 29.82 %, 8.77 %, 3.51 %, 1.75 %, and 1.75 %, respectively. A comprehensive list of registered EPF biopesticides with their target pests, producer and country of production is provided in Faria & Wraight (2007), Maina et al. (2018), Bamisile et al. (2021a), Chaurasiya et al. (2021), Sharma et al. (2021), and Liu et al. (2023).

Employing EPF in the field can be challenging (Singh et al. 2017). Multiple papers point to the fact that the efficacy of the fungal pathogens decreases exponentially in the field, compared to laboratory trials (Moore 2002, Batta 2003, Singh et al. 2017, Maina et al. 2018, Quesada-Moraga et al. 2023). The persistence of these fungal biopesticides, which is an integral component in determining the success of the microbial biocontrol programme (Jackson 1999), is influenced by

abiotic factors, such as temperature, humidity, and solar radiation (ultraviolet (UV) light) (Wu et al. 2020, Quesada-Moraga et al. 2023). However, fungal isolates within the hypogeal habitat mainly face temperature and humidity as limiting factors, whereas isolates from the epigeal environment are limited by temperature, humidity and UV radiation (Quesada-Moraga et al. 2023). For example, the infection process is heavily dependent on environmental conditions, demonstrated by the fact that conidium germination, infection, and sporulation all require an excess of 95 % relative humidity, whilst the virulence of the strain and the kill rate is influenced by temperature (Roy et al. 2006).

Acheampong et al. (2020a) demonstrated that isolates of both *B. bassiana* and *M. anisopliae*, regardless of conidial concentration, successfully infected FCM fifth instars when exposed to humidity levels below 95 %. However, sporulation of the two fungal species was only recorded on cadavers exposed to 98 % relative humidity.

Furthermore, the survival success of foliar-applied EPF is affected by solar radiation. More specifically, the conidial viability decreases exponentially when exposed to sunlight (UV-B solar radiation) (Wu et al. 2020, Quesada-Moraga et al. 2023). Although species and isolate dependant, fungal species within the genus *Beauveria* have shown greater resistance to UV radiation compared to some fungal species within the *Metarhizium* genus (further demonstrating that the presence of dark-pigmented conidia does not infer the presence of melanin nor greater UV protection) (Fernández-Bravo et al. 2017). It has been speculated that the increased UV tolerance in *Beauveria* spp. is likely due to the presence of melanin-like structures, absorbing UV radiation and ultimately acting as a sunscreen (Quesada-Moraga et al. 2023). Meyling et al. (2011) reported differences between entomopathogenic fungal communities pertaining to aboveground and belowground niches. The authors mainly documented *Metarhizium* within the belowground

community, whilst *Beauveria* dominated the aboveground environment. The localisation (epigeal or hypogeal environment) of these different EPF species, therefore, may be correlated to their ability to tolerate UV radiation. Research suggests that entomopathogenic fungal strains evolve to exist and survive based on habitat, ultimately determining environmental tolerance levels. The isolation habitat of EPF (e.g. agricultural vs forested) (Bidochka et al. 2001), as well as the geographical origins (e.g. latitudinal differences) (Braga et al. 2001b) have been shown to influence the UV resistance of EPF. However, these findings have not been consistent. Fernández-Bravo et al. (2017) demonstrated that *Metarhizium* isolates recovered from the phylloplane (leaf surface) did not display a greater UV-B radiation tolerance than isolates recovered from the soil.

A review by Quesada-Moraga et al. (2023) thoroughly discusses the abiotic factors that influence EPF efficacy and emphasises that different strains (below species level) demonstrate different tolerance levels to these environmental factors. The fact that abiotic factors highly influence the efficacy of EPF ultimately highlights that the selection of an environmentally competent entomopathogenic fungal strain is crucial for the success of a microbial biocontrol programme (Quesada-Moraga et al. 2023).

## 1.3 EPF AS BIOCONTROL AGENTS IN SOUTH AFRICAN CITRUS ORCHARDS

In South Africa, a few EPF products have been registered for use. Commercial products, such as Broadband™ (BASF, South Africa) and Eco-Bb® (Plant Health products, South Africa), both consist of *B. bassiana*, whilst Real Metarhizium 69 (Real IPM, Nairobi, Kenya) consists of *M. anisopliae*. In pursuit of creating a mycoinsecticide tailored for use against key citrus pests of

South African citrus orchards, numerous investigations were conducted collaboratively by Citrus Research International and the Centre for Biological Control, Department of Zoology and Entomology at Rhodes University. Consequently, 62 strains of EPF, recovered by Goble et al. (2010), were sourced from the soil within and around conventionally and organically managed citrus orchards in the Eastern Cape. Of the various strains recovered, Goble et al. (2011) established the comparative virulence of 21 strains against the subterranean life stages of the three key citrus pests: FCM, the Mediterranean fruit fly and the Natal fruit fly. Under laboratory conditions, 12 isolates that caused over 80 % mortality of FCM soil-dwelling life stages were identified.

Through field trials, Coombes et al. (2013, 2016) identified three isolates (*M. anisopliae* sensu lato G 11 3 L6, *M. pinghaense* FCM Ar 23 B3 and *B. bassiana* sensu lato G Ar 17 B3) that demonstrated effective control of FCM for at least six months following soil application. These virulent isolates were further tested against citrus thrips (Chartier-FitzGerald 2014), citrus mealybug (Chartier-FitzGerald 2014, Chartier-FitzGerald et al. 2016) and red scale (*A. aurantii*) (Upfold et al. 2016) through laboratory trials, as well as field trials against mealybug and thrips (Grout et al. 2015).

As previously mentioned, field trials against subterranean FCM were effective. Laboratory trials against citrus mealybug displayed great control potential (Chartier-FitzGerald et al. 2016). However, control of citrus thrips and mealybug, for which effective control is greatly sought, has been highly disappointing in field trials (Grout et al. 2015). Subsequently, Acheampong et al. (2020a, 2020b) demonstrated how abiotic factors (temperature, relative humidity, and solar radiation) influence the efficacy of seven of the most promising soil-recovered isolates (*M. anisopliae* s.l. G 11 3 L6, *M. pinghaense* FCM Ar 23 B3, *M. anisopliae* s.l. G OL R8, *B. bassiana*

G Ar 17 B3, *B. bassiana* s.l. G B Ar 23 B3, *B. bassiana* s.l. G 14 2 B5 and *B. bassiana* s.l. FCM 10 13 L1). They found that the poor performance of these entomopathogens in the field is mainly owed to their susceptibility to UV radiation, as 2 h exposure to simulated radiation resulted in death for all of the fungal isolates. Their extremely low tolerance to UV radiation could explain why these isolates did not significantly reduce these two arboreal pests in the field. This ultimately hinders the possibility of foliar application for foliar pest control using EPF without developing a suitable UV protectant formulation.

## 1.4 RESEARCH AIMS

Variability in susceptibility to UV radiation appears to be correlated to the habitat from where fungi are isolated (Fernandes et al. 2015). For example, Fernandes et al. (2009) demonstrated that *B. bassiana* isolates recovered from low latitudes, where warmer regions experience stronger UV radiation, were more UV-B tolerant than those recovered further away from the equator. Furthermore, fungal isolates recovered from agricultural settings showed greater UV tolerance than isolates recovered from forested areas (Fernandes et al. 2015). As a result, it is presumed that EPF isolated from epigeal habitats experience more intense abiotic challenges and thus exhibit higher UV tolerance than isolates recovered from hypogeal habitats. (Bidochka et al. 2001).

Different strains (below species level) demonstrate different tolerance levels to environmental stressors. The fact that these abiotic factors highly influence the efficacy of EPF ultimately highlights that the selection of an environmentally competent (particularly to UV irradiation) entomopathogenic fungal strain is crucial for the success of a microbial biocontrol programme (Quesada-Moraga et al. 2023). Critical steps in the identification of promising biological control agents involve the selection of strains exhibiting elevated virulence towards the target pest and

choosing candidate isolates demonstrating exceptional natural UV tolerance (Fernandes et al. 2015), in addition to environmental competence (Quesada-Moraga et al. 2023). This highlights the importance of continuous local bioprospecting for novel, virulent strains that exhibit environmental competence.

This thesis aims to (1) bioprospect for novel EPF from infected foliar pests and endophytic EPF from leaf material of organically and conventionally managed citrus plants (epigeal environment). Some EPF can asymptomatically colonise certain plant/crop species and exist as endophytes (Bamisile et al. 2021a, Mantzoukas et al. 2022). Endophytic fungi have been reported to enhance the environmental tolerance of EPF, making these entomopathogenic fungal isolates persist significantly longer in the field than non-endophytic EPF (Mantzoukas & Eliopoulos 2020). As a result, the alternative use of EPF as endophytes has become prominent in research (Arnoldi et al. 2022). Furthermore, this thesis aims to (2) establish the virulence of isolated EPF against an aboveground citrus pest and (3) establish the UV sensitivity of the most promising virulent isolates in comparison to EPF isolated from the hypogeal environment.

# CHAPTER 2: EPF ASSOCIATED WITH FOLIAR PESTS: A SURVEY FOR POTENTIAL ENTOMOPATHOGENIC FUNGI

## 2. 1 INTRODUCTION

As previously outlined in Chapter 1, the listing of the most common and economically prominent South African citrus pests follows that of Bedford et al. (1998), in which the authors list 100 insect pests and 10 mite pests. More recently, Grout & Moore (2015) established an inventory of 63 pests associated with citrus in South Africa, the type of damage imposed, and the suggested management for that pest. Moore & Duncan (2017) inventoried citrus pests and the associated EPF recorded attacking the pests. The authors highlight the abundance of EPF attacking these pests but also the lack of commercialised EPF products registered for citrus pest control in South Africa, specifically for the control of arboreal citrus pests. Subterranean citrus pests, or pests with subterranean life stages, in South Africa, have shown great potential to be controlled by EPF in the field (Coombes et al. 2013, 2016). It is also becoming increasingly important to find alternatives to pesticides, owing to more stringent EU export market regulations and the observed insecticide resistance, for the control of South African arboreal citrus pests.

There is a consistent rise in the use of microbial biopesticides to control agricultural pests; however, the efficacy of these, including EPF in the field, is limited by abiotic factors such as temperature, humidity, and UV radiation (Jackson et al. 2010, Jaronski 2010, Vega et al. 2012, Bamisile et al. 2021a, Quesada-Moraga et al. 2023).

The effects of temperature on the efficacy of entomopathogenic fungal isolates have been well documented (Quesada-Moraga et al. 2023), with most research exacerbating the interspecies and

intraspecies variability in tolerance to different temperature ranges. However, temperature is still considered a crucial factor as it affects growth, spore germination and host penetration and, thus, the pathogenic ability, virulence, and viability of the EPF isolate in the field (Zimmermann 2007, Tumuhaise et al. 2018, Quesada-Moraga et al. 2023). Whilst some studies, e.g., Fargues et al. (1997), show no relationship between climatic origin and relative growth rates of isolates from different climatic regions, several other studies suggest this influences the temperature tolerance ranges of isolates. Though 25 to 35 ℃ is considered the optimal temperature range for growth rate and germination for most EPF (Luz & Fargues 1997, Quesada-Moraga et al. 2023), there is major variability between strains. For example, Tumuhaise et al. (2018) demonstrated temperature differences between two *Metarhizium anisopliae* strains (ICIPE 18 and ICIPE 69) regarding growth rate, conidial germination percentage and virulence against *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) larvae. Similarly, Dimbi et al. (2004) found that temperature varyingly influences the mycelial growth rate, conidial germination as well as percentage mortality against *C. capitata, C. cosyra* and *C. fasciventris* for six different isolates of *Metarhizium anisopliae*. Vidal et al. (1997) demonstrated that *Isaria* (=*Paecilomyces*) *fumosorosea* displayed intraspecific variability of temperature ranges for growth in relation to the habitat origin of the isolate. Strains isolated from Europe (temperate environment) had a temperature growth range from 8 to 30 ℃, whereas strains isolated from the southern United States (humid and dry subtropical climates) and west Asia (humid tropical climate) had a slightly broader range from 8 to 35 ℃. Optimal growth rates, however, for all 37 isolates of *I. fumosorosea* were between 20 and 30 ℃ (Vidal et al. 1997). Similar trends are observed in *Beauveria bassiana*, where temperature also influences radial growth, germination percentage and virulence based on the originating habitat of the isolated EPF strain. Seid et al. (2019) showed that isolates recovered from tropical environments grew more

profusely and showed greater conidial germination at higher temperatures as opposed to arctic isolates displaying a greater hyphal growth rate and conidial germination at lower temperatures. As shown, there is a common trend between optimal temperature range tolerance and the isolation habitat of the strain (Inglis et al. 2001).

Humidity is considered a crucial factor influencing conidial germination, sporulation and efficacy. As with temperature, there is variability in relative humidity (RH) requirements for different isolates of EPF (Jaronski 2010, Vega et al. 2012, Quesada-Moraga et al. 2023). However, > 90 % humidity is considered optimal for most EPF (Luz & Fargues 1997, Quesada-Moraga et al. 2023). Shipp et al. (2003) found that the virulence of the commercially formulated *B. bassiana* strain GHA [Botanigard® ES, (Emerald BioAgriculture Corporation, USA)] varied based on different humidity regimes. Conducted in Petri dishes, the authors found that a humidity of 97.5 % caused significantly more mortalities of several greenhouse pests compared to RH of 75 and 80 %. Similarly, another *B. bassiana* (Bb INRA 297) isolate caused significantly higher mortalities and increased kill speed in *Rhodnius prolixus* Ståhl (Hemiptera: Reduviidae) at 96 % RH compared to lower RH levels (Luz & Fargues 1999). Interestingly, Hu et al. (2021) found that the conidia of two strains of *B. bassiana* (Bb10331 and Bb7725) had a germination rate of over 70 % at a RH of 70 % in the absence of a host. However, both strains' optimum humidity for conidial germination was recorded at 95 – 100 % RH. Athanassiou et al. (2017) found no relationship between the virulence of an isolate of *M. anisopliae* at two different relative humidity levels (55 and 75 %) for the control of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). In saying this, the isolate was still able to germinate at a considerably lower RH than what is said to be optimal. Conversely, Acheampong et al. (2020a) found that a relative humidity range of 12 – 98 % has no effect on the

virulence of four *M. anisopliae* and five *B. bassiana* isolates (including two formulated commercial strains) against fifth-instar FCM.

Ultraviolet (UV) radiation is considered to be the most detrimental factor inhibiting EPF efficacy following foliar application in field settings (Quesada-Moraga et al. 2023). Similar to temperature and humidity, EPF species and strains display inter- and intra-species variation in tolerance to UV radiation (Fernandes et al. 2015). UV radiation as an abiotic factor influencing EPF is further discussed in Chapter 4, section 4.1.

Above and belowground communities of entomopathogenic fungal isolates may differ in terms of the taxa present as well as the frequency of that specific taxa present (Meyling et al. 2011). For example, Meyling et al. (2011) surveyed both soil-borne and aboveground EPF in conventionally and organically managed cropping systems and found a clear distinction between the below- and aboveground communities of fungal entomopathogens. *Metarhizium anisopliae* was the most common in the belowground environment but absent aboveground, whereas *B. bassiana* was the most common aboveground (Meyling et al. 2011). Some entomopathogenic fungal genera, limited to the groups Ascomycota and Basidiomycota, can asymptomatically colonise plants and exist as endophytes (Bamisile et al. 2021a, Mantzoukas et al. 2022). Similarly, endophytic *Beauveria* strains have been found predominantly to colonise the stems and leaves of plants, whereas endophytic strains of *Metarhizium* are more abundant in the rhizosphere of plants (Behie et al. 2015). With these differences in taxa localisation, it can be deduced that certain taxa of EPF have evolved and adapted to exist in that certain environment. Endophytic fungi have been reported to enhance the environmental tolerance of EPF, making these entomopathogenic fungal isolates persist significantly longer in the field compared to non-endophytic EPF (Mantzoukas & Eliopoulos 2020). Endophytic EPF have also been shown to promote plant growth in several plant

species (Bamisile et al. 2018, 2021b). As a result, the alternative use of EPF as endophytes has become prominent in research (Arnoldi et al. 2022). There have been a few records of naturally colonised EPF isolated from agriculturally important host plants. For example, Vega et al. (2010), Reay et al. (2010), and Rajab et al. (2023) isolated different *B. bassiana* strains from *Coffee arabica* L. (Rubiaceae), *Pinus radiata* L. (Pinaceae), and *Cucumis sativus* L. (Cucurbitaceae), respectively and Kambrekar & Aruna (2018) recovered a *B. bassiana* strain from a *Citrus* spp. (Rutaceae).

EPF isolates from different habitats likely have different tolerances to environmental parameters (humidity, temperature and UV radiation), as suggested in the above examples, and this variability in susceptibility to UV radiation may reflect the natural adaptations of EPF isolates to their different environmental conditions (Fernandes et al. 2015). Selecting an environmentally competent strain to increase persistence in the aboveground environment is most desirable for field application.

Therefore, the aims of this chapter were (1) to recover indigenous entomopathogenic fungi from infected insects found in the aboveground environment and foliar endophytes from both conventionally and organically managed farms, (2) to morphologically and genetically identify the novel EPF isolates and (3) to ultimately establish a collection of epigeal-isolated EPF that could be used as a potential biological control agent against foliar citrus pests.

## 2. 2 MATERIALS AND METHODS

### 2. 2.1 Entomopathogenic fungal isolation and identification

#### 2.2.1.1 Sampling and isolation from infected insects

Epizootics are most often observed when insect infestations occur (Onstad & Carruthers 2003). Since infestations are hard to predict, regular scouting was undertaken during the summer season from February to April 2024 in a nearby, easily accessible cabbage farm, Varnam Farm (Belmont Valley, Makhanda, Eastern Cape), to increase the probability of locating infected aboveground insects (Table 2.1). Scouting events occurred every two weeks. Both sporulating insect cadavers and healthy pests were taken back to the laboratory. Healthy insects were kept until death to observe if fungal infection would present itself at any point. When sampling for foliar endophytes (section 2.2.1.2), scouting for infected insects in the citrus orchards occurred simultaneously. Suspected EPF-infected insect cadavers were placed into an Eppendorf tube filled with sterile distilled water (sd $H_2O$) supplemented with 0.05 % Tween® 20. The Eppendorf was then vortex-mixed for 30 s, and two subsequent 10-fold serial dilutions were made. 100 µl of the suspensions were pipetted onto Sabouraud dextrose agar plates supplemented with 1 ml 50 mg/ml chloramphenicol (SDAc) and surface spread with a sterilised glass rod, parafilm sealed and incubated for five days at 26 °C (12 h photoperiod) to allow single colonies to form. The hyphae from single colonies were further sub-cultured on SDAc plates to achieve pure cultures. Furthermore, the same sporulating cadaver was surface sterilised in 70 % ethanol for 30 s and subsequently placed on a separate SDAc plate, sealed with parafilm, and incubated at 26 °C with a 12 h photoperiod. All saprophytic fungi and non-EPF fungi were discarded. Recovered EPF were labelled using the following system: the first part describes the host insect (e.g. Ha = *Helicoverpa armigera*); the second is the farm from which the isolate was sampled (e.g. LM = Lower Melrose);

and the third is a given isolate number (e.g., 1). All isolates were grown on SDAc plates and incubated at 26 °C with a 12 h photoperiod. Once pure cultures were obtained, potential EPF isolates were stored at 4 °C in a refrigerated unit.

**Table 2.1:** Potential EPF isolates recovered from aboveground insect pests that exhibited mycosis.

| Isolate | Origin farm (orchard #) | Coordinates | Host/substrate | Collection date |
|---|---|---|---|---|
| Ha LM 2 | Lower Melrose | 33º19'43"S 26º38'41"E | *Helicoverpa armigera*/ Cabbage | 21/02/2023 |
| Px LM 4 | Lower Melrose | 33º19'43"S 26º38'41"E | *Plutella xylostella*/ Cabbage | 21/02/2023 |
| Pc HV 9 | Hillview (# 5) | 33º19'41"S 25º35'21"E | *Planococcus citri*/ Eureka lemon | 07/03/2023 |
| Ha LM 11 | Lower Melrose | 33º19'43"S 26º38'41"E | *Helicoverpa armigera*/ Cabbage | 18/04/2023 |
| Ha LM 12 | Lower Melrose | 33º19'43"S 26º38'41"E | *Helicoverpa armigera*/ Cabbage | 18/04/2023 |
| Ha LM 14 | Lower Melrose | 33º19'43"S 26º38'41"E | *Hellula undalis*/ Cabbage | 18/04/2023 |
| Px LM 15 | Lower Melrose | 33º19'43"S 26º38'41"E | *Plutella xylostella*/ Cabbage | 18/04/2023 |

#### 2.2.1.2 Sampling and isolation of foliar endophytic fungi

The study was conducted in six citrus orchards consisting of three different cultivars (Newhall Navels, Palmer Navels, Delta Valencias) under different spray regimes (three conventional and three organic). Each cultivar was apparent across both farming regimes (Table 2.2).

Between June 2023 and April 2024, six-leaf collection events occurred every second month. From each orchard, seven random trees were chosen from which two healthy and non-symptomatic leaves were collected from each individual tree, resulting in 14 leaves per orchard. 84 leaf samples were collected across all orchards per sampling event, giving a total of 504 leaves sampled for the

study (252 leaves from conventionally managed farms and 252 from organically managed farms). Leaf samples were processed within 48 h of collection and held in a refrigerator at 4 °C until use. In the laboratory, sampled leaves were first washed with tap water to remove any excess chemical application, dust and/or debris. Each leaf sample was then cut into four fragments of approximately 10 × 8 mm, and then immersed in sd $H_2O$ for 3 min, followed by 2 min in each of two 0.5 % bleach solutions (3.5 % NaOCL) containing 0.05 % Tween® 20. This was followed by a 2 min immersion in 70 % ethanol and three consecutive 2 min rinses in sd $H_2O$. The sterilants and rinse waters were replaced after every orchard was processed. The effectiveness of the sterilisation process was assessed by plating 100 µL aliquots of the final rinsing water on both SDAc and Malt extract agar (MEA) plates supplemented with chloramphenicol (1 ml 50 mg/ml ) (MEAc) (Greenfield et al. 2016). The entirety of the sterilisation process occurred within a laminar flow hood to prevent microbial contamination. Sterile fragment samples were placed in sterile plastic Petri dishes lined with filter paper and allowed to dry under the laminar flow hood for 5 min. Dried fragments were then placed on MEAc plates (4 leaf fragments per agar plate). All plates, including aliquots of the last rinse water, were incubated at ± 27 °C, 30 % RH, for three weeks with a 12 h photoperiod. Plates were inspected daily, and any fungal growth was isolated and supplemented on MEAc and SDAc plates for further morphological and genetic identification after pure cultures were obtained. Any fungal growth observed on the last rinse plates indicated ineffective aseptic control, and the corresponding fungi from the plated leaf samples were excluded from endophytic analysis (Greenfield et al. 2016, Oliveira et al. 2020). Recovered fungal isolates were refrigerated at 4 °C for genetic identification. Based on morphology, non-EPF were discarded from this study.

**Table 2.2:** Farm details from where foliar endophytic fungi were sampled.

| Location | Orchard # | Cultivar | Farming system | Coordinates |
|---|---|---|---|---|
| Huguenot | 38 | Delta Valencia | Conventional | 33°36'43.55"S<br>25°39'39.57"E |
| Miskruier | 210 | Newhall navels | Conventional | 33°23'19.41"S<br>25°26'26.94"E |
| Westhaven | 43 | Palmer navels | Conventional | 33°24'18.00"S<br>25°39'39.57"E |
| Olifantskop | 18 | Delta Valencia | Organic | 33°37'16.08"S<br>25°40'27.58"E |
| Coerney | 1 | Newhall navels | Organic | 33°26'21.65"S<br>25°41'34.93"E |
| Hippo Pools | 11 | Palmer navels | Organic | 33°24'35.64"S<br>25°24'26.28"E |

#### 2.2.1.3 Morphological identification

EPF isolates were initially identified using morphological characteristics and classified by colony colour (above and inverted), and conidial structures were microscopically observed. Slide mounts were prepared by pipetting 5 µl of sd $H_2O$ supplemented with 0.05 % Tween® 20 onto a glass slide. From each isolate, a fine-tipped needle was used to collect a minimal amount of material from two- to three-week-old cultures grown on SDAc. The mycelia were teased apart to be viewed effectively. A plastic coverslip was placed over the mounting material. A microscope at 40 X magnification was used for initial identifications and the EPF were assigned to genera based on the classification system outlined by Humber (1997, 2012). EPF that were tentatively speculated to be entomopathogenic in nature based on morphology were conclusively identified to the lowest taxonomic level via genetic techniques.

#### 2.2.1.4 Molecular identification of recovered EPF isolates

DNA was obtained from fungal cultures using a modified salting method (Sunnucks & Hales 1996). Mycelia and spores were removed from SDAc plates using a 1000 µl tip and added to a 1.5 ml Eppendorf tube containing 100 µl of sd $H_2O$ supplemented with 0.05 % Tween® 20 (Merck KGaA, Darmstadt, Germany). The fungal suspension was homogenised through centrifugation, and 180 µl of ATL buffer (Qiagen, Broadacres, Johannesburg) and 15 µl of proteinase K were added. Samples were then placed in a heat block at 56 °C (to digest tissue) and incubated overnight. The following day, samples were removed and centrifuged for 5 min at 13000 rpm. The supernatant of each sample was then pipette transferred to a clean Eppendorf tube, followed by the addition of 65 µl of 5M NaCl to each new tube. Samples were then vortexed for 30 s and centrifuged for 5 min at 13000 rpm. Each sample's supernatant was again transferred to a clean Eppendorf tube, and 150 µl of cooled 98 % isopropanol was added. Samples were mixed gently by inverting the samples 30 times and placed in the freezer overnight. On day three, the samples were centrifuged for 5 min at 13000 rpm. The supernatant of each sample was removed and discarded, leaving a DNA pellet behind that was further supplemented with 250 µl of cooled 70 % ethanol. The samples were then vortexed for 30 s and centrifuged for 5 min at 13000 rpm, after which the ethanol was removed. Eppendorf tubes were placed in a heat block at 40 °C for 15 min to ensure all ethanol had evaporated from the samples. DNA pellets were then re-suspended in 20 µl AE buffer (elution buffer) (Qiagen). A Nanodrop spectrophotometer (ThermoFisher Scientific®, Waltham, MA U.S.A) was used to determine the DNA concentration of each sample.

#### 2.2.1.5 PCR amplification of rDNA-ITS region sequences and phylogenetic analysis

Universal fungal primers ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3') and ITS4 (5' – TCC TCC GCT TAT TGA TAT GC – 3') (White et al. 1990) were used to amplify the internal transcriber space region using a Veriti 96 Well Thermal Cycler PCR machine (Thermo Fisher Scientific). A 25 µl reaction was prepared (Table 2.3) and subjected to the following cycles; a 10 min initial denaturation at 94 °C, followed by 36 cycles, each involving 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C, and a final 7 min extension step at 72 °C for 5 min. Successful amplification was confirmed by running 5 µl of each sample on a 1 % Agarose Gel Electrophoresis (AGE). The PCR samples were then sent to Macrogen, Inc. for sequencing. The sequences obtained from Macrogen were analysed and were put through ABI Base Recall for automatic trimming of regions with poor quality located at the sequence ends (Elyazghi et al. 2017). Once trimmed and error-corrected, the sequences were submitted to the NCBI BLAST to identify similar sequences available on the GenBank database (Table 2.4). For further genetic analysis, phylogenetic trees for *Beauveria* and *Metarhizium* were constructed independently with reference sequences from the ITS region selected from Bich et al. (2021) and were obtained from the GenBank database. Sequences were aligned using Muscle and analysed using the maximum likelihood method in MEGA (v 11). To enhance the molecular identification of the recovered isolates, separate phylogenetic analyses were conducted for the *Beauveria* and *Metarhizium* strains. To further identify and compare the recovered isolates, representative sequences from Bich et al. (2021) were obtained from GenBank (NCBI). Our own reference sequence was also incorporated for both analyses (Eco-Bb® and Mp FCM Ar 23 B3). 1000 bootstraps were used to support the topology of the tree using the Kimura

2-parameter and Gamma distribution model (K2 + G) and *Cordyceps javanica* (strain CBS 134.22) was used to root the trees.

**Table 2.3:** Reagents used for the amplification of the ITS region of fungal isolates.

| **Reagent** | **Quantity (µl)** | |
|---|---|---|
| | **Negative control** | **Samples** |
| Terra™ PCR Direct Red Dye Premix | 12.5 | 12.5 |
| ITS1 (10 µm) | 2 | 2 |
| ITS4 (10 µm) | 2 | 2 |
| Template DNA (50 ng/µl) | 0 | 2 |
| $MgCl_2$ | 1 | 1 |
| $ddH_2O$ | 8.5 | 6.5 |
| Total | 25 | 25 |

## 2. 3 RESULTS

### 2. 3. 1 Isolation of EPF from aboveground insects or citrus leaf samples

Initially, 15 isolates were recovered from infected insects. Only 7 morphologically presented as EPF. Six of these isolates were recovered from four foliar pests, *Helicoverpa armigera* (50 %), *Plutella xylostella* (25 %), *Hellula undalis* (12.5 %), and *Planococcus citri* (12.5 %), with the former three found on cabbage and the latter one found on Eureka lemon. Out of 504 leaves sampled across the six different farms, only one endophytic entomopathogenic fungal strain was isolated from Newhall navels, from the organically managed Coerney farm.

### 2. 3. 2 Morphological identification of EPF

Initially, strains were identified morphologically based on conidial shape and size and colony morphology, according to Humber (1997, 2012) to genus. From the seven novel isolates recovered, six of the strains displayed typical characteristics of EPF (except for isolate Ha LM 2, which did not morphologically present as an EPF, but passaged through FCM and was, thus, included). Four of the isolates corresponded to species within *Beauveria* (Px LM 4, Ha LM 11, Ha LM 12 and Coe 18), one strain corresponded to species within *Metarhizium* (Hu LM 14), and one strain corresponded to species within *Fusarium* (Pc HV 9) (Figure 2.1).

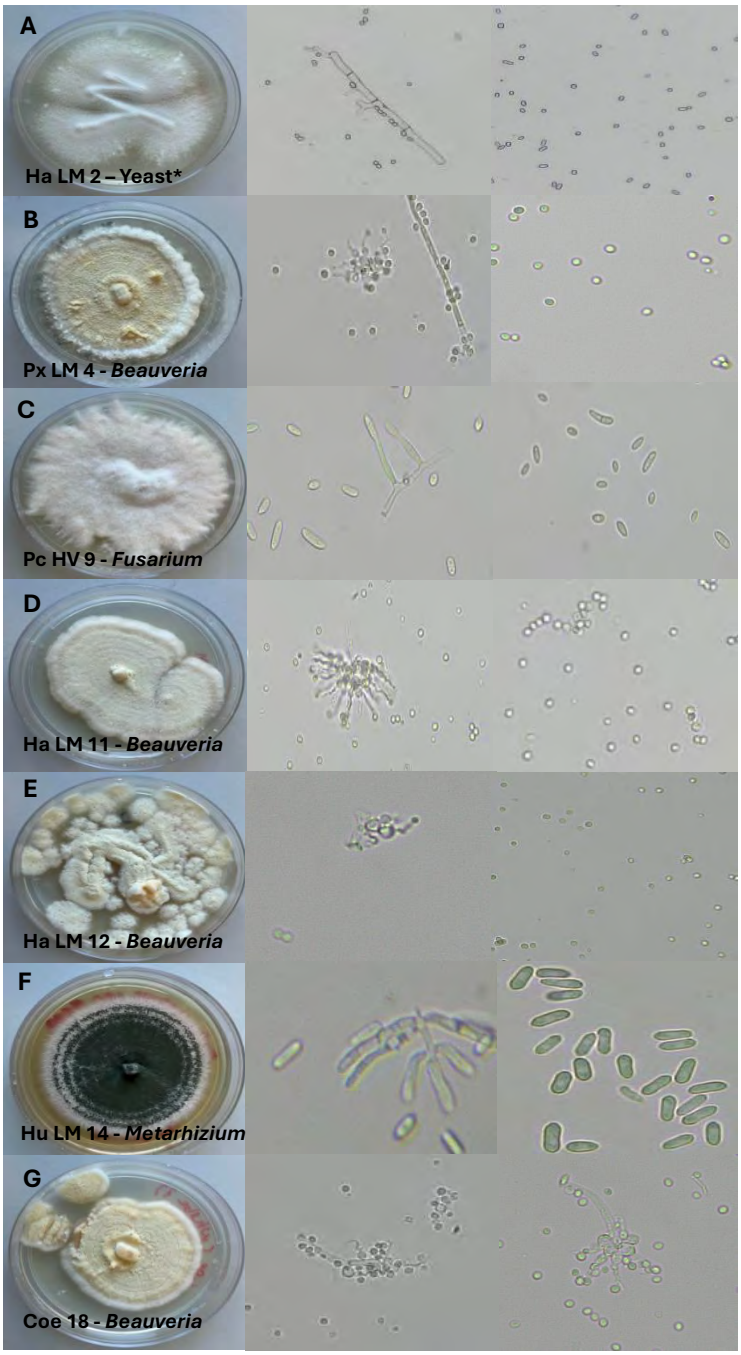


**Figure 2.1:** Morphological characteristics (left to right: surface morphology and conidia) of the seven novel EPF isolates. Isolates A-F were recovered from infected foliar pest cadavers and isolate G was recovered as a foliar endophyte from Newhall navels of an organically managed citrus farm in the Eastern Cape. *Included as it successfully passaged through FCM.

### 2. 3. 3 Molecular identification of EPF

Based on the BLAST analysis, morphological characterisation was confirmed. Isolates Px LM 4, Ha LM 11, Ha LM 12, and Coe 18 were confirmed as *Beauveria* species, most likely *Beauveria bassiana*. The isolate Hu LM 14 displayed similarity indices of 98 % corresponding to the *Metarhizium* genus. Isolate Pc HV 9 corresponded to the *Fusarium* genus with a 99 % similarity index. Isolate Ha LM 2 belonged to the yeast *G. candidum* (teleomorph *Galactomyces*), which has been shown to have EPF properties against mosquitoes (Accoti et al. 2021) (Table 2.5). Isolates Px LM 4, Ha LM 11, Ha LM 12, and Coe 18 were clustered with several reference *B. bassiana* strains, and whilst similar, likely different isolates (Figure 2.2). EPF isolate Hu LM 14 was clustered with reference *M. anisopliae* strains, and whilst similar, different to Mp FCM Ar 23 B3 (Figure 2.3).

**Table 2.4:** BLAST search results for each isolate with the top three most similar sequences and their GenBank accession numbers.

| Isolate | Scientific Name | Max Score | Total Score | Query Cover | E-value | % ID | Accession # |
|---|---|---|---|---|---|---|---|
| Ha LM 2 | *Galactomyces* sp. | 472 | 472 | 100 % | $5 \times 10^{-128}$ | 96.81 | JQ437602.1 |
| | *Geotrichum candidum* | 460 | 460 | 100 % | $1 \times 10^{-124}$ | 96.1 | PP905104.1 |
| | *G. candidum* | 460 | 460 | 100 % | $1 \times 10^{-124}$ | 96.1 | OP758535.1 |
| Px LM 4 | *Beauveria bassiana* | 953 | 953 | 99 % | 0 | 100 | KX263271.1 |
| | *B. bassiana* | 952 | 952 | 100 % | 0 | 99.81 | MH754663.1 |
| | *B. bassiana* | 952 | 952 | 100 % | 0 | 99.81 | OR251920.1 |
| Pc HV 9 | *Fusarium* sp. | 907 | 907 | 100 % | 0 | 99.8 | PQ302438.1 |
| | *Colletotrichum dematium* | 907 | 907 | 99 % | 0 | 100 | MG798697.1 |
| | *F. oxysporum* | 907 | 907 | 100 % | 0 | 99.8 | KU170717.1 |
| Ha LM 11 | *B. bassiana* | 948 | 948 | 100 % | 0 | 99.81 | HQ222967.1 |
| | *B. bassiana* | 944 | 944 | 100 % | 0 | 99.61 | MK346246.1 |
| | *B. bassiana* | 944 | 944 | 99 % | 0 | 99.81 | KX263272.1 |
| Ha LM 12 | *B. bassiana* | 948 | 948 | 100 % | 0 | 99.81 | MK346246.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Beauveria* sp. | 948 | 948 | 100 % | 0 | 99.81 | OP497959.1 |
| | *Beauveria* sp. | 948 | 948 | 100 % | 0 | 99.81 | OP497958.1 |
| Hu LM 14 | *Metarhizium anisopliae* | 904 | 904 | 99 % | 0 | 98.63 | KU364600.1 |
| | *M. anisopliae* | 902 | 902 | 99 % | 0 | 98.62 | OM372875.1 |
| | *M. anisopliae* | 902 | 902 | 99 % | 0 | 98.62 | OM372844.1 |
| Cv 17* | *Akanthomyces lecanii* | 970 | 970 | 98 % | 0 | 99.62 | OR304366.1 |
| | *A. lecanii* | 970 | 970 | 99 % | 0 | 99.44 | OR304365.1 |
| | *A. lecanii* | 970 | 970 | 98 % | 0 | 99.62 | OR304364.1 |
| Coe 18 | *B. bassiana* | 972 | 972 | 99 % | 0 | 95.89 | MT703031.1 |
| | *Beauveria* sp. | 942 | 942 | 86 % | 0 | 99.24 | ON573344.1 |
| | *B. bassiana* | 941 | 941 | 85 % | 0 | 99.42 | KC121560.1 |
| FCM Ar 23 B3* | *M. anisopliae* | 920 | 920 | 99 % | 0 | 100 | OP597533.1 |
| | *M. anisopliae* | 920 | 920 | 99 % | 0 | 100 | OM372875.1 |
| | *Metarhizium* sp. | 915 | 915 | 99 % | 0 | 99.8 | KU593541.1 |

*Isolates which had previously been molecularly identified, Cv 17 as *Akanthomyces lecanii* and FCM Ar 23 B3 as *Metarhizium pinghaense* (based on sequencing of the EF1 alpha region).

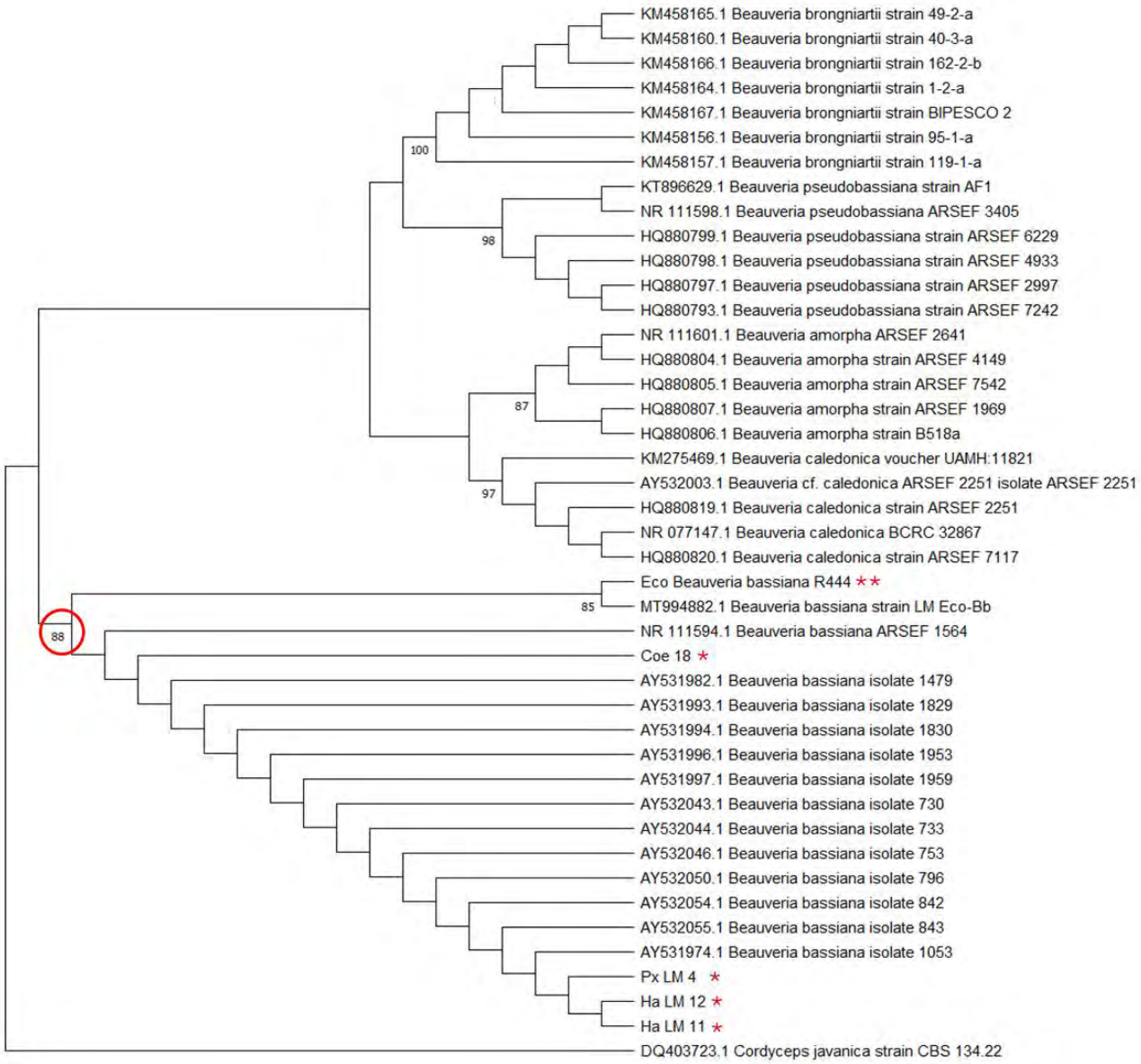


**Figure 2.2:** Phylogenetic analysis of ITS region of *Beauveria* isolates recovered in this study [denoted by a single asterisk (*)] and reference sequences obtained from GenBank (NCBI). A provided reference sequence of known species identification was also included in the analysis [denoted by a double asterisk (**)]. Bootstrap values next to nodes are based on 1000 replicates. The tree was rooted using isolate *Cordyceps javanica* strain CBS 134.22 as the outgroup. The circle indicates the bootstrap value obtained for the group of interest. Bootstrap values ≥ 80 % are labelled.

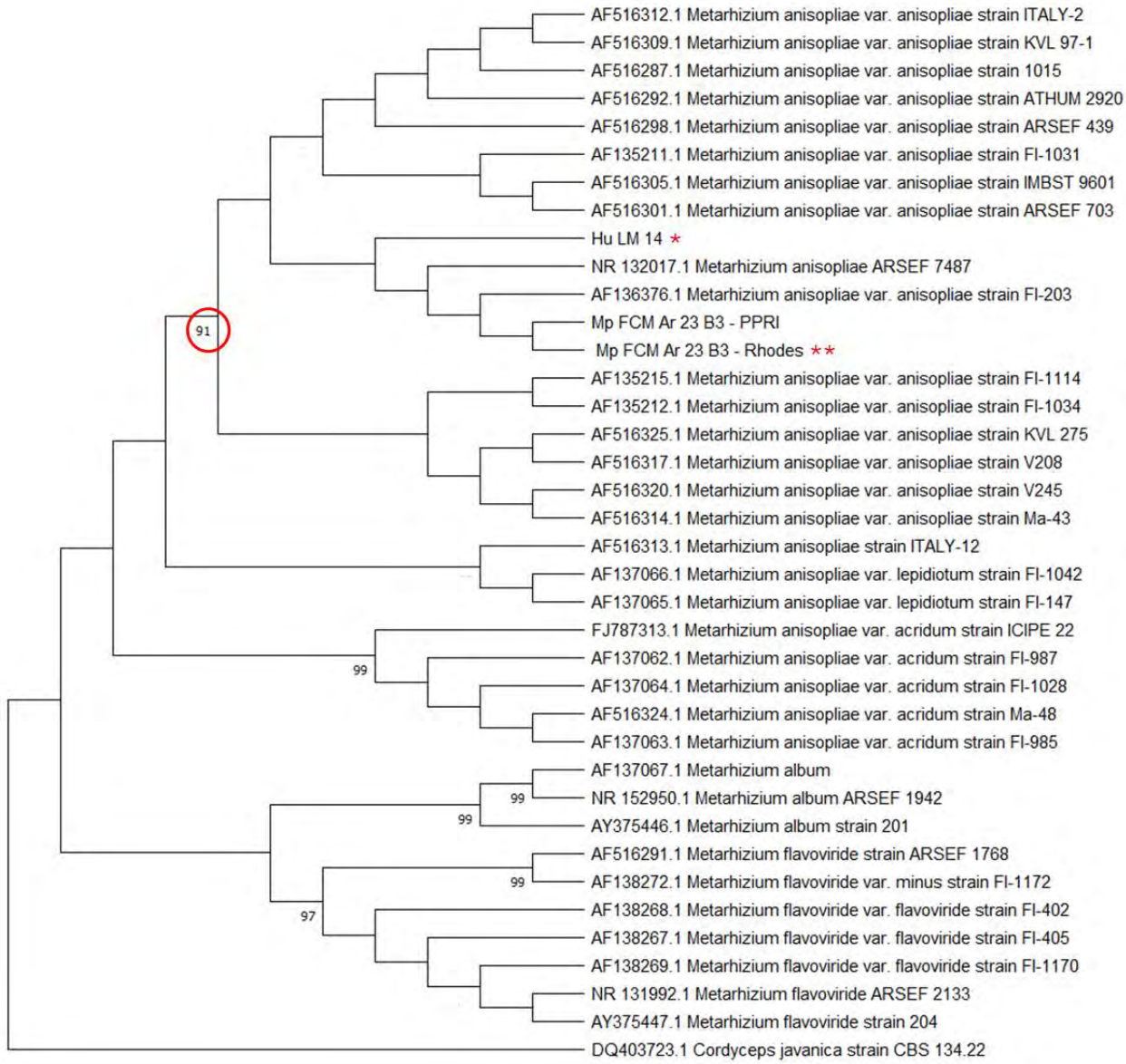


**Figure 2.3:** Phylogenetic analysis of ITS region of *Metarhizium* isolates recovered in this study [denoted by a single asterisk (*)] and reference sequences obtained from GenBank (NCBI). A provided reference sequence of known species identification was also included in the analysis [denoted by a double asterisk (**)]. Bootstrap values next to nodes are based on 1000 replicates. The tree was rooted using *Cordyceps javanica* strain CBS 134.22 as the outgroup. The circle indicates the bootstrap value obtained for the group of interest. Bootstrap values ≥ 80 % are labelled.

## 2. 4 DISCUSSION

*Beauveria* was the most detected EPF species (57.14 %), followed by *Metarhizium* (14.29 %). The fact that *B. bassiana* was the most abundant EPF is interesting as research highlights that EPF taxa may be preferably localised to certain niches (i.e. above and belowground environments) and thus, these results conform with other studies. *Beauveria bassiana* has been reportedly recovered more frequently from the aboveground environments compared to *M. anisopliae* (Meyling et al. 2011). This may be because *B. bassiana* has melanin-like features (not inferring dark pigments), allowing this species to tolerate the pressures imposed by UV radiation (Braga et al. 2015, Quesada-Moraga et al. 2023).

Most EPF were isolated from cabbage pests rather than citrus pests. However, some of these cabbage pests are also considered pests of citrus, as seen with *Helicoverpa armigera*. While the insect from which the isolates were recovered may influence the virulence of the isolate against different pest species (host range differences) (Anderson et al. 2011), the main focal point of this thesis is to investigate the isolates' capability of being more tolerant to aboveground environmental conditions. Thus, emphasis is placed on the fact that the isolates were recovered from an aboveground environment and not the insect host from which the EPF was recovered. There has been a myriad of reports that support the fact that EPF tend to be more common in organically managed farms compared to those conventionally managed. For example, Klingen et al. (2002), Goble et al. (2010), and Clifton et al. (2015) recovered more EPF isolates from organically managed farms as opposed to conventionally managed. Afandhi et al. (2022) also found there to be a higher abundance of EPF from organic fields. However the most virulent isolates against *S. litura* were recovered from the conventional farm. Tkaczuk et al. (2019) did not find a significant difference between the two farm management styles. All these studies focused on soil-inhabiting

EPF, whereas Meyling et al. (2011) looked at the differences in aboveground EPF abundance between organic and conventional farms and, in agreement with our study, found there to be more infected insect cadavers recovered from conventional than organic farms. All of the infected insect cadavers recovered in this study originated from farms under conventional management.

The recovery of the *Fusarium* isolate from an insect cadaver was interesting as many species within this genus are well-known plant pathogens, and therefore research on *Fusarium*-insect interactions has been limited (Geiser et al. 2013; Sharma & Marques 2018). However, some *Fusarium* species have been reported to possess entomopathogenic properties and cause infection in some insect hosts (Santos et al. 2020). For example, using the *Galleria* bait method, several isolates of *Fusarium oxysporum* were recovered from soil samples (Santos et al. 2020). Mushore et al. (2023) also recovered four *Fusarium oxysporum* isolates from soil with *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) used as the bait-insect. These isolates induced between 10-60 % mortality in nymphs and adults of *Molopopterus* spp. Jacobi (Hemiptera: Cicadellidae). Furthermore, strains of *Fusarium* have also been isolated from foliar insect cadavers. For example, Sharma et al. (2018) isolated *F. oxysporum* from *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), which was also able to induce up to 50 % mortality in *P. ficus* under laboratory conditions. This demonstrates that EPF species within *Fusarium* have the potential for biocontrol. However, due to their ability to be plant pathogens, further evaluations in terms of ecological impact need to be considered, more so than with species of *Beauveria* and *Metarhizium*, which are well-known entomopathogenic species already used commercially in various mycopesticides.

The recovery of *Geotrichum candidum* from an infected insect in an agricultural setting is unusual as this yeast has only been recorded to be recovered and infect mosquitoes (Scholte et al. 2004, Accoti et al. 2021). However, this yeast has also been recorded to be ubiquitous in nature and often

saprophytes of soils, fruits and vegetables (Corbu et al. 2023). As such, this yeast could be opportunistic and saprophytic to insect cadavers and thus may have been a contaminant. Even though it did passage through FCM, not all fifth-instar FCM that were subjected to treatment by this isolate became infected. Certain individuals of FCM may have been damaged prior to treatment due to handling errors which may have made them more susceptible to host the saprophytic yeast.

Such as seen with the different localisation (above- or belowground) of EPF of infected insects (Meyling et al. 2011), endophytic EPF inhabit different tissues of host plants. As seen with infected foliar pests, *B. bassiana* generally dominates plant tissue localised aboveground (i.e. leaves and stems), whilst *M. anisopliae* tend to dominate plant tissue localised belowground (Behie et al. 2015). Based on this information and the fact that foliar samples were collected, it is logical that a *B. bassiana* isolate was recovered rather than *M. anisopliae.*

This sole *Beauveria* endophytic EPF isolate was recovered from an organically managed farm. In agreement with our research, endophytic EPF tend to be recovered more frequently from organic rather than conventional farms (Radić et al. 2014, Ramos et al. 2017). This may be because the conventionally managed farms are typically managed extremely intensely (i.e., excessive use of pesticides, herbicides, and fungicides) as opposed to organic farms which would likely promote the establishment of naturally occurring endophytic EPF, as seen with soil-inhabiting EPF (Clifton et al. 2015). In South African citrus orchards, many fungicides utilised are copper based. When pre-washing leaves for endophyte sampling, it was clear that many of the orchards used copper-based fungicides (evident from blue-stained leaf samples). Notably, copper-based fungicides have been shown to decrease EPF radial growth, sporulation and conidial germination (Majchrowicz & Poprawski 1993, Shah et al. 2009, Martins et al. 2012). Retrieving spray regimes from farmers has

been a challenge, and thus, determining if there is a correlation between the recovery and isolation of fungal endophytes and the timing of fungicide application is not possible. The above is important to mention as the application time of copper-based fungicides may have influenced fungal recovery in general, but more specifically EPF isolation.

Morphological characterisation is the basis of fungal identification but should never be solely relied upon for accurate species identification as it is limited in terms of genetic variability (Chaithra et al. 2022). ITS primers are generally used to determine the species to which an isolate belongs. However, using several specific primers (such as EF1-α and Bbchit1) would provide a more accurate species identification (Chaithra et al. 2022). More specifically, to assess the diversity within the genus *Beauveria*, primers such as RPB1, RPB2, TEF, and Bloc have been suggested (Rehner et al. 2011); and primers such as EF1-α, RPB1, RPB2 and beta-tubulin have been suggested for *Metarhizium* (Bischoff et al. 2009). Ultimately, employing a multigene phylogenetic approach when identifying EPF to species and strain level is important.

The recovery of EPF from the aboveground environment (foliar insect cadavers and citrus leaves) provides an opportunity to identify strains with enhanced resilience to environmental stressor, particularly to UV radiation, a focal point of this thesis.

# CHAPTER 3: THE USE OF NOVEL EPF ISOLATES TO CONTROL AN ARBOREAL CITRUS PEST, CITRUS MEALYBUG (*PLANOCOCCUS CITRI*): LABORATORY BIOASSAYS

## 3.1 INTRODUCTION

Many research papers use the words 'pathogenicity' and 'virulence' interchangeably (Thomas & Elkinton 2004). In the context of EPF, pathogenicity and virulence are related, but distinct concepts. It is, therefore, important to make the distinction between the two terms. 'Pathogenicity' can be described as the innate genetic ability of a pathogen to infect an organism and produce disease. It is a qualitative measure, meaning that it addresses whether the fungus can cause disease in an insect host or not (Onstad et al. 2006). Whereas 'virulence' is a quantitative measure as it is the degree or extent of pathogenicity. It measures the severity of the disease caused by the EPF once it infects the host and is influenced by factors such as the rate of fungal growth within the haemolymph of the host and the speed of infection (Onstad et al. 2006). Pathogenicity and virulence differ between species, strains, and target hosts. For example, a strain of *B. bassiana* was more virulent to the eggs, larvae, and pupae of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) by inducing mortalities of 12 %, 32.50 %, and 35 %, respectively, compared to a strain of *M. anisopliae* which induced 9 %, 15 %, and 17.5 % mortality during the egg, larval and pupal stage, respectively (Omar et al. 2021). Likewise, Idrees et al. (2022) demonstrated that different strains of *B. bassiana* induced significantly different percentage mortalities against the egg and second instar larval stage of *Spodoptera frugiperda*, JE Smith, 1797, (Lepidoptera: Noctuidae), whilst Goble et al. (2011) investigated the pathogenicity of 21 native isolates against

*Ceratitis rosa, C. capitata* and FCM and found that the same isolates differed in virulence toward all three citrus pests.

Whilst this research is aimed at identifying an environmentally competent strain, particularly to UV radiation, isolates that are not virulent will not be suitable candidates for a microbial biopesticide control programme or field application. Therefore, the first crucial step is to establish the relative virulence of these novel entomopathogenic isolates.

This chapter used citrus mealybug as the target pest for the bioassays of the novel EPF isolates, as it is considered a widely distributed and important economic pest of citrus in South Africa, is readily available in laboratory culture, and there is a major need for the control of this pest in the field (Grout et al. 2015, Chartier-FitzGerald et al. 2016).

Citrus mealybug (*Planococcus citri)* is a sporadic, damaging polyphagous pest found in most citrus-producing countries. In South Africa, this pest is found in all citrus-producing regions throughout the year on all types of citrus cultivars. On citrus fruit, crawlers and adults are typically localised cryptically (although this is not the case under heavy infestations) (Grout & Moore 2015). Through feeding, this pest causes significant damage to all parts of the plant besides the roots (Canhilal et al. 2001). Crawlers and nymphs are usually located under the calyx of the fruit, whilst the adults are found under curled leaves or between two or more touching fruits. Individual adult females can produce 300 – 587 eggs over six to 10 days (Grout & Moore 2015). Eggs are produced in an ovisac (i.e. white filamentous protective covering), and once eggs hatch, mobile female crawlers go through three instar stages before developing into sessile, wingless females. The males undergo holometabolous metamorphosis and pupate as winged adults, which are considerably smaller than females. The life cycle from egg to adult stage takes approximately four weeks

(summer) to several months (winter) depending on climatic conditions (Ahmed & Abd-Rabou 2010, Grout & Moore 2015).

Populations tend to increase in spring, peak in summer and decline at the beginning of winter. Their piercing mouthparts allow them to feed by sucking plant sap from the fruit, leaves, stems and branches. When populations proliferate, mealybugs produce copious amounts of honeydew, which supports the growth of black sooty mould, consequently staining the fruit and leaves and hindering the plant's ability to photosynthesise and produce exportable fruit (Ahmed & Abd-Rabou 2010, Demirci et al. 2011, Muller & Pountney 2013). Additionally, severe infestations can lead to premature fruit drop, fruit deformation and other cosmetic damage (Muller & Pountney 2013, Grout & Moore 2015). Although ants can act as important predators of arboreal citrus pests that pupate in the soil (i.e. FCM) (Bownes et al. 2014), they can be problematic as certain ant species have been recorded to defend honeydew-producing pests, such as citrus mealybug, from their natural enemies, thus further precipitating outbreaks (Samways et al. 1982, Grout & Moore 2015, Moore 2021). Furthermore, control of citrus mealybug relies heavily on both the use of broad-spectrum insecticides and on their natural enemies. In turn, many of their natural enemies (i.e. predators and parasitoids) suffer the consequences of these insecticides and tend to die off. Mealybug has also been recorded to develop insecticidal resistance (Venkatesan et al. 2016, Mruthunjayaswamy et al. 2019).

Its waxy layer, cryptic lifestyle, disrupted natural enemy complex, and resistance to chemical pesticides have made citrus mealybug extremely challenging to control via the use of chemical insecticides in citrus orchards, resulting in the search for alternative environmentally sustainable control methods. Entomopathogenic fungi offer an environmentally sustainable alternative to suppress mealybug populations. Mathulwe et al. (2022) demonstrated that EPF isolates,

*Metarhizium robertsii* and *M. pinghaense*, can both cause over 90 % mortality in obscure mealybug adult females (*Pseudococcus viburni*) under laboratory conditions, and Demirci et al. (2011) found that *Isaria farinosa,* isolated from *Rhizococcus spinifera* (Hemiptera: Coccidae), caused significant mortality in citrus mealybug of various developmental stages. Goble et al. (2010) recovered numerous isolates of EPF from several South African citrus orchards and surrounding refugia. Three of the isolates, two *Beauveria bassiana* sensu lato and one *Metarhizium pinghaense* (FCM Ar 23 B3), were effective against citrus mealybug under laboratory conditions (Chartier-FitzGerald et al. 2016) but not in the field (Grout et al. 2015).

Therefore, using the seven novel isolates identified in the previous chapter, the virulence of these isolates at an initial screening dose was determined. An additional isolate, *Akanthomyces lecanii* PPRI 7179 (Cv 17), previously isolated from a foliar citrus pest, along with two isolates for comparative purposes - *M. pinghaense* (FCM Ar 23 B3) and the active ingredient of a commercially available product, Eco-Bb® (a.i., *B. bassiana* R444), were included. Isolates that induced mortality of 60 % or greater were further analysed to determine the lethal concentration required to kill 50 % of the test population ($LC_{50}$).

## 3.2 MATERIALS AND METHODS

### 3.2.1 Mealybug culture

A culture of citrus mealybug was reared on organically grown butternuts, provided by Insectec, (Tzaneen, Limpopo), in Tupperware containers covered with fine mesh lids. The containers were kept in a constant environment room at 26 ℃ with a relative humidity of 30 % on a 12 h photoperiod. Female adult mealybugs were gently brushed onto new butternuts and placed on

moistened egg cartons. When approximately 30 % of crawlers covered the butternut, it was moved into a new container. Female adults were used in subsequent experiments.

### 3.2.2 Culture of entomopathogenic fungal isolates

EPF isolates previously identified in Chapter 2 (Chapter 2, section 2.3.3) were used in the screening bioassays against adult females of citrus mealybug. One isolate (Cv 17), previously isolated from *Coccus viridis* (Green) (Hemiptera: Coccidae), was obtained from the South African National Collection of Fungi at the Plant Protection Research Institute (PPRI 7179), and one isolate (FCM Ar 23 B3), previously collected by Goble et al. (2010) [which has shown to be infective against citrus mealybug and several other citrus pests), (Chartier-FitzGerald et al. 2016)], was obtained from stock cultures stored at Rhodes University. An unformulated isolate was obtained from the commercial product Eco-Bb® (Plant Health products, South Africa) (i.e. *B. bassiana* strain R444) by pipetting 100 µl of the formulated product in 900 µl of sterile distilled water (sd $H_2O$) in an Eppendorf tube. The suspension was vortex-mixed for another 30 s. 100 µl was pipetted onto three SDAc, and independent colonies of the EPF were isolated onto a new plate to achieve pure cultures. The labelling system, previously described in Chapter 2, section 2.2.1.1, which correlates to the species identification after molecular analysis, is used throughout.

### 3.2.3 Screening bioassays

#### 3.2.3.1 Passaging of entomopathogenic fungal isolates

Since entomopathogenic fungal isolates may exhibit a loss of virulence due to the process of successive *in vitro* sub-culturing to achieve culture purification (Butt et al. 2006), it was deemed necessary to pass the EPF isolates through an insect host. Highly concentrated conidial suspensions were prepared in sterile 40 ml McCartney bottles containing 10 ml sd $H_2O$ supplemented with 0.05 % Tween® 20. Two-week-old fungal cultures were flooded with the contents from the McCartney bottle, and the conidia were dislodged by gently scraping with a sterile 1000 µl pipette tip. The content was then poured back into the McCartney bottles and vortex-mixed for 30 s. Five fifth-instar FCM and five adult female mealybugs (per isolate) were placed in sterile plastic Petri dishes lined with moistened filter paper and were inoculated with 50 µl of the conidial suspension. The Petri dishes were parafilmed and stored in a controlled environment room at 26 °C, 30 % RH with a 12 h photoperiod. After five days, the Petri dishes were observed for insect mortality. To confirm the cause of death, insect cadavers (now FCM pupae) were surface sterilised using 70 % ethanol and then rinsed in sterile distilled water, each for 30 s. The surface sterilised cadavers were then placed on SDAc plates. After five days of incubating at 26 °C, mycosis was assessed by observing if the fungal isolate began sporulating on the insect cadavers. EPF were once again re-isolated onto new SDAc plates, and conidial suspension stocks for the bioassays were acquired from these plates following two to three weeks of growth at 26 °C, 30 % RH with a 12 h photoperiod. Only isolates that successfully passaged (Ha LM 2, Px LM 4, Pc HV 9, Ha LM 11, Ha LM 12, Hu LM 14, Cv 17, Coe 18, Eco-Bb®, and FCM Ar 23 B3) were used in the subsequent screening bioassays.

#### 3.2.3.2 Preparation of the conidial suspensions

Fungal conidia from 2- to 3-week-old fungal cultures were collected using the same method as described for passaging of isolates above. Collected conidia were suspended in 20 ml of sd $H_2O$ supplemented with 0.05 % Tween® 20 in sterile 40 ml McCartney bottles. Once sealed, the containers were vortex-mixed for 2 min to ensure a homogeneous suspension. The conidia concentration and viability of the suspension were determined.

##### 3.2.3.2.1 Determination of concentration

To determine the concentration of the conidiospores in the stock suspension, a haemocytometer (Neubauer improved) (MARIENFELD) was used following appropriate dilution. Two counts were made per isolate, and the average was used to determine the concentration of the original stock (conidia/ml). Desired concentrations were then prepared accordingly.

##### 3.2.3.2.2 Assessment of viability

The viability of each fungal isolate was assessed by spread plating 100 µl of a $10^5$ conidia/ml suspension onto each of three SDA plates. After incubating for 24 h, the percentage germination was determined by counting 100 conidia on each SDA plate (Ekesi et al. 2002). Conidia were inspected under a microscope at 20 X magnification and deemed germinated if a germ tube was present. The average percentage germination per sample was determined. Viability was assessed after each bioassay.

### 3.2.3.3 Experimental setup

Eight novel isolates (including the *A. lecanii* isolate, Cv 17) were used in screening bioassays to determine the most virulent EPF strain. All isolates were screened at a standard conidial concentration of $1 \times 10^7$ conidia/ml. Two comparative control isolates, the unformulated active ingredient from the commercial Eco-Bb® product (a.i. *B. bassiana*) and the *M. pinghaense* (FCM Ar 23 B3) and a negative control consisting of sd $H_2O$ supplemented with 0.05 % Tween® 20 were utilised in the bioassays. A total of 60 adult female citrus mealybugs (12 insects in each of five 24-well plates) were used for each isolate treatment. Individual citrus mealybugs were positioned in an alternate well within a 24-well bioassay plate equipped with 11 × 11 mm square of sterile filter paper (Figure 3.1).

**Figure 3.1:** 24-well plate filled with filter paper pieces placed in every alternate well.

Conidial suspensions of 50 µl were pipette applied to each of the adults (Chartier-FitzGerald et al. 2016, Mathulwe et al. 2022). During the inoculation process, the inoculums were consistently

vortex-mixed for 30 s to prevent conidia settlement at the bottom of the suspension and to retain a homogenous suspension. Subsequently, glass covers were placed between the top of the wells and the lid to seal the treatment well plates tightly. The lid was further secured with elastic bands. The bioassay plates were placed in sterile 2 L plastic Tupperware containers fitted with moistened paper towels to maintain a high humidity throughout the incubation period. The plastic containers were then placed in controlled environment rooms (26 ℃, 30 % RH, with a 12 h photoperiod) for seven days, after which mealybugs from each plate were observed under a dissecting microscope. Dead adults were washed in 70 % ethanol for 1 min followed by three 30 s rinses in sd $H_2O$ and placed in a Petri dish lined with moistened paper, incubated at 26 ℃, to monitor for mycosis and record mortality (Figure 3.2). Each treatment was replicated three times with new conidial suspensions, and the viability of each suspension was determined as described in section 3.2.3.2.

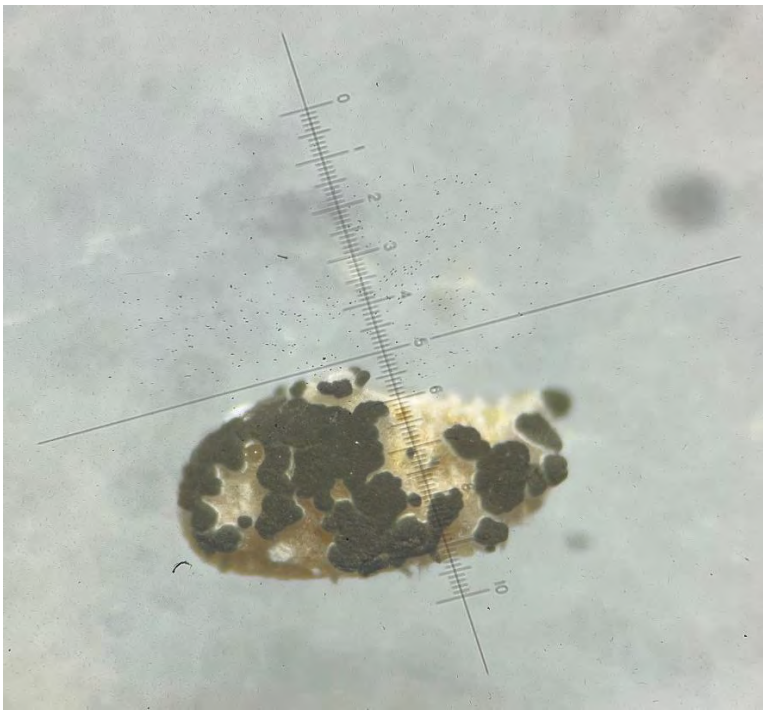

**Figure 3.2:** Adult female citrus mealybug displaying signs of mycosis as a result of *Metarhizium* infection.

### 3.2.4 Dose-response bioassays

Fungal isolates showing a cumulative mortality of less than 60 % were not selected for dose-response bioassays. Six isolates were used in the dose-response assays (Px LM 4, Ha LM 11, Ha LM 12, Hu LM 14, Coe 18, and FCM Ar 23 B3) to determine the $LC_{50}$ values. Preparation of the inoculums followed the same procedure described in section 3.2.3.2, with conidial suspensions tested at five different concentrations ($10^3$, $10^4$,$10^5$, $10^6$ and $10^7$ conidia/ml). A negative control, consisting of sd $H_2O$ supplemented with 0.05 % Tween® 20, was used. Since the glass covers were successful in inhibiting the mealybugs from escaping and moving between wells, there was no need to place the insects alternatively in the 24-well bioassay plates. Thus, 24 adult female citrus mealybugs were used for each dose (a total of 120 insects per isolate) and followed the same inoculation procedure as described in section 3.2.3.3. The experiment was terminated after seven days, and mycosis and mortality were recorded as described in section 3.2.3.3 above. Each treatment was replicated three times with new conidial suspensions, and the viability of each suspension was determined as described in section 3.2.3.2.2.

### 3.2.5 Statistical analysis

The Shapiro-Wilk test was used to check for normality. A one-way analysis of variance (ANOVA) was used to compare the differences in the mean mortality of female adult citrus mealybugs between 10 different EPF isolate treatments. A Tukey HSD test was used to determine where significant differences were detected between the different isolate treatments.

To evaluate the potential for dose-dependent effects on mealybug mortality, a series of GLMM's were used. Insect mortality (proportion of insects that died per replicate) was modelled as a function of the dose (EPF concentrations of $1 \times 10^{3,4,5,6,7}$) and isolate.

Two generalised linear mixed models (GLMMs) were fitted with a binomial error distribution and a probit link function using the glmmTMB package (Brooks et al. 2017), with replicates treated as a random effect to account for possible between-replicate variation (Bolker at el. 2009).

The first model was an additive model, which included fixed effects for isolate and dose and the second model was a multiplicative model, which included an interaction term between isolate and dose to allow the effect of dose on mortality to vary by isolate.

The two models were compared using a Wald $\chi^2$ test to assess whether the inclusion of the interaction term significantly improved the model fit. If there was a significant difference between the two models fitted, the model with the lower Akaike information criterion (AIC) value was used. Where no significant improvement in model fit was calculated due to the inclusion of the multiplicative interaction effect, the simpler additive model was used for model inference.

The lethal concentration ($LC_{50}$) for each isolate was calculated using GLMs fitted to the dose-response data. For each isolate, a model was fitted with dose as the numeric fixed effect variable, and the $LC_{50}$ was estimated using the MASS::dose.p function (Venables & Ripley 2002). Pairwise differences in $LC_{50}$ values between isolates were assessed using lethal ratio tests, using the ecotox::ratio_test function (Wheeler et al. 2006). These analyses were done in R ver. 4.4.1 (R Core Team, 2024).

## 3.3 RESULTS

### 3.3.1 Screening bioassays

Isolate Px LM 15 did not successfully pass through FCM or citrus mealybug and was therefore discarded from screening and dose-response trials. Of the remaining isolates, conidial viability on SDA plates was > 85 %. Control mortality was low, 7 ± 3.2 %. The mean mortality differed significantly between treatments ($F_{10, 22} = 23.05$, $P < 0.0001$) (Figure 3.3). The average percentage mortality ranged from 15 % to 90 %. Isolates Px LM 4 and FCM Ar 23 B3 induced the highest mortalities of 90 ± 1.2 % and 90 ± 4.2 %, respectively. Isolates Ha LM 11, Ha LM 12, Hu LM 14, Coe 18 and the commercial strain Eco-Bb® induced mortalities greater than 60 %. Ha LM 2, Pc HV 9, and Cv 17 induced the lowest mortalities of 15 ± 3.3 %, 28 ± 2.7 %, and 41 ± 8 %, respectively, and were therefore excluded from further analysis. Eco-Bb® was also excluded as further analyses solely focused on the novel isolates. Isolate FCM Ar 23 B3 was included in the following analysis owing to the high level of mortality it was able to induce in the test populations.

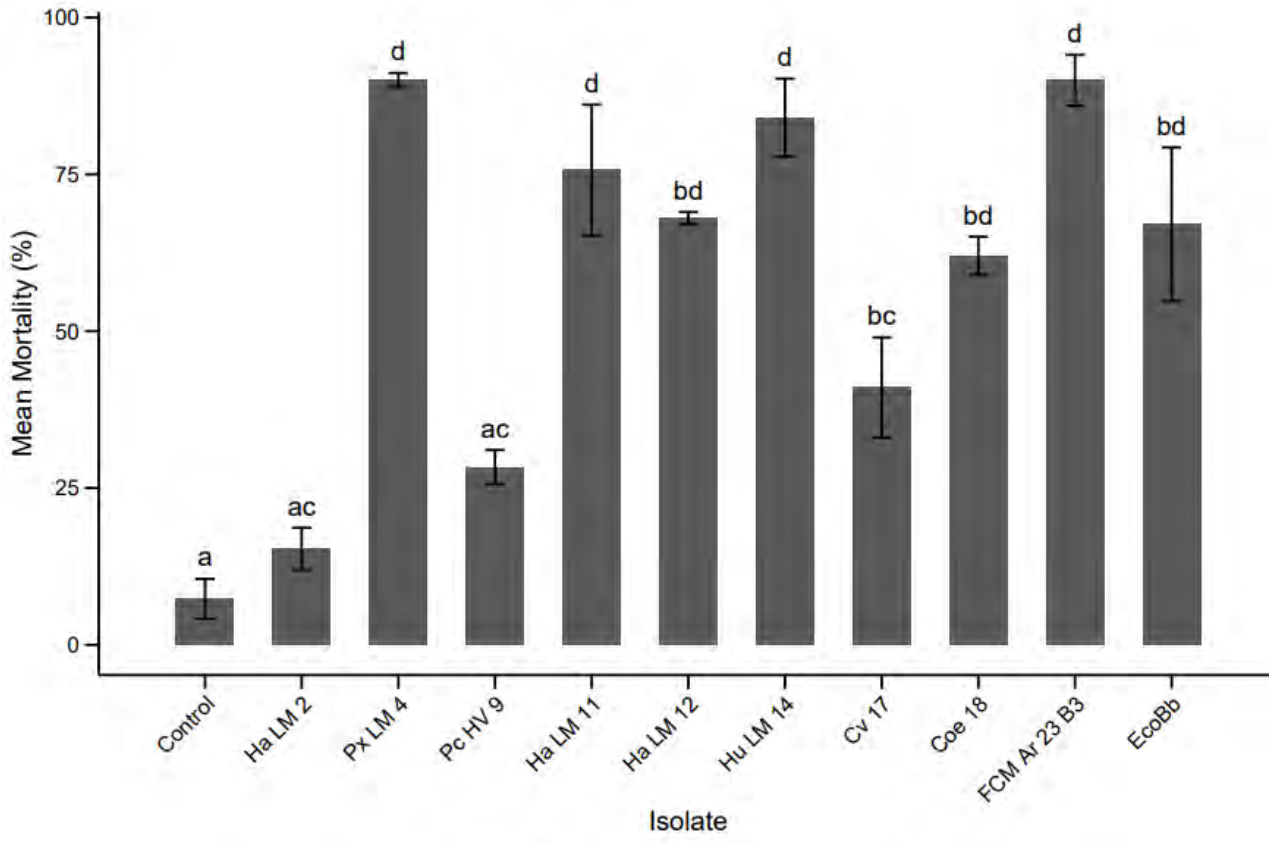


**Figure 3.3:** Percentage mortality (± standard error) of adult female citrus mealybug, *Planococcus citri,* seven days after treatment with the novel, aboveground isolates applied at a concentration of $1 \times 10^7$ conidia/ml. Different letters above the bars indicate significant differences between isolates according to the Tukey HSD test ($P < 0.05$) ($F_{10, 22} = 23.05$, $P < 0.001$).

### 3.3.2 Dose-response bioassays

The Wald $\chi^2$ test indicated statistical support for a multiplicative effect of dose and isolate on mortality rates ($\chi^2 = 12.27$, df = 5, $P = 0.031$). This suggests that the mortality rate of the isolates was dose-dependent, and the magnitude of dose-dependence varied between isolates. Model predictions (with 95 % confidence intervals) showed that mortality increased with dose for all

isolates, but the rate of increase and the final mortality levels differed by isolate. For example, some isolates exhibited strong mortality responses at low doses, while others induced greater mortality at higher doses only (Figure 3.4).

Of the six isolates measured for $LC_{50}$, FCM Ar 23 B3 stood out as the only isolate with an $LC_{50}$ value in the $\times 10^5$ range ($5.25 \times 10^5$ conidia/ml). In contrast, the remaining isolates required concentrations in the $\times 10^6$ range to achieve 50 % mortality. Within this range, Px LM 4 ($1.09 \times 10^6$ conidia/ml) and Hu LM 14 ($1.32 \times 10^6$ conidia/ml) showed lower $LC_{50}$ values compared to Ha LM 11 ($2.68 \times 10^6$ conidia/ml), Coe 18 ($3.57 \times 10^6$ conidia/ml), and Ha LM 12 ($4.40 \times 10^6$ conidia/ml) (Figure 3.4).

The lethal ratio test indicated that some isolates exhibited significantly different $LC_{50}$ values while other isolates displayed comparable values. For example, Coe 18 exhibited a significantly higher $LC_{50}$ than FCM Ar 23 B3 ($z = 3.22$, $P < 0.001$). Additionally, significant differences were detected between isolates Ha LM 11 and Ha LM 12 ($z = 2.24$, $P = 0.025$) and between Ha LM 12 and Hu LM 14 ($z = 3.78$, $P < 0.001$). Conversely, no significant differences were found between isolates such as FCM Ar 23 B3 and Px LM 4 ($z = 1.09$, $P = 0.275$) (Table 3.1).

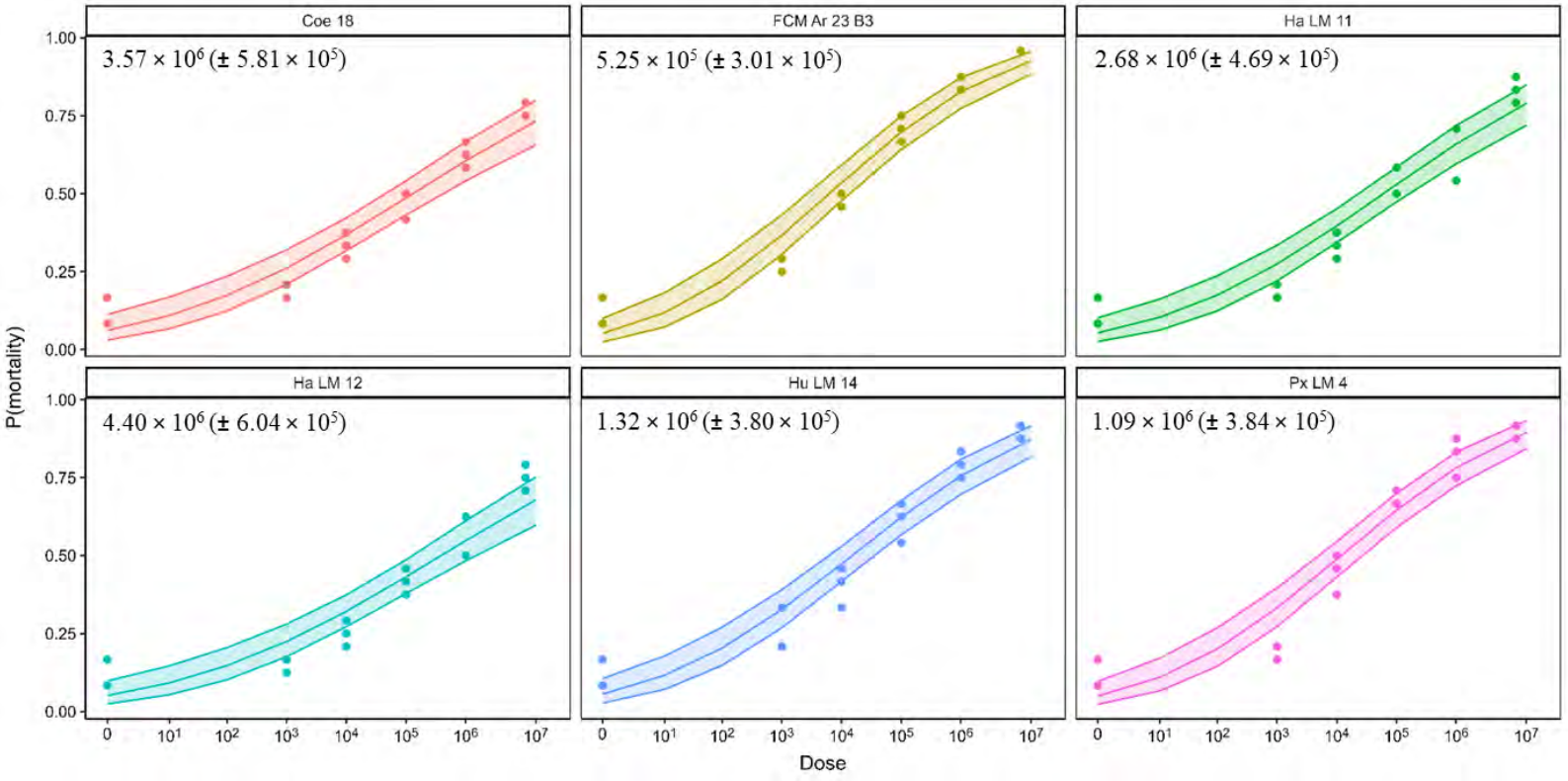


**Figure 3.4:** Probit regression dose-response curves for mortality of female *P. citri* adults exposed to different concentrations (log dose) for the six most virulent isolates used in the dose-response bioassays. Central lines indicate mean probability of mortality [P(mortality)], shaded areas represent the 95 % confidence interval of the mean and the dots indicate raw data points. Each panel represents the individual fungal isolates. The values provided are the median lethal concentration measures ($LC_{50}$) (± S.E.) of the six most virulent isolates used in the dose-response bioassays against female *Planococcus citri* adults.

**Table 3.1:** Pairwise comparisons of the lethal concentration ($LC_{50}$) values between the six most virulent isolates used in the dose-response bioassays against female *Planococcus citri* adults. The estimate is the difference in the $LC_{50}$ values for each pairwise comparison and statistical differences are indicated by the asterisk (*) ($P < 0.05$).

| **Isolate** | **Estimate** | ***z*-ratio** | ***P*** |
|---|---|---|---|
| Coe18 - FCM Ar 23 B3 | $3.04 \times 10^6$ | 3.22 | 0.001* |
| Coe18 - Px LM 4 | $2.48 \times 10^6$ | 3.05 | 0.002* |
| Coe18 - Ha LM 11 | $8.92 \times 10^5$ | 1.20 | 0.229 |
| Coe18 - Ha LM 12 | $-8.35 \times 10^5$ | 0.99 | 0.323 |
| Coe18 - Hu LM 14 | $2.24 \times 10^6$ | 3.01 | 0.003* |
| FCM Ar 23 B3 - Px LM 4 | $-5.69 \times 10^5$ | 1.09 | 0.275 |
| FCM Ar 23 B3 - Ha LM 11 | $-2.15 \times 10^6$ | 2.72 | 0.007* |
| FCM Ar 23 B3 - Ha LM 12 | $-3.88 \times 10^6$ | 3.61 | 0.001* |
| FCM Ar 23 B3 - Hu LM 14 | $-7.99 \times 10^5$ | 1.44 | 0.148 |
| Px LM 4 - Ha LM 11 | $-1.58 \times 10^6$ | 2.28 | 0.023* |
| Px LM 4 - Ha LM 12 | $-3.31 \times 10^6$ | 3.69 | 0.001* |
| Px LM 4 - Hu LM 14 | $-2.31 \times 10^5$ | 0.42 | 0.673 |
| Ha LM 11 - Ha LM 12 | $-1.73 \times 10^6$ | 2.24 | 0.025* |
| Ha LM 11 - Hu LM 14 | $1.35 \times 10^6$ | 2.09 | 0.036* |
| Ha LM 12- Hu LM 14 | $3.08 \times 10^6$ | 3.78 | 0.001* |

## 3.4 DISCUSSION

EPF serve as a promising alternative to chemical insecticides for pest control and are currently incorporated into many IPM regimes. Given that environmental competence is known to differ

between species and isolates, it was deemed necessary to include isolates which exhibited lower percentage mortalities in comparison to the most virulent ones based solely on a laboratory assay. The most virulent isolates may not be the most UV tolerant; thus, finding the most tolerant isolates that still exhibit high virulence allows for a competent microbial biocontrol agent in the field.

Isolates Px LM 4 and FCM Ar 23 B3 both induced the highest mortalities of 90 % in the screening trials. Even though the novel *Beauveria* isolates performed well in this study, both the *Metarhizium* strains induced significantly higher mortality levels. Similarly, Mathulwe et al. (2022) found that two isolates of *Metarhizium* (*M. robertsii* and *M. pinghaense*) caused over 90 % mortality of the obscure mealybug whereas the strains of *Beauveria* used in their study did not offer nearly as much control over obscure mealybug as the indigenous *Beauveria* isolates used against citrus mealybug in this study. Manjushree & Chellappan (2019) demonstrated under laboratory conditions that, following *A. lecanii* (previously known as *Lecanicillium lecanii*), *B. bassiana,* at a high conidial concentration of $1 \times 10^9$ conidia/ml, induced a significantly higher percentage mortality of the pink mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae) on pineapple when compared to *M. anisopliae.*

The active ingredient of Eco-Bb® (which is registered for use against mealybugs in general) did relatively poorly in controlling citrus mealybug compared to most of the novel strains. It is notable that the active ingredient in Eco-Bb® also performed poorly against the obscure mealybug under laboratory conditions compared to other isolates tested (Mathulwe et al. 2022). Coombes et al. (2015) also found that Eco-Bb® did not offer as effective control against fifth-instar FCM compared to the indigenous isolates collected by Goble et al. (2010).

A lower $LC_{50}$ value offered by an isolate infers greater virulence to the insect host and is prominent in terms of commercial production as it means that a smaller concentration of that specific isolate

will offer the same control compared to an isolate used at a higher concentration. Of the six isolates investigated, three (FCM Ar 23 B3, Hu LM 14 and Px LM 4) performed well in the dose-response bioassays with relatively low $LC_{50}$ values. Chartier-FitzGerald et al. (2016) also found FCM Ar 23 B3 to perform the best against citrus mealybug crawlers and adults, as it had the lowest $LC_{50}$ value of $4.96 \times 10^6$ conidia/ml. This specific isolate induced a mortality of 90 % and had the lowest $LC_{50}$ of $5.25 \times 10^5$ conidia/ml and thus may be considered the most virulent to adult female citrus mealybugs. This may be due to the fact that isolates were passaged through an insect host (FCM or mealybug) in this study, whereas the authors state that the isolates used in their study may have experienced an attenuation of virulence, due to lack of passaging (Chartier-FitzGerald et al. 2016). In agreement with Chartier-FitzGerald et al. (2016) in the dose-response bioassays, a $1 \times 10^7$ conidia/ml dose, induced higher mortalities of adults when compared to the same dose used in the screening bioassays. As commonly reported, the mortality was positively correlated to the dose for all isolates (i.e., as the dose increased, so did the percentage mortality) (e.g., Mohamed 2016, Chartier-FitzGerald et al. 2016, Mathulwe et al. 2022, 2023).

Although mortality rates are the primary measure of EPF efficacy, sub-lethal effects such as reduced fecundity, slower development and ultimately, a reduced overall fitness can contribute to pest suppression. Notably, the fecundity and hatchability of the citrus mealybug's eggs appeared to be reduced in treatment plates. However, this cannot be accurately confirmed as egg counts were not distinguished between the control and treatments. Nevertheless, Gopal et al. (2021) supports this observation as the authors documented a significant decrease in both the fecundity of *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae) and hatchability of the eggs following *A. lecanii* treatment at a concentration of $1 \times 10^8$ conidia/ml. Udayakumar et al. (2014) also reported a reduced fecundity of adult female *M. hirsutus* as well as decreased hatchability of

the eggs when treated with either *A. lecanii*, *B. bassiana* or *M. anisopliae*. These sublethal effects warrant further investigation to enhance the effectiveness of the overall pest control strategy.

The hydrophobic property of the wax enables some of the mealybugs to escape drowning or becoming swamped by water in their typical cryptic sites. They are covered with a powdery wax that repels water-based insecticide solutions (Venkatesan et al. 2016). This could explain why some of the EPF isolates did not infect some of the adults as their waxy coverings prevented conidia, which was inoculated in Tween water, from attaching to its integument. Even at the adult stage of development, some citrus mealybugs are waxier than others. The lack of waxy coverings on the crawlers may provide an understanding of why crawlers could be more susceptible to EPF infection. Future studies investigating the potential of EPF against mealybugs should consider using lipid degraders or surfactants to reduce the waxy layer and improve fungal germination, however, these results have been contradictory (Herrick & Cloyd 2023).

Environmental conditions (temperature and relative humidity) under laboratory conditions directly influence how EPF perform (Jackson et al. 2010, Demirci et al. 2011, Quesada-Moraga et al. 2023). Even though the CE room was kept at a RH of 30 %, the glass cover over the 24-well plates and the fact that the plates were parafilmed and kept in containers lined with moistened filter paper, which would significantly increase the relative humidity. The high relative humidity is conducive to fungal germination and thus, infection. As a result, greenhouse and field trial results may differ significantly. The most virulent isolates from the laboratory bioassays should be further tested in greenhouse and field trials to validate the efficacy of the EPF isolate under natural conditions. However, Acheampong et al. (2020a) found that the isolate FCM Ar 23 B3 infected fifth-instar FCM across a variety of humidity levels and states that this abiotic factor will unlikely hinder the efficacy of this isolate in the field. It would be beneficial to assess whether humidity, as well as

temperature, influence the efficacy of the novel, most virulent isolates. Environmental abiotic factors influence the ability of EPF to control pests, however, the most detrimental factor on this entomopathogens' efficacy is UV radiation (Fernandes et al. 2015).

# CHAPTER 4: VARIABILITY IN TOLERANCE OF NOVEL EPF ISOLATES TO STIMULATED SOLAR RADIATION

## 4.1 INTRODUCTION

UV radiation, particularly UV-B (280 – 315 nm), is considered the most prominent factor, particularly in epigeal habitats, contributing to conidial inactivation and influencing the persistence of EPF in the field (Fernandes et al. 2007, Vega et al. 2012, Fernández-Bravo et al. 2017, Quesada-Moraga et al. 2023). Even though the UV spectrum comprises three wavelengths (UV-A, UV-B, and UV-C), only UV-A and UV-B radiation reach the Earth's surface, constituting 95% and 5%, respectively (Braga et al. 2015). Whilst the UV-A component is associated with delayed conidial germination and death, the UV-B component is considered to impose the most negative biological effects on EPF, despite only a small percentage reaching the Earth's surface (Braga et al. 2001a, Jaronski 2010). Therefore, considerable research on the negative effects associated with UV radiation on EPF has focused on UV-B (Jaronski 2010). Attempts to increase the persistence of EPF in the field have mainly relied on protectant formulations (Fernandes et al. 2015) but screening for an environmentally competent EPF strain before formulation may offer a more sustainable alternative.

Variability in the tolerance to UV radiation between EPF species and isolates of the same species has been found. For example, species in the genus *Beauveria* have been shown to exhibit a greater inherent UV tolerance than species in the genus *Metarhizium* (Quesada-Moraga et al. 2023), but the darker pigments, such as those seen in the *Metarhizium* genus, do not necessarily infer greater UV tolerance (Tseng et al. 2011, Quesada-Moraga et al. 2023). The variation in tolerance may be

due to the fact that *B. bassiana* is composed of UV-resistant structures (UV-absorbing metabolites) that act as a sunscreen and absorb UV-B radiation, which is absent in *Metarhizium* species (Tseng et al. 2011, Braga et al. 2015, Quesada-Moraga et al. 2023). *Isaria fumosorosea* isolates originating from warm climates exhibited significantly greater resilience to a 1 h artificial light exposure, compared to those from cooler, temperate regions (Fargues et al. 1996), and strains isolated near the equator, and therefore exposed to more UV, demonstrated higher UV-B tolerance (Fernandes et al. 2007, 2009). Isolates of *M. anisopliae* originating from agricultural habitats demonstrated a greater UV tolerance than isolates of the same species obtained from forested environments, with the latter being considered a more protective environment against the damaging effects of UV radiation. Strains from forested habitats may therefore be less adapted to UV radiation (Bidochka et al. 2001). This research underscores the varying UV tolerance among isolates from diverse habitats and that such differences in UV susceptibility likely represent the natural adaptations of EPF to their specific environments (Fernandes et al. 2015). It stands to reason that EPF isolated from the foliar environment may be more UV tolerant than isolates recovered from the soil environment. For field applications and foliar pest management, selecting a strain with innate high UV tolerance is preferable to enhance persistence, and in turn, may also offset the cost of formulation.

Acheampong et al. (2020b) determined the UV tolerance of some strains of *M. anisopliae* and *B. bassiana*, previously isolated by Goble et al. (2010). This study indicated that all these strains were unable to germinate after a 2 h exposure to simulated solar radiation at a spectrum of 0.3 $W/m^2$. However, these strains were isolated from the belowground environment where UV radiation is not considered a key abiotic stressor (Jaronski 2010). Under laboratory conditions, *Metarhizium pinghaense* FCM Ar 23 B3 used in these studies was previously demonstrated to infect citrus

mealybug (Chartier-FitzGerald et al. 2016), as was also shown in this study (Chapter 3). However, under field conditions, the same isolates have proven to be ineffective against this foliar pest (Grout et al. 2015). Selecting an environmentally competent EPF isolate is highly desirable as it directly relates to the ability of the biocontrol agent to persist and potentially proliferate within the targeted pest's environment (Jackson et al. 2000, Quesada-Moraga et al. 2023).

The first step in selecting a microbial biocontrol agent is to (1) establish the virulence and (2) establish the ecological competence of the microbe. In Chapter 3, the virulence of these novel isolates was established against citrus mealybug (a common foliar pest of citrus in South Africa). As UV radiation is a key determining factor in EPF success, an increased understanding of the variance in UV tolerance of these novel isolates allows strains with enhanced UV tolerance to be identified and, thus, select the most suitable candidate for mycoinsecticide development for foliar pest management.

Therefore, this chapter aimed to determine the UV tolerance of five indigenous novel isolates using simulated solar radiation and compare their susceptibility to the belowground isolate (FCM Ar 23 B3) [previously collected by Goble et al. (2010), of which UV tolerance has been established, and found to be poor (Acheampong et al. 2020b)]. Using this isolate allows one to draw comparisons between different UV chambers and further observe the potential differences in UV tolerance between isolates recovered from the soil environment and the novel isolates collected from the foliar environment.

## 4. 2 MATERIALS AND METHODS

### 4.2.1 Fungal isolates and culture condition prior to irradiation

The UV tolerance of the five novel isolates (Px LM 4, Ha LM 11, Ha LM 12, Hu LM 14, and Coe 18) and the FCM Ar 23 B3 isolate are investigated in this chapter. Following one sub-culturing (incubated at 26 ℃, 30 % RH with a 12 h photoperiod for 2- to 3-weeks) onto SDAc plates from sporulating cadavers (see Chapter 3, section 3.2.3.1 for passaging details), stock cultures were acquired. Conidia were harvested from 2- to 3-week-old cultures from each isolate and conidial suspensions were adjusted to $1 \times 10^5$ conidia/ml as described in Chapter 3, section 3.2.3.2 and 3.2.3.2.1.

### 4.2.2 EPF inoculation and exposure to simulated solar radiation

For each of the six isolates, 50 µl of the conidial suspension was spread onto two SDA plates, one corresponding to the exposure treatment and the other the control. Inoculated control plates were wrapped in aluminium foil to block UV radiation. Following inoculation, plates were exposed to simulated UV radiation in the Atlas SUNTEST CPS+ UV chamber (Germany) at 0.3 $W/m^2$ for 15, 30 and 60 min (Acheampong et al. 2020b) with the corresponding total doses at these exposure periods being 0.83 ± 5.66, 0.85 ± 4.07, and 1.08 ± 0.48 $KJ/m^2$, respectively. Due to space limitations within the UV chamber, only five isolates, with their corresponding control plate, could be assessed at a time, without the belowground isolate. The belowground isolate [FCM Ar 23 B3, for which UV tolerance had previously been established (Acheampong et al. 2020b)], was then tested on its own. The Atlas UV chamber (weathering instrument) can be used for assessing UV variability in EPF due to its specific control over irradiance and spectrum (Rycobel) (Saga

Instruments Pte Ltd) (Figure 4.1). The UV chamber is equipped with a Xenon lamp and a coated quartz filter segment which regenerates full spectrum solar radiation in the range of 300 – 800 nm. Irradiance is measured by a photodiode, and the test chamber temperature is measured by a temperature sensor. During the trials, the UV chamber had a temperature of 24.58 ± 0.68 ℃ with a RH of 51.08 ± 1.04 %. Post irradiation, plates were stored in darkness at approximately 25 ℃. After 24 and 48 h following UV exposure, the number of germinated and non-germinated conidia, out of 300 conidia per plate, were evaluated under a microscope at 20 X magnification. The subsequent assessment period was intended to determine if conidial germination could be restored through potential photoreactivation (Alves et al. 1998, Tong & Feng 2022). Conidial germination was assessed as described in Chapter 3, section 3.2.3.2.2. The experiment was replicated three times for each isolate, using conidial suspensions prepared from fresh cultures.

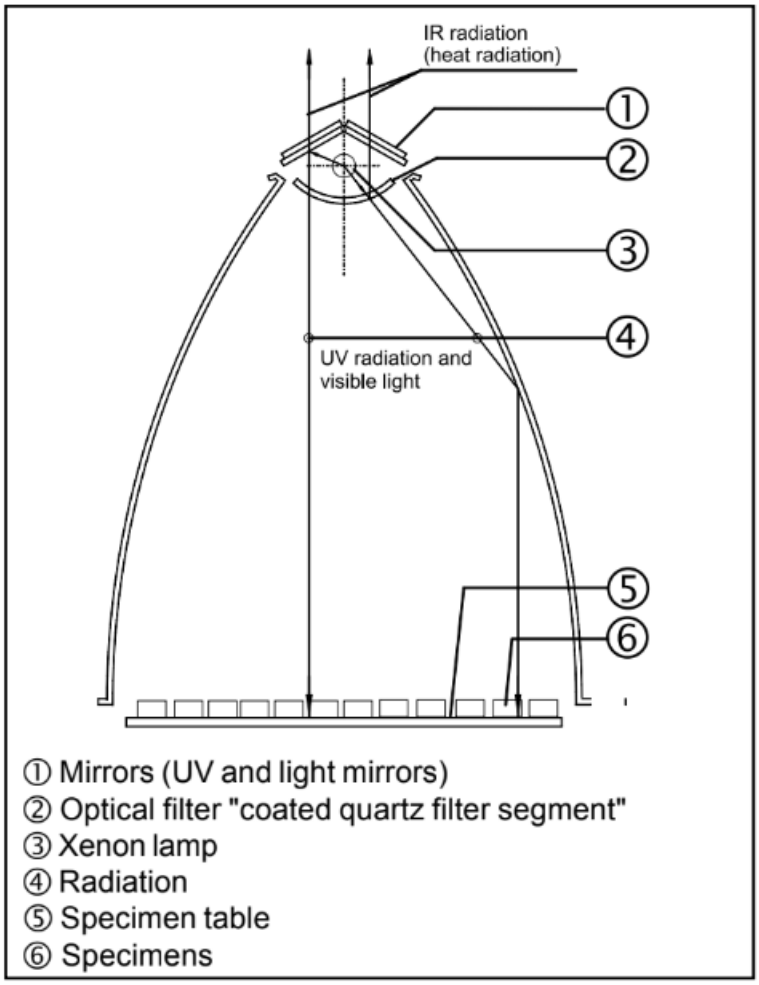


**Figure 4.1:** Atlas SUNTEST CPS+ (UM_suntest_cps_plus.pdf).

### 4.2.3 Statistical analysis

The relative percentage conidial germination to non-irradiated conidial germination (control) was calculated for each isolate using the following equation:

Relative germination (%) = $(G_i/G_c) \times 100$

where $G_i$ is the number of germinated conidia in the irradiated plate (for that certain exposure period) and $G_c$ the number of germinated conidia in the non-irradiated plates (control). The relative

percentage germination can range between 0 (no germination) to 1 (germination equal to or greater than the control) (Braga et al. 2001a).

To determine whether the effect of exposure time on relative germination rates varied between isolates, two generalised linear mixed models (GLMMs) were fitted using the glmmTMB package (Brooks et al. 2017). Both models used a beta distribution for the response variable, relative germination rate (bounded between 0 and 1). The models included fixed effects for isolate and exposure time, and replicates were treated as a random intercept term to account for repeated measurements (Bolker et al. 2009).

The first model (additive) included main effects for isolate and exposure time, while the second model (multiplicative) included an interaction term to allow the effect of exposure time on germination to vary between isolates.

The two models were compared using a Wald $\chi^2$ test to assess whether the inclusion of the interaction term significantly improved the model fit. If there was a significant difference between the two models fitted, the model with the lowest log likelihood value was used for model inference. Where no significant improvement in model fit was calculated due to the inclusion of the multiplicative interaction effect, the simpler additive model was used for model inference.

Separate models were specified to compare relative germination at 24 and 48 h time intervals. Post-hoc means separation for between-isolate and within-isolate effects was performed using the 'emmeans' R package (Lenth, 2024) and adjusted for multiple comparisons using the Bonferroni correction ($P < 0.05$). All statistical analyses were conducted in R ver. 4.4.1 (R Core Team, 2024).

## 4.3 RESULTS

### 4.3.1 Simulated solar radiation effect on conidial germination

*24 h assessment*

All control plates demonstrated a greater percentage of conidial germination (> 82 %) than plates exposed to simulated sunlight, regardless of exposure time.

The Wald $\chi^2$ test indicated statistical support for a multiplicative effect of the different exposure times on relative germination across the different fungal isolates ($\chi^2 = 22.4$, df = 5, $P < 0.001$). This suggests that the impact of exposure time on relative germination varied between isolates. Thus, both isolate and exposure time were found to have statistically significant effects on relative germination, with the interaction term showing that the effect of exposure time was isolate-specific. Averaged across all isolates, the relative germination decreased by 15 % when the exposure time increased from 15 min to 30 min. The relative germination decreased by an average of 25 % (for all isolates) when the exposure time increased from 30 min to 60 min. The biggest difference in relative germination for most of the isolates was observed between the increase in exposure period from 30 min to 60 min (Figure 4.2). However, isolate FCM Ar 23 B3 had the greatest change in relative germination between the 15- and 30-min exposure periods (decreased by 43 %) as opposed to the 30- and 60-min exposure period, where a decrease of relative germination by 6 % was recorded (Figure 4.2).

After 60 min exposure to simulated sunlight, the most UV-tolerant isolate was Ha LM 11 (46.51 % relative germination) whereas the most UV-susceptible isolate was FCM Ar 23 B3 (1.42 % relative germination) (Figure 4.2).

*48 h assessment*

All control plates demonstrated a greater percentage of conidial germination (> 90 %) compared to plates exposed to simulated sunlight, regardless of exposure time.

The Wald $\chi^2$ test comparing the additive and multiplicative models revealed no statistical support for a multiplicative effect of exposure time on germination rates across isolates ($\chi^2 = 3.28$, df = 5, $P = 0.657$). Thus, the additive model was used for model inference.

Subsequent analysis of the additive model revealed a significant effect of isolate on germination rates ($\chi^2 = 116.25$, df = 1, $P < 0.001$) averaged across all exposure periods. The mean relative germination of isolate Hu LM 14 (63 % ± 0.04) was significantly lower than that of the other five isolates, which had germination rates ranging from 77 % to 82 % with isolate Ha LM 12 displaying the highest relative germination (Figure 4.3).

There was also a significant effect of exposure time on relative germination ($\chi^2 = 106.69$, df = 1, $P < 0.001$). Averaged across isolates, germination was highest at an exposure time of 15 min (86 ± 2 %), decreasing by 8 % at 30 min (79 ± 2 %). The largest decrease in germination occurred between 30- and 60-min of exposure (65 ± 3 %), with an average reduction of 14 %.

The estimated effect of exposure time was -0.042, and the exponent of this coefficient [exp (-0.027) = 0.973] indicated that for an average 1 min increase in exposure time, the relative germination decreased by approximately 2.7 %, averaged across isolates (Figure 4.3).

After 60 min exposure to simulated sunlight, the most UV tolerant isolate was Ha LM 12 (73.52 ± 3.34 % relative germination), whereas the most UV susceptible isolate was Hu LM 14 (50.22 ± 5.06 % relative germination) (Figure 4.3).

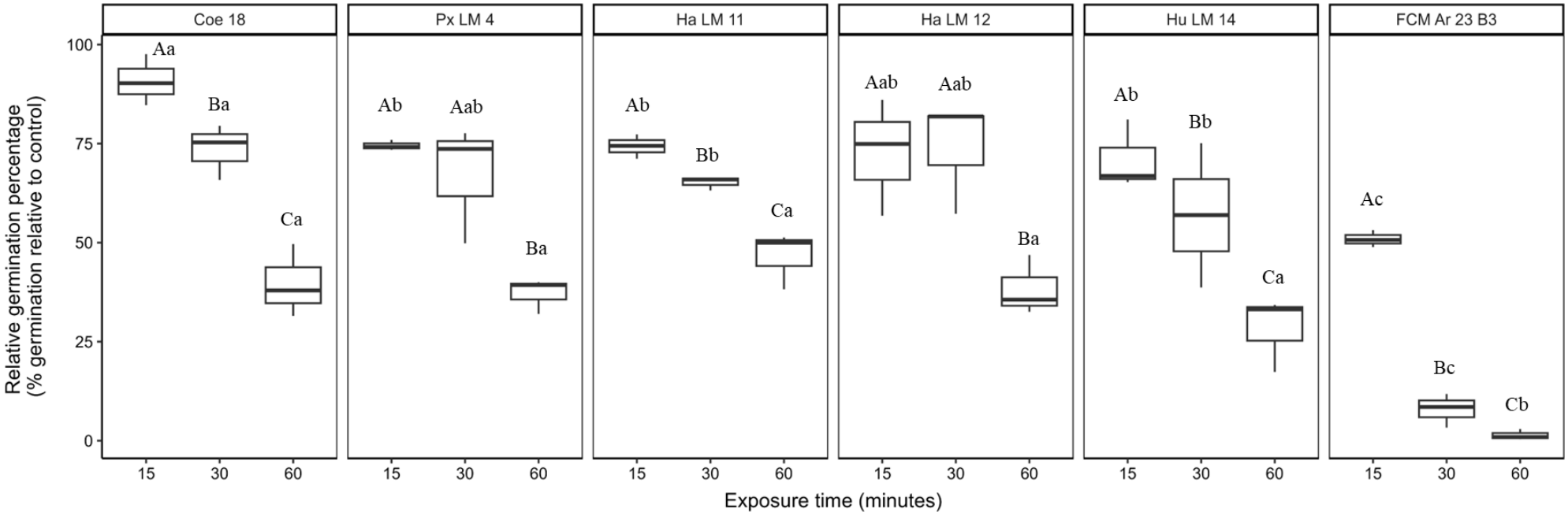


**Figure 4.2:** Boxplots displaying relative germination percentage assessed 24 h following exposure to Xenon lamps (300 – 800 nm) at 0.3 W/m$^2$ at different time intervals, at 24.6 ± 0.68 ℃ and 51.1 ± 1.04 % RH. Samples were incubated at 25 ℃. Each plot represents the median (line inside the box), interquartile range (IQR, the height of the box), and potential outliers (points outside the whiskers). Isolate FCM Ar 23 B3, which was previously isolated by Goble et al. (2010) and UV tolerance demonstrated by Acheampong et al. (2020b), was recovered from the soil environment whereas the rest of the novel isolates were recovered from the aboveground environment. Means within each isolate panel (within isolate comparisons) with the same uppercase letter are not significantly different ($P > 0.05$). Means within each exposure time (between isolate comparisons) with the same lowercase letter are not significantly different ($P > 0.05$).

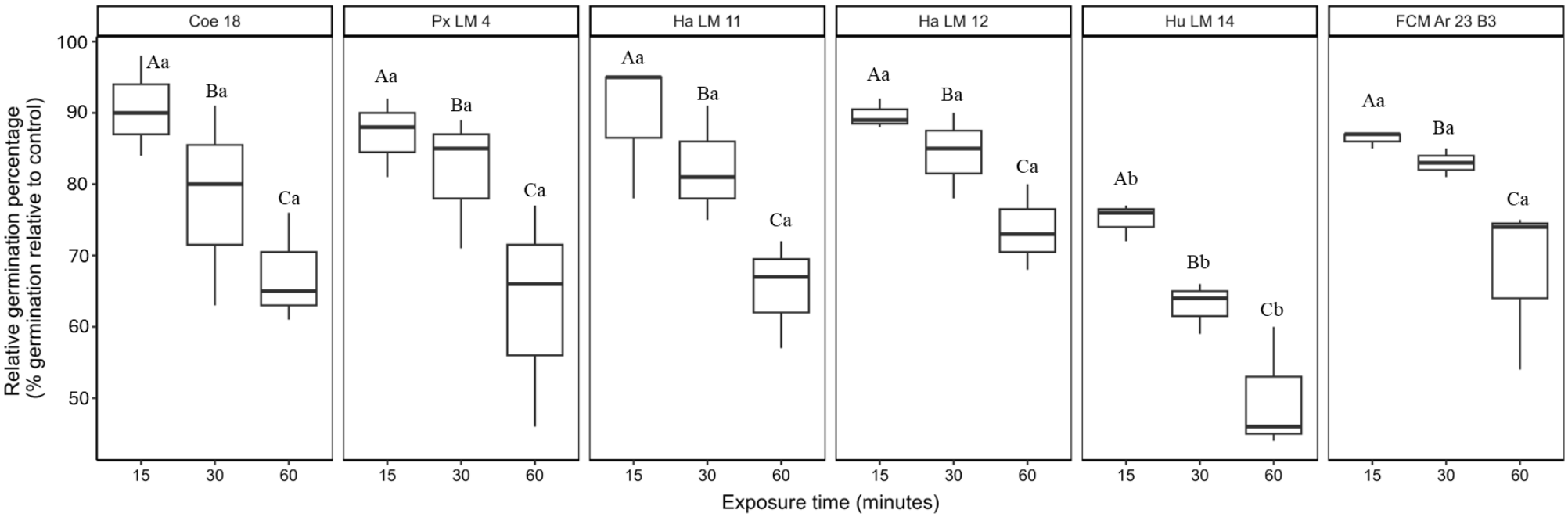


**Figure 4.3:** Boxplots displaying relative germination percentage assessed 48 h following exposure to Xenon lamps (300 – 800 nm) at 0.3 W/m$^2$ at different time intervals, at 24.6 ± 0.68 ℃ and 51.1 ± 1.04 % RH. Samples were incubated at 25 ℃. Each plot represents the median (line inside the box), interquartile range (IQR, the height of the box), and potential outliers (points outside the whiskers). Isolate FCM Ar 23 B3, which was previously isolated by Goble et al. (2010) and UV tolerance demonstrated by Acheampong et al. (2020b), was recovered from the soil environment whereas the rest of the novel isolates were recovered from the aboveground environment. Means within each isolate panel (within isolate comparisons) with the same uppercase letter are not significantly different ($P > 0.05$). Means within each exposure time (between isolate comparisons) with the same lowercase letter are not significantly different ($P > 0.05$).

## 4.4 DISCUSSION

EPF have demonstrated great control of a wide variety of pests and in turn, decreased the reliance on chemical pesticides (Vega et al. 2009). However, their susceptibility to UV radiation, particularly UV-B, hinders their efficacy as biological control agents (Inglis et al. 1993, Braga et al. 2015, Fernández-Bravo et al. 2024).

Most notably, comparing the UV tolerance of EPF isolates from different studies is extremely challenging as many other conditions and factors, such as the methodology, dose, UV source, exposure or restriction to light before and following UV exposure, the temperature of culture incubation, the medium on which the isolates are grown and culture age, may influence their susceptibility to UV radiation (Chelico et al. 2006, Fernandes et al. 2015). As such, this study incorporated an isolate that was recovered from the belowground environment (isolate FCM Ar 23 B3), for which the UV tolerance had previously been demonstrated (Acheampong et al. 2020b) to determine if the aboveground isolates demonstrated greater tolerance to UV in comparison. This study aimed to address whether isolates recovered from the aboveground environment, where UV radiation is considered the most detrimental abiotic factor hindering EPF efficacy, are more tolerant to simulated sunlight than an isolate recovered from the belowground environment where UV radiation is limited.

Following 15 and 30 min UV exposure 24 h post-incubation, the conidial viability of isolate FCM Ar 23 B3 was 47.68 % and 89.14 % higher, respectively, in the study conducted by Acheampong et al. (2020b) in comparison to this study. However, at 60 min exposure and the same incubation period, the FCM Ar 23 B3 isolate performed similarly (0.01 % higher in this study). Similarly, following 15 and 30 min UV exposure and incubation for 48 h, the isolate had a higher percentage conidial germination by 12.28 % and 16.21 %, respectively in the Acheampong et al. (2020b)

study. Yet at 60 min exposure and 48 h post-incubation, the same isolate performed better in this study (65.23 % more conidial germination). Again, this highlights that comparing the UV tolerance of the same isolate at the same UV irradiation levels across different studies is challenging. These differences in relative percentage conidial germination may be owed to differences in temperature in the UV chamber as well as the temperature during the incubation periods. In the UV chamber, the temperature was 3.84 ℃ higher in the study conducted by Acheampong et al. (2020b), but the incubation temperature was 4 ℃ higher in this study. However, based on the results of the study conducted by Acheampong et al. (2020a), these relatively small temperature differences should not have influenced the growth (i.e., germination) of this isolate. As previously mentioned, differences in UV tolerance of isolate FCM Ar 23 B3 may be due to various factors, such as the use of different UV chambers.

*In vitro* research has extensively documented the effects of UV radiation on conidial viability, demonstrating significant inter- and intra-specific variability in UV susceptibility (Braga et al. 2001b, Jackson et al. 2010, Fernández-Bravo et al. 2016, 2017, 2024). These studies reveal that UV radiation affects each fungal genus, species, and different strains or isolates within the same species to differing extents. Inter and intraspecies variability in tolerance to UV radiation amongst isolates was apparent across all exposure periods but became pronounced after 60 min exposure, as delayed conidial germination was observed at 24 h post-incubation, which is in agreement with Braga et al. (2002). This was also recorded at 48 h post-incubation, as seen in the study conducted by Alves et al. (1998). Specifically, after 24 h incubation and at all exposure periods, both *Metarhizium* isolates (FCM Ar 23 B3 and Hu LM 14), regardless of recovery habitat, were much more susceptible to simulated sunlight than the *Beauveria* isolates. This is in agreement with several other studies (Fernández-Bravo et al. 2016, 2017) and well reported (Quesada-Moraga et

al. 2023). This may be because *Metarhizium* species tend to be more susceptible to UV inactivation than *Beauveria* species (Fernández-Bravo et al. 2016, 2017). This is most likely due to the melanin-like structures (metabolites) absorbing or blocking UV irradiation of *B. bassiana* strains (Tseng et al. 2011, Braga et al. 2015, Quesada-Moraga et al. 2023). Notably, even though both *Metarhizium* isolates were more susceptible to simulated sunlight than the *Beauveria* strains, the isolate Hu LM 14, which was recovered aboveground from a foliar pest, was more UV tolerant (at all exposure periods) than the other *Metarhizium* isolate FCM Ar 23 B3, 24 h post-incubation, which was recovered from belowground. However, following incubation for 48 h, the *Metarhizium* isolates, more specifically FCM Ar 23 B3, demonstrated relatively high germination percentages across all exposure periods, comparable to most of the *Beauveria* isolates. However, the *Metarhizium* isolate Hu LM 14 was the most susceptible across all the exposure periods, demonstrating that this isolate did not recover. Even though photoreactivation, a repair mechanism serving to repair damaged DNA induced by UV radiation, is present in the *Metarhizium* genus (Chelico et al. 2006, Fang & Leger 2012, Rossouw et al. 2023), the ability to exercise this mechanism may differ between different species and strains of the same species.

This research ultimately demonstrated that UV tolerance varied amongst all isolates, regardless of isolation habitat. Similarly, there was no correlation found between the geoclimatic origins of strains of different EPF species nor the isolation habitat (foliar insect cadavers or soil environment) and the UV tolerance (Fargues et al. 1996). Likewise, Fernández-Bravo et al. (2016) found no correlation between *B. bassiana* isolates recovered from both the phylloplane and soil environment and UV susceptibility.

UV radiation certainly delayed conidial germination of all the isolates investigated which is in agreement with several other studies (Braga et al. 2001a, 2001b, Fernández-Bravo et al. 2016,

2017, 2024, Acheampong et al. 2020b). This is an important factor as it implicitly influences the efficacy of isolates as some insects, such as aphids and some lepidopteran species, experience rapid ecdysis which may prevent conidial attachment and thus infection (Jaronski 2010). As reported by Acheampong et al. (2020b), recovery from UV damage is possible via DNA repair mechanisms. Conidial germination increased post-irradiation for most of the isolates when assessed after 48 h compared to the 24 h assessment time. This may be due to the process of photoreactivation of UV-damaged conidia (Quesada-Moraga et al. 2023). However, this was not implicitly accounted for in this study.

The conidial viability of the isolates was determined following UV exposure, however, a high or low percentage of viable conidia does not necessarily infer that isolates retain or lose pathogenicity and virulence (Fernández-Bravo et al. 2024). For example, Wu et al. (2020) found that isolates in which no germination occurred following 10 min exposure to UV were still virulent against *Galleria mellonella*. It would be beneficial to test whether the pathogenic ability and virulence of the isolates in this study were influenced, as this would provide a more comprehensive assessment of how well these EPF isolates would perform under field conditions. Similarly, the effect of UV radiation on EPF is also less apparent when conidia are inoculated onto insect bodies as opposed to flat surfaces (Fernández-Bravo et al. 2017, Quesada-Moraga et al. 2023). For example, the irradiation time needed to reduce mortality to 50 % for *C. capitata* adults treated with $1.0 \times 10^8$ conidia/ml was 34.69 h for *B. bassiana* isolate EABb 10/225-Fil whereas conidial viability was reduced to 50 % in only 9.89 h when exposed to at an irradiance level of 1200 mW $m^{-2}$ for 6 h (Fernández-Bravo et al. 2024).

Considerable research has focussed on formulating EPF isolates to increase UV tolerance, with the understanding that formulating conidia with UV protectants increases persistence (Fernandes

et al. 2015). For example, the *B. bassiana* strain BbZJ1 was demonstrated to be more UV resistant when formulated in ultraviolet protective agents (groundnut oil, soybean oil, and Triton X-100) than an inoculant with only Tween® 80. Even after 4 h of UV exposure, the groundnut oil and Triton X-100 formulants provided enough protection for the isolate to germinate to 35.0 % and 38.3 %, respectively (Jia et al. 2023). Kaiser et al. (2019) also showed that the formulation of a *B. bassiana* strain ART2587 with humic acid sodium increased the relative colony forming unit (CFU) numbers by 64.5 % (compared to the unformulated control) on agar plates after exposure to UV-B radiation for 4 h. Moving forward with the isolates tested in this study, it would be beneficial to investigate whether any UV protectant formulations would promote a higher UV tolerance, as well as to observe which different formulants provides the most protection against UV radiation.

In summary, following 60 min exposure to simulated sunlight and incubation for 24 h, isolate Ha LM 11 (46.51 % relative conidial germination) was demonstrated to be the most UV tolerant of the isolates tested, and isolate FCM Ar 23 B3 was the most susceptible to UV (1.42 % relative germination). However, following 60 min exposure and incubation for 48 h, isolate Ha LM 12 was demonstrated to be the most UV tolerant (73.52 %) and isolate Hu LM 14 was the most susceptible to UV (50.22 %). After 60 min of UV exposure and 24 h post-incubation, all the isolates from this study outperformed seven out of the nine unformulated, indigenous isolates in the study conducted by Acheampong et al. (2020b). However, after 60 min of UV exposure and 48 h post-incubation, all isolates from this study, except for FCM Ar 23 B3, were more UV susceptible than the isolates tested in the Acheampong et al. (2020b) study, demonstrating that the isolates tested in this study, besides FCM Ar 23 B3, did not recover as effectively. Arguably, the Coe 18 isolate demonstrated both strong initial UV tolerance and sustained a relatively high germination percentage over time.

The ability to maintain reliable performance across both exposure time and incubation period establishes Coe 18 as the most UV-tolerant isolate. Demonstrating strong initial UV tolerance indicates that the conidia are more viable compared to UV susceptible isolates. This increased conidial viability provides a significant advantage for infection to occur as more tolerant isolates tend to be better equipped for the process of attachment to the host's integument, germinate and thus, ultimately infect their insect host. In comparison, even the most virulent isolates will not be able to infect a host if their conidia are inactivated by UV radiation. Notably, all control plates demonstrated a greater percentage conidial germination than plates exposed to simulated sunlight, regardless of exposure time, ultimately confirming that the UV radiation emitted by this UV chamber delayed germination of all isolates tested.

# CHAPTER 5: GENERAL DISCUSSION

## 5. 1 THESIS SYNTHESIS

EPF are globally recognised for their ability to infect insects percutaneously and provide sustainable control over agriculturally important pests. As a result, this entomopathogenic group has been incorporated into many pest management programmes (Faria & Wraight 2007, Maina et al. 2018, Liu et al. 2023). In the South African citrus industry, EPF are being developed and used as additional control measures in IPM programmes. Several *B. bassiana* strains and *Metarhizium* species were isolated from citrus orchards and neighbouring refugia in the Eastern Cape Province of South Africa and demonstrated significant potential as microbial biocontrol agents for key pests, including FCM, citrus thrips, and citrus mealybugs under laboratory conditions (Goble et al. 2010, 2011, Coombes et al. 2015, Chartier-FitzGerald et al. 2016). Under field conditions, some of these isolates proved to be effective against the soil-dwelling life stages of FCM following soil application (Coombes et al. 2016), however, these same isolates provided insufficient control of citrus thrips and mealybug following foliar application (Grout et al. 2015). Acheampong et al. (2020a, 2020b) investigated the biological attributes of these isolates, specifically the temperature and humidity tolerance as well as the UV susceptibility. Finding that varying temperature ranges and humidity levels would not hinder the isolates' efficacy in the field, the authors concluded that the ineffective control of these foliar pests is most likely attributed to the conidial inactivation induced by UV radiation. As these strains were recovered from the soil environment, it stands to reason that EPF isolates recovered from the foliar environment may be more suited for foliar application. A strain that is relatively virulent but displays ecological competence in the targeted environment is highly desired. Thus, bioprospecting for isolates from the aboveground

environment was initiated and was the focal point of this thesis. Following the isolation and identification, the virulence and UV tolerance of these novel strains were established. Findings from this research will be briefly highlighted, and implications for advancing the development and application of these novel isolates are discussed.

## *Isolation and identification*

Of the isolates recovered from the aboveground environment, four were *B. bassiana* (Px LM 4, Ha LM 11, Ha LM 12, Coe 18), one *M. anisopliae* (Hu LM 14), one *F. oxysporum* (Pc HV 9), and one *G. candidum* yeast (Ha LM 2). The majority were isolated from insect cadavers but one (Coe 18) was isolated as a foliar endophyte from an organically managed citrus farm in the Eastern Cape. Based on ecological niche preferences, it was expected that *B. bassiana* was the most common EPF recovered from the aboveground environment (Meyling & Eilenberg 2007, Meyling et al. 2011).

## *Laboratory bioassays*

Laboratory bioassays to determine the pathogenic ability and virulence of isolates are generally the first factor investigated for the potential development and implementation of a microbial biocontrol agent. Using standard protocols and conidial doses, the virulence of the isolates was established against a common foliar pest of citrus, citrus mealybug. Isolate FCM Ar 23 B3 was included as a comparative control in this study as the virulence against citrus mealybug has previously been established (Chartier-FitzGerald et al. 2016). The initial screening of the isolates ranged between 15 and 90 % mortality. Isolates Px LM 4 and FCM Ar 23 B3 both induced an average mortality of 90 %. Isolates Ha LM 11, Ha LM 12, Hu LM 14, and Coe 18 mortalities greater than 60 % and were further investigated under dose-response assays. Of the six isolates

measured for $LC_{50}$, FCM Ar 23 B3 was the most virulent ($5.25 \times 10^5$ conidia/ml), congruent with the study conducted by Chartier-FitzGerald et al. (2016). Px LM 4 ($1.09 \times 10^6$ conidia/ml) and Hu LM 14 ($1.32 \times 10^6$ conidia/ml) showed lower $LC_{50}$ values compared to Ha LM 11 ($2.68 \times 10^6$ conidia/ml), Coe 18 ($3.57 \times 10^6$ conidia/ml), and Ha LM 12 ($4.40 \times 10^6$ conidia/ml). The percentage mortality of citrus mealybug was dose-dependent for each of the isolates. Even the most virulent isolates will not be able to induce infection in target hosts if conidia viability is drastically reduced by ecological incompetence, such as UV radiation, thus, the UV susceptibility of these six isolates was investigated.

### *UV tolerance*

Isolate FCM Ar 23 B3 was included as a standard isolate to validate the assays, as the UV susceptibility of this strain is already known (Acheampong et al. 2020b). UV radiation certainly delayed conidial germination of all the isolates investigated which is in agreement with several other studies (Braga et al. 2001b, 2001a, Fernández-Bravo et al. 2016, 2017c, 2024, Acheampong et al. 2020b). However, all the strains isolated from the aboveground environment, even the *M. anisopliae* isolate (Hu LM 14), demonstrated significant initial tolerance to UV radiation compared to the most virulent *M. pinghaense* isolate (FCM Ar 23 B3) (Chapter 3), which was recovered from the soil environment. However, this susceptible isolate was able to recover after 48 h following UV exposure. Arguably, *B. bassiana* Coe 18, was the most UV tolerant as it demonstrated both initial tolerance and sustained a relatively high germination percentage.

For the implementation of these isolates, further research is warranted, specifically for foliar application. This is discussed further in the subsequent sections.

## 5. 2 FORMULATION FOR FOLIAR APPLICATION

Formulation of EPF is mainly aimed at increasing economic viability (improved stability and shelf life), efficiency against target pests and increasing efficacy and persistence in the field by providing protection against environmental factors (Jackson et al. 2010, Mascarin & Jaronski 2016, Sharma et al. 2023).

It is important to note that the targeted environment or habitat in which certain pests reside influences the type of formulation. For example, granular or pellet formulations are aimed at targeting soil-dwelling pests, or pests with a subterranean developmental stage such as FCM. As a result, these formulations usually do not require UV protective additives as this abiotic factor does not significantly influence EPF in the soil environment (Quesada-Moraga et al. 2023). However, oil-based formulations, including encapsulation, tend to provide better UV protection for foliar applications to control arboreal pests.

Oil-based formulations have been shown to enhance the UV tolerance of EPF. For instance, *B. bassiana* conidia formulated in sesame oil exhibited a higher colony-forming unit (CFU) count compared to the control after UV exposure in two separate experiments: 4 h on agar and 5 h on leaf discs (Kaiser et al. 2019). Jia et al. (2023) also found that 4 h of exposure to simulated sunlight was lethal to *B. bassiana* (BbZJ1 strain) conidia suspended in Tween® 80, whereas fungal agents formulated in groundnut oil and soybean oil had increased germination percentages. Posadas et al. (2012) investigated whether the addition of three plant-based oils (soybean, sunflower and corn oil) and one mineral oil (petrolatum) increased the UV tolerance of six fungal isolates. The authors found that all the formulations significantly increased the percentage conidial germination of five of the six isolates relative to the control after 4 h exposure to simulated UV. It is important to note

that the one isolate possessed innate UV tolerance and that is why the additives of the formulants did not statistically increase germination percentage relative to the control (Posadas et al. 2012). Due to the radiation absorption properties of the oil, Moore et al. (1993) found that conidia of *M. flavoviride* formulated in either mineral or groundnut oil offered higher levels of UV protection to simulated sunlight compared to conidia suspended in water. Superior UV protection was also observed with the addition of 1 % oxybenzone (organic compound used in sunscreens) in groundnut oil formulation of *M. flavoviride* (Moore et al. 1993). Similarly, the addition of oxybenzone (at 0.5 % (w/v)) to conidial suspensions of *B. bassiana* isolates improved the UV tolerance of four of the six isolates investigated (Posadas et al. 2012).

Encapsulation of EPF involves enclosing fungal spores or mycelium in a protective material to enhance their stability, viability, and efficacy as biopesticides. Encapsulation is particularly useful for protecting the fungi from environmental stresses (e.g., UV radiation, desiccation, and temperature extremes) (Preininger et al. 2018). Nanoparticles of zinc oxide, titanium oxide, silica and graphene oxide may be used for increased UV tolerance and prevent conidial inactivation by sunlight (Dunlap & Schisler 2014, Maghsoudi & Jalali 2017, Preininger et al. 2018). Microencapsulation has also been explored for the formulation of the bacteria *Bacillus thuringiensis*. Research has shown that this form of formulation is viable and significantly increases resistance to UV radiation (Zhang et al. 2016). This method has also been explored for EPF and offered promising results. For example, Felizatti et al. (2021) investigated the optimisation of *B. bassiana* formulations using a spray-drying technique and compared their stability and bioactivity against *Spodoptera cosmioides* Walker (Lepidoptera: Noctuidae) with those of ionic gelatinisation formulations. Both methods produced conidia that demonstrated greater stability under exposure to UV light compared to non-formulated *B. bassiana*. Qiu et al.

(2019) also demonstrated the potential for microencapsulation of *M. anisopliae* in gelatin and found that the germination rate of microencapsulated conidia was significantly higher (44 %) than that of bare conidia following 3 h of UV exposure. Other notable studies exhibiting Pickering emulsions of EPF have been demonstrated in Yaakov et al., 2018, Birnbaum et al., 2021, Feldbaum et al., 2021, and Kotliarevski et al., 2021.

The science of formulation is complex. Selecting an appropriate method depends on the biocontrol agent's characteristics and the desired application, as well as achieving a balance between scalability, cost-effectiveness, and compatibility with the biocontrol agents (Saberi Riseh et al. 2022). Based on this dynamic complexity, research into the optimal method of formulation of these isolates is desirable, whilst maintaining the virulence of the isolates. The tree canopy serves as the primary breeding and infestation site for mealybugs. Therefore, incorporating an effective sunscreen into formulations, despite its added cost, could be highly advantageous and potentially offer control to other important foliar citrus pests. Enhanced persistence under UV exposure would not only improve efficacy but also promote secondary infection, making it a worthwhile investment for managing these pests. It would be worthwhile investigating different formulations for increased UV protection of these novel isolates for foliar applications.

## 5. 3 OTHER DELIVERY MECHANISMS OF EPF

Effective control of arboreal pests like thrips and mealybugs remains a challenge, particularly because their life stages predominantly occur in tree canopies. This necessitates the use of foliar applications of mycoinsecticides to target these pests effectively. Enhancing the persistence of fungal isolates under canopy conditions is crucial, and the incorporation of UV protectants, such

as sunscreens, holds promise for improving their efficacy. Beyond UV protection, exploring additional strategies to reinforce the efficacy of these biocontrol agents warrants further investigation and is outlined in the sections that follow.

### *Endophytes*

It is well documented that some endophytic EPF, such as *B. bassiana* and *M. anisopliae* isolates, increase herbivory resistance (Jaber & Vidal 2010, Quesada-Moraga et al. 2014, 2022, Sánchez-Rodríguez et al. 2018), but they offer various other agriculturally important benefits too, such as plant-pathogen antagonism and promoting plant growth. Pimenta et al. (2012) isolated four different endophytic fungal strains which demonstrated significant fungal pathogenic antagonistic activity, through inhibiting volatiles, against *Monilinia fructicola* (a necrotrophic pathogen causing brown rot in stone fruits). Sui et al. (2023) demonstrated that tomato plants (*Solanum lycopersicum* var. BEAUTY), once colonised with *B. bassiana,* were significantly more resistant to the colonisation and proliferation of grey mould caused by *Botrytis cinerea.* Tomato (*Solanum lycopersicum* var. BEAUTY) and field corn (*Zea mays* L.), when inoculated with *B. bassiana* and *M. anisopliae*, respectively*,* had a significant increase in plant biomass compared to controls (Kabaluk & Ericsson 2007, Sui et al. 2023). The overall plant growth of *Vicia faba*, commonly known as broad or fava bean, also increased significantly following seed-soaking treatments with either *B. bassiana* (NATURALIS) or *M. brunneum* (BIPESCO5) (Jaber & Enkerli 2016). Sasan & Bidochka (2012) shows that *M. robertsii* colonisation of the rhizosphere in *Panicum virgatum* (switchgrass) and *Phaseolus vulgaris* (haricot beans) results in the proliferation of root hairs, promoting plant growth through direct nutrient acquisition. Multiple *Trichoderma* spp. have been shown to prevent and control plant diseases by inhibiting the growth of pathogenic fungi (Yao et

al. 2023). Liu et al. (2022) demonstrated, through dual-culture antagonism assays, that various *Trichoderma* strains showed antagonistic effects against oomycete pathogens. For example, *T. asperellum* suppressed the plant pathogen *Globiosporangium ultimum* in peas and ultimately promoted overall vegetative growth of endophytically-colonised plants (Moussa et al. 2023). Much research has been undertaken into the ability of EPF to artificially colonise agriculturally important crop species and proven successful with some EPF isolates and citrus (Mantzoukas & Eliopoulos 2020, Aguila et al. 2021, Arnoldi et al. 2022). Due to the fact that citrus is a perennial crop, repeated applications may offer some of these same benefits reported for other crops above. However, this aspect needs to be explored further.

Based on the above evidence, it would be highly beneficial to investigate whether these newly isolated strains can artificially colonise citrus plants and offer control to accompanying pests.

### *Integration with natural enemies*

Predators, such as *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae) (commonly known as the mealybug destroyer) and parasitoids, such as *Anagyrus vladimiri* (Hymenoptera: Encyrtidae), are used for the control of citrus mealybugs. These two biological control agents are mass-reared and commercially available to citrus farmers in South Africa and generally augment released (Moore & Hattingh 2004, 2022). Thus, considering whether these isolates are IPM-compatible for the control of this pest is highly desirable and further research should be conducted on whether these novel EPF isolates may infect and thus hinder the ability of citrus mealybugs' natural enemies. Besides biocontrol agents released for mealybugs, several other parasitoids are released for other key citrus pests, such as red scale or FCM. Whilst the focus was on mealybug, it is paramount that the application of one treatment does not hinder that of another.

Some EPF isolates have been shown to exhibit relatively low pathogenicity and virulence towards *C. montrouzieri*. Using a range of conidial concentrations ($1 \times 10^6$ - $1 \times 10^9$ conidia/ml), Brown & Khan (2009), treated *Malachra alceifolia* leaf discs with conidial suspensions of four *M. anisopliae* isolates. Among these, isolate ARSEF 954 demonstrated the lowest virulence against adult *C. montrouzieri*, with a median lethal time ($LT_{50}$) of about 12 days at a $LC_{50}$ of $6.53 \times 10^8$ conidia/ml. However, the same isolate was the most infective against *Anagyrus kamarli* (Hymenoptera: Encyrtidae), a parasitoid of the pink hibiscus mealybug (Brown & Khan 2009). Conversely, Aghaeepour et al. (2022) found that two *B. bassiana* strains (AM-118 and BB3) caused significant mortality of the third-instars and adult *C. montrouzieri*. Nevertheless, two isolates, one *B. bassiana* and another *M. anisopliae*, needed extremely high concentrations (which is unlikely under field conditions) to cause 50 % mortality of *C. montrouzieri* under laboratory conditions (Mohamed 2016). The above evidence highlights that EPF infections of these natural enemies are isolate-dependent, and thus, future studies testing whether the isolates from this study are pathogenic and virulent to these natural enemies are recommended.

Furthermore, if resistance to infection by these isolates is found, it would be advisable to investigate whether these natural enemies could act as vectors of the EPF strains obtained in this study. Several laboratory and controlled field studies have demonstrated enhanced pest management effectiveness against species such as aphids (Roy et al. 2001), the western flower thrips [(*Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)] (Down et al. 2009), and the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Zhang et al. 2015). This improvement is attributed to predators of these pests serving as vectors of EPF conidia, facilitating their spread to uninfected pest populations (Gonzalez et al. 2016, Lin et al.

2017). Finding the balance between a lethal dose to pests but sublethal to their natural enemies should be explored.

### *Compatibility and formulation with other entomopathogens*

In South Africa, several products containing different entomopathogens have been produced for the control of certain key citrus pests. For example, the commercially available product CryptoMax™ and Cryptogran™ (River Bioscience, South Africa) contains an entomopathogenic virus used to target FCM larvae and is incorporated into many IPM programmes. Similarly, several South African strains of the entomopathogenic nematode species belonging to the genus *Steinernema* have been reported to be pathogenic to citrus mealybug (van Niekerk & Malan 2012). However, combining EPF and entomopathogenic nematodes for the control of pests can have three outcomes: additive, synergistic, or antagonistic. An additive effect occurs when two entomopathogens are combined but work independently from one another inside the host (Koppenhöfer & Grewal 2005). Synergism between EPF and nematodes is characterised as the combination of the two biological controls that are able to induce a greater control than the sum of the individual agents acting alone (Devi 2019). An antagonistic interaction occurs when one or both of the entomopathogens are negatively affected, generally through competition for resources (Půža & Tarasco 2023). A synergistic interaction is the most favourable type for pest management. Research has shown that the combination of EPF with entomopathogenic nematodes has proven to be more effective towards two fruit fly species than individual treatments under laboratory, glasshouse and field cage conditions, ultimately displaying a synergistic interaction (Wakil et al. 2022). Similarly, Prinsloo et al. (2022) found a synergistic interaction between EPF and nematodes when applied for the control of fifth instar FCM. When individually applied, the *Steinernema*

*yirgalemense* nematode strain induced a mortality of 43.33 % whilst the *M. pinghaense* EPF (strain FCM Ar 23 B3) induced a mortality of 65.83 %. When combined, the two strains induced 100 % mortality (Prinsloo et al. 2022). Future research should explore the potential for certain strains of these entomopathogens to act synergistically with the EPF strains currently under investigation in controlling mealybugs and other citrus arboreal pests in general, such as citrus thrips. Such investigations could provide valuable insights into whether these entomopathogens will function synergistically, additively or antagonistically within IPM strategies for citrus crops.

## 5. 4 CONCLUSION & FUTURE RECOMMENDATIONS

This study investigated whether novel EPF strains isolated from the aboveground environment were infective to a common foliar pest (citrus mealybug) and whether these isolates demonstrated improved UV tolerance. Five novel isolates were demonstrated to be virulent against this common pest as bioassays. Furthermore, all five virulent isolates demonstrated some level of UV tolerance, especially initial tolerance which is extremely important for foliar applications as even the most virulent isolate will not be able to induce infection if conidia are inactivated by UV radiation. Isolate Coe 18 was established as the most UV-tolerant isolate as this strain demonstrated both initial UV tolerance and sustained a relatively high germination percentage.

However, formulation to improve the UV tolerance of these isolates for foliar application for control of arboreal pests is still recommended for the development of these isolates as mycopesticides, whilst continuing to search for more UV-tolerant isolates may be beneficial. It is also recommended that these isolates are tested for their pathogenic ability and virulence toward other key foliar citrus pests as these factors may vary based on the targeted pest. Since many citrus

orchards in South Africa are conventionally managed with insecticides and fungicides, [especially considering that citrus black spot (CBS) is heavily controlled for], it would be highly beneficial to investigate how these isolates perform in the presence of these synthetic control measures.

# CHAPTER 6: REFERENCES


Accoti, A., Engdahl, C. S. & Dimopoulos, G. 2021. Discovery of novel entomopathogenic fungi for mosquito-borne disease control. Frontiers in Fungal Biology 2:637234.

Acheampong, M. A., Coombes, C. A., Moore, S. D. & Hill, M. P. 2020a. Temperature tolerance and humidity requirements of select entomopathogenic fungal isolates for future use in citrus IPM programmes. Journal of Invertebrate Pathology 174:107436.

Acheampong, M. A., Hill, M. P., Moore, S. D. & Coombes, C. A. 2020b. UV sensitivity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates under investigation as potential biological control agents in South African citrus orchards. Fungal Biology 124:304–310.

Afandhi, A., Choliq, F. A., Fernando, I., Marpaung, Y. M. A. N. & Setiawan, Y. 2022. Occurrence of soil-inhabiting entomopathogenic fungi within a conventional and organic farm and their virulence against *Spodoptera litura*. Biodiversitas Journal of Biological Diversity 23(2):1172–1180.

Aghaeepour, S., Zibaee, A., Ramzi, S. & Hoda, H. 2022. Host-pathogen interactions of the two native isolates of *Beauveria bassiana* to a predatory coccinellid, *Cryptolaemous montrouzieri* Mulsant (Coleoptera: Coccinellidae). Invertebrate Survival Journal 53–68.

Aguila, L., Akutse, K., Bamisile, B., Sánchez Moreano, J., Javaria, H., Zhou, C., Li, X. & Wang, L. 2021. Endophytically colonized Citrus limon seedlings by *Beauveria bassiana* hampered development, reproduction and progeny fitness of *Diaphorina citri*. Journal of Applied Entomology 146:229–242.

Ahmed, N. & Abd-Rabou, S. 2010. Host plants, geographical distribution, natural enemies and biological studies of the citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae). Egyptian Academic Journal of Biological Sciences. A, Entomology 3:39–47.

Alves, R. T., Bateman, R. P., Prior, C. & Leather, S. R. 1998. Effects of simulated solar radiation on conidial germination of *Metarhizium anisopliae* in different formulations. Crop Protection 17:675–679.

Anderson, R. D., Bell, A. S., Blanford, S., Paaijmans, K. P. & Thomas, M. B. 2011. Comparative growth kinetics and virulence of four different isolates of entomopathogenic fungi in the house fly (*Musca domestica* L.). Journal of Invertebrate Pathology 107:179–184.

Arnoldi, M., Duren, E. B., Avery, P. B. & Rossi, L. 2022. Assessing the endophytic potential of a commercially available entomopathogenic *Beauveria bassiana* strain in various citrus rootstocks. Applied Microbiology 2:561–571.

Athanassiou, C. G., Kavallieratos, N. G., Rumbos, C. I. & Kontodimas, D. C. 2017. Influence of temperature and relative humidity on the insecticidal efficacy of *Metarhizium anisopliae* against larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on wheat. Journal of Insect Science 17(1):22.

Bamisile, B. S., Akutse, K. S., Siddiqui, J. A. & Xu, Y. 2021a. Model application of entomopathogenic fungi as alternatives to chemical pesticides: prospects, challenges, and insights for next-generation sustainable agriculture. Frontiers in Plant Science 12:741804.

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., Afolabi, O. G., Hussain, M., Qasim, M. & Wang, L. 2018. Prospects of endophytic fungal entomopathogens as biocontrol and plant growth promoting agents: an insight on how artificial inoculation methods affect endophytic colonization of host plants. Microbiological Research 217:34–50.

Bamisile, B. S., Siddiqui, J. A., Akutse, K. S., Ramos Aguila, L. C. & Xu, Y. 2021b. General limitations to endophytic entomopathogenic fungi use as plant growth promoters, pests and pathogens biocontrol agents. Plants 10(10):2119.

Barzman, M., Bàrberi, P., Birch, A., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J. E., Kiss, J., Kudsk, P., Lamichhane, J. R., Messean, A., Moonen, A. C., Ratnadass, A., Ricci, P., Sarah, J.-L. & Sattin, M. 2015. Eight principles of integrated pest management. Agronomy for Sustainable Development 35:1199–1215.

Batta, Y. A. 2003. Production and testing of novel formulations of the entomopathogenic fungus *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes). Crop Protection 22:415–422.

Bedford, E.C. 1998. Introduction (Part I) and Methods of controlling citrus pests (Part II), In: E.C.G Bedford, M.A. Van den Berg & E.A. de Villiers (Eds.) Citrus Pests in the Republic of South Africa. 1-22. Dynamic Ad, Nelspruit, South Africa.

Behie, S., Jones, S. & Bidochka, M. 2015. Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. Fungal Ecology 13:112–119.

Bich, G. A., Castrillo, M. L., Kramer, F. L., Villalba, L. L. & Zapata, P. D. 2021. Morphological and molecular identification of entomopathogenic fungi from agricultural and forestry crops. Floresta Ambiente 28(2): e20180086.

Bidochka, M. J., Kamp, A. M., Lavender, T. M., Dekoning, J. & De Croos, J. N. A. 2001. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? Applied and Environmental Microbiology 67(3):1335–1342.

Birnbaum, N., Reingold, V., Matveev, S., Kottakota, C., Davidovitz, M., Mani, K. A., Feldbaum, R., Yaakov, N., Mechrez, G., & Ment, D. 2021. Not only a formulation: The effects of Pickering emulsion on the entomopathogenic action of *Metarhizium brunneum*. Journal of Fungi 7(7):499.

Bischoff, J. F., Rehner, S. A. & Humber, R. A. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101(4):512–530.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. & White, J.-S. S. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology & Evolution 24(3):127–135.

Bownes, A., Moore, S. & Villet, M. 2014. My enemy's enemies: Recruiting hemipteran-tending generalist ants for biological control in citrus orchards by spatial partitioning of foraging webs. African Entomology 22(3):519–529.

Braga, G. U., Flint, S. D., Miller, C. D., Anderson, A. J. & Roberts, D. W. 2001a. Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. Photochemistry and Photobiology 74:734–739.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J. & Roberts, D. W. 2001b. Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitudes from 61°n to 54°s. Journal of Invertebrate Pathology 78:98–108.

Braga, G. U. L., Rangel, D. E. N., Fernandes, É. K. K., Flint, S. D. & Roberts, D. W. 2015. Molecular and physiological effects of environmental UV radiation on fungal conidia. Current Genetics 61(3):405–425.

Braga, G., Rangel, D., Flint, S., Miller, C., Anderson, A. & Roberts, D. 2002. Damage and recovery from UV-B exposure in conidia of the entomopathogens *Verticillium lecanii* and *Aphanocladium album*. Mycologia 94(6):912–20.

Brooks, M. E., Kristensen, K., Benthem, K. J. Van, Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M. & Bolker, B. M. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9(2):378–400.

Brown & Khan, A. 2009. Pathogenicity and virulence of four isolates of *Metarhizium anisopliae* on selected natural enemies: *Cryptolaemus montrouzieri, Anagyrus kamali, Lysiphlebus testaceipes* and *Bracon thurberiphagae*. Journal of Biopesticides 2(2):199–203.

Butt, T. M., Coates, C. J., Dubovskiy, I. M. & Ratcliffe, N. A. 2016. Entomopathogenic Fungi: New insights into host–pathogen interactions (Chapter 9). In: Lovett, B. & St. Leger, R. J. (Eds.). Advances in Genetics. Academic Press. 94:307–364.

Butt, T.M., Wang, C., Shah, F.A. & Hall, R. 2006. Degeneration of entomogenous fungi (Chapter 10). In: J. Eilenberg & H.M.T. Hokkanen (Eds.). An Ecological and Societal Approach to Biological Control. 213–226. Springer, The Netherlands.

Canhilal, R., Uygun, N. & Carner, G. R. 2001. Effects of temperature on development and reproduction of a predatory beetle, *Nephus includens* Kirsch (Coleoptera: Coccinellidae). Journal of Agricultural and Urban Entomology 18(2):117–125.

Castrillo, L., Roberts, D. & Vandenberg, J. 2005. The fungal past, present, and future: Germination, ramification, and reproduction. Journal of Invertebrate Pathology 89:46–56.

Chaithra, M., Prameeladevi, T., Bhagyasree, S. N., Prasad, L., Subramanian, S. & Kamil, D. 2022. Multilocus sequence analysis for population diversity of indigenous entomopathogenic fungus *Beauveria bassiana* and its bio-efficacy against the cassava mite, *Tetranychus truncatus* Ehara (Acari: Tetranychidae). Frontiers in Microbiology 13: 1007017.

Chartier-Fitzgerald, V. 2014. Screening of entomopathogenic fungi against citrus mealybug *(Planococcus citri* (Risso)) and citrus thrips (*Scirtothrips aurantii* (Faure)). MSc thesis. Rhodes University, Grahamstown, South Africa.

Chartier-Fitzgerald, V., Hill, M. P., Moore, S. D. & Dames, J. F. 2016. Screening of entomopathogenic fungi against citrus mealybug, *Plannococcus citri* (Risso) (Hemiptera: Pseudococcidae). African Entomology 24(2):343–351.

Chaurasiya, D., Singh, S., Kumar, M., Singh, A. & Sahni, S. 2021. Bio-control of insect pests through entomopathogenic fungi: A novel approach. (Chapter 4). p. 79–99.

Chelico, L., Haughian, J. L. & Khachatourians, G. G. 2006. Nucleotide excision repair and photoreactivation in the entomopathogenic fungi *Beauveria bassiana, Beauveria brongniartii, Beauveria nivea, Metarhizium anisopliae, Paecilomyces farinosus* and *Verticillium lecanii.* Journal of Applied Microbiology 100(5):964–972.

Citrus Growers' Association (CGA). 2023. Key Industry Statistics 2023 Export Season. Available at: https://www.citrusresourcewarehouse.org.za/home/document-home/information/cga-key-industry-statistics/9834-cga-key-industry-statistics-2024/file

Clifton, E. H., Jaronski, S. T., Hodgson, E. W. & Gassmann, A. J. 2015. Abundance of Soil-Borne Entomopathogenic Fungi in Organic and Conventional Fields in the Midwestern USA with an Emphasis on the Effect of Herbicides and Fungicides on Fungal Persistence. PloS one 10(7): e0133613.

Coombes, C. A., Hill, M. P., Moore, S. D. & Dames, J. F. 2016. Entomopathogenic fungi as control agents of *Thaumatotibia leucotreta* in citrus orchards: field efficacy and persistence. BioControl 61(6):729–739.

Coombes, C. A., Hill, M. P., Moore, S. D., Dames, J. F. & Fullard, T. 2013. Persistence and virulence of promising entomopathogenic fungal isolates for use in citrus orchards in South Africa. Biocontrol Science and Technology 23(9):1053–1066.

Coombes, C. A., Hill, M. P., Moore, S. D., Dames, J. F. & Fullard, T. 2015. *Beauveria* and *Metarhizium* against false codling moth (Lepidoptera: Tortricidae): A step towards selecting isolates for potential development of a mycoinsecticide. African Entomology 23:239–242.

Corbu, V. M., Gheorghe-Barbu, I., Dumbravă, A. Ștefania, Vrâncianu, C. O. & Șesan, T. E. 2023. Current insights in fungal importance—a comprehensive review. Microorganisms 11(6):1384.

Demirci, F., Muştu, M., Bora Kaydan, M. & Ülgentürk, S. 2011. Laboratory evaluation of the effectiveness of the entomopathogen; *Isaria farinosa*, on citrus mealybug, *Planococcus citri.* Journal of Pest Science 84(3):337–342.

de Roode, J. C. & Lefèvre, T. 2012. Behavioral immunity in insects. Insects 3(3):789–820.

Devi, G. 2019. Interaction between entomopathogenic nematodes and entomopathogenic fungi in biocontrol mechanism. Journal of Entomology and Zoology Studies 7(6):959–964.

Dimbi, S., Maniania, N. K., Lux, S. A. & Mueke, J. M. 2004. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. BioControl 49:83–94.

Down, R., Cuthbertson, A., Mathers, J. & Walters, K. 2009. Dissemination of the entomopathogenic fungi, *Lecanicillium longisporum* and *L. muscarium*, by the predatory bug, *Orius laevigatus*, to provide concurrent control of *Myzus persicae, Frankliniella occidentalis* and *Bemisia tabaci*. Biological Control 50(2):172–178.

Dunlap, C. A. & Schisler, D. A. 2014. Characterization of the surface properties of wheat spikelet components grown under different regimens and the biocontrol yeast *Cryptococcus flavescens*. Journal of Agricultural and Food Chemistry 62(4):809–815.

Ekesi, S., Maniania, N. K. & Lux, S. A. 2002. Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. Biocontrol Science and Technology 12(1):7–17.

Elkhateeb, W. A., Mousa, K. M., Elnahas, M. O. & Daba, G. M. 2021. Fungi against insects and contrariwise as biological control models. Egyptian Journal of Biological Pest Control 31(1):13.

Elyazghi, Z., Yazouli, L. E., Sadki, K. & Radouani, F. 2017. ABI Base Recall: Automatic correction and ends trimming of DNA sequences. IEEE Transactions on Nanobioscience 16(8):682–686.

Fang, W. & Leger, R. J. S. 2012. Enhanced UV resistance and improved killing of malaria mosquitoes by photolyase transgenic entomopathogenic fungi. PloS one 7(8): e43069.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A. & Rougier, M. 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. Mycologia 89(3):383–392.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L. A., Lomer, C. J. & Rougier, M. 1996. Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. Mycopathologia 135(3):171–181.

Faria, M. & Wraight, S. 2007. Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biological Control 43(3):237–256.

Feldbaum, R. A., Yaakov, N., Mani, K. A., Yossef, E., Metbeev, S., Zelinger, E., Belausov, E., Koltai, H., Ment, D., & Mechrez, G. 2021. Single cell encapsulation in a Pickering emulsion stabilized by $TiO_2$ nanoparticles provides protection against UV radiation for a biopesticide. Colloids and Surfaces. B, Biointerfaces 206:111958.

Felizatti, A. P., Manzano, R. M., Rodrigues, I. M. W., Da Silva, M. F. Das G. F., Fernandes, J. B. & Forim, M. R. 2021. Encapsulation of *B. bassiana* in biopolymers: improving microbiology of insect pest control. Frontiers in Microbiology 12:704812

Fernandes, É. K. K., Moraes, Á. M. L., Pacheco, R. S., Rangel, D. E. N., Miller, M. P., Bittencourt, V. R. E. P. & Roberts, D. W. 2009. Genetic diversity among Brazilian isolates of *Beauveria bassiana*: comparisons with non-Brazilian isolates and other *Beauveria* species. Journal of Applied Microbiology 107:760–774.

Fernandes, É. K. K., Rangel, D. E. N., Braga, G. U. L. & Roberts, D. W. 2015. Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation. Current Genetics 61(3):427–440.

Fernandes, É. K. K., Rangel, D. E. N., Moraes, Á. M. L., Bittencourt, V. R. E. P. & Roberts, D. W. 2007. Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. Journal of Invertebrate Pathology 96:237–243.

Fernández-Bravo, M., Bonnet, J., Quesada-Moraga, E. & Garrido-Jurado, I. 2024. Imperfect match between radiation exposure times required for conidial viability loss and infective capacity reduction attenuate UV-B impact on *Beauveria bassiana*. Pest Management Science 80(3):1557–1565.

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

Fernández-Bravo, M., Garrido-Jurado, I., Valverde-García, P., Enkerli, J. & Quesada-Moraga, E. 2016. Responses to abiotic environmental stresses among phylloplane and soil isolates of *Beauveria bassiana* from two holm oak ecosystems. Journal of Invertebrate Pathology 141:6–17.

Follett, P. A. & Neven, L. G. 2006. Current trends in quarantine entomology. Annual Review of Entomology 51(1):359–385.

Franco, J. C., Suma, P., Da Silva, E. B., Blumberg, D. & Mendel, Z. 2004. Management strategies of mealybug pests of citrus in Mediterranean countries. Phytoparasitica 32(5):507–522.

Geiser, D. M., Aoki, T., Bacon, C. W., Baker, S. E., Bhattacharyya, M. K., Brandt, M. E., Brown, D. W., Burgess, L. W., Chulze, S., Coleman, J. J., Correll, J. C., Covert, S. F., Crous, P. W., Cuomo, C. A., De Hoog, G. S., Di Pietro, A., Elmer, W. H., Epstein, L., Frandsen, R. J. N., Freeman, S., Gagkaeva, T., Glenn, A. E., Gordon, T. R., Gregory, N. F., Hammond-Kosack, K. E., Hanson, L. E., Jímenez-Gasco, M. Del M., Kang, S., Kistler, H. C., Kuldau, G. A., Leslie, J. F., Logrieco, A., Lu, G., Lysøe, E., Ma, L.-J., Mccormick, S. P., Migheli, Q., Moretti, A., Munaut, F., O'donnell, K., Pfenning, L., Ploetz, R. C., Proctor, R. H., Rehner, S. A., Robert, V. A. R. G., Rooney, A. P., Bin Salleh, B., Scandiani, M. M., Scauflaire, J., Short, D. P. G., Steenkamp, E., Suga, H., Summerell, B. A., Sutton, D. A., Thrane, U., Trail, F., Van Diepeningen, A., Vanetten, H. D., Viljoen, A., Waalwijk, C., Ward, T. J., Wingfield, M. J., Xu, J.-R., Yang, X.-B., Yli-Mattila, T. & Zhang, N. 2013. One fungus, one name: Defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. Phytopathology 103(5):400–408.

Goble, T. A., Dames, J. F., Hill, M. P. & Moore, S. D. 2010. The effects of farming system, habitat type and bait type on the isolation of entomopathogenic fungi from citrus soils in the Eastern Cape Province, South Africa. BioControl 55(3):399–412.

Goble, T. A., Dames, J. F., Hill, M. P. & Moore, S. D. 2011. Investigation of native isolates of entomopathogenic fungi for the biological control of three citrus pests. Biocontrol Science and Technology 21(10):1193–1211.

Gonzalez, F., Tkaczuk, C., Dinu, M. M., Fiedler, Ż., Vidal, S., Zchori-Fein, E. & Messelink, G. J. 2016. New opportunities for the integration of microorganisms into biological pest control systems in greenhouse crops. Journal of Pest Science 89(2):295–311.

Gopal, G. S., Venkateshalu, B., Nadaf, A. M., Guru, P. N. & Pattepur, S. 2021. Management of the grape mealy bug, *Maconellicoccus hirsutus* (Green), using entomopathogenic fungi and botanical oils: a laboratory study. Egyptian Journal of Biological Pest Control 31(1):100.

Grafton-Cardwell, E., Ouyang, Y., Striggow, R. & Vehrs, S. 2001. Armored scale insecticide resistance challenges San Joaquin Valley citrus growers. California Agriculture 55(5):20–25.

Greenfield, M., Gómez-Jiménez, M. I., Ortiz, V., Vega, F. E., Kramer, M. & Parsa, S. 2016. *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. Biological Control 95:40–48.

Grout, T. 2015. The status of citrus IPM in South Africa. Acta Horticulturae 1065:1091–1095.

Grout, T. G. & Moore, S. D. 2015. Citrus. In: G.L. Prinsloo & V.M. Uys (Eds.) Insects of Cultivated Plants and Natural Pastures in Southern Africa. 447–499. Entomological Society of Southern Africa, Hatfield, South Africa.

Grout, T. G., Richards, G. I. & Stephen, P. R. 1997. Further non-target effects of citrus pesticides on *Euseius addoensis* and *Euseius citri* (Acari: Phytoseiidae). Experimental & Applied Acarology 21:171-177.

Grout, T., Moore, S. D., Stephen, P. R. & Kirkman, W. 2015. Evaluation of entomopathogenic fungi and new chemicals against thrips and mealybug. Citrus Research International, Nelspruit, South Africa. Internal Report:1–11.

Gul, H., Saeed, S. & Khan, F. Z. A. 2014. Entomopathogenic fungi as effective insect pest management tactic: a review. Applied Sciences and Business Economics. 1:10–18.

Hajek, A. E. & van Frankenhuyzen, K. 2017. Use of entomopathogens against forest pests (Chapter 21). In: Lacey, L. A. (Eds.). Microbial Control of Insect and Mite Pests. 313–330. Academic Press, USA.

Herrick, N. J. & Cloyd, R. A. 2023. Performance of entomopathogenic fungal-based insecticides against the citrus mealybug (Hemiptera: Pseudococcidae) on *Coleus* (Lamiales: Lamiaceae) plants under greenhouse conditions. Journal of Entomological Science 58(2):187–200.

Hofmeyr, J. H., Hofmeyr, M., Carpenter, J. E., Bloem, S. & Slabbert, J. P. 2016. Sterile insect releases for control of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae): An assessment on semi-commercial scale. African Entomology 24(1):80–89.

Hofmeyr, J. H. & Pringle, K. L. 1998. Resistance of false codling moth, *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), to the chitin synthesis inhibitor, triflumuron: short communication. African Entomology 6(2):373–375.

Hu, R., Bai, P., Liu, B. & Yu, J. 2021. On the virulence of two *Beauveria bassiana* strains against the fall webworm, *Hyphantria cunea* (Durry) (Lepidoptera: Erebidae), larvae and their biological properties in relation to different abiotic factors. Egyptian Journal of Biological Pest Control 31(1):107.

Humber, R. A. 1997. Fungi: identification. (Chapter V-1). In Lacey, L. A. (Eds.). Manual of Techniques in Insect Pathology. 153–185. Academic Press, London. https://doi.org/10.1016/B978-012432555-5/50011-7.

Humber, R. A. 2012. Identification of entomopathogenic fungi. In Lacey, L. A. (Eds.). Manual of Techniques in Insect Pathology. 153–185. Academic Press, London. https://doi/10.1016/B978-0-12-386899-2.00006-3.

Idrees, A., Afzal, A., Qadir, Z. A. & Li, J. 2022. Bioassays of *Beauveria bassiana* isolates against the Fall Armyworm, *Spodoptera frugiperda*. Journal of Fungi 8(7):717.

Inglis, G. D., Goettel, M. S. & Johnson, D. L. 1993. Persistence of the entomopathogenic fungus, *Beauveria bassiana*, on phylloplanes of crested wheatgrass and alfalfa. Biological Control 3:258–270.

Inglis, G.D., Goettel, M., Butt, T. & Strasser, H. 2001. Use of hyphomycetous fungi for managing insect pests. In: T.M. Butt, C.W. Jackson & N. Magan (Eds.). Fungi as Biocontrol Agents: progress, problems and potential. 23-70. CABI Publishing, Wallingford, United Kingdom.

Islam, S. M. N., Chowdhury, M. Z. H., Mim, M. F., Momtaz, M. B. & Islam, T. 2023. Biocontrol potential of native isolates of *Beauveria bassiana* against cotton leafworm *Spodoptera litura* (Fabricius). Scientific Reports 13(1):8331.

Jaber, L. & Enkerli, J. 2016. Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. Biological Control 103:187–195

Jaber, L. R. & Vidal, S. 2010. Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. Ecological Entomology 35:25–36.

Jackson, M. A., Dunlap, C. A. & Jaronski, S. T. 2010. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55:129–145.

Jackson, T. A. 1999. Factors in the success and failure of microbial control agents for soil dwelling pests. Integrated Pest Management Reviews 4(4):281–285.

Jackson, T. A., Alves, S. B. & Pereira, R. M. 2000. Success in biological control of soil-dwelling insects by pathogens and nematodes. In: Gurr, G. & Wratten, S. (Eds.). Biological Control: Measures of Success. 271–296. Springer, Netherlands, Dordrecht.

Jaronski, S. T. 2010. Ecological factors in the inundative use of fungal entomopathogens. BioControl 55(1):159–185.

Jia, H., Camara, I., Zhang, Z., Gao, Y., Yang, X., Sangbaramou, R., Zhen, C., Shi, W. & Tan, S. 2023. Effect of ultraviolet radiation on *Beauveria bassiana* virulence and development of protective formulations. Archives of Microbiology 205(4):112.

Kabaluk, J.T. & Ericsson, J.D. 2007. *Metarhizium anisopliae* seed treatment increases yield of field corn when applied for wireworm control. Agronomy Journal 99(5): 1377- 1381.

Kaiser, D., Bacher, S., Mène-Saffrané, L. & Grabenweger, G. 2019. Efficiency of natural substances to protect *Beauveria bassiana* conidia from UV radiation. Pest Management Science 75(2):556–563.

Kambrekar, D. & Aruna, J. 2018. Screening for endophytic *Beauveria bassiana* from different plants and its pathogenecity against chickpea pod borer, *Helicoverpa armigera* (Hubner). Journal of Experimental Zoology, India 21(2):727–731.

Khan, S., Guo, L., Maimaiti, Y., Mijit, M. & Qiu, D. 2012. Entomopathogenic fungi as microbial biocontrol agent. Molecular Plant Breading 3(7):63–79.

Klingen, I., Eilenberg, J. & Meadow, R. 2002. Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. Agriculture, Ecosystems & Environment 91:191–198.

Koppenhöfer, A.M. & Grewal, P.S. 2005. Compatibility and interactions with agrochemicals and other biocontrol agents. In: P.S. Grewal, R. U. Ehlers & D. Shapiro-Ilan (Eds.). Nematodes as Biocontrol Agents. 362–381. CABI Publishing, Wallingford, UK.

Kotliarevski, L., Mani, K. A., Feldbaum, R. A., Yaakov, N., Belausov, E., Zelinger, E., Ment, D., & Mechrez, G. 2021. Single-conidium encapsulation in oil-in-water Pickering emulsions at high encapsulation yield. Frontiers in Chemistry 9:726874.

Lacey, L. A., Frutos, R., Kaya, H. K. & Vail, P. 2001. Insect pathogens as biological control agents: do they have a future? Biological Control 21(3):230–248.

Lacey, L. & Shapiro-Ilan, D. 2003. The potential role for microbial control of orchard insect pests in sustainable agriculture. Food, Agriculture and Environment 1(2):326331.

Lenth, R. V. 2024, October 20. emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.10.5-0900003. https://rvlenth.github.io/emmeans/.

Lin, G., Tanguay, A., Guertin, C., Todorova, S. & Brodeur, J. 2017. A new method for loading predatory mites with entomopathogenic fungi for biological control of their prey. Biological Control 115:105–111.

Litwin, A., Nowak, M. & Różalska, S. 2020. Entomopathogenic fungi: unconventional applications. Reviews in Environmental Science and Biotechnology 19:23–42.

Liu, D., Smagghe, G. & Liu, T. X. 2023. Interactions between Entomopathogenic Fungi and Insects and Prospects with Glycans. Journal of Fungi 9(5):575.

Liu, Y., He, P., He, P., Munir, S., Ahmed, A., Wu, Y., Yang, Y., Lu, J., Wang, J., Yang, J., Pan, X., Tian, Y. & He, Y. 2022. Potential biocontrol efficiency of *Trichoderma* species against oomycete pathogens. Frontiers in Microbiology 13:974024

Love, C. N., Hill, M. P. & Moore, S. D. 2014. *Thaumatotibia leucotreta* and the Navel orange: ovipositional preferences and host susceptibility. Journal of Applied Entomology 138:600–611.

Luz, C. & Fargues, J. 1997. Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. Mycopathologia 138(3):117–125.

Luz, C. & Fargues, J. 1999. Dependence of the entomopathogenic fungus*, Beauveria bassiana,* on high humidity for infection of *Rhodnius prolixus*. Mycopathologia 146(1):33–41.

Ma, M., Luo, J., Li, C., Eleftherianos, I., Zhang, W. & Xu, L. 2024. A life-and-death struggle: interaction of insects with entomopathogenic fungi across various infection stages. Frontiers in Immunology 14:1329843.

Maghsoudi, S. & Jalali, E. 2017. Noble UV protective agent for *Bacillus thuringiensis* based on a combination of graphene oxide and olive oil. Scientific Reports 7(1):11019.

Maina, U., Galadima, I., Gambo, F. & Dauda, Z. 2018. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. Journal Of Entomology and Zoology Studies 6(1):27-32

Majchrowicz, I. & Poprawski, T. J. 1993. Effects in vitro of nine fungicides on growth of entomopathogenic fungi. Biocontrol Science and Technology 3:321–336.

Majumder, D., Deb, L. & Malakar, P. 2018. Entomopathogenic fungi: potential tool for biological control of plant diseases. In: Khan, M. R., Mukhopadhyay, A. N., Pandey, R. N., Thakur, M. P., Singh, D., Siddiqui, M. A., Akram, M., Mohiddin, F. A., & Haque, Z. (Eds.). Bio-intensive Approaches: Application and effectiveness in the management of plant nematodes, insects and weeds. 549–580. Today & Tomorrow's Printer & Publishers, New Delhi, India.

Malan, A., Von Diest, J., Moore, S. D. & Addison, P. 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomology 26(1):14–29.

Manjushree, G. & Chellappan, M. 2019. Evaluation of entomopathogenic fungus for the management of pink mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae) on pineapple in Kerala. Journal of Entomology and Zoology Studies. 7(1):1215–1222.

Mannino, M. C., Huarte-Bonnet, C., Davyt-Colo, B. & Pedrini, N. 2019. Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. Journal of Fungi 5(2):33.

Manrakhan, A., Daneel, J.-H., Beck, R., Theron, C. D., Weldon, C. W., Moore, S. D. & Hattingh, V. 2018. Non-host status of commercial export grade lemon fruit (*Citrus limon* (L.) Burman f.cv. Eureka) for *Ceratitis capitata, Ceratitis rosa, Ceratitis quilicii* and *Bactrocera dorsalis* (Diptera: Tephritidae) in South Africa. African Entomology 26(1):202–214.

Mantzoukas, S. & Eliopoulos, P. A. 2020. Endophytic entomopathogenic fungi: a valuable biological control tool against plant pests. Applied Sciences 10(1):360.

Mantzoukas, S., Kitsiou, F., Natsiopoulos, D. & Eliopoulos, P. A. 2022. Entomopathogenic fungi: interactions and applications. Encyclopedia 2(2):646–656.

Martins, F., Soares, M. E., Oliveira, I., Pereira, J. A., De Lourdes Bastos, M. & Baptista, P. 2012. Tolerance and bioaccumulation of copper by the entomopathogen *Beauveria bassiana* (Bals. - Criv.) Vuill. exposed to various copper-based fungicides. Bulletin of Environmental Contamination and Toxicology 89(1):53–60.

Mascarin, G. M. & Jaronski, S. T. 2016. The production and uses of *Beauveria bassiana* as a microbial insecticide. World Journal of Microbiology and Biotechnology 32(11):177.

Mathulwe, L. L., Malan, A. P. & Stokwe, N. F. 2022. Laboratory screening of entomopathogenic fungi and nematodes for pathogenicity against the obscure mealybug, *Pseudococcus viburni* (Hemiptera: Pseudococcidae). Biocontrol Science and Technology 32(4):397–417.

Mathulwe, L. L., Malan, A. P. & Stokwe, N. F. 2023. The occurrence of entomopathogenic fungi in apple orchards and their biocontrol potential against *Eriosoma lanigerum*. African Entomology 31:1–9.

Meyer, M., Mwatawala, M., Copeland, R. & Virgilio, M. 2016. Description of new *Ceratitis* species (Diptera: Tephritidae) from Africa, or how morphological and DNA data are complementary in discovering unknown species and matching sexes. European Journal of Taxonomy 233: 1–23.

Meyling, N. V. & Eilenberg, J. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. Biological Control 43(2):145–155.

Meyling, N. V., Thorup-Kristensen, K. & Eilenberg, J. 2011. Below- and aboveground abundance and distribution of fungal entomopathogens in experimental conventional and organic cropping systems. Biological Control 59(2):180–186.

Mo, X., Chen, C., Riaz, M., Moussa, M. G., Chen, X., Wu, S., Tan, Q., Sun, X., Zhao, X., Shi, L. & Hu, C. 2022. Fruit characteristics of citrus trees grown under different soil Cu levels. Plants 11(21):2943.

Mohamed, G. 2016. Virulence of entomopathogenic fungi against the vine mealy bug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). Egyptian Journal of Biological Pest Control 26(1):47–51.

Moore, D., Bridge, P. D., Higgins, P. M., Bateman, R. P. & Prior, C. 1993. Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. Annals of Applied Biology 122(3):605–616.

Moore, G.A. 2001. Orange and lemons: clues to the taxonomy of Citrus from molecular markers. Trends in Genetics 17(9): 536-540.

Moore, S. D. 2002. Entomopathogens & microbial control of citrus pests in South Africa: a review. SA Fruit Journal 1:30–32.

Moore, S. D. 2021. Biological control of a phytosanitary pest (*Thaumatotibia leucotreta*): a case study. International Journal of Environmental Research and Public Health 18(3):1198.

Moore, S. D. & Duncan, L. W. 2017. Chapter 19 - Microbial Control of Insect and Mite Pests of Citrus. In Lacey, L. A. (Eds.). Microbial Control of Insect and Mite Pests. 283–298. Academic Press. https://doi:10.1016/B978-0-12-803527-6.00019-6.

Moore, S. D. & Hattingh, V. 2004. Augmentation of natural enemies for control of citrus pests in South Africa: A guide for growers. South African Fruit Journal 3(4):45–53.

Moore, S. & Hattingh, V. 2012. A review of current pre-harvest control options for false codling moth in citrus in southern Africa. South African Fruit Journal 11(4):82–85.

Moore, S. D. & Hattingh, V. 2022. Mealybugs. Citrus Research International IPM Production Guidelines 3(7):1–5.

Moore, S. & Kirkman, W. 2008. Citrus orchard sanitation with emphasis on false codling moth control. South African Fruit Journal 7(6):57–60.

Moore, S., Kirkman, W. & Hattingh, V. 2015. The host status of lemons for the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) with particular reference to export protocols. African Entomology 23(2):519–525.

Moore, S. & Manrakhan, A. 2022. Postharvest disinfestation treatments for false codling moth and fruit flies in citrus from South Africa. Horticulturae 8(3):221.

Mora, M. A. E., Castilho, A. M. C. & Fraga, M. E. 2018. Classification and infection mechanism of entomopathogenic fungi. Arquivos do Instituto Biológico 84:1–10.

Moussa, Z., Alanazi, Y. F., Khateb, A. M., Eldadamony, N. M., Ismail, M. M., Saber, W. I. A. & Darwish, D. B. E. 2023. Domiciliation of *Trichoderma asperellum* suppresses *Globiosporangium ultimum* and promotes pea growth, ultrastructure, and metabolic features. Microorganisms 11(1):198.

Mruthunjayaswamy, P., Venkatesan, T. & Jalali, S. 2019. Detection of insecticide resistance in field populations of citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae). Indian Journal of Experimental Biology 57:435–442.

Muller, L. & Pountney, C. A. 2013. Mealybug control in citrus orchards and vineyards: an Integrated Pest Management approach. South African Fruit Journal. Available at: https://www.citrusresourcewarehouse.org.za/home/document-home/news-articles/south-african-fruit-journal-safj/safj-2012/2607-sa-fruit-journal-dec-2012-jan-2013-9/file.

Mushore, T., Coombes, C. & Hill, M. 2023. Entomopathogenic fungi associated with cultivated honeybush, *Cyclopia* spp., in South Africa and their pathogenicity towards a leafhopper pest, *Molopopterus* sp. (Hemiptera: Cicadellidae). African Entomology 31: e14183.

Nicolosi, E., Deng, Z. N., Gentile, A., La Malfa, S., Continella, G. & Tribulato, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics 100(8):1155–1166.

Oliveira, R. J. V. de., Sousa, N. M. F. de., Pinto Neto, W. de P., Bezerra, J. L., Silva, G. A. da., & Cavalcanti, M. A. de Q. (2020). Seasonality affects the community of endophytic fungi in coconut (*Cocos nucifera*) crop leaves. Acta Botanica Brasilica 34(4), 704–711.

Omar, G., Ibrahim, A. & Hamadah, K. 2021. Virulence of *Beauveria bassiana* and *Metarhizium anisopliae* on different stages of the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae). Egyptian Journal of Biological Pest Control 31:102.

Onstad, D. & Carruthers, R. 2003. Epizootiological models of insect diseases. Annual Review of Entomology 35(1):399–419.

Onstad, D., Fuxa, J., Humber, R., Oestergaard, J., Shapiro-Ilan, D., Gouli, V., Anderson, R., Andreadis, T. & Lacey, L. 2006. An abridged glossary of terms used in invertebrate pathology. United States Department of Agriculture 3:1-70. Available at: https://www.researchgate.net/publication/280669583_An_Abridged_Glossary_of_Terms_Used_in_Invertebrate_Pathology

Ortiz-Urquiza, A. & Keyhani, N. O. 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. Insects 4(3):357–374.

Paschapur, A., Subbanna, A. R. N. S., Singh, A. K., Jeevan, B., Stanley, J., Rajashekhar, H. & Mishra, K. K. 2021. Unraveling the importance of metabolites from entomopathogenic fungi in insect pest management (Chapter 5). In: Khan, Md. A. & Ahmad, W. (Eds.). Microbes for Sustainable lnsect Pest Management: Hydrolytic Enzyme & Secondary Metabolite 2:89–120 Springer International Publishing, Cham. https://doi: 10.1007/978-3-030-67231.

Pimenta, R. S., Da Silva, J. F. M., Buyer, J. S. & Janisiewicz, W. J. 2012. Endophytic fungi from plums (*Prunus domestica*) and their antifungal activity against *Monilinia fructicola*. Journal of Food Protection 75(10):1883–1889.

Posadas, J. B., Angulo, L. M., Mini, J. I. & Lecuona, R. E. 2012. Natural tolerance to UV-B and assessment of photoprotectants in conidia of six native isolates of *Beauveria bassiana* (Bals-Criv) Vuillemin. World Applied Sciences Journal 20(7): 1024-1030.

Preininger, C., Sauer, U., Bejarano, A. & Berninger, T. 2018. Concepts and applications of foliar spray for microbial inoculants. Applied Microbiology and Biotechnology 102(17):7265–7282.

Prinsloo, S., Hill, M. P., Moore, S. D., Malan, A. P. & Coombes, C. A. 2022. Interaction between an entomopathogenic fungus and entomopathogenic nematodes for increased mortality of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). Biocontrol Science and Technology 32(10):1194–1207.

Půža, V. & Tarasco, E. 2023. Interactions between entomopathogenic fungi and entomopathogenic nematodes. Microorganisms 11(1):163.

Qiu, H.-L., Fox, E. G. P., Qin, C.-S., Zhao, D.-Y., Yang, H. & Xu, J.-Z. 2019. Microcapsuled entomopathogenic fungus against fire ants, *Solenopsis invicta*. Biological Control 134:141–149.

Quesada-Moraga, E., Garrido-Jurado, I., Yousef-Yousef, M. & González-Mas, N. 2022. Multitrophic interactions of entomopathogenic fungi in BioControl. BioControl 67:457–472.

Quesada-Moraga, E., González-Mas, N., Yousef-Yousef, M., Garrido-Jurado, I. & Fernández-Bravo, M. 2023. Key role of environmental competence in successful use of entomopathogenic fungi in microbial pest control. Journal of Pest Science 97:1–15

Quesada-Moraga, E., López-Díaz, C. & Landa, B. B. 2014. The hidden habit of the entomopathogenic fungus *Beauveria bassiana*: first demonstration of vertical plant transmission. PloS one 9(2): e89278.

Radić, T., Likar, M., Hančević, K., Bogdanović, I. & Pasković, I. 2014. Occurrence of root endophytic fungi in organic versus conventional vineyards on the Croatian coast. Agriculture, Ecosystems & Environment 192:115–121.

Rajab, L., Habib, W., Gerges, E., Gazal, I. & Ahmad, M. 2023. Natural occurrence of fungal endophytes in cultivated cucumber plants in Syria, with emphasis on the entomopathogen *Beauveria bassiana*. Journal of Invertebrate Pathology 196:107868.

Ramos, Y., Portal, O., Lysøe, E., Meyling, N. V. & Klingen, I. 2017. Diversity and abundance of *Beauveria bassiana* in soils, stink bugs and plant tissues of common bean from organic and conventional fields. Journal of Invertebrate Pathology 150:114–120.

Reay, S. D., Brownbridge, M., Gicquel, B., Cummings, N. J. & Nelson, T. L. 2010. Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. Biological Control 54(1):52–60.

Rehner, S., Minnis, A., Sung, G.-H., Luangsa-Ard, J. & Devotto, L. 2011. Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. Mycologia 103(5):1055–73.

Rossouw, S., Mathulwe, L. L., Stokwe, N. F. & Malan, A. P. 2023. Effect of visible light and ultraviolet light on the pathogenicity of entomopathogenic fungi to false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) larvae. African Entomology 31:1–8.

Roy, H. E., Pell, J. K. & Alderson, P. G. 2001. Targeted dispersal of the aphid pathogenic fungus *Erynia neoaphidis* by the aphid predator *Coccinella septempunctata*. Biocontrol Science and Technology 11(1):99–110.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. & Pell, J. K. 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annual Review of Entomology 51:331–357.

Saberi Riseh, R., Hassanisaadi, M., Vatankhah, M., Soroush, F. & Varma, R. S. 2022. Nano/microencapsulation of plant biocontrol agents by chitosan, alginate, and other important

biopolymers as a novel strategy for alleviating plant biotic stresses. International Journal of Biological Macromolecules 222:1589–1604.

Samways, M., Nel, M. & Prins, A. J. 1982. Ants (Hymenoptera: Formicidae) foraging in citrus trees and attending honeydew-producing Homoptera. Phytophylactica 14:155–157.

Sánchez-Rodríguez, A. R., Raya-Díaz, S., Zamarreño, Á. M., García-Mina, J. M., Del Campillo, M. C. & Quesada-Moraga, E. 2018. An endophytic *Beauveria bassiana* strain increases spike production in bread and durum wheat plants and effectively controls cotton leafworm (*Spodoptera littoralis*) larvae. Biological Control 116:90–102.

Santos, A. C., Diniz, A., Tiago, P. & Oliveira, N. 2020. Entomopathogenic *Fusarium* species: a review of their potential for the biological control of insects, implications and prospects. Fungal Biology Reviews 34:41–57.

Sasan, R. K. & Bidochka, M. J. 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. American Journal of Botany 99(1):101–107.

Scholte, E.-J., Knols, B. G. J., Samson, R. A. & Takken, W. 2004. Entomopathogenic fungi for mosquito control: A review. Journal of Insect Science 4:19.

Seid, A. Md., Fredensborg, B. L., Steinwender, B. M. & Meyling, N. V. 2019. Temperature-dependent germination, growth and co-infection of *Beauveria* spp. isolates from different climatic regions. Biocontrol Science and Technology 29(5):411–426.

Shah, F. A., Ansari, M. A., Watkins, J., Phelps, Z., Cross, J. & Butt, T. M. 2009. Influence of commercial fungicides on the germination, growth and virulence of four species of entomopathogenic fungi. Biocontrol Science and Technology 19(7):743–753.

Sharma, L. & Marques, G. 2018. Fusarium, an Entomopathogen—A Myth or Reality? Pathogens 7:93.

Sharma, A., Sharma, S. & Yadav, P. K. 2023. Entomopathogenic fungi and their relevance in sustainable agriculture: a review. Cogent Food & Agriculture 9(1):2180857.

Sharma, L., Bohra, N., Rajput, V. D., Quiroz-Figueroa, F. R., Singh, R. K. & Marques, G. 2021. Advances in entomopathogen isolation: a case of bacteria and fungi. Microorganisms 9(1):16.

Sharma, L., Gonçalves, F., Oliveira, I., Torres, L. & Marques, G. 2018. Insect-associated fungi from naturally mycosed vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). Biocontrol Science and Technology 28(2):122–141.

Shipp, J. L., Zhang, Y., Hunt, D. W. A. & Ferguson, G. 2003. Influence of humidity and greenhouse microclimate on the efficacy of *Beauveria bassiana* (Balsamo) for control of greenhouse arthropod pests. Environmental Entomology 32(5):1154–1163.

Singh, D., Raina, T. K. & Singh, J. 2017. Entomopathogenic fungi: an effective biocontrol agent for management of insect populations naturally. Journal of Pharmaceutical Sciences and Research 9(6):830–839

Smith, D. & Peña, J. E. 2002. Tropical citrus pests. In: J.E. Peña, J.L. Sharp & M. Wysoki (Eds.) Tropical Fruit Pests and Pollinators: Biology, Economic Importance, Natural Enemies, and Control. 57-101. CABI Publishing, Wallingford, United Kingdom.

Stotter, R. L., Samways, M. J. & Hattingh, V. 2014. Preparing the way for sterile insect release: Determination of false codling moth distribution across a landscape mosaic. Crop Protection 60:1–4.

Sui, L., Lu, Y., Zhou, L., Li, N., Li, Q. & Zhang, Z. 2023. Endophytic *Beauveria bassiana* promotes plant biomass growth and suppresses pathogen damage by directional recruitment. Frontiers in Microbiology 14:1227269.

Sunnucks, P. & Hales, D. F. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). Molecular Biology and Evolution 13(3):510–24.

Theron, C. D., Kotzé, Z., Manrakhan, A. & Weldon, C. W. 2023. Oviposition by the oriental fruit fly, (Hendel) (Diptera: Tephritidae), on five citrus types in a laboratory. Austral Entomology 62:503–516.

Thomas, S. R. & Elkinton, J. S. 2004. Pathogenicity and virulence. Journal of Invertebrate Pathology 85(3):146–151.

Tkaczuk, C. A., Tipping, C., Majchrowska-Safaryan, A. B., Król, A., Wyrzykowska, M. J. & Wegensteiner, R. 2019. The occurrence of entomopathogenic fungi in soils from apple orchards protected in different farming systems. Fresenius Environmental Bulletin 28(11):7906–7914.

Tong, S.-M. & Feng, M.-G. 2022. Molecular basis and regulatory mechanisms underlying fungal insecticides' resistance to solar ultraviolet irradiation. Pest Management Science 78(1):30–42.

Tseng, M. N., Chung, P. C. & Tzean, S. S. 2011. Enhancing the stress tolerance and virulence of an entomopathogen by metabolic engineering of dihydroxynaphthalene melanin biosynthesis genes. Applied and Environmental Microbiology 77(13):4508–4519.

Tumuhaise, V., Ekesi, S., Maniania, N. K., Tonnang, H. E. Z., Tanga, C. M., Ndegwa, P. N., Irungu, L. W., Srinivasan, R. & Mohamed, S. A. 2018. Temperature-dependent growth and virulence, and mass production potential of two candidate isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin for managing *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) on cowpea. African Entomology 26(1):73–83.

Udayakumar, A., Chinniah, C. C. H., Sawant, I., Muthiah, C. & N, M. 2014. Bio-efficacy and lethal reproductive effects of three entomopathogenic fungi against pink mealybug, *Maconellicoccus hirsutus* Green infesting grapes. Green Farming 5(4):697–700.

Upfold, J., Coombes, C. A. & Hill, M. P. 2016. Entomopathogenic fungi for the control of California red scale (*Aonidiella aurantii*) Maskell (Hemiptera: Diapsidae) a pest of citrus in South Africa. Unpublished BSc graduate report, Rhodes University, Grahasmtown.

Urquhart, P. 1999. IPM and the citrus industry in South Africa. Gatekeeper Series No. 86. International Institute for Environment and Development, Sustainable Agriculture and 140 Rural Livelihoods Programme, London, United Kingdom. [Available: http://pubs.Iied .org/6338IIED.html] accessed: 23/02/2023.

van Niekerk, S. & Malan, A. P. 2012. Potential of South African entomopathogenic nematodes (*Heterorhabditidae* and *Steinernematidae*) for control of the citrus mealybug, *Planococcus citri* (Pseudococcidae). Journal of Invertebrate Pathology 111(2):166–174.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N. K., Monzón, A., Ownley, B. H., Pell, J. K., Rangel, D. E. N. & Roy, H. E. 2009. Fungal entomopathogens: new insights on their ecology. Fungal Ecology 2(4):149–159.

Vega, F.E., Meyling, N.V., Luangsa-Ard, J.J. & Blackwell, M. 2012. Fungal entomopathogens. In: F. Vega & H.K. Kaya (Eds.). Insect Pathology. 171–220. Academic Press, San Diego, USA.

Vega, F., Simpkins, A., Aime, M., Posada-Florez, F., Peterson, S., Rehner, S., Infante, F., Castillo, A. & Arnold, A. 2010. Fungal endophyte diversity in coffee plants from Colombia, Hawai'i, Mexico and Puerto Rico. Fungal Ecology 1:122–138.

Venables, B. & Ripley, B. 2002. Modern Applied Statistics with S. Springer. http://doi:10.1007/b97626.

Venkatesan, T., Jalali, S. K., Ramya, S. L. & Prathibha, M. 2016. Insecticide resistance and its management in mealybugs. 223–229. In: Mani, M. & Shivaraju, C. (Eds.). Mealybugs and their management in agricultural and horticultural crops. Springer India, New Delhi.

Vidal, C., Fargues, J. & Lacey, L. A. 1997. Intraspecific variability of *Paecilomyces fumosoroseus*: effect of temperature on vegetative growth. Journal of Invertebrate Pathology 70(1):18–26.

Wakil, W., Usman, M., Piñero, J. C., Wu, S., Toews, M. D. & Shapiro-Ilan, D. I. 2022. Combined application of entomopathogenic nematodes and fungi against fruit flies, *Bactrocera zonata* and *B. dorsalis* (Diptera: Tephritidae): laboratory cups to field study. Pest Management Science 78(2):2779–2791.

Wheeler, M. W., Park, R. M. & Bailer, A. J. 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environmental Toxicology and Chemistry 25(5):1441–1444.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: M. Innis, D. Gelfand, J. Sninsky & T. White (Eds.). PCR protocols: a guide to methods and applications. 315–322. Academic Press, London, United Kingdom.

Wu, G. A., Sugimoto, C., Kinjo, H., Azama, C., Mitsube, F., Talon, M., Gmitter, F. G. & Rokhsar, D. S. 2021. Diversification of mandarin citrus by hybrid speciation and apomixis. Nature Communications 12(1):4377.

Wu, S., Toews, M. D., Oliveira-Hofman, C., Behle, R. W., Simmons, A. M. & Shapiro-Ilan, D. I. 2020. Environmental tolerance of entomopathogenic fungi: a new strain of *Cordyceps javanica* isolated from a whitefly epizootic versus commercial fungal strains. Insects 11(10):711.

Yaakov, N., Mani, K. A., Felfbaum, R., Lahat, M., Da Costa, N., Belausov, E., Ment, D., & Mechrez, G. 2018. Single cell encapsulation via Pickering emulsion for biopesticide applications. ACS omega 3(10):14294–14301.

Yao, X., Guo, H., Zhang, K., Zhao, M., Ruan, J. & Chen, J. 2023. *Trichoderma* and its role in biological control of plant fungal and nematode disease. Frontiers in Microbiology 14:1160551

Zhang, Y.-X., Sun, L., Lin, G., Lin, J.-Z., Chen, X., Ji, J., Zhang, Z. & Saito, Y. 2015. A novel use of predatory mites for dissemination of fungal pathogen for insect biocontrol: The case of *amblyseius swirskii* and *neoseiulus cucumeris* (Phytoseiidae) as vectors of *Beauveria bassiana* against *diaphorina citri* (Psyllidae). Systematic and Applied Acarology 20(2):177–187.

Zhang, L., Zhang, X., Zhang, Y., Wu, S., Gelbič, I., Xu, L. & Guan, X. 2016. A new formulation of *Bacillus thuringiensis*: UV protection and sustained release mosquito larvae studies. Scientific Reports 6(1):39425.

Zimmermann, G. 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Science and Technology 17(9):879–920.

Zimmermann, G., Papierok, B. & GLARE, T. 1995. Elias Metschnikoff, Elie Metchnikoff or Ilya Ilich Mechnikov (1845-1916): A pioneer in insect pathology, the first describer of the entomopathogenic fungus *Metarhizium anisopliae* and how to translate a Russian name. Biocontrol Science and Technology 5(4):527–530.