The efficacy of selected indigenous entomopathogenic fungal strains against the grapevine mealybug, *Planococcus ficus*

By

Ilyaas Rhoda

Thesis presented in partial fulfilment of the requirements for the degree Master of Science in the Department of Horticultural Sciences at Cape Peninsula University of Technology.

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TITLE PAGE

THE EFFICACY OF SELECTED INDIGENOUS ENTOMOPATHOGENIC FUNGAL STRAINS AGAINST THE GRAPEVINE MEALYBUG, *PLANOCOCCUS FICUS*

DECLARATION

I, Ilyaas Rhoda, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

07/04/2023

SIGNED **DATE**

DEDICATION

I dedicate this thesis to:

- The Rhoda Family, may this dissertation serve as a prosperous accomplishment for the past, present and future generations of Rhoda's.
- May this study be an inspiration for future legacies to amplify their knowledge and make a difference in the world, in whichever area they may find themselves.

'Education is power'

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LIST OF ACRONYMS

ANOVA – Analysis of Variance

CA – Corresponding Analysis

DNA – Deoxyribonucleic Acid

EPF – Entomopathogenic Fungi

GLRV – Grapevine Leaf-Roll Virus

GVA – Grapevine Virus A

IPM – Integrated Pest Management

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

pH – Potential Hydrogen

PHA – Phillipi Horticultural Area

RH – Relative Humidity

spp. - Species

THESIS STRUCTURE

This study includes four concisely written chapters.

Chapter 1 – It comprises of the introduction, background to the research problem and literature review.

Chapter 2 – This chapter focuses on, the influence of season and land-use on the occurrence of entomopathogenic fungi in the Cape Peninsula region, South Africa.

Chapter 3 – This chapter focuses on, does insect-based oil formulation enhance the pathogenicity of indigenous entomopathogenic fungal isolates from the Cape Peninsula region against *Planococcus ficus*, grapevine mealybug?

Chapter 4 – It comprises of the general discussion and an overview of the results obtained in the study, as well as recommendations for future studies in this area of research.

ABSTRACT

Soil-borne micro-organisms are crucial in maintaining ecological functionality and stability of soils. More recently, a group of fungi, entomopathogenic fungi (EPF), are gaining prominence due to their versatility – they are rhizopheric, endophytic and arthropod pathogenic. EPF are being used for pest management in many countries as a biological control and plant growth promoting agents. Despite a plethora of evidences of bio-efficacy of EPF against insects under greenhouse and field conditions, inconsistent efficacies in field studies and stringent regulatory requirements on the use of imported EPF strains are hampering their widespread use. To overcome these setbacks, it is necessary to isolate and identify virulent indigenous strains of EPF, understand how ecological factors factors influence the occurrence, persistence, and virulence of EPF, and develop more effective conidial formulations. However, few studies have isolated and investigated the relationship between EPF and the ecological factors in Africa in general and the Cape Peninsula region in particular. This study explored the relationships between the occurrence of indigenous EPF and land use and season of the Cape Peninsula region. The study also examines the enhancing effect of insect oil in conidial formulations on the bioactivity activity of EPF against *P. ficus in vitro*. To investigate the ecological relationships between entomopathogenic fungal occurrence in the Cape Peninsula region and season and land use, soils were collected from randomly selected sampling sites within randomly selected nature reserves (undisturbed sites) and agricultural farms (disturbed sites) in the Cape Peninsula region. Soil samples from the same sites were collected in different seasons (winter and summer). The isolates were identified morphologically and molecularly. In total, 96 EPF strains were identified. The associations among land use, season and fungal occurrence were determined by correspondence analysis (CA). The number of fungal strains occurring in the sites, the differences in the number of fungal species and strains found in the two seasons and in the farms (disturbed sites) and reserves (undisturbed sites) were compared using the Fischer Chi-square test. The results revealed that season positively influenced EPF occurrence, with significantly ($\chi 2 = 10.286$; $p < 0.01$) higher number of EPF isolates occurring in winter than in summer, as well as in farms than in reserves. Furthermore, a higher EPF species diversity occurred in winter compared to summer — up to 20 EPF species were isolated in winter compared with only eight species in summer. *Metarhizium* spp. were more frequently isolated from agricultural farms than in reserves. In addition, this study aimed to screen indigenous fungal strains against the grapevine mealybug, *Planococcus ficus* and assess the prospect of using insect-based oil for formulating EPF against the mealybug *in vitro*. The study focused on screening the 96 fungal isolates collected from soils in the Cape Peninsula region against grapevine mealybug using a dipping method. Insect mortalities caused by the fungal strains, varied significantly ($\chi 2 = 204.8$, $p < 0.01$, dF = 380). *Metarhizium* isolates induced the highest mortalities (ranging from 50% - 80% kill rate) within three days. Based on high insect mortality, two fungal isolates (Isolate 71 and 74) were selected and formulated in oil (Ento-oil®) obtained from black soldier fly at varying conidial concentrations of 1×10^7, 1×10^5, and 1×10^3 conidia mL^{-1}. *Metarhizium brunneum* isolate (Isolate 74), tested at 1×10^7 conidia/mL and formulated in 20% Ento-oil (20% oil and 0.5% aqueous Tween 80 mixture) yielded the highest mealybug mortality (83%) within 3 days. The addition of oil obtained from black soldier fly larvae to the formulation increased the virulence of the *M. brunneum* isolate.

This study presents interesting insights into the influence of seasonal change on the occurrence of EPF under different land use conditions in the Cape Peninsula region. Furthermore, the formulation of selected entomopathogenic fungal conidia with insect oil and water emulsion enhances insecticidal activity against *P. ficus*. The findings, suggest that indigenous EPF could play a major role in the future of biological control and sustainable farming practices.

Keywords: Biological control, Cape Peninsula, ecology, entomopathgenic fungi, formulations, grapevine mealybug, indigenous fungal strains, isolation, micro-organisms

Chapter 1

General Introduction

1.1 Introduction

The rapid human population growth and development is placing undue pressures on natural resources and causing environmental degradation. The human population is estimated to increase and reach 9.8 billion by 2050, worsening the high demand for food, which is fueling the adoption of unsustainable farming practices and soil degradation (Kopittke *et al*., 2019). Concerns over the widespread degradation of arable lands globally have led to the search of sustainable food production strategies. However, achieving sustainable food production warrants an in-depth knowledge of the soil constituents and their roles. Many groups of microbes including, nutrient cycling bacteria and, mycorrhiza, have been widely studied. More recently, another group of fungi, entomopathogenic fungi (EPF), are gaining prominence due to their versatility and agro-ecological roles – rhizopheric, endophytic and arthropod pathogenic (Msimbira & Smith, 2020).

Many EPF originate from the order Entomophthorales and Hypocreales. These fungi are considered as pathogens of mites, ticks and insects (Benny *et al*., 2014). According to phylogenetic studies, the taxonomy of fungi has been adjusted recently with the reclassification of Entomophthorales within the subphylum of Entomophthoromycotina, as well as the classes Neozygitomycetes, Basidiobolomycetes, and Entomophthoromycetes (Humber, 2012). Entomopathogenic fungi occur in many ecosystems and are widely distributed, however, insufficient studies have focused on their ecology, which is hindering the widespread application of these fungi in mainstream pest programmes (Abaajeh & Nchu, 2015). Entomopathogenic fungi offers many benefits; some EPF species (*Metarhizium* spp. *and Beauveria* spp.*)* [Hypocreales] are highly effective against arthropod pests. Furthermore, some EPF specie, such as *Trichoderma* spp. positively influence soil rehabilitation and overall plant growth and development (Harman *et al*., 2004). These entomopathogens have a wide distribution and are ubiquitous, having the aptitude to survive in various terrestrial ecosystems worldwide. Entomopathogenic fungi have adapted to living in soils, where most of their life-cycles occur (Rajula *et al*., 2021; Kovač *et al*., 2021). Entomopathogenic fungi can survive as saprophytes, living in the soil and colonizing insect cadavers and organic matter. Soil is a constant structure, which alleviates the oscillation of their populations and shielding them from abiotic factors, such as solar radiation, temperature, humidity or drying (Hummadi *et* al., 2021; Kovač *et al*., 2021). There is increasing evidence that most EPF are able to colonize some plant species. Moreover, depending on the fungal isolate and plant species, interactions between an endophytic fungal strain and a plant species are agriculturally beneficial (Vidal & Jaber, 2015). Fungi are sensitive to contaminants (e.g., insecticides, fungicides, herbicides and heavy metals) which cause delayed or decreased growth, damage to cellular structures and aberrations of cellular metabolic pathways. These compounds can cause adverse changes within the cells. In the presence of contaminants, several fungi have developed mechanisms, which allows for their survival (Litwin *et al*., 2020).

Many factors can influence the occurrence and diversity of plants and microorganisms. As reported by Zhou *et* al. (2020), soil microbes were affected by land-use. Frequently, microbes are influenced by temperature and moisture changes among the different seasons. Hence, these changes, link directly to the occurrence and diversity of the microbial populations (Luo *et al*., 2019). However, few studies have specifically looked at the influence of land-use and season on the occurrence of EPF in the Cape Peninsula region. The region is home to one of the most important agricultural production lands in SA. Knowledge

of the factors that influence EPF can contribute toward achieving conservation of EPF and optimization of conservation biological control.

Endophytic fungi can be valuable to host plants through their mutualistic symbiosis. These endophytic microbes can help with the utilization and uptake of soil nutrients. These nutrients promote plant growth and development, resulting in better yields (Xia *et al*., 2019). Many endophytic fungi can improve plant resistance, as well as their resistance to abiotic stress, like drought or excessive salt in soils, making relationships between endophytic fungi and plants significant, because they can be exploited to achieve, sustainability of agro-ecosystems (Xia *et al*., 2019; Quesada-Moraga, 2020). The Cape Peninsula region is host to distinctive terrains consisting of mountains, forests, agricultural lands, and the fynbos biome. It is blessed with high and unique fauna and flora diversities in the world. The geographical gradients of some of these locations allow for exceptional biodiversity. Additionally, the flora has a high degree of rarity due to the plant-species richness (Simmons & Cowling, 1996). The climatic conditions are quite unique as well, and the Mediterranean-climate is cool wet winters and hot dry summers (Loundou, 2008).

Planococcus ficus, infects several plant species, but it is most damaging to grapevines. It has detrimental effects on the wine and table grape industries, influencing fruit quality and productivity (Moloinyane, 2018). The mealybug belongs to the family Pseudococcidae. There are approximately 2 240 mealybug species, 68 of which are indigenous to South Africa. According to Walton. (2003), the grapevine mealybug, *P. ficus*, is the most abundant mealybug species in South Africa. They are soft-bodied insects which looks like white cotton masses on stems, fruit, and leaves of grapevines. These insect pests cause direct damage to the phloem sieve tubes, by using their stylets (long sucking mouthparts) and sucking out plant fluids, hence, resulting in wilting, leaf stunting and possible plant death. In addition, mealybugs are vectors for grapevine leafroll virus; they may cause indirect damage by secretion of honey mildew, acting as a substrate for sooty mold disease. Subsequently, it results in the reduction of the plants' photosynthetic rate (Moloinyane, 2018). For years grape farmers have depended on synthetic chemicals, for controlling this insect pest. The insecticides primarily used were organophosphate, such as fipronil and cypermethrin. While synthetic insecticides can substantially reduce the insect infestation levels, they are environmentally hazardous and acquired pest resistance to chemicals is not uncommon (Bostanian *et al*., 2012).

Consequently, alternative insect control methods are being explored. Interestingly, the endophytic ability of some EPF allows fungal endophytes to systematically overcome the cryptic nature of the mealybug, including sap-sucking, high reproductive rates, and ability to hide in crevices. The outcomes of this study could serve as an insight, into the occurrence and diversity of indigenous fungal strains. Some of the strains, may be highly effective and could be used as control agents within the Cape Peninsula region and South Africa. Locally isolated EPF strains might be better adapted to local environmental conditions and soil properties.

1.2 Thesis rationale

Soil properties are recognized to influence the mobility, availability and virulence of fungal conidia in the soil. Therefore, physical and chemical soil properties, has an impact on performance of EPF. The use of organic matter and fertilizers, enhance the soil health, which then directly effects the changes in EPF growth, diversity and activity. In addition, it will improve the fungi-plant relationship (Presa-Parra *et al*., 2020). Screening of EPF is important to filter out why a fungus can be effective, and at what concentrations it should be, to achieve its optimum capabilities. As proven by Mathulwe *et al.* (2022), the use of local

fungal strains is effective because they are adapted to the local environmental conditions, making it easier for these strains to reach their maximum potential. Whereas, using a fungal strain from abroad might not be effective in this environment. Hence, the use of indigenous EPF would automatically make a difference in terms of accessibility, as well as efficacy (Sutanto *et* al., 2021; Mathulwe *et al*., 2022). Although, the fungal strains are effective under *in vivo* conditions, various climatic changes can influence their performance. Mathulwe *et al.* (2022) reported formulations of EPF, using different adjuvants, can assist in extending their tolerance and persistence when applied in the field. Moreover, when using different formulations there are a few things to consider. Using a dry or liquid formulation are some options to explore. Liquid formulations can be made in various mixes. According to Batta (2016), oil-based formulations is highly effective and invert emulsion (water-oil-based) is the most suitable formulation. Dry formulations include natural dusts such as, charcoal, chalk, wheat flour, etc. However, mixing dry conidia with diatomaceous earth, has been the most promising. The key is optimizing the ingredients within the formulations, to enhance fungal strains (Batta, 2016).

This study aims to isolate EPF from different locations in the Cape Peninsula region and understand the influence of seasonal changes and land-use on the occurrence and to screen indigenous fungal isolates against *P. ficus* and assess the prospect of enhancing efficacy of EPF formulations using insect oil. The study is expected to contribute towards the collection of new insecticidal fungal strains and the development of environmentally friendly pest management strategies. Current control mostly relies on chemical control. However, the efficacy of synthetic chemicals to control grapevine mealybug is low to moderate due to reasons such as, enigmatic natures of the larvae and reported cases of resistance to insecticides are rampant (Platt *et al*., 2019). According to Rajula *et al*. (2021), EPF offers one of the most dependable alternatives. The majority of EPF naturally regulate insect populations. They can be mass-produced and commercialised due to the ability to be cultured on artificial medium. It has been investigated that they control a wide range of pests. The use of EPF in bio-pesticides has many benefits such as, they are harmless to beneficial and non-target organisms and pose minimum risk to humans and animals (Goble, 2009; Skinner *et al*., 2014; Rajula *et al*., 2021).

Despite all these potential benefits of EPF, their ecology is not fully understood. Little is known on the effects of land-use and season, on their bioactivities and occurrence in the context of South Africa. This is a major knowledge gap that this study addressed. Investigating the link among the variations of indigenous EPF occurrences in soils between summer and winter seasons and between disturbed and undisturbed sites in the Cape Peninsula region, Western Cape Province, South Africa. In addition, screening the isolated indigenous fungal strains against the grapevine mealybug and assess the prospect of using insect oil for formulating EPF against mealybug, *in vitro*.

1.3 Research objectives

- To investigate the influence of season on the occurrence of indigenous isolates of EPF in the Cape Peninsula region.
- To investigate the influence of land-use on the occurrence of indigenous isolates of EPF in the Cape Peninsula region.
- To evaluate the pathogenicity of isolated fungal strains in *in vitro* insect mortality bioassay.
- To evaluate the effect of formulating EPF in insect oil against *P. ficus in-vitro*.

1.4 Statement of the research problem

The lack of sufficient knowledge of the ecological factors, such as season and land-use, which influence the occurrence of entomopathogenic fungi and the urgent need to find indigenous fungal isolates and formulations that could be developed into a biopesticide for control of *P. ficus*, a well-known destructive pest, inspired this study. The Cape Peninsula region has the famous fynbos biome, with a rich and unique diversity of flora and fauna species, dry summers and temperate, wet winters and diverse land-uses, which present an interesting opportunity to study the two important ecological factors (season and land-use) on the occurrence of EPF. Entomopathogenic fungi were isolated from reserves and farms in the Cape Peninsula region during winter and summer months, and the effects of season and land use on the occurrence of EPF were determined. Furthermore, the virulence of the fungal isolates and the prospects of using insect oil in EPF was assessed in laboratory studies. The study led to the collection of indigenous fungal strains and a better understanding of how ecological factors influence EPF distribution.

1.5 Alternative Hypotheses

- EPF occurrence in the Cape Peninsula region is influenced by season and land use.
- EPF virulence varies with indigenous fungal strains and species.
- A formulation of endophytic fungal conidia and insect oil positively influences the mortality of *P. ficu*s *in vitro*.

1.6 Thesis Outline

Chapter One covers the theoretical justification of the study and a detailed literature review on the topic. Chapters Two and Three present the thesis's research components, which were carried out in two parts: field (Chaper Two) and laboratory (Chapter Three) experiments. For the field investigation, soil-borne EPF were randomly sampled from vegetable farms and reserves to establish the influence of land use on EPF occurrence. The sites were also sampled in two seasons (winter and summer) to determine the effect of seasonal change on fungal occurrence. The EPF in the soil samples was isolated using insect bait and selective medium methods. The isolated fungal strains were screened for pathogenicity against adult female *P. ficus* in the laboratory. The conidia of the most virulent EPF strains were further formulated in insect oil-water emulsion and insect oil and tested at three concentrations ($1{\times}10^7$, $1{\times}10^5$, and $1{\times}10^3$ conidia mL^{-1}) to determine the effect of insect oil and concentration on pathogenicity against *P. ficus*. An overview of the study is given in Chapter Four, which links everything togther.

Literature Review

1.7 Biocontrol Industry in South Africa

Biocontrol agents are being developed in Africa for the control of arthropod pests. Farmers in Africa producing high-value crops for exportation, are increasingly reducing synthetic fungicides and insecticides (Cherry & Gwynn., 2007). However, the uptake of biological agents for pest control in Africa is relatively slower in Africa compared to Europe, Asia, and the Americas. A few biopesticides are registered in Kenya, Ghana, and South Africa for commercial use. In Africa 80% of food produced is by small-scale farmers, with areas less than 2 hectares, with limited resources (Steveson *et al*., 2017) and knowledge on pesticide use and application (Grzywacz *et al*. 2014). Considering the complexity of how biological agents work, the farmers need to be educated to ensure efficiency.

The use of microbial solutions to pest problems is not new in Africa. The 'Green Muscle' was the first practical example of the use mycoinsecticide in IPM in Africa. In 1998, Biological Control Products of South Africa, was the first company to acquire a license to sell this product. Subsequently, many Eastern and Southern African countries have used the product to control locusts (Maina *et al*., 2018). Hence, there is potential for growth in this sector. Once the products are formulated properly and are efficacious, they could be distributed throughout the continent and abroad. Another hindrance to biopesticide use in Africa is difficult regulatory framework. Regulations regarding the registrations of biological products in Africa is quite complex because it is based on synthetic chemicals. This requires extensive toxicology reports, and this may be beyond local biocontrol companies because of the high costs (Grzywacz *et al*., 2014).

Nevetheless organic agriculture, which is a system of production that works exclusively without the use of synthetic fertilizers and pesticides, is growing, and Africa can benefit immensely from huge opportunities that it brings (Behera *et al*., 2012; Binta & Barbier, 2015).

1.8 Land-uses in the Cape Peninsula (reserves, forests, farms)

Table Mountain is World Heritage Site. It is globally known for its extraordinary rich, unique, and diverse flora and fauna. However, its ecosystems are endangered, with metals being the main cause of environmental contamination (Krüger *et al.*, 2019). Because microorganisms depend on the chemical (pH, C/N ratio) and physical properties (soil texture), organic matter biophysicochemical changes in ecosystems may affect their occurrence, for example, agricultural activities may promote increased abundance and diversity of EPF species because of the higher nutrient profiles compared to reserves (Qayyum *et al.,* 2021).

1.8.1 Reserves

The Cape Floristic region is recognised as an international biodiversity hotspot. However, the occurrence of microbial populations in these soils remains poorly understood (Mager & Hui, 2012). The primary natural vegetation type, which occurs throughout the Cape Peninsula is the Peninsula sandstone fynbos, followed by the Peninsula granite fynbos. The fynbos soils are typically acidic to neutral (pH ranging from 4 – 7). The typical vegetation types found are as follows; sandy nutrient-poor soil, granite-derived clay soils, dense calcium-rich soils, light-grey quartzite (Pryke & Samways, 2008; Schnetler *et al*., 2021).

1.8.2 Forests

The Cape Peninsula is of substantial biological importance, mostly because of the endemism. It has forests that are found within urban ecosystems and plays a key role in biodiversity conservation (Krüger *et al*, 2019). As reported by Wyse *et al.* (2015) and Krüger *et al.* (2019), urban ecosystems must be actively protected because they are crucial for the removal of air pollutants, as well as conservation of water and soil resources. Regrettably, soil biodiversity is profoundly threatened by human activities and economic development. According to Breure *et al.* (2012), a reduction in soil biodiversity would directly affect the above-ground biodiversity, potentially having a huge impact on the forest ecosystems' functionality (Krüger *et al*., 2019).

1.8.3 Farming

Agriculture is a key contributor to the economy of the Western Cape Province. The province has a diverse production capacity. A minimum of 11 commercial agricultural crops are cultivated in the province. Some of these include grains, fruit, viticulture, vegetables, etc. (Murray, 2010). Cape Town city has a long history of supporting urban agriculture and creating more jobs (Kanosvamhira, 2019).

1.8.4 Climate of the Cape Peninsula region

Cape Peninsula region has an annual rainfall of around 500 – 650 mm, with temperatures reaching a high of 24 ºC and a low of 9 ºC (Pryke & Samways, 2008; Schnetler *et al*., 2021). It has a Mediterranean climate, characterized by warm, dry summers and cool, wet winters.

1.9 Environmentally sustainable farming

Environmentally sustainable farming uses fewer toxic insecticides, such as biological control agents to mitigate insect infestations. The increasing recognition of the benefits of environmentally friendly farming approaches by farmers and consumers have created the need to develop bio-control technologies, some of which are based on microbial agents (Stokwe, 2016). One of the well-known groups of biocontrol agents is entomopathogenic fungi (EPF) and among these are species in the genera *Beauveria, Clonostachys* and *Metarhizium*; they are among the most successful genera of entomopathogens used commercially. *B. bassiana* are being used for pest management in many countries. Moreover, there are no major reports of substantial negative effects when using this EPF as a biocontrol (El Kichaoui *et al*., 2016). Generally, organic farming practices focus on manipulating the microbial composition of soils to promote diversity.

1.10 Organic farming versus non-organic farming

As reported by Skinner *et al.* (2019), producing enough food, while preserving ecosystems is challenging. Generally, organic farming practices enhance microbial persistence and to promote diversity compared to non-organic farming. There are increased differences on diversity and sustainability of plant health with the presence of microbial populations in organic farming compared to conventional farming schemes (Lupatini, 2017). Futhermore, it includes the use of biopesticides, biofertilizers, compost, etc. which all enhances soil fertility, and improves overall soil organic matter (Goel *et al.,* 2021; Mukherjee *et al.,* 2022). According to Goel *et al.* (2021) organic farming systems have several challenges which includes; long-term productivity, sutainable use of natural resources and feasibility compared to conventional farming practices.

1.10.1 Organic crop cultivation

Demand for organic products is growing throughout the world, particularly in developed countries (Tal, 2018). Over the past two decades, organic agriculture has grown considerably. A previous study by Shennan *et al.* (2017) on organic farming showed that organic farming provides greater ecosystems and social benefits. Hence, organic farming schemes need to be encouraged (Skinnet *et al.*, 2019). It is worth noting that cultivated soils do not only serve as plant growth medium but also serve as suitable habitats for soil organisms (Hill *et al.,* 2000; *Skinner et al.*, 2019). Organic agriculture usually increases enzymatic activity and improves soil health through the accumulation of organic matter using farmyard manure, catch crops and reduced tillage practices (Kwiatkowski *et al.*, 2020).

1.10.2 Conventional crop cultivation

Conventional farming systems depend on intensive use of agrochemicals, such as synthetic fertilizers and pesticides to increase crop productivity (Lupatini *et al.*, 2017). Agro-ecosystems regularly face numerous problems including infestations by plant pathogens, nematodes, and harmful soil-borne phytopathogenic fungi and bacteria, which effect crop productivity. Synthetic chemical pesticides are often used to control these pests causing toxicity to non-target organisms (Lupatini *et al.*, 2017).

1.11 Mealybug as a pest

1.11.1 Biology of mealybug

Mealybugs are among the most problematic and invasive vineyard pests. Many mealybug species are economically destructive, for example, the long-tailed mealybug (*Pseudococcus longispinus*), citrophilus mealybug (*Pseudococcus calceolariae*), obscure mealybug (*Pseudococcus viburni*), citrus mealybug (*Planococcus citri*) and vine mealybug (*Planococcus ficus*) (Daane *et al.,* 2018; Ji *et al.,* 2020). Once they feed, they emit carbohydrate rich honeydew, which accrues on grape bunches and leaves, acting as a substrate for growth of sooty mould. Any occurrence of mealybugs, sooty moulds, or honeydew on grape bunches, especially for table growers, affects the marketability of the produce negatively (Daane *et al.,* 2012; Daane *et al.,* 2018). The density of the vine mealybug is influenced by cultural practices such as, fertilization, soil management and irrigation, potentially affecting population growth by modifying fertility, fecundity, and could lead to pest outbreaks (Cocco *et al.*, 2018)

Conventional control schemes of broad-spectrum insecticides are often inadequate, due to the cryptic habits of *P. ficus*. Environmentally-friendly control has become increasingly popular with strategies such as, mating disruption, which could be a good alternative to chemical control (Cocco *et al.*, 2018). If conditions are favourable *P. ficus* can thrive throughout the growing season. Interactions between mealybug and grapevine in field conditions are complex, unpredictable, and interdependent (Timm, 2014).

1.11.2 Life cycle

Mealybug development and persistence is commonly favoured at 30 ºC (Varikou *et al*, 2010).
A mealybug lifecycle from egg to egg takes approximately four weeks in summer and longer in winter. The females lay pale yellow eggs of about 300 to 580, within a cottony ovisac, which juts under the female. Eggs are laid over an interval of one to two weeks, and they hatch after six to ten weeks (FitzGerald, 2014). Males have four larval instars, while female have three, which comprise the first, second, prepupal and pupal stages. The first larval instar is light yellow and very mobile, also known as crawlers. Within the second larval instar, females and male can be categorized from each, by perceiving the eye spot variation in males. Males have substantial development in their third and fourth instars, being concealed in a cotton cocoon. They are relatively smaller compared to the females, reaching about 1 mm in size, yellowish in colour with two long white anal filaments and hyaline wings. Females are much bigger, around 3 mm, also yellow, covered in a mealy wax layer. They have 18 pairs of wax filaments, lengthening over time. Towards the end of the life cycle, mostly after ovipositing, adult females become lethargic. The larval life stages last about two weeks each, and females start to oviposit around two weeks after their last molt (Daane *et al.,* 2012; FitzGerald, 2014; Moloinyane 2018).

1.11.3 Damages caused by mealybug

The mealybug efficaciously transmits numerous viruses including grapevine leafroll-associated viruses (GLRaVs), grapevine virus A (GVA) and corky bark disease. Mealybug feeding on leaves may prevent photosynthesis, resulting in discolouration, defoliation and ultimately death of the vines (Timm & Reineke, 2014)

1.12 Management of Mealybug

1.12.1 Cultural Control

Cultural control is a classic biological control used to eliminate the mealybug, making it an effective method of control. Generally, *P. ficus* is susceptible to a variety of enemies, such as *Anagyrous pseudococci*

(Girault) and *Coccidox-enoides perminutus* (Timberlake), which are the most dominant parasitoids. Additionally, there are voracious beetles such as *Nephus binaevatus* (Mulsant), *Cydonia lunata montrouzieri* Mulant and *Hippodamia* sp. It is crucial to protect natural enemies by lessening chemical applications (Walton & Pringle, 2005; Varikou *et al.*, 2010; Mansour *et al.*, 2018). As reported by Varikou *et al.* (2010), awareness of these cultural practices is important to determine the seasonal pest presence and developing mitigation strategies using this type of integrated pest management. The suitable choice of grapevine cultivars could be valuable in decreasing *P. ficus* infestations. Early-harvested cultivars habitually have lower pest density levels compared to late-harvested cultivars. Dormant grape cuttings immersed in hot water for five minutes at 51 ℃, efficiently kill 99 % of *P. ficus* life stages. Additionally, improved nitrogen fertilization efforts could prevent pest outbreaks and help diminish the grapevine mealybug population densities (Mansour *et al.*, 2018).

1.12.2 Biological control methods

There is a growing interest in evaluating the prospective role of native natural enemies in reducing plant pest populations. Biological, cultural, and biotechnological approaches for pest management is becoming fashionable, which is mostly driven by awareness that the continual use of chemical insecticides might harm non-target organisms along with the environment, and it is not sustainable. Furthermore, several important insect pests have developed resistance to chemical insecticides (Sharma, 2008; El Kichaoui *et al*., 2016; Alemu, 2020).

As stated by Pell *et al.* (2010), conservation biological control depends on management practices and modification of the environment to encourage and protect natural enemies. These strategies focused on reducing factors such as, extensive use of pesticides, agronomic interventions and tillage, etc. (Begg *et al.,* 2017). Conservation biological control using EPF includes, the maniplution of both, above and below ground factors such as, soil health, crop production, environmental factors, etc. Understanding their ecology and complex relationships with EPF hosts and habitats, may enhance their abilites to control pest populations resourcefully (Pell *et al.,* 2010).

Semiochemicals are defined as mixtures of substances released by animals, plants and other organisms that induce a physiological or behavioural response in individuals of different or the same species. For instance, insect sex pheromones are often emitted by females, attracting males for mating. Hence, this lures the males and traps are setup accordingly (Mansour *et al.*, 2018). Traps containing lure and EPF could be used to attract targeted insect to EPF conidia, facilitating autodissemination. The use of sex pheromone traps as bait is beneficial for monitoring insect movement and that makes it easier to plan IPM schemes (Lucchi *et al.*, 2019).

1.12.3 Chemical control

Synthetic chemical pesticides remain the main tool for eradication of pests. However, composite issues have been generated from using chemical pesticides in insect pest control including: contamination of groundwater, safety risks for humans and domestic and wild animals, and decrease in biodiversity (El Kichaoui *et al*., 2016; Ojo, 2016; Rani *et al.*, 2021).

Previously Bell & Walker (2009) conducted a field trial using insecticides, buprofezin (Applaud™) and prothiofos (Tokuthion®), on a commercial vineyard. The insecticides successfully reduced the mealybug

numbers. Chemical control is measured to be the most common control approach used against *P. ficus*. Consequently, frequent application of pesticides can completely compromise the efficacy of IPM schemes, harming non-target organisms such as, bees, predatory species, etc. (Mansour *et al.*, 2018).

1.13 Entomopathogenic Fungi (EPF)

1.13.1 History of Entomopathogenic Fungi (EPF)

In 1888, the first revolutionary field trails with EPF began with a microbiologist named, Elie Metchnikoff (Taliyan *et al*., 2020). He started mass producing fungal conidia on sterilized brewer's mash and then combined cultures with granules of sand for distribution on field crops. However, although the results were inconsistent, Metchnikoff's work inspired programs in United States and Europe for experimentation of "friendly fungi" on insect pests (Maina *et al*., 2018). Many EPF originate from the order Entomophthorales and Hypocreales. These fungi are pathogens of mites, ticks, and insects (Benny *et al*., 2014). According to phylogenetic studies, the taxonomy of fungi has been adjusted recently with the reclassification of Entomophthorales within the subphylum of Entomophthoromycotina, as well as the classes Neozygitomycetes, Basidiobolomycetes, and Entomophthoromycetes (Humber, 2012). Entomopathogenic fungi infect insects of nearly all orders; commonly Diptera, Lepidoptera, Hymenoptera, Coleoptera, Orthoptera and Hemiptera (Maina *et al*., 2018; Petersen *et al*., 2019).

1.13.2 Reproductive Stage and Life Cycle

Majority of EPF have life cycles that synchronize with environmental conditions and insect host stages. Spore germination vastly depends on moisture (Stokwe, 2016). While fungal pathogens have a lot in common with bacteria, viruses, and other pathogenic insect microbes, they are, however, distinctive in many ways. The most substantial variation lies within their mode of infection. Fungi naturally penetrate the insect cuticle (Gul *et al.,* 2014; Stokwe, 2016).

The life cycle of EPF compromises of two growth phases (Khan *et al.,* 2012): a mycelial growth phase, which happens outside the host insect body and a yeast-like budding phase (blastospores), which happens inside the insect host haemocoel (Mathulwe *et al*., 2021). Once the spores gain entry to the host insect haemolymph, the growth morphology of the EPF changes to the second growth phase (yeast-like growth phase). After the death of the insect host, the fungus arises from the cadaver, concluding its life cycle via sporulation on the exterior of the cadaver. The fungal conidia are dispersed to a new host, where the infection cycle resumes (Khan *et al.,* 2012; Stokwe, 2016).

1.13.3 Infection Process

Entomopathenic fungi must overcome several host challenges, to produce sufficient new spores to maintain viable populations within every generation (Mora *et al*., 2018). Many EPF are soil-borne organisms, and all have a comparable mode of infection (Maina *et al*., 2018). When fungal conidia come into contact with a host, they attach to the cuticle of the insect through hydrophobic mechanisms and germinate, forming germ tubes under optimum conditions (Figure 1). Through this process, the fungus produces specialized infection structures which includes, penetration pegs and/or appressoria. This facilitates growing hyphae to breach the host integument (Singh *et al*., 2017; Islam *et al*., 2021).

1.13.4 Hemocoel penetration and replication

For successful infections, the EPF must penetrate the cuticle of the insect, which is made up of a cluster of polysaccharide polymers integrated in a protein matrix. The cuticle is split into three segments. The outermost layer is termed the envelope with a 10 – 30 nm thickness. Beneath the envelope is the epicuticle,

with a 0.5 – 2 mm thickness. Followed by the last section, the procuticle, which is significantly wider than the epicuticle and envelope, as well as where the proteins and chitin form a matrix. The procuticle if further split into an outer layer (exocuticle) and inner layer called the endocuticle (10 – 200 mm thick). By the base of the procuticle, the epidermal cells are found, and beneath the epidermal cells is the hemocoel (Stokwe, 2016; Mora *et al*., 2018; Islam *et al*., 2021). By mechanical pressure, the cuticle is penetrated, with the fungus producing enzymes to degrade the cuticle and ultimately colonize the insect. However, the way an insect is penetrated by the EPF depends on the cuticle properties, such as sclerotization, thickness and antifungal substances (Mora *et al*., 2018). According to Khan *et al.* (2012) and Sánchez-Pérez *et al.* (2014), enzymes involved in the degradation of the cuticle include chitinases, proteases, and lipases, which degrade chitin, proteins, and lipids, respectively. Over-expression of some enzymes in the EPF species can accelerate death of insects and is essential for selecting the best strains for biological pesticides (Maina *et al.*, 2018; Mora *et al.,* 2018).

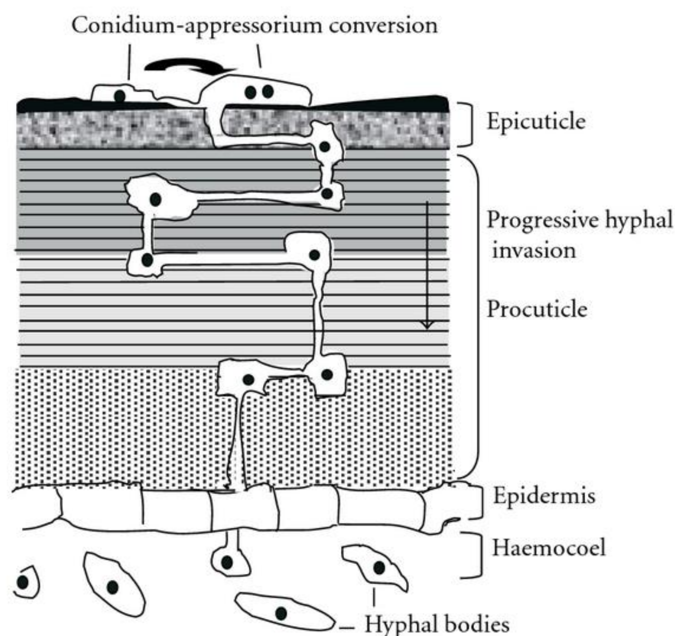

Figure 1: A depiction of the infection process: EPF invades and proliferates the insect hosts, causing various abnormalities within insect hosts (Sandhu et al., 2012).

Once the hemocoel is reached, most fungi go into a dimorphic transition from mycelium into yeast. This occurs often without cell wall development, but rather forming in protoplasts (blastospores), evading recognition by circulating hemocytes within the hemocoel. The benefits of this cellular formation allow the fungal cells to proliferate in the hemocoel, while the insect immune system cannot detect it. Usually, the insect responds to infection through cellular and humoral mechanisms (Strasser, 2001; Islam *et al*., 2021). Physiological symptoms of abnormalities in insects are induced by mycosis causing altered behavior, lack of coordination, seizures, and paralysis. Death occurs from the following: physical damage of tissues, dehydration of cells causing loss of fluids, toxicity, and consumption of nutrients (Mora *et al*., 2018). As reported by Pal *et al.* (2007), once inside the insect, the fungus must collapse the defense mechanisms to conclude the infection process by amalgamating other proteases that abolish the humoral immune system, as well as using, destruxins and cyclic depsipeptides to cause paralysis. It furthermore deters their ability to move and feed. Hence, this leads to the death of the insect, approximately taking 3 – 14 days after (Maina

et al., 2018). Environmental factors play a huge role in the production of conidia and their survival. It also plays a crucial role in their germination and development of epizootics. Sporulation typically occurs in insect cadavers, but may also occur in live insects. The dispersal of spores can be a passive or active process, depending on the characteristics of the sporangium or spore (Litwin *et al.*, 2020; Mantzoukas *et al*., 2020).

1.13.5 EPF (Beauveria) as biopesticides

Beauvaria is a diverse genus of soil-borne EPF and is eminent for producing a large range of biologically active secondary metabolites which includes non-ribosomally synthesized peptides, non-peptide pigments and polyketides. In addition, it secretes metabolites associated in pathogenesis and virulence, which, incidentally, have potential for pharmaceutical, industrial, and agricultural uses (Goble, 2009; Xiao *et al*., 2012). *Beauvaria bassiana* is a significant natural pathogen of various insect species, infecting more than 700 species in nine orders, primarily Coleoptera and Lepidoptera (Feng *et al*., 1994; Goble, 2009). Its hosts consist of several economically important pests and its extensive variation in virulence towards various insect hosts makes it more versatile for biological control (Goble, 2009). *Beauvaria* is one of the fungi species that can be mass produced commercially. Hence, it is currently being evaluated against urban and agricultural insect pests (Stokwe, 2016).

1.13.6 EPF (Metarhizium) as biopesticides

Metarhizium species have a broad range of virulence, which infects more than 200 insect species from over 14 various orders, which mainly consist of several major agricultural pests. The genus is morphologically demarcated based on the arrangement of phialides which have columns and chains of dry, green and slightly ovoid conidia (Goble, 2009; St. Leger & Wang, 2020). Some commercial endeavours have listed strains of *Metarhizium* used as an active ingredient of many pest management products namely: Green Muscle® (Biological Control Products, South Africa) against grasshopers, locusts, *Locustana pardalina* and *Metarhizium* 50® (AgoBiocontrol) for control of garden pests in Columbia. Several other *Metarhizium* registered products are currently available commercially (de Faria & Wraight, 2007). There are numerous fundamental criteria for selecting a fungal strain for commercialisation, such as virulence of isolates and whether isolates are indigenous to a specific location (Fernandes *et al.,* 2012; Tomer *et al.,* 2018).

1.13.7 Occurrence, bioactivity and ecology of EPF

Development of epizootics is influenced by host range and environmental factors. Environmental factors affect the efficacy of fungi as well as the stability of EPF in the field. Ambient temperature impacts the rate of infection and might limit EPF use. Most EPF are mesophilic, but has the best growth at temperatures between 25 °C and 35 °C. Adaptation to environmental fluctuations is an essential consideration in the selection and development of commercial fungal strains. Interestingly, strains which come from hotter zones often perform well at higher temperatures and similarly, strains from colder areas perform well at lower temperatures (Goble, 2009; Khan *et al.,* 2012). Several EPF are soil-borne microorganisms, and the environment embodies numerous abiotic and biotic factors which might affect the persistence and efficacy of fungi. These abiotic factors consist of soil type (organic matter content, texture, pH, cation exchange capacities, etc.) and occurrence of soil microflora and moisture. Soil texture and organic matter seem to be the most significant factors favouring occurrence of fungi (Quesada-Moraga *et al.,* 2007). Soil moisture affects vertical movement of conidia within the soil, and it has been shown to adversely affect persistance of *Beauvaria* and *Metarhizium* (Singh *et al*., 2017; Litwin *et al*., 2020; Mantzoukas *et al*., 2020; Islam *et al.,* 2021). According to Bueno-Pallero *et al.* (2020), soil granulometry can influence EPF communities;

higher porosity soils like sandy soils improve the movement of the fungal hyphae, while clay soils block the passage for conidial movement.

Management practices, for instance tillage, can affect the presence of EPF considerably because during the process conidia can be moved to the soil surface, where they are exposed to higher temperatures and UV-B radiation. Furthermore, biotic factors like additional soil microorganisms might influence the tenacity of EPF species. Biological activity in the soil effects fungal species because both *Metarhizium* and *Beauvaria* are poor contenders for organic resources (Clifton *et al*., 2015). The rhizosphere environment is crucial for the primary establishment of endophytes (Elsheikh *et al.,* 2021). Vidal & Jaber (2015) reported that when coffee plants were inoculated with an EPF isolate by direct injection, the interaction between the fungus and the host plant was crucial in the establishment of the entomopathogens in the plants. Depending on the fungal isolate and plant species, interactions between both the fungus and the plant are beneficial (Nchu *et al.*, 2022).

1.13.8 EPF formulation

Liquid formulation of EPF, water and oil are quite effective for IPM (Batta, 2016). However, to achieve maximum efficacy of the fungal activity, different formulations need to be tested. Generally, the presence of water helps with conidial germination and oils help obtain stability of the formulation. A study carried out by Sedighi *et al*. (2013) indicated an increase in virulence of conidia formulated in oil; the fungal spores were more active and could penetrate the insect cuticle with ease. Oil-based formulation may assist in lessening the influence of UV irradiation under open field, and it is cheap to maintain (ElShafei *et al*., 2010). Invert emulsions are water-in-oil formulations, where water is homogenized with oil, resulting in the water droplets to be covered with oil, averting evaporation when applied. It has been proven to show a higher efficacy against agricultural pests (Batta, 2016).

1.14 References

1. Abaajeh, A.R., 2014. *Evaluation of entomopathogenic fungi (Ascomycota) for the control of Cydia pomonella (Lepidoptera: Tortricidae)* (Doctoral dissertation, Cape Peninsula University of Technology).
2. Abaajeh, A.R. and Nchu, F., 2015. Isolation and pathogenicity of some South African entomopathogenic fungi (Ascomycota) against eggs and larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). *Biocontrol Science and Technology*, *25*(7), pp.828-842.
3. Batta, Y.A., 2016. Recent advances in formulation and application of entomopathogenic fungi for biocontrol of stored-grain insects. *Biocontrol Science and Technology*, *26*(9), pp.1171-1183.
4. Begg, G.S., Cook, S.M., Dye, R., Ferrante, M., Franck, P., Lavigne, C., Lövei, G.L., Mansion-Vaquie, A., Pell, J.K., Petit, S. and Quesada, N., 2017. A functional overview of conservation biological control. *Crop Protection*, *97*, pp.145-158.
5. Behera, K.K., Alam, A., Vats, S., Sharma, H.P. and Sharma, V., 2012. Organic farming history and techniques. *Agroecology and strategies for climate change*, pp.287-328.
6. Bell, V.A. and Walker, J.T.S., 2009. Maximising the effectiveness of insecticides to control mealybugs in vineyards. *New Zealand Plant Protection*, *62*, pp.296-301.
7. Benny, G.L., Humber, R.A. and Voigt, K., 2014. 8 Zygomycetous fungi: phylum entomophthoromycota and subphyla kickxellomycotina, mortierellomycotina, mucoromycotina, and zoopagomycotina. In *Systematics and evolution* (pp. 209-250). Springer, Berlin, Heidelberg.

8. Binta, B.A. and Barbier, B., 2015. Economic and environmental performances of organic farming system compared to conventional farming system: a case study of the horticulture sector in the Niayes region of Senegal. *Procedia Environmental Sciences*, 29, pp.17-19.
9. Bostanian, N.J., Wise, J.C. and Isaacs, R., 2012. Pesticides for arthropod control in vineyards. In *Arthropod Management in Vineyards:* (pp. 53-90). Springer, Dordrecht.
10. Breure, A.M., De Deyn, G.B., Dominati, E., Eglin, T., Hedlund, K., Van Orshoven, J. and Posthuma, L., 2012. Ecosystem services: a useful concept for soil policy making!. *Current Opinion in Environmental Sustainability*, *4*(5), pp.578-585.
11. Bueno-Pallero, F.Á., Blanco-Pérez, R., Vicente-Díez, I., Rodríguez Martín, J.A., Dionísio, L. and Campos-Herrera, R., 2020. Patterns of Occurrence and Activity of Entomopathogenic Fungi in the Algarve (Portugal) Using Different Isolation Methods. *Insects*, *11*(6), p.352.
12. Cherry, A.J. and Gwynn, R.L., 2007. Perspectives on the development of biological control agents in Africa. *Biocontrol Science and Technology*, *17*(7), pp.665-676.
13. Cilliers, C.D., Botha, A., Esler, K.J. and Boucher, C., 2005. Effects of alien plant management, fire and soil chemistry on selected soil microbial populations in the Table Mountain National Park, South Africa. *South African Journal of Botany*, *71*(2), pp.211-220.
14. Clifton, E.H., Jaronski, S.T., Hodgson, E.W. and 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), p.e0133613.
15. Cocco, A., Muscas, E., Mura, A., Iodice, A., Savino, F. and Lentini, A., 2018. Influence of mating disruption on the reproductive biology of the vine mealybug, *Planococcus ficus* (Hemiptera: Pseudococcidae), under field conditions. *Pest management science*, *74*(12), pp.2806-2816.
16. Daane, K.M., Almeida, R.P., Bell, V.A., Walker, J.T., Botton, M., Fallahzadeh, M., Mani, M., Miano, J.L., Sforza, R., Walton, V.M. and Zaviezo, T., 2012. Biology and management of mealybugs in vineyards. *Arthropod management in vineyards: Pests, approaches, and future directions*, pp.271-307.
17. Daane, K.M., Middleton, M.C., Sforza, R.F., Kamps-Hughes, N., Watson, G.W., Almeida, R.P., Correa, M.C., Downie, D.A. and Walton, V.M., 2018. Determining the geographic origin of invasive populations of the mealybug *Planococcus ficus* based on molecular genetic analysis. *PloS one*, *13*(3), p.e0193852.
18. de Faria, M.R. and Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological control*, *43*(3), pp.237-256.
19. El Kichaoui, A.Y., El-Shafai, A., Muheisen, H., Mosleh, F. and El Hindi, M., 2016. Safe approach to the Biological Control of the Tomato Leafminer *Tuta absoluta* by entomopathogenic fungi *Beauveria bassiana* isolates from Gaza Strip. *Safe approach to the Biological Control of the Tomato Leafminer Tuta absoluta by entomopathogenic fungi Beauveria bassiana isolates from Gaza Strip*, *2*(4), pp.351-354.
20. Elsheikh, E.A., El-Keblawy, A., Mosa, K.A., Okoh, A.I. and Saadoun, I., 2021. Role of endophytes and rhizosphere microbes in promoting the invasion of exotic plants in arid and semi-arid areas: A review. *Sustainability*, *13*(23), p.13081.

21. ElShafei, G.M., El-Said, M.M., Attia, H.A. and Mohammed, T.G.M., 2010. Environmentally friendly pesticides: Essential oil-based w/o/w multiple emulsions for anti-fungal formulations. *Industrial Crops and Products*, *31*(1), pp.99-106.
22. Feng, M.G., Poprawski, T.J. and Khachatourians, G.G., 1994. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol science and technology*, *4*(1), pp.3-34.
23. Fernandes, É.K., Bittencourt, V.R. and Roberts, D.W., 2012. Perspectives on the potential of entomopathogenic fungi in biological control of ticks. *Experimental parasitology*, *130*(3), pp.300-305.
24. FitzGerald, V.C., 2014. *Screening of entomopathogenic fungi against citrus mealybug (Planococcus citri (Risso)) and citrus thrips (Scirtothrips aurantii (Faure))* (Doctoral dissertation, Rhodes University).
25. Goble, T.A., 2009. *Investigation of entomopathogenic fungi for control of false codling moth, Thaumatotibia leucotrata, Mediterranean fruit fly, Ceratitis capitata and Natal fruit fly, C. rosa in South African citrus* (Doctoral dissertation, Rhodes University).
26. Goel, R., Debbarma, P., Kumari, P., Suyal, D.C., Kumar, S. and Mahapatra, B.S., 2021. Assessment of soil chemical quality, soil microbial population and plant growth parameters under organic and conventional rice–wheat cropping system. *Agricultural Research*, 10, pp.193-204.
27. Grzywacz, D., Stevenson, P.C., Mushobozi, W.L., Belmain, S. and Wilson, K., 2014. The use of indigenous ecological resources for pest control in Africa. *Food Security*, *6*(1), pp.71-86.
28. Gul, H.T., Saeed, S. and Khan, F.A., 2014. Entomopathogenic fungi as effective insect pest management tactic: A review. *Appl. Sci. Bus. Econ,* 1(1), pp.10-18.
29. Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M., 2004. Trichoderma species—opportunistic, avirulent plant symbionts. *Nature reviews microbiology*, *2*(1), pp.43-56.
30. Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T. and Nelson, E.B., 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied soil ecology*, *15*(1), pp.25-36.
31. Humber, R.A., 2012. Entomophthoromycota: a new phylum and reclassification for entomophthoroid fungi. *Mycotaxon*, *120*(1), pp.477-492.
32. Hummadi, E.H., Dearden, A., Generalovic, T., Clunie, B., Harrott, A., Cetin, Y., Demirbek, M., Khoja, S., Eastwood, D., Dudley, E. and Hazir, S., 2021. Volatile organic compounds of *Metarhizium brunneum* influence the efficacy of entomopathogenic nematodes in insect control. *Biological Control*, *155*, p.104527.
33. Islam, W., Adnan, M., Shabbir, A., Naveed, H., Abubakar, Y.S., Qasim, M., Tayyab, M., Noman, A., Nisar, M.S., Khan, K.A. and Ali, H., 2021. Insect-fungal-interactions: A detailed review on entomopathogenic fungi pathogenicity to combat insect pests. *Microbial Pathogenesis*, *159*, p.105122.
34. Ji, W., Han, K., Lu, Y. and Wei, J., 2020. Predicting the potential distribution of the vine mealybug, *Planococcus ficus* under climate change by MaxEnt. *Crop protection*, *137*, p.105268.
35. Kanosvamhira, T.P., 2019. The organisation of urban agriculture in Cape Town, South Africa: A social capital perspective. *Development Southern Africa*, *36*(3), pp.283-294.
36. Khan, S., Guo, L., Maimaiti, Y., Mijit, M. and Qiu, D., 2012. Entomopathogenic fungi as microbial biocontrol agent. *Molecular Plant Breeding*, *3*(7), pp.63-73.

37. Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A. and Lombi, E., 2019. Soil and the intensification of agriculture for global food security. *Environment international*, *132*, p.105078.
38. Kovač, M., Tkaczuk, C. and Pernek, M., 2021. First Report of Entomopathogenic Fungi Occurrence in Forest Soils in Croatia. *Forests*, *12*(12), p.1690.
39. Krüger, A.L., Snyman, R. and Odendaal, J., 2019. The impact of urban pollution on metal contamination of selected forest pockets in Cape Town, South Africa. *Environmental Science and Pollution Research*, *26*(12), pp.12537-12549.
40. Kwiatkowski, C.A., Harasim, E., Feledyn-Szewczyk, B. and Antonkiewicz, J., 2020. Enzymatic activity of loess soil in organic and conventional farming systems. *Agriculture*, *10*(4), p.135.
41. Litwin, A., Nowak, M. and Różalska, S., 2020. Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Bio/Technology*, *19*(1), pp.23-42.
42. Loundou, P.M., 2008. *Medicinal plant trade and opportunities for sustainable management in the Cape Peninsula, South Africa* (Doctoral dissertation, Stellenbosch: Stellenbosch University).
43. Lucchi, A., Suma, P., Ladurner, E., Iodice, A., Savino, F., Ricciardi, R., Cosci, F., Marchesini, E., Conte, G. and Benelli, G., 2019. Managing the vine mealybug, *Planococcus ficus*, through pheromone-mediated mating disruption. *Environmental Science and Pollution Research*, *26*, pp.10708-10718.
44. Luo, X., Wang, M.K., Hu, G. and Weng, B., 2019. Seasonal change in microbial diversity and its relationship with soil chemical properties in an orchard. *PLoS One*, *14*(12), p.e0215556.
45. Lupatini, M., Korthals, G.W., De Hollander, M., Janssens, T.K. and Kuramae, E.E., 2017. Soil microbiome is more heterogeneous in organic than in conventional farming system. *Frontiers in microbiology*, *7*, p.2064.
46. Mager, D.M. and Hui, C., 2012. A first record of biological soil crusts in the Cape Floristic Region. *South African Journal of Science*, *108*(7), pp.1-4.
47. Maina, U.M., Galadima, I.B., Gambo, F.M. and Zakaria, D., 2018. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *J. Entomol. Zool. Stud*, *6*(1), pp.27-32.
48. Mansour, R., Brévault, T., Chailleux, A., Cherif, A., Grissa-Lebdi, K., Haddi, K., Mohamed, S.A., Nofemela, R.S., Oke, A., Sylla, S. and Tonnang, H.E., 2018. Occurrence, biology, natural enemies and management of *Tuta absoluta* in Africa. *Entomol Gen*, *38*(2), pp.83-112.
49. Mansour, R., Belzunces, L.P., Suma, P., Zappalà, L., Mazzeo, G., Grissa-Lebdi, K., Russo, A. and Biondi, A., 2018. Vine and citrus mealybug pest control based on synthetic chemicals. A review. *Agronomy for Sustainable Development*, *38*(4), pp.1-20.
50. Mathulwe, L.L., Malan, A.P. and Stokwe, N.F., 2021. A review of the biology and control of the obscure mealybug, *Pseudococcus viburni* (Hemiptera: Pseudococcidae), with special reference to biological control using entomopathogenic fungi and nematodes. *African Entomology*, *29*(1), pp.1-16.
51. Mathulwe, L.L., Malan, A.P. and 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), pp.397-417.
52. Mantzoukas, S. and Eliopoulos, P.A., 2020. Endophytic entomopathogenic fungi: A valuable biological control tool against plant pests. *Applied Sciences*, *10*(1), p.360.
53. Moloinyane, S., 2018. *Bioefficacy of selected entomopathogenic fungal endophytes (Ascomycota) against grapevine mealybug (Planococcus ficus)* (Doctoral dissertation, Cape Peninsula University of Technology).

54. Mora, M.A.E., Rouws, J.R.C and Fraga, M.E., 2016. Occurrence of entomopathogenic fungi in Atlantic forest soils. *Microbiol. Discov, 4*(1), pp.1-7.
55. Mora, M.A.E., Castilho, A.M.C. and Fraga, M.E., 2018. Classification and infection mechanism of entomopathogenic fungi. *Arquivos do Instituto Biológico*, 84.
56. Msimbira, L.A. and Smith, D.L., 2020. The roles of plant growth promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. *Frontiers in Sustainable Food Systems*, *4*, p.106.
57. Mukherjee, K., Konar, A. and Ghosh, P., 2022. Organic farming in India: A brief review. *International Journal of Research in Agronomy*, 5(2), pp.113-118.
58. Murray, M., 2010. Key Trends in the agricultural economy of the Cape Winelands District Municipality.
59. Nchu, F., Macuphe, N., Rhoda, I., Niekerk, L.A., Basson, G., Keyster, M. and Etsassala, N.G., 2022. Endophytic *Beauveria bassiana* Induces Oxidative Stress and Enhances the Growth of *Fusarium oxysporum*-Infected Tomato Plants. *Plants*, *11*(22), p.3182.
60. Ojo, J., 2016. Pesticides use and health in Nigeria. *Ife Journal of Science*, *18*(4), pp.981-991.
61. Pal, S., Leger, R.J.S. and Wu, L.P., 2007. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. *Journal of Biological Chemistry*, *282*(12), pp.8969-8977.
62. Pell, J.K., Hannam, J.J. and Steinkraus, D.C., 2010. Conservation biological control using fungal entomopathogens. *BioControl*, *55*, pp.187-198.
63. Petersen, M., Armisén, D., Gibbs, R.A., Hering, L., Khila, A., Mayer, G., Richards, S., Niehuis, O. and Misof, B., 2019. Diversity and evolution of the transposable element repertoire in arthropods with particular reference to insects. *BMC Ecology and Evolution*, *19*(1), pp.1-15.
64. Platt, T., Stokwe, N.F. and Malan, A.P., 2019. Foliar application of Steinernema yirgalemense to control *Planococcus ficus*: Assessing adjuvants to improve efficacy. *South African Journal of Enology and Viticulture*, *40*(1), pp.1-7.
65. Presa-Parra, E., Hernández-Rosas, F., Bernal, J.S., Valenzuela-González, J.E., Altúzar-Molina, A. and Birke, A., 2020. Occurrence, identification, and virulence of native fungal pathogens isolated from Mexican fruit fly (Diptera: Tephritidae) larvae from soils of three cropping Systems. *Journal of Economic Entomology*, 113(3), pp.1088-1096.
66. Pryke, J.S. and Samways, M.J. 2008. Conservation of the insect assemblages of the Cape Peninsula biodiversity hotspot. *Journal of Insect Conservation*, 13, pp. 627-641.
67. Qayyum, M.A., Saeed, S., Wakil, W., Nawaz, A., Iqbal, N., Yasin, M., Chaurdhry, M.A., Bashir, M.A., Ahmed, N., Riaz, H. and Bilal, H., 2021. Diversity and correlation of entomopathogenic and associated fungi with soil factors. *Journal of King Saud University-Science*, *33*(6), p.101520.
68. Quesada-Moraga, E., Navas-Cortés, J.A., Maranhao, E.A., Ortiz-Urquiza, A. and Santiago-Álvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological research*, *111*(8), pp.947-966.
69. Rajula, J., Pittarate, S., Suwannarach, N., Kumla, J., Ptaszynska, A.A., Thungrabeab, M., Mekchay, S. and Krutmuang, P., 2021. Evaluation of Native Entomopathogenic Fungi for the Control of Fall Armyworm (Spodoptera frugiperda) in Thailand: A Sustainable Way for Eco-Friendly Agriculture. *Journal of Fungi*, *7*(12), p.1073.
70. Rajula, J., Karthi, S., Mumba, S., Pittarate, S., Thungrabeab, M. and Krutmuang, P., 2021. Current status and future prospects of entomopathogenic fungi: A potential source of biopesticides. *Recent advancement in microbial biotechnology*, pp.71-98.

71. Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A.S., Srivastav, A.L. and Kaushal, J., 2021. An extensive review on the consequences of chemical pesticides on human health and environment. *Journal of Cleaner Production*, *283*, p.124657.
72. Sánchez-Pérez, L.D.C., Barranco-Florido, J.E., Rodríguez-Navarro, S., Cervantes-Mayagoitia, J.F. and Ramos-López, M.Á., 2014. Enzymes of entomopathogenic fungi, advances and insights. *Advances in Enzyme Research*, *2*(02), pp.65-76.
73. Sandhu, S.S., Sharma, A.K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A.K. and Malhotra, S., 2012. Myco-biocontrol of insect pests: factors involved, mechanism, and regulation. *Journal of pathogens*, 2012.
74. Schnetler, A.K., Radloff, F.G. and O'Riain, M.J., 2021. Medium and large mammal conservation in the City of Cape Town: factors influencing species richness in urban nature reserves. *Urban Ecosystems*, 24(2), pp.215-232.
75. Sedighi, N., Abbasipour, H., Askary, H. and Sheikhi Gorjan, A., 2013. Effect of oil suspended conidia of *Metarhizium anisopliae* var. major on mortality of the sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). *Archives of Phytopathology and Plant Protection*, *46*(2), pp.128-140.
76. Simmons, M.T. and Cowling, R.M., 1996. Why is the Cape Peninsula so rich in plant species? An analysis of the independent diversity components. *Biodiversity & Conservation*, *5*(5), pp.551-573.
77. Singh, D., Raina, T.K. and Singh, J., 2017. Entomopathogenic fungi: An effective biocontrol agent for management of insect populations naturally. *Journal of Pharmaceutical Sciences and Research*, *9*(6), p.833.
78. Sharma, H.C., 2008. *Biotechnological approaches for pest management and ecological sustainability*. CRC Press. pp. 4-14.
79. Shennan, C., Krupnik, T.J., Baird, G., Cohen, H., Forbush, K., Lovell, R.J. and Olimpi, E.M., 2017. Organic and conventional agriculture: A useful framing?. *Annual Review of Environment and Resources*, *42*, pp.317-346.
80. Skinner, M., Parker, B.L. and Kim, J.S., 2014. Role of entomopathogenic fungi in integrated pest management. *Integrated pest management*, pp.169-191.
81. Skinner, C., Gattinger, A., Krauss, M., Krause, H.M., Mayer, J., Van Der Heijden, M.G. and Mäder, P., 2019. The impact of long-term organic farming on soil-derived greenhouse gas emissions. *Scientific reports*, *9*(1), pp.1-10.
82. Stevenson, P.C., Isman, M.B. and Belmain, S.R., 2017. Pesticidal plants in Africa: A global vision of new biological control products from local uses. *Industrial Crops and Products*, 110, pp.2-9.
83. St. Leger, R.J. and Wang, J.B., 2020. Metarhizium: Jack of all trades, master of many. *Open biology*, 10(12), p.200307.
84. Stokwe, N.F., 2016. *Efficacy of entomopathogenic nematodes and fungi as biological control agents of woolly apple aphid, Eriosoma lanigerum (Hausmann)(Hemiptera: Aphididae) under South African conditions* (Doctoral dissertation, Stellenbosch: Stellenbosch University).
85. Strasser, H., 2001. Use of hyphomycetous fungi for managing insect pests. *Fungi as biocontrol agents: progress problems and potential*, p.23.
86. Sutanto, K.D., Husain, M., Rasool, K.G., Al-Qahtani, W.H. and Aldawood, A.S., 2021. Pathogenicity of local and exotic entomopathogenic fungi isolates against different life stages of red palm weevil (*Rhynchophorus ferrugineus). Plos one*, *16*(7), p.e0255029.

87. Tal, A., 2018. Making conventional agriculture environmentally friendly: moving beyond the glorification of organic agriculture and the demonization of conventional agriculture. *Sustainability*, *10*(4), p.1078.
88. Taliyan, A., Kumar, A., Singh, R., Rana, R. and Rana, S., 2020. Pathogenicity *of Metarhizium anisopliae* on *Helicoverpa armigera* larvae instars. *International Journal of Agriculture Invention*, *5*, pp.105-109.
89. Timm, A.E. and Reineke, A., 2014. First insights into grapevine transcriptional responses as a result of vine mealybug *Planococcus ficus* feeding. *Arthropod-Plant Interactions*, *8*(6), pp.495-505.
90. Tomer, H., Blum, T., Arye, I., Faigenboim, A., Gottlieb, Y. and Ment, D., 2018. Activity of native and commercial strains of *Metarhizium* spp. against the poultry red mite *Dermanyssus gallinae under* different environmental conditions. *Veterinary parasitology*, *262*, pp.20-25.
91. Varikou, K., Birouraki, A., Bagis, N. and Kontodimas, D.C., 2010. Effect of temperature on the development and longevity of *Planococcus ficus* (Hemiptera: Pseudococcidae). *Annals of the Entomological Society of America*, *103*(6), pp.943-948.
92. Vidal, S. and Jaber, L.R., 2015. Entomopathogenic fungi as endophytes: plant–endophyte–herbivore interactions and prospects for use in biological control. *Current science*, pp.46-54.
93. Walton, V.M., 2003. *Development of an integrated pest management system for vine mealybug, Planococcus ficus (Signoret), in vineyards in the Western Cape Province, South Africa* (Doctoral dissertation, Stellenbosch: Stellenbosch University).
94. Walton, V.M. and Pringle, K.L., 2005. Developmental biology of vine mealybug, Planococcus ficus (Signoret)(Homoptera: Pseudococcidae), and its parasitoid Coccidoxenoides perminutus (Timberlake)(Hymenoptera: Encyrtidae). *African entomology*, *13*(1), pp.143-147.
95. Wyse, S.V., Beggs, J.R., Burns, B.R. and Stanley, M.C., 2015. Protecting trees at an individual level provides insufficient safeguard for urban forests. *Landscape and urban planning*, *141*, pp.112-122.
96. Xia, Y., Sahib, M.R., Amna, A., Opiyo, S.O., Zhao, Z. and Gao, Y.G., 2019. Culturable endophytic fungal communities associated with plants in organic and conventional farming systems and their effects on plant growth. *Scientific reports*, *9*(1), pp.1-10.
97. Xiao, G., Ying, S.H., Zheng, P., Wang, Z.L., Zhang, S., Xie, X.Q., Shang, Y., Leger, R.J.S., Zhao, G.P., Wang, C. and Feng, M.G., 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific reports*, *2*, p.483.
98. Zhou, Z., Wang, C. and Luo, Y., 2020. Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality. *Nature communications*, *11*(1), pp.1-10

Chapter 2

The influence of season and land use on the occurrence of entomopathogenic fungi in the Cape Peninsula region, South Africa

Abstract
Soil-borne microorganisms are crucial in maintaining the ecological functionality and stability of soils. Entomopathogenic fungi (EPF) are increasingly being recognized for their wide range of applications, including pest management and soil amendment. To investigate the ecological relationships between entomopathogenic fungal occurrence in the Cape Peninsula region and season and land use, soils were collected from randomly selected sampling sites within randomly selected nature reserves (undisturbed sites) and agricultural farms (disturbed sites) in the Cape Peninsula region. Soil samples from the same sites were collected in different seasons (winter and summer). Relative humidity and temperature play a significant role in EPF efficacy, as it can influence spore virulence. There is a link between various soil nutrients and EPF, as well as different EPF species requires different nutrients to thrive. The isolates were identified morphologically and molecularly. The associations among land use, season and fungal occurrence were determined by correspondence analysis (CA). The number of fungal strains occurring in the sites, the differences in the number of fungi found in the two seasons and in the number of fungi found in the farms (disturbed sites) and reserves (undisturbed sites) were compared using the Fischer Chi-square test. The results revealed that season positively influenced EPF occurrence, with significantly ($\chi 2 = 10.286$; $p < 0.01$) higher number of EPF isolates occurring in winter than in summer, as well as in farms than in reserves. Furthermore, a higher EPF species diversity occurred in winter compared to summer — up to 20 EPF species were isolated in winter compared with only eight species in summer. *Metarhizium* spp. were more frequently isolated from agricultural farms than in reserves. This study presents interesting insights into the influence of seasonal change on the occurrence of EPF under different land use conditions in the Cape Peninsula region, which is characterized by wet winters and dry summers.

2.1 Introduction
Soil-borne microorganisms are crucial in maintaining a soil ecological functions and stability (Garcia-Sanchez & Szakova, 2016). Many studies have revealed some fascinating roles of soil microbes, which include the breakdown of organic wastes, cycling of nutrients, forming symbiotic relationships with plants, protecting them against pests and diseases, and denaturing toxins (Pavao-Zuckerman, 2008; Garcia-Sanchez & Szakova, 2016; Jacoby *et al*., 2017). In recent years, however, among the beneficial fungi, a group stands out from the rest — entomopathogenic fungi (EPF). These ubiquitous organisms, whose natural habitats include soil, plant tissues, insects, and rhizospheres, can infect and kill insect pests (Balla *et al*., 2021).

Entomopathogenic fungi are increasingly gaining prominence for their wide range of applications in agriculture, with species in the genera *Beauveria, Clonostachys,* and *Metarhizium* being among the most extensively studied and used in the biological control of insects (Sandhu *et al*., 2012; Qiu *et al*., 2020). As the opportunities to apply these fungi for sustainable agriculture increases, the need to sufficiently understand the ecology of EPF becomes even more crucial. Gaining insights into which factors drive their occurrence could enhance the efficiency and efficacy of EPF-based insecticides and conservation biological control programs.

An understanding of the ecology of EPF is crucial not only because it provides an insight into factors that drives the occurrence of fungi within the Cape Peninsula, but it also provides the knowledge needed to optimize conservation biological control. Quesada-Moraga *et al.* (2007) argued that fungal biocontrol agents perform inconsistently within soils because of poor ecological knowledge on these fungi. So far, only a handful of studies have focused on the ecology and isolation of EPF in South Africa (Goble, 2009; Abaajeh & Nchu, 2015; Moloinyane *et al*., 2020).

Many EPF are soil-borne, and the soil environment is affected by numerous abiotic and biotic factors, which may affect fungi's occurrence, persistence, and efficacy. Abiotic factors, including organic matter content, texture, pH, cation exchange capacities, soil microflora, and moisture are among the most significant factors influencing the occurrence of fungi (Tkaczuk *et al*., 2014). Soil moisture affects the vertical movement of conidia within the soil, and lack thereof adversely affects the persistence of species such as *Beauveria* and *Metarhizium* spp. (Goble, 2009). Soil granulometry can influence EPF communities; the high porosity of sandy soils improves the movement of the fungal hyphae, while clay soils reduce conidial movement (Bueno-Pallero *et al*., 2020). Anthropogenic activities play a vital role in soil-plant-environment interactions; harmful substances introduced into the environment, such as pesticides and inorganic fertilizers, can have deleterious effects on living organisms and the balance of the ecosystem (Litwin *et al*., 2020; Yang *et al*., 2021). A few studies have reported that the occurrence of fungi is influenced by the presence of animal or plant species, for example, a high abundance of *Metarhizium, Pochonia* and *Purpureocillium* species are associated with ant nests (Angelone & Bidochka, 2018). Maltz *et al*. (2017), showed that the effects of fragmentation on plants influenced resources and function in fragmented shrubland in southern California and argued that a greater litter resource produced by diverse plant assemblages might provide a greater ecological niche space, which might support larger numbers of fungal taxa (Maltz *et al*., 2017). Previous studies by Quesada-Moraga *et al*. (2007) and Moloinyane *et al*. (2020) highlights the association of soil nutrients with the occurrence and abundance of EPF. There was a positive correlation found between EPF species and the level of soil organic matter. González-Guzmán *et al.* (2020) observed there is a key relationship with soil properties and EPF, testing various nutrients to detect which soil properties promotes EPF abudance. Moloiyane *et al.* (2020) reported optimum C, N and high Ca were present in soil samples, where *C. rosea f. catenulate* were isolated from. This corroborates the link between various soil nutrients and EPF, as well as different EPF species requires different nutrients to thrive.

The Cape Peninsula region, located within the Western Cape Province of South Africa, has a Mediterranean climate with cool, wet winters and hot, dry summers (Simmons & Cowling, 1996). The region has relatively high summer temperatures and UV-B radiation, which can negatively affect EPF persistence (Santos *et al*., 2011; Sutanto *et al*., 2022). Fires are rampant in the region. Higher temperatures may influence microbial populations and structure, their activities, and growth (Cairney & Bastias, 2007), for example, Cilliers *et al.* (2005) reported that fungal populations vary between burnt and unburnt areas and argued that the variation may be due to alterations in macronutrients and soil textures. According to Mager & Hui (2012), the fynbos soil type of the Cape Peninsula is primarily nutrient-poor and usually acid to neutral (pH from 4 – 7). However, the microbial communities in these soil types remain poorly understood. Furthermore, biotic factors such as plants and microorganisms may influence the persistence of EPF species. The Cape Peninsula is also host to unique and important plant species diversity and a large part of the Cape Floral Kingdom and is recognized as one of the most botanically diverse regions in the world (Loundou, 2008).

The disintegration of the landscape, reduction of patch size and intensive management practices, including excessive use of synthetic herbicides and fungicides could influence the occurrence of EPF in the region (Tkaczuk *et al*., 2019; Sharma *et al*., 2021). Vegetable farming activities in the region is mostly concentrated in the Phillipi Horticultural Area (PHA). Interestingly, many vegetable farmers in the PHA amend the soil with manures and composts, which could favour increased microbial activity (Battersby-Lennard & Haysom, 2012). Wakil *et al.* (2013) reported higher diversity of EPF in soil samples from forests compared to crop fields in various geographical areas of Punjab, Pakistan, with *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces lilacinus*, *Beauveria brongniartii*, *Paecilomyces chlamydosporia* and *Lecanicillium attenuatum*, being the species most recovered. Fungi tend to proliferate under humid conditions but are adversely affected by high temperatures and UV radiation and low humidity (Birnbaum *et al*., 2021). Generally, EPF is recommended for high RH conditions, as it assists fungi to complete development and spore production (Athanassiou *et al*., 2017; Abdul Qayyum *et al.,* 2021). Moreover, RH plays a significant role in EPF efficacy, as it can influence spore virulence. However, there are some EPF species which can thrive in low-level RH and have high control rates (Rumbos & Athanassiou, 2017). According to Mora *et al.* (2016), the occurrence of EPF varies during the rainy and dry seasons. More recent studies have revealed that some fungal species, such as *Metarhizium* spp. thrive or are more abundant in disturbed ecosystems than in undisturbed ecosystems (Sharma *et al*., 2018). However, only a handful of studies have focused on the ecology and isolation of EPF in South Africa (Goble, 2009; Abaajeh & Nchu, 2015; Moloinyane *et al*., 2020; Mathulwe *et al*., 2021). It is fascinating to investigate the associations of EPF occurrence in soils with seasonal change (winter and summer) and land use (disturbed and undisturbed sites) in the Cape Peninsula region. The objective of this study was to investigate the influence of two ecological factors, season, and land-use, on the occurrence of indigenous EPF, in the Cape Peninsula region, Western Cape Province, South Africa.

2.2 Methods and Materials

2.2.1 Research Design

To investigate the ecological relationships between fungal occurrence in the Cape Peninsula region, season and land use, soils were collected from randomly selected sampling sites within randomly selected nature reserves (undisturbed sites) and agricultural farms (disturbed sites) in the Cape Peninsula region, Western Cape Province, South Africa. The crops grown on the farms were Onion (*Allium cepa*), Potatoe (*Solanum tuberosum*), Broccoli (*Brassica oleracea*) and, Carrot (*Daucus carota*). The reserves had some well-known species such as, Peninsula Conebush (*Leucadendron strobilinum*), Berry Heath (*Erica baccans*), Heady Capegorse (*Aspalathus capitata*) and, Cluster Pine (*Pinus pinaster*). Soil samples from the same sites were collected in different seasons (winter and summer). A total of 90 soil samples were collected per season (winter and summer). A composite soil sample from reserve and farm sites were analysed to determine the nutrient content, pH and texture. Soil plating and insect bait methods were used to isolate the EPF in the soil samples (Figure 2). The isolates were identified morphologically and molecularly. The association among land use, season and fungal occurrence was determined with a correspondence analysis (CA). The number of fungal strains occurring in the sites, the differences in the number of fungi found in the two seasons, and the differences in the number of fungi found in the various land uses were compared using the Fischer Chi-square test.

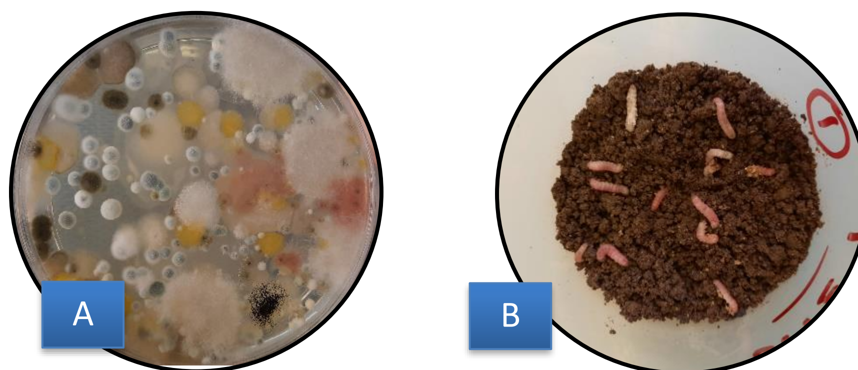

Figure 2: Fungal isolation methods: A – Soil plating method, B – Insect bait method.

2.2.2 Soil Sampling

Soil samples were collected from randomly selected reserve sites within Cape Town, and on the outskirts of the city and from vegetable farm sites within the PHA (Figure 1). Soil samples (300 g – 500 g) were collected from 10 randomly selected points, 100 – 300 m apart, on each site with a garden spade at a depth of 15 – 20 cm. The surface debris was removed, and each soil sample was transferred into a paper bag. The soil-filled paper bags were packed into a large plastic container. The soil samples were transported to the laboratory and were sieved using a 2 mm sieve. The sieved soil samples were used for fungal isolation within 24 h of collection. A total of 90 soil samples were collected per season (winter and summer). In total, five nature reserves and four farms were sampled. The geographical coordinates of each sampling point were recorded (Figure 3). This study was granted site permits by SANParks (Permit No. CRC/2021-2022/006—2021/V1) (Appendix 2) and Cape Nature (Permit No. CN32-87-17235) (Appendix 3) for access to protected sites and farmers for access to agricultural sites.

2.2.3 Soil analysis

The physicochemical properties of the soil were determined using the methods described by Moloinyane *et al.* (2020). The soil samples obtained from the different sites were air dried and sieved (2 mm sieve) prior to tests. The pH and total P, K, Ca, Mg analyses were based on The Non-Affiliated Soil Analyses Work Committee (1990) and Campbell & Plank (1998) with slight modifications. Meanwhile, optical C and N were determined using total combustion using a LecoTruspec C/N analyzer.

2.2.4 Rainfall data

The rainfall data (mm) for the different seasons were obtained from weather stations located within of closest to the study sites coutersy of the South African Weather Services.

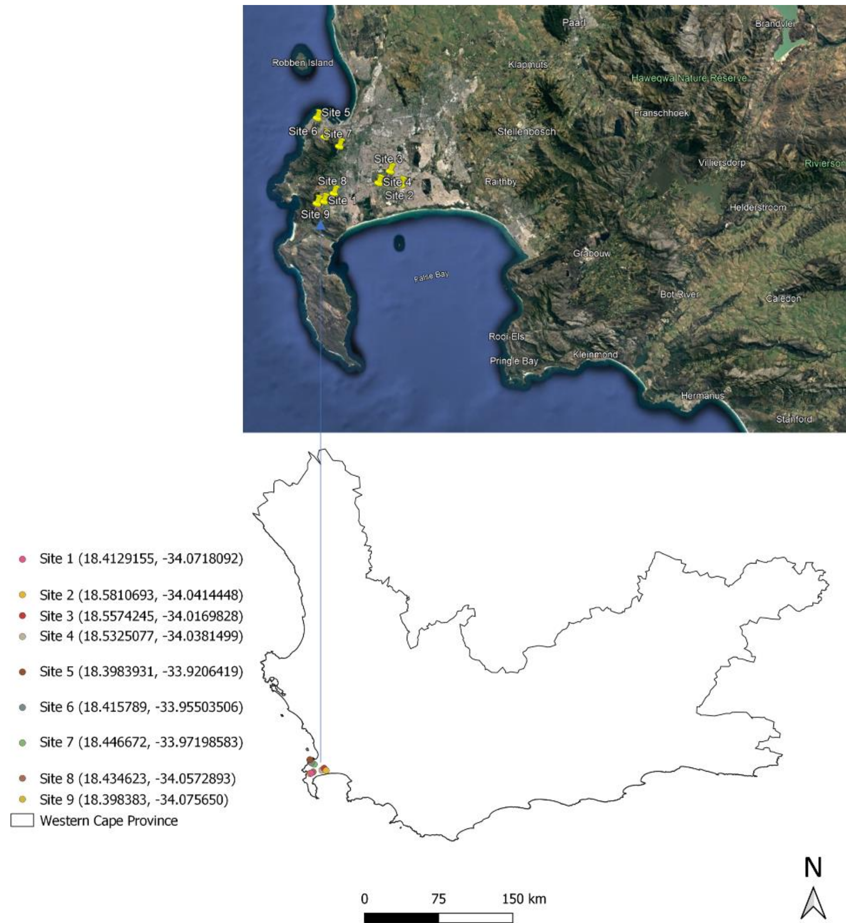

Figure 3: A map of the sampled sites in the Cape Peninsula region was created using QGIS and Google Earth software.

2.2.5 Insect bait method

The protocols described by Moloinyane *et al.* (2020) were used to isolate the fungal strains. Fifth instar larvae of *Cydia pomonella* (Codling Moth) sourced from a laboratory culture held at Entomon PTY. LTD. (Stellenbosch, Western Cape, South Africa) was used.

One hundred grams of each soil sample was sieved using a metal sieve with a mesh size of 4 mm, into a plastic container and mixed with sterile distilled water until damp. Ten 5th instar larvae were placed in the plastic container on the surface of each soil sample, closed with a perforated plastic lid and placed in a dark room. Containers were momentarily inverted and returned to an upright position once per day for the first week to increase interaction between soil particles and insects. Containers were inspected for dead larvae every three to four days for three weeks. Dead larvae were surface sterilized with 70% ethanol for 30 s, rinsed with sterile distilled water for one minute, placed on dampened filter paper, and incubated at 25 °C. An inoculation loop was used to scrap off conidia from fungi which sporulated from dead larvae and transferred onto a half-strength selective medium of Potato dextrose agar (PDA) (Sigma-Aldrich PTY.LTD., South Africa) (19.5 g/L) supplemented with 0.04 g/L streptomycin and 0.02 g/L ampicillin (Moloinyane *et al*., 2020).

2.2.6 Soil plating method

Fungal strains were also isolated using the plating soil method. A mixture of 100 mg soil sample, 10 ml sterile distilled water and 0.05% (v/v) Tween 80 (Sigma-Aldrich PTY.LTD., South Africa) was vigorously mixed for 5 minutes to form a suspension. One milliliter of the suspension was plated onto half-strength PDA (19.5 g/L) supplemented with 0.04 g /L streptomycin and 0.02 g/L ampicillin per soil sample in a 9 cm Petri dish and spread out using a cell spreader. Each soil sample had three replicates (Moloinyane *et al*., 2020).

2.2.7 Isolation of fungal DNA and multi-locus sequence analysis

To acquire single spore isolates the technique described by Ho & Ko (1997), was followed. Conidia from the cultures of each fungal isolate were collected and then surface cultured on half-strength PDA at 25 ± 2 °C; 70 ± 2% relative humidity (RH) for 2 – 3 months (Figure 3). Once fungal cultures were successfully isolated, the isolates were prepared for DNA extraction. Fungal conidia were scraped into 2 ml Eppendorf tubes using a sterilized micro spatula. The spores were mixed into 1.5 ml nutrient broth (13 g/L) supplemented with 0.02 g/L ampicillin and 0.04 g/L streptomycin. Clean fungal cultures were incubated 24h for spores to multiply. The fungal isolates were identified based on morphology using light microscopy techniques and cultivation characteristics on agar plates as described in the literature (Nelson *et al*., 1983; Bischoff *et al*., 2009; Kepler *et al*., 2014; Anwar *et al*., 2018).

The fungal cell mass was pelleted by centrifugation at 10 000 rpm for 5 minutes. The cell pellet was used for DNA isolation using the *Quick*-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, California, USA) as per the manufacturer's instructions. DNA concentration and purity were determined using a Genova Nano Micro-Volume Spectrophotometer (Jenway, Staffordshire, United Kingdom) prior to utilization in polymerase chain reaction (PCR) setup. For the multi-locus sequence analysis, three genes (DNA topoisomerase I, beta-tubulin II, translation elongation factor 1 alpha), and the internal transcribed spacer (ITS) region were amplified using the primers listed in Table 2.

Table 1: PCR primers used in this study for the amplification of three target genes and the internal transcribed spacer (ITS) region.

DNA barcoding marker	Acronym	Primers	Reference
Internal transcribed spacer	ITS	ITS1 5'-TCCGTAGGTGAACCTGCGG-3' ITS4 5'-TCCTCCGCTTATTGATATGC-3'	White *et al*., (1990)
DNA topoisomerase I	TOP1	TOP1-501F 5'-ACTGCCAAGGTTTTCCGTACHTACAACGC-3' TOP1-501R 5'-CCAGTCCTCGTCAACWGACTTRATRGCCCA-3'	Stielow *et al*., (2015)
Translation elongation factor I alpha	TEF1	EF1-983F 5'-GCYCCYGGHCAYCGTGAYTTYAT-3' EF1-1567R 5'-ACHGTRCCRATACCACCRATCTT-5'	Stielow *et al*., (2015)
β-tubulin II	BTUB	Btub2Fd 5'-GTBCACCTYCARACCGGYCARTG-3' Btub4Rd 5'-CCRGAYTGRCCRAARACRAAGTTGTC-3'	Woudenberg *et al*., (2009)

Each PCR reaction contained the following: 12.5 μL MyTaq™ HS Mix (Bioline, Meridian Bioscience, Tennessee, USA), 1 μL 10 μM forward and reverse primers, 1 μL DNA, 0.8 μL dimethylsulfoxide (DMSO), 8.7 μL molecular grade distilled water (final volume of 25 μL). Touchdown PCR was performed, which consisted of two phases. Phase 1: Initial denaturation at 95 °C for 5 min, followed by ten cycles of denaturation at 95 °C for 45 s, annealing at 68 °C with a decrease in 1°C for each cycle (68-58 °C), and extension at 72 °C for 1 min. Phase 2: 30 cycles of denaturation (95 °C for 45 s), annealing at 58 °C for 45 s and 60 °C for 45 s, followed by extension at 72 °C for 1 min. A final elongation step of 5 min at 72 °C completed the PCR process. Amplicons generated were analysed using a 1 % (w/v) agarose gel in 1xTAE buffer. Ethidium bromide (8 μg/mL final concentration) was added before to running the gel. Amplicons were visualised using a Bio-Rad Gel Doc XR+ system (Bio-Rad, Johannesburg, South Africa), followed by purification using the MSB Spin PCRapace PCR purification kit (Invitek) according to the manufacturer's instructions. Purified amplicons were submitted to the Central Analytical Facility (CAF) at Stellenbosch University for Sanger sequencing.

2.2.8 Sequence processing and analysis

Sequence data were analysed using SnapGene® Viewer 6.1.1 (Snapgene Software, www.snapgene.com) and consensus sequences prepared from the forward and reverse sequences. Consensus sequences were submitted to BLAST to identify the nearest hit for each sequence and were used for phylogenetic analysis. Sequences were aligned with that of known fungal sequences obtained from the GenBank database and analysed using the neighbour-joining (Saitou and Nei, 1987; test of phylogeny: 1000 bootstrap resamplings), maximum parsimony (Takahashi and Nei, 2000), and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA X (Appendex 1) (Kumar et al., 2018). All sequences were submitted to GenBank, and accession numbers were assigned (Table 2).

2.2.9 Statistical Analysis

The associations among land use, season and fungal occurrence were determined with correspondence analyses (CA) using PAST (Version 3.22) (Hammer *et al*., 2001). The number of fungal strains occurring in the sites, the differences in the number of fungi found in the two seasons, and the differences in the number of fungi found in disturbed and undisturbed sites were compared using the Fischer Chi-square test in PAST (Hammer *et al*., 2001). The pH levels and soil micronutrient and macronutrient content were compared using the Kruskall-Wallis test followed by a posthoc Man-Whitney test.

2.3 Results

2.3.1 Soil physicochemical properties and seasonal rainfall

The results showed that macronutrients, P, Ca and N (NO_3) were significantly higher (dF = 4; $\chi 2$ = 9,85; p < 0.007) in farms than in the reserves (Table 2). However, N (NH_4) was significantly higher in the reserve than in the farm sites (dF = 7,32; $\chi 2$ = 10,36; p < 0.01). K and Mg did not significantly vary between reserves and farms. Among the micronutrients (B, Cu, Fe and Mn), only B contents varied significantly (dF = 6,3; $\chi 2$ = 9,41; p < 0.02) between reserve and farm sites (Table 3). Generally, season had minimal

effect on the nutrient contents. The pH levels of the reserves' soils were lower that those from the farms, and was significantly different (dF = 6,7; $\chi2$ = 12,88; $p < 0.004$). All the study sites had sandy soils. The rainfall recorded during the study period were consistently higher during the winter season. The average winter rainfall (mm) ranged from 2.65 ± 0.99 mm to 7.68 ± 2,23 mm, while the summer average ranged from 0.88 ± 0.71 mm to 2.43 ± 1.16 mm (Table 4)

2.3.2 Winter EPF occurrence

A total of 54 EPF isolates were collected from the soil during the winter months. The following species were isolated: *Aspergillus* spp., *Clonostachys* spp., *Fusarium* spp., *Metarhizium* spp., *Penicilium* spp., *Pochonia* spp., *Talaromyces* spp., *Trichoderma* spp., and *Umbelopsis* spp. The results showed that over 70% of the isolates collected were from the farms in the Cape Peninsula, whereas 30% of the total isolates came from the reserves ($\chi2$ = 21.33; $p < 0.01$) (Table 5A). It is worth noting that the macronutrients, P, K, Mg, Ca and N (NO_3) were consistently higher in farms than in the reserves during the winter and summer months (Table 2). The results also showed that over 55% were isolated using insect-baiting ($\chi2$ = 30.25; $p < 0.01$) compared with the soil plating method. It was observed that Site 4, farm site, had the greatest number of isolates collected (12), while Site 8, forest site, had the least number of isolates collected (2).

Table 2: The macronutrient (mean ± SE) in composite soil samples obtained from reserves and farms in the Cape Peninsula region, South Africa.

Season	Sites	P mg/kg	K mg/kg	Ca mg/kg	Mg mg/kg	NO3 mg/kg	NH_4 mg/kg	C %
Winter	Farms	505.35 ± 194.47b	128.88 ± 71.94a	2386.82 ± 576.29b	270.48 ± 156.73a	5.23 ± 1.96a	4.58 ± 0.95a	1.99 ± 0.38a
	Reserves	45.54 ± 21.52a	82.73 ± 19.46a	657.35 ± 88.03a	141.86 ± 10.74a	1.47 ± 0.59a	16.6 ± 2.9b	2.93 ± 0.37a
Summer	Farms	514.2 ± 203.61b	205.25 ± 109.41a	2274.69 ± 486.10b	285.55 ± 124.75a	4.03 ± 0.58b	4.35 ± 1.7a	2.12 ± 0.71a
	Reserves	29.1 ± 7.98a	80.73 ± 19.91a	812.2 ± 221.18a	152.04 ± 14.87a	1.61 ± 0.59a	11.86 ±2.94b	2.82 ± 0.53a

Means with the lowercase letter 'a' are not significantly different between reserves and farms for each season following Mann-Whitney's test at P < 0.05.

Table 3: The micronutrient and pH level (mean ± SE) in composite soil samples obtained from reserves and farms in the Cape Peninsula region, South Africa.

Season	Sites	pH KCl	Cu mg/kg	Zn mg/kg	Mn mg/kg	B mg/kg	Fe mg/kg
Winter	Farms	7.15 ± 0.42a	2.43 ± 0.61a	28.53 ± 7.0a	12.43 ± 2.86a	1.11 ± 0.41b	60.3 ± 18.44a
	Reserves	4.46 ± 0.19a	2.86 ± 1.62a	11.34 ± 5.28a	23.12 ± 10.34a	0.32 ± 0.03a	111.7 ± 45.42a
Summer	Farms	7.35 ± 0.51a	1.88 ± 0.23a	28.53 ± 8.0a	15.34 ± 2.98a	1.04 ±0.41b	53.78 ± 19.55a
	Reserves	4.7 ± 0.31a	1.14 ± 0.5a	10.36 ± 4.27a	27.46 ± 16.18a	0.31 ± 0.05a	106.5 ± 39.39a

Means with the lowercase letter 'a' are not significantly different between reserves and farms for each season following Mann-Whitney's test at P < 0.05.

Table 4: Rainfall data (mean ± SE) recorded during the winter and summer months by weather stations within or near the study sites in the Cape Peninsula region, South Africa.

Weather Stations	Collection Sites	Land use	Co-ordinates	Average Winter rainfall (mm)	Average Summer rainfall (mm)
Silvermine Nature Reserve	Site 9	Reserve	-34.0860 18.4200	5,71 ± 2,99	0,88 ± 0,71
Groot Constantia Wine Estate	Site 1 & 8	Farm & Reserve	-34.0280 18.4190	3,14 ± 2,07	1,53 ± 1,09
Table Mountain House	Site 6	Reserve	-33.9750 18.4010	6,99 ± 1,91	2,43 ± 1,16
Moltento Reservoir	Site 5	Reserve	-33.9370 18.4100	4,32 ± 1,53	1,88 ± 0,87
Kirstenbosch	Site 7	Reserve	-33.9860 18.4300	7,63 ± 2,23	2,41 ± 1,08
CT-Aws	Site 2 - 4	Farms	-33.9780 18.6000	2,65 ± 0,99	1,68 ± 0,74

Table 5A: Soil samples collected during winter at various sites, within the Cape Peninsula, fungal isolates obtained. Genbank accession numbers assigned, and fungal species identification.

No. of Isolate	Source of Isolation	Location	Land-use	GPS Co-ordinates	GenBank Accession numbers	Fungal Species Identification
1	Soil	Site 1	Agriculture	-32.084874 18.423375	OP497867	*Penicillium simplicissimum*
2	Soil	Site 1	Agriculture	-32.084874 18.423376	OP497868	*Talaromyces cellulolyticus*
3	Soil	Site 1	Agriculture	-34.073828 18.423021	OP497869	*Clonostachys rosea*
4	Soil	Site 1	Agriculture	-34.073828 18.423021	OP497870	*Clonostachys rosea*
5	Soil	Site 1	Agriculture	-34.073663 18.424859	OP497871	*Metarhizium anisopliae*
6	Soil	Site 1	Agriculture	-34.074971 18.424566	OP497872	*Penicillium restrictum*
7	Soil	Site 1	Agriculture	-34.074971 18.424566		*Fusarium* sp.
8	Soil	Site 1	Agriculture	-34.074971 18.424566	OP497873	*Penicillium restrictum*
9	Insect	Site 2	Agriculture	-34.040717 18.580460	OP497874	*Metarhizium anisopliae*
10	Soil	Site 2	Agriculture	-34.039208 18.580647	OP497875	*Metarhizium anisopliae*
11	Soil	Site 2	Agriculture	-34.039208 18.580648	OP497876	*Fusarium oxysporum*
12	Soil	Site 2	Agriculture	-34.039208 18.580649	OP497877	*Pochonia chlamydosporia*
13	Insect	Site 2	Agriculture	-34.039208 18.580650	OP497878	*Metarhizium guizhouense*
14	Insect	Site 2	Agriculture	-34.039208 18.580651	OP497879	*Metarhizium anisopliae*
15	Soil	Site 2	Agriculture	34.039934 18.581148		*Metarhizium* sp.
16	Soil	Site 2	Agriculture	34.039934 18.581148	OP497880	*Talaromyces cellulolyticus*
17	Insect	Site 2	Agriculture	34.039934 18.581148	OP497881	*Metarhizium guizhouense*
18	Insect	Site 2	Agriculture	-34.041544 18.582249	OP497882	*Metarhizium brunneum*

19	Insect	Site 3	Agriculture	-34.018103 18.557428	OP497883	*Metarhizium robertsii*
20	Insect	Site 3	Agriculture	-34.018103 18.557428	OP497884	*Metarhizium anisopliae*
21	Insect	Site 3	Agriculture	-34.017793 18.557612	OP497885	*Fusarium oxysporum*
22	Insect	Site 3	Agriculture	-34.0173701 18.557090	OP497886	*Metarhizium guizhouense*
23	Insect	Site 3	Agriculture	-34.016975 18.558143	OP497887	*Metarhizium robertsii*
24	Insect	Site 3	Agriculture	-34.016975 18.558143	OP497888	*Metarhizium anisopliae*
25	Insect	Site 3	Agriculture	-34.017303 18.558631	OP497889	*Metarhizium anisopliae*
26	Insect	Site 3	Agriculture	-34.017303 18.558631	OP497890	*Talaromyces aculeatus*
27	Insect	Site 3	Agriculture	-34.017410 18.558708	OP497891	*Metarhizium robertsii*
28	Insect	Site 4	Agriculture	-34.037853 18.532446	OP497892	*Metarhizium anisopliae*
29	Insect	Site 4	Agriculture	-34.037853 18.532447	OP497893	*Fusarium foetens*
30	Insect	Site 4	Agriculture	-34.037836 18.532918	OP497894	*Metarhizium guizhouense*
31	Insect	Site 4	Agriculture	-34.037807 18.533138	OP497895	*Metarhizium brunneum*
32	Insect	Site 4	Agriculture	-34.037947 18.532915	OP497896	*Metarhizium guizhouense*
33	Insect	Site 4	Agriculture	-34.037967 18.533076	OP497897	*Metarhizium brunneum*
34	Insect	Site 4	Agriculture	-34.037967 18.533077	OP497898	*Talaromyces cellulolyticus*
35	Insect	Site 4	Agriculture	-34.038129 18.533305	OP497899	*Metarhizium guizhouense*
36	Insect	Site 4	Agriculture	-34.038129 18.533306	OP497900	*Metarhizium robertsii*
37	Insect	Site 4	Agriculture	-34.038104 18.532756	OP497901	*Metarhizium robertsii*
38	Insect	Site 4	Agriculture	-34.038167 18.532897	OP497902	*Metarhizium anisopliae*
39	Insect	Site 4	Agriculture	-34.038167 18.532898	OP497903	*Trichoderma virens*

40	Soil	Site 5	Reserve	-33.938023 18.393001	OP497904	*Aspergillus* sp.
41	Soil	Site 5	Reserve	-33.936861 18.394758	OP497905	*Penicillium chalabudae*
42	Soil	Site 5	Reserve	-33.936861 18.394759	OP497906	*Penicillium philippinense*
43	Insect	Site 6	Reserve	-33.955736 18.41610	OP497907	*Fusarium oxysporum*
44	Soil	Site 6	Reserve	-33.954718 18.415303	OP497908	*Fusarium oxysporum*
45	Soil	Site 6	Reserve	-33.954718 18.415304	OP497909	*Fusarium oxysporum*
46	Insect	Site 7	Reserve	-33.97595 18.447537	OP497910	*Metarhizium* sp.
47	Soil	Site 7	Reserve	-33.970514 18.447378	OP497911	*Talaromyces stipitatus*
48	Soil	Site 7	Reserve	-33.970514 18.447379	OP497912	*Talaromyces stipitatus*
49	Soil	Site 7	Reserve	-33.970201 18.447417	OP497913	*Fusarium oxysporum*
50	Soil	Site 8	Reserve	-34.057600 18.434654	OP497914	*Umbelopsis ramanniana*
51	Soil	Site 8	Reserve	-34.057600 18.434655		*Micromucor* sp.
52	Insect	Site 9	Reserve	-34.074747 18.399280	OP497915	*Metarhizium guizhouense*
53	Insect	Site 9	Reserve	-34.074747 18.399281	OP497916	*Penicillium sanguifluum*
54	Insect	Site 9	Reserve	-34.074747 18.399282	OP497917	*Metarhizium pinghaense*

2.3.2 Summer EPF occurrence

A total of 42 isolates were collected in the summer. Some of the species found belonged to the genera, *Clonostachys*, *Fusarium*, *Metarhizium*, and *Trichoderma*. Sixty-nine percent of the total isolates was isolated from farm sites. While the remaining 31% were isolated from reserves in the Cape Peninsula ($\chi2 = 12.19$; $p < 0.01$) (Table 2B). The results showed that 67% were isolated from soil using the soil-plating method compared with 33% for the insect-baiting method ($\chi2 = 20.571$; $p < 0.01$). However, farm sites 2 and 3 yielded the highest number of isolates, while site 9 had the least number of isolates.

Table 5B: Soil samples collected during summer at various site, within the Cape Peninsula, fungal isolates obtained. Genbank accession numbers assigned, and fungal species identification.

No. of Isolate	Source of Isolation	Location	Land-use	GPS Co-ordinates	GenBank Accession Numbers	Fungal Species Identification
55	Soil	Site 1	Agriculture	-32.074404 18.423000		*Fusarium* sp.
56	Soil	Site 1	Agriculture	-34.073828 18.423021	OP497918	*Clonostachys rosea*
57	Soil	Site 1	Agriculture	-34.073487 18.423816		*Clonostachys* sp.
58	Soil	Site 1	Agriculture	-34.074971 18.424566	OP497919	*Fusarium foetens*
59	Soil	Site 2	Agriculture	34.039934 18.581148	OP497920	*Fusarium solani*
60	Insect	Site 2	Agriculture	34.039934 18.581148	OP497921	*Metarhizium guizhouense*
61	Soil	Site 2	Agriculture	-34.040337 18.581483	OP497922	*Clonostachys rosea*
62	Insect	Site 2	Agriculture	-34.041544 18.582249	OP497923	*Metarhizium guizhouense*
63	Insect	Site 2	Agriculture	-34.042082 18.582677	OP497924	*Fusarium oxsporum*
64	Soil	Site 2	Agriculture	-34.042526 18.582969	OP497925	*Fusarium oxysporum*
65	Soil	Site 2	Agriculture	-34.042997 18.583101	OP497926	*Clonostachys rosea*
66	Insect	Site 2	Agriculture	-34.042997 18.583102	OP497927	*Metarhizium brunneum*
67	Soil	Site 2	Agriculture	-34.04337 18.583209		*Fusarium* sp.
68	Insect	Site 2	Agriculture	-34.04337 18.583210	OP497928	*Metarhizium anisopliae*
69	Insect	Site 3	Agriculture	34.018296 18.557834	OP497929	*Metarhizium guizhouense*
70	Soil	Site 3	Agriculture	34.018103 18.557645	OP497930	*Fusarium solani*
71	Soil	Site 3	Agriculture	34.018103 18.557646	OP497931	*Fusarium solani*
72	Insect	Site 3	Agriculture	-34.017793 18.557612	OP497932	*Metarhizium guizhouense*

73	Insect	Site 3	Agriculture	-34.017680 18.557096	OP497933	*Metarhizium guizhouense*
74	Insect	Site 3	Agriculture	-34.017301 18.557090	OP497934	*Metarhizium brunneum*
75	Soil	Site 3	Agriculture	-34.016975 18.558143	OP497935	*Fusarium oxysporum*
76	Insect	Site 3	Agriculture	-34.016975 18.558144	OP497936	*Metarhizium anisopliae*
77	Insect	Site 3	Agriculture	-34.016980 18.558523	OP497937	*Metarhizium robertsii*
78	Soil	Site 3	Agriculture	-34.01735 18.558631	OP497938	*Fusarium oxysporum*
79	Soil	Site 4	Agriculture	-34.037990 18.523520	OP497939	*Fusarium solani*
80	Soil	Site 4	Agriculture	-34.037990 18.523521	OP497940	*Fusarium oxysporum*
81	Insect	Site 4	Agriculture	-34.037990 18.523522	OP497941	*Metarhizium robertsii*
82	Soil	Site 4	Agriculture	-34.037817 18.533344	OP497942	*Fusarium oxysporum*
83	Insect	Site 4	Agriculture	-34.037817 18.533345	OP497943	*Metarhizium anisopliae*
84	Soil	Site 5	Reserve	-33.939982 18.393157	OP497944	*Fusarium oxysporum*
85	Soil	Site 5	Reserve	-33.938023 18.393001	OP497945	*Trichoderma simplex*
86	Soil	Site 6	Reserve	-33.955941 18.416378	OP497946	*Fusarium oxysporum*
87	Soil	Site 6	Reserve	-34.955899 18.416378	OP497947	*Fusarium oxysporum*
88	Soil	Site 6	Reserve	-34.955899 18.416379	OP497948	*Trichoderma simplex*
89	Soil	Site 7	Reserve	-34.979203 18.44781	OP497949	*Fusarium oxysporum*
90	Soil	Site 7	Reserve	-34.971349 18.447938	OP497950	*Fusarium oxysporum*
91	Insect	Site 7	Reserve	-34.971349 18.447939	OP497951	*Metarhizium brunneum*
92	Soil	Site 7	Reserve	-34.971526 18.447749	OP497952	*Fusarium oxysporum*
93	Soil	Site 8	Reserve	-34.057600 18.434655	OP497953	*Fusarium oxysporum*

94	Soil	Site 8	Reserve	-33.57436 18.434972	OP497954	*Fusarium oxysporum*
95	Soil	Site 8	Reserve	-33.056362 18.433446	OP497955	*Fusarium oxysporum*
96	Soil	Site 9	Reserve	-34.073296 18.398715	OP497956	*Fusarium oxysporum*

The relationship between the different land uses and the occurrence of the isolated fungal strains is presented in Figure 4. Strains of *Clonostachys* spp., *Aspergillus* spp., *Metarhizium* spp., and *Pochonia* spp., mostly occurred in the agriculture sites, which were associated with higher levels of macronutrients (Table 2). In the reserve sites, a wide variety of fungal species were isolated, including, *Fusarium* spp., *Penicillium* spp., *Talaromyces* spp., *Trichoderma* spp., and *Umbelopsis* spp.

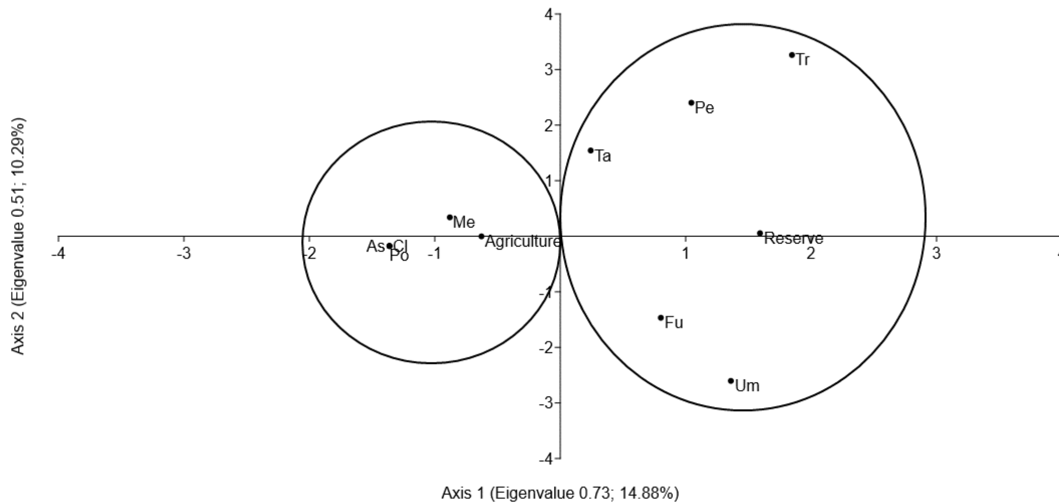

***Figure 4: Corresponding Analysis of the relationship between the occurrence of fungal species and land-use diversity. Fungal species isolated for the above figure include:* As – *Aspergillius* spp., Cl – *Clonostachys* spp., Fu – *Fusarium* spp., Me – *Metarhizium* spp., Pe – *Penicillium* spp., Po – *Pochnia* spp., Ta – *Talaromyces* spp., Tr – *Trichoderma* spp. *and* Um – *Umberlopsis* spp.**

Correspondence analysis revealed that winter correlated with *Metarhizium* spp. occurrence and *Fusarium* spp. were more abundant in summer (Figure 5) within the Cape Peninsula region. The most predominant EPF genus found in this study was *Metarhizium.* Overall, a significantly higher number of EPF species was isolated in winter (20) than in summer (8), $\chi2 = 10.29$; $p < 0.01$ (Table 6). As expected, higher rainfall data were recorded in winter than in summer (Table 4). Significantly ($p < 0.01$) higher numbers of EPF isolates occurred in the farms (39) than in the reserves (15) during the winter and summer (29 and 13, respectively) seasons (Table 6).

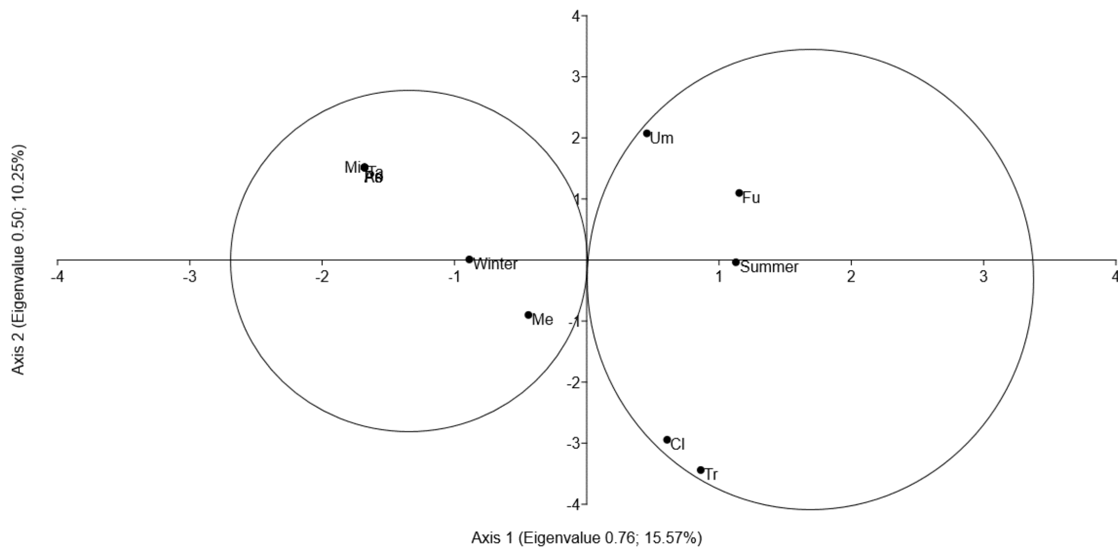

Figure 5: Corresponding analysis of the relationship between season and the occurrence of fungal isolates. Fungal species isolated for the above figure include: **As– *Aspergillius* spp., Cl – *Clonostachys* spp., Fu – *Fusarium* spp., Me – *Metarhizium* spp., Mi – *Micromucor* spp., Pe – *Penicillium* spp., Ta – *Talaromyces* spp., Tr – *Trichoderma* spp. *and* Um – *Umberlopsis* spp.**

Table 6: Comparing the effects of land use and season, on the number of isolated fungal strains collected from the Cape Peninsula region using soil plating and insect-baiting methods.

Season	Sites	Fungal isolation method		Total no. of fungal isolates	Total no. of different fungal species isolated
		Soil-plating	Insect-baiting		
Winter	Reserves	10aA	5aB	**15a**	**20a**
	Farms	12aA	27bB	**39b**	
Summer	Reserves	12aB	1aA	**13a**	**8b**
	Farms	16aA	13bA	**29b**	

Means with the same lowercase letters are not significantly different between reserves and farms for each season following Fischer Chi-square test at $P < 0.05$. Means with the same uppercase letters are not significantly different between soil and insect bait isolation methods.

2.4 Discussion

Understanding the ecology of EPF is crucial in revealing insight into factors that drives the occurrence of fungi, as well as providing knowledge to conserve these species, isolate virulent strains, and promote biological control (Quesada- Moraga et al., 2007). Several studies have demonstrated that EPF can colonize plant tissues symbiotically, and these non-pesticidal activities are crucial for fungal evolution and survival without the presence of an insect host (Dara, 2019; Bamisile *et al*., 2022).

This study revealed that seasonal change influence EPF occurrence, with a higher number of EPF isolates occurring in winter. The winter season yielded a higher diversity of fungal species — 20 different EPF species were isolated compared with only 8 species in summer (Table 5A and B and 6). In a previous study, Manfrino *et al.* (2014) showed that lower temperatures and humid conditions tend to be more propitious for fungal occurrence. Tkaczuk *et al.* (2014) also reported that fungal growth is more diverse in rainy seasons than in summer. During this study, a higher average rainfall was recorded in winter than in summer. Given that the Cape Peninsula has wet winter months, it is probable that the higher water availability in winter is conducive to the occurrence of EPF. Furthermore, since all the current study sites had sandy soil, which is characterised by high percolation and low water holding capacity, it is reasonable to expect that the influence of rainfall is important factor to EPF occurrence.

Although not investigated in this study, the frequent fire occurrences in the fynbos biome, especially in summer, can lead to higher soil temperatures, negatively affecting microbial occurrences and populations, including EPF (Cilliers *et al*., 2005; Moroenyane *et al*., 2016). This argument corroborates the results obtained in this study, where less diverse fungal species were found, especially in these fynbos-rich reserves where fires are more prevalent. Many of the studied reserve sites are in Table Mountain National Park, which is a hotspot for seasonal fires (Dubay, 2018). In addition, because various fungal species prefer different plant species for root colonization, seasonal fires can destroy many plant species, resulting in the loss of fungal species (Forsyth & Van Wilgen, 2008; Maulana *et al*., 2021).

Metarhizium isolates can remarkably adapt to various habitats including degraded soils. *Metarhizium* spp. are highly resilient species which can persist in harsh habitats, making them versatile (St. Leger & Wang, 2020). Some *Metarhizium* species obtained in this study are *M. robertsii, M. brunneum, M. guizhouense, and M. pinghaense*. There was a direct correlation between *Metarhizium* species occurrence and the winter season. Kryukov *et al.* (2017) reported that *Metarhizium* communities differ among locations and habitat distribution, and some *Metarhizium* species prefer colder temperatures.

Pfordt *et al.* (2020) conducted a study during the dry season, where temperatures are generally higher, and showed that some *Fusarium* spp. were more abundant than other *Fusarium* spp. In the current study, *F. foetens*, *F. oxysporum,* and *F. solani* were isolated in the summer. Interestingly, some of the other effective EPF species, such as *Clonostachys* spp. and *Trichoderma* spp. occurred more in the summer season. According to Morandi *et al*. (2008) microclimate factors such as relative humidity (RH) and temperature, can affect the persistence of *Clonostachys* spp. The warmer temperatures do not affect fungal species and strains equally; some are more adapted to warmer temperatures and less moisture (Samish *et al*., 2014; Litwin *et al.,* 2021). According to Hawkes *et al.* (2011), fungal species may be reliant on water for metabolic activity, and reduced water availability hot, dry summer in the Cape Peninsula region may be adversely, resulting in a decline in EPF occurrence. These varied rainfall patterns between winter and summer, play a significant role in the range of species occurrence and enables specific fungal species to reveal their resilience and dominance (Hawkes *et al*, 2011; Shi *et al*, 2020).

In the current study, the results show that land use influences EPF occurrence. For example, *Metarhizium* spp. occurred more abundantly in the agricultural sites compared to reserves (Table 5A and B). Many studies have reported that *Metarhizium* spp. are more abundant and diverse in agricultural lands because of several factors, such as tillage, mycorrhizal relationships, as well as arthropod populations (Fernández-Bravo *et al.,* 2021). *Metarhizium* spp. become more virulent when they can colonize insect hosts and reproduce. Soil structure can also contribute to the occurrence of EPF (Fernández-Bravo *et al.,* 2021), especially organic farming practices have been found to have a positive correlation with the abundance of EPF species (Qayyum *et al*., 2021). Furthermore, the availability of high concentrations of some soil macro- and micro-nutrients and organic matter in farms could increase the persistence of some EPF species (Frac *et al*., 2018). Interestingly, the farm sites in this study had significantly higher levels of macronutrients. Moloinyane *et al.* (2020) observed that potassium significantly improved the occurrence of *Metarhizium* spp. High levels of macronutrients, such as Ca and Mg, in agricultural soils can increase the pH level. In the current study, the farm sites had moderate or neutral pH levels ranging from 7.15-7.35, which could be more conducive for EPF occurrence (Hallouti *et al*., 2020). Bidochka (1998) isolated EPF from sites across Ontario, Canada and postulated that the occurrence of *M. anisopliae* and *B. bassiana* was not related to soil pH. While *Metarhizium* spp. were more prevalent in farms, interestingly, *Trichoderma* spp. were more prevalent in the nature reserve sites in this study. *Trichoderma* spp. have become popular in recent years as being effective soil-rehabilitation specialist. *Trichoderma* spp. are highly interactive in soil, root and foliar environments, where they produce antibiotics (Harman *et al*., 2004).

Generally, there was a significant difference between fungal isolates found on farms compared to reserves. About 71 % of the fungi were isolated from farms, whereas 29 % were isolated from reserve soils (Table 5 A and B & 6). According to Xia *et al.* (2019) land use plays a fundamental role in fungal occurrence. Besides agricultural land (disturbed land) offering favourable conditions for EPF to flourish, fire incidences are more frequent in the reserves in the Cape Peninsula region.

While there were significant differences between soil plating and insect bait methods when the number of isolates for farm and reserves were compared separately, no pattern was observed. There were no significant differences in the total number of EPF isolates collected using insect baiting or soil plating methods. In total, 50 isolates were obtained using the soil plating method, and 46 were isolated using the insect baiting method (Tables 5A and B & 6). Studies performed by Tuininga *et al.* (2014) and Bueno-Pallero *et al.* (2020) reported that their significance may not be measured accurately. Hence, it is difficult to determine which might be the better isolation method. However, using both methods optimises the isolation of EPF.

2.5 Conclusion

In conclusion, this study revealed that (i) more EPF strains occurred in agricultural land (disturbed land) than in reserves (undisturbed soil) and (ii) more EPF strains occurred in winter than in summer in the Cape Peninsula region. Vegetable farmers can use this information to optimize conservation biological control of pests. Although this study was limited to the Cape Peninsula region, this is the first and most comprehensive study of EPF occurrence and its relationship with land use and seasonal change in the region. However, it is recommended that the study sites be expanded to cover more reserves and agricultural lands in the Cape floristic region. The next chapter focuses on the pathogenicity of the isolated EPF strains and evauating the effect of insect oil-based formulation on conidial activity against *P. ficus in vitro*.

2.6 References

1. Abaajeh, A.R. and Nchu, F., 2015. Isolation and pathogenicity of some South African entomopathogenic fungi (Ascomycota) against eggs and larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). *Biocontrol Science and Technology*, *25*(7), pp.828-842.
2. Abdul Qayyum, M., Bilal, H., Ali, H., Raza, H. and Wajid, M., 2021. Factors affecting the epizootics of entomopathogenic fungi-A review. *Journal of Bioresource Management*, 8(4), p.5.
3. Angelone, S. and Bidochka, M.J., 2018. Diversity and abundance of entomopathogenic fungi at ant colonies. *Journal of invertebrate pathology*, *156*, pp.73-76.
4. Anwar, W., Ali, S., Nawaz, K., Iftikhar, S., Javed, M.A., Hashem, A., Alqarawi, A.A., Abd_Allah, E.F. and Akhter, A., 2018. Entomopathogenic fungus *Clonostachys rosea* as a biocontrol agent against whitefly (*Bemisia tabaci*). *Biocontrol science and technology*, 28(8), pp.750-760.
5. Athanassiou, C.G., Kavallieratos, N.G., Rumbos, C.I. and 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), p.22.
6. Balla, A., Silini, A., Cherif-Silini, H., Chenari Bouket, A., Moser, W.K., Nowakowska, J.A., Oszako, T., Benia, F. and Belbahri, L., 2021. The threat of pests and pathogens and the potential for biological control in forest ecosystems. *Forests*, *12*(11), p.1579.
7. Bamisile, B.S., Akutse, K.S., Siddiqui, J.A. and Xu, Y., 2022. genera *Beauveria*, *Metarhizium*, *Isaria*, *Hirsutella*, and *Lecanicillium*. Their application is usually through an inundative approach, which inherently involves exposure of the fungal spores to unfavorable humidity, temperature, and solar radiation conditions. These abiotic factors reduce the persistence and efficacy of these insect-pathogenic fungi. Despite these limitations, over 170 strains have been formulated as mycopesticides and are. *Bio-based Solutions for Sustainable Development of Agriculture*, p.9.
8. Battersby-Lennard, J. and Haysom, G., 2012. Philippi Horticultural Area: A City asset or potential development node?. [online] Academia.edu. Available at: https://www.academia.edu/1871167/Philippi_Horticultural_Area_A_City_asset_or_potential_development_node [Accessed 6 April 2022].
9. Bidochka, M.J., Kasperski, J.E. and Wild, G.A., 1998. Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany, 76*(7), pp.1198-1204.
10. Birnbaum, N., Reingold, V., Matveev, S., Kottakota, C., Davidovitz, M., Mani, K.A., Feldbaum, R., Yaakov, N., Mechrez, G. and Ment, D., 2021. Not only a formulation: The effects of pickering emulsion on the entomopathogenic action of *Metarhizium brunneum*. *Journal of Fungi*, *7*(7), p.499.
11. Bischoff, J.F., Rehner, S.A. and Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 101(4), pp.512-530.
12. Bueno-Pallero, F.Á., Blanco-Pérez, R., Vicente-Díez, I., Rodríguez Martín, J.A., Dionísio, L. and Campos-Herrera, R., 2020. Patterns of Occurrence and Activity of Entomopathogenic Fungi in the Algarve (Portugal) Using Different Isolation Methods. *Insects*, *11*(6), p.352.
13. Cairney, J.W. and Bastias, B.A., 2007. Influences of fire on forest soil fungal communities. *Canadian Journal of Forest Research*, *37*(2), pp.207-215.
14. Campbell, C.R. and Plank, C.O., 1998. Preparation of plant tissue for laboratory analysis. *Methods for Plant Analysis*, 37.

15. Cilliers, C.D., Botha, A., Esler, K.J. and Boucher, C., 2005. Effects of alien plant management, fire and soil chemistry on selected soil microbial populations in the Table Mountain National Park, South Africa. *South African Journal of Botany*, *71*(2), pp.211-220.
16. Dara, S.K., 2019. Non-entomopathogenic roles of entomopathogenic fungi in promoting plant health and growth. *Insects*, *10*(9), p.277.
17. Dubay, S., 2018. *Behavioural and physiological responses of chacma baboons (Papio ursinus) to wildfire in the Cape Peninsula of South Africa* (Master's thesis, University of Cape Town).
18. Fernández-Bravo, M., Gschwend, F., Mayerhofer, J., Hug, A., Widmer, F. and Enkerli, J., 2021. Land-use type drives soil population structures of the entomopathogenic fungal genus *Metarhizium*. *Microorganisms*, *9*(7), p.1380.
19. Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of molecular evolution*, *17*(6), pp.368-376.
20. Forsyth, G.G. and Van Wilgen, B.W., 2008. The recent fire history of the Table Mountain National Park and implications for fire management. *Koedoe: African Protected Area Conservation and Science*, *50*(1), pp.3-9.
21. Frąc, M., Hannula, S.E., Bełka, M. and Jędryczka, M., 2018. Fungal biodiversity and their role in soil health. *Frontiers in microbiology*, *9*, p.707.
22. García-Sanchez, M. and Szakova, J., 2016. Biological Remediation of Mercury-Polluted Environments. In *Plant metal interaction*, pp. 311-334.
23. Goble, T.A., 2009. *Investigation of entomopathogenic fungi for control of false codling moth, Thaumatotibia leucotrata, Mediterranean fruit fly, Ceratitis capitata and Natal fruit fly, C. rosa in South African citrus* (Doctoral dissertation, Rhodes University).
24. González-Guzmán, A., Sacristán, D., Sánchez-Rodríguez, A.R., Barrón, V., Torrent, J. and Del Campillo, M.C., 2020. Soil nutrients effects on the performance of durum wheat inoculated with entomopathogenic fungi. *Agronomy*, 10(4), p.589.
25. Hallouti, A., Ait Hamza, M., Zahidi, A., Ait Hammou, R., Bouharroud, R., Ait Ben Aoumar, A. and Boubaker, H., 2020. Diversity of entomopathogenic fungi associated with Mediterranean fruit fly (*Ceratitis capitata* (Diptera: Tephritidae)) in Moroccan Argan forests and nearby area: impact of soil factors on their distribution. *BMC ecology*, *20*(1), pp.1-13.
26. Hammer, Ø., Harper, D.A. and Ryan, P.D., 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica*, *4*(1), p.9.
27. Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M., 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nature reviews microbiology*, *2*(1), pp.43-56.
28. Hawkes, C.V., Kivlin, S.N., Rocca, J.D., Huguet, V., Thomsen, M.A. and Suttle, K.B., 2011. Fungal community responses to precipitation. *Global Change Biology*, *17*(4), pp.1637-1645.
29. Ho, W.C. and Ko, W.H., 1997. A simple method for obtaining single-spore isolates of fungi. *Botanical Bulletin-Academia Sinica Taipei*, *38*, pp.41-44.
30. Jacoby, R., Peukert, M., Succurro, A., Koprivova, A. and Kopriva, S., 2017. The role of soil microorganisms in plant mineral nutrition—current knowledge and future directions. *Frontiers in plant science*, *8*, p.1617.
31. Kepler, R.M., Humber, R.A., Bischoff, J.F. and Rehner, S.A., 2014. Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia*, 106(4), pp.811-829.
32. Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A. and Lombi, E., 2019. Soil and the intensification of agriculture for global food security. *Environment international*, *132*, p.105078.
33. Kryukov, V., Yaroslavtseva, O., Tyurin, M., Akhanaev, Y., Elisaphenko, E., Wen, T.C., Tomilova, O., Tokarev, Y. and Glupov, V., 2017. Ecological preferences of *Metarhizium* spp.

from Russia and neighboring territories and their activity against Colorado potato beetle larvae. *Journal of Invertebrate Pathology*, *149*, pp.1-7.

34. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), p.1547.
35. Litwin, A., Nowak, M. and Różalska, S., 2020. Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Bio/Technology*, *19*(1), pp.23-42.
36. Litwin, A., Bernat, P., Nowak, M., Słaba, M. and Różalska, S., 2021. Lipidomic response of the entomopathogenic fungus *Beauveria bassiana* to pyrethroids. *Scientific reports*, *11*(1), pp.1-11.
37. Loundou, P.M., 2008. *Medicinal plant trade and opportunities for sustainable management in the Cape Peninsula, South Africa* (Doctoral dissertation, Stellenbosch: Stellenbosch University).
38. Mager, D. and Hui, C. (2012) "A first record of biological soil crusts in the Cape Floristic Region", *South African Journal of Science*, 108(7/8), p. 4 Pages. Available at: https://sajs.co.za/article/view/9812 (Accessed: 21 March 2022).
39. Maltz, M.R., Treseder, K.K. and McGuire, K.L., 2017. Links between plant and fungal diversity in habitat fragments of coastal shrubland. *Plos One*, *12*(9), p.e0184991.
40. Manfrino, R.G., Hatting, J.L., Humber, R., Salto, C.E. and Lopez Lastra, C.C., 2014. Natural occurrence of entomophthoroid fungi (Entomophthoromycota) of aphids (Hemiptera: Aphididae) on cereal crops in Argentina. *Annals of applied biology*, *164*(1), pp.151-158.
41. Mathulwe, L.L., Jacobs, K., Malan, A.P., Birkhofer, K., Addison, M.F. and Addison, P., 2021. Characterisation of *Metarhizium majus* (Hypocreales: Clavicipitaceae) isolated from the Western Cape Province, South Africa. *Plos one*, *16*(2), p.e0240955.
42. Maulana, A.F., Turjaman, M., Hashimoto, Y., Cheng, W. and Tawaraya, K., 2021. Nitrogen and phosphorus concentrations in growth media affect the relationship between root endophytic fungi and host plant. *Archives of Microbiology*, *203*, pp.2411-2418.
43. Moloinyane, S., Addison, P., Achiano, K.A. and Nchu, F., 2020. Association between chemical properties of vineyard soils and occurrence of entomopathogenic fungi causing different levels of mortality in *Planococcus ficus*. *BioControl*, pp.1-13.
44. Mora, M.A.E., Rouws, J.R.C and Fraga, M.E., 2016. Occurrence of entomopathogenic fungi in Atlantic Forest soils. *Microbiol. Discov, 4*(1).
45. Morandi, M.A., Mattos, L.P., Santos, E.R. and Bonugli, R.C., 2008. Influence of application time on the establishment, survival, and ability of *Clonostachys rosea* to suppress Botrytis cinerea sporulation on rose debris. *Crop Protection*, *27*(1), pp.77-83.
46. Moroenyane, I., Chimphango, S.B.M., Wang, J., Kim, H.K. and Adams, J.M., 2016. Deterministic assembly processes govern bacterial community structure in the Fynbos, South Africa. *Microbial ecology*, *72*, pp.313-323.
47. Msimbira, L.A. and Smith, D.L., 2020. The roles of plant growth-promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. *Frontiers in Sustainable Food Systems*, *4*, p.106.
48. Nelson, P.E., 1983. *Fusarium* species. *An illustrated manual for identification*.
49. Pavao-Zuckerman, M.A., 2008. Soil ecology.
50. Pfordt, A., Ramos Romero, L., Schiwek, S., Karlovsky, P. and von Tiedemann, A., 2020. Impact of environmental conditions and agronomic practices on the prevalence of *Fusarium* species associated with ear-and stalk rot in maize. *Pathogens*, *9*(3), p.236.
51. Qayyum, M.A., Saeed, S., Wakil, W., Nawaz, A., Iqbal, N., Yasin, M., Chaurdhry, M.A., Bashir, M.A., Ahmed, N., Riaz, H. and Bilal, H., 2021. Diversity and correlation of

entomopathogenic and associated fungi with soil factors. *Journal of King Saud University-Science*, *33*(6), p.101520.

52. Quesada-Moraga, E., Navas-Cortés, J.A., Maranhao, E.A., Ortiz-Urquiza, A. and Santiago-Álvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological research*, *111*(8), pp.947-966.
53. Qiu, P.L., Liu, S.Y., Bradshaw, M., Rooney-Latham, S., Takamatsu, S., Bulgakov, T.S., Tang, S.R., Feng, J., Jin, D.N., Aroge, T. and Li, Y., 2020. Multi-locus phylogeny and taxonomy of an unresolved, heterogeneous species complex within the genus *Golovinomyces* (Ascomycota, Erysiphales), including *G. ambrosiae*, *G. circum*fusus and *G. spadiceus*. *BMC Microbiology*, *20*, pp.1-16.
54. Saitou, N. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, *4*(4), pp.406-425.
55. Samish, M., Rot, A., Ment, D., Barel, S., Glazer, I. and Gindin, G., 2014. Efficacy of the entomopathogenic fungus *Metarhizium brunneum* in controlling the tick *Rhipicephalus annulatus* under field conditions. *Veterinary Parasitology*, *206*(3-4), pp.258-266.
56. Sandhu, S.S., Sharma, A.K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A.K. and Malhotra, S., 2012. Myco-biocontrol of insect pests: factors involved, mechanism, and regulation. *Journal of pathogens*, 2012.
57. Santos, M.P., Dias, L.P., Ferreira, P.C., Pasin, L.A. and Rangel, D.E., 2011. Cold activity and tolerance of the entomopathogenic fungus *Tolypocladium* spp. to UV-B irradiation and heat. *Journal of invertebrate pathology*, *108*(3), pp.209-213.
58. Sharma, L., Oliveira, I., Torres, L. and Marques, G., 2018. Entomopathogenic fungi in Portuguese vineyards soils: Suggesting a '*Galleria-Tenebrio*-bait method'as bait-insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium* (*robertsii*) and *Beauveria* (*bassiana*). *MycoKeys*, (38), p.1.
59. Sharma, L., Oliveira, I., Gonçalves, F., Raimundo, F., Singh, R.K., Torres, L. and Marques, G., 2021. Effect of soil chemical properties on the occurrence and distribution of entomopathogenic fungi in Portuguese grapevine fields. *Pathogens*, *10*(2), p.137.
60. Shi, Y., Zhang, K., Li, Q., Liu, X., He, J.S. and Chu, H., 2020. Interannual climate variability and altered precipitation influence the soil microbial community structure in a Tibetan Plateau grassland. *Science of the Total Environment*, *714*, p.136794.
61. Simmons, M.T. and Cowling, R.M., 1996. Why is the Cape Peninsula so rich in plant species? An analysis of the independent diversity components. *Biodiversity & Conservation*, *5*(5), pp.551-573.
62. Stielow, J.B., Levesque, C.A., Seifert, K.A., Meyer, W., Irinyi, L., Smits, D., Renfurm, R., Verkley, G.J.M., Groenewald, M., Chaduli, D. and Lomascolo, A., 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, *35*(1), pp.242-263.
63. St. Leger, R.J. and Wang, J.B., 2020. Metarhizium: jack of all trades, master of many. *Open Biology*, *10*(12), p.200307.
64. Sutanto, K.D., Husain, M., Rasool, K.G., Malik, A.F., Al-Qahtani, W.H. and Aldawood, A.S., 2022. Persistency of indigenous and exotic entomopathogenic fungi isolates under ultraviolet B (UV-B) irradiation to enhance field application efficacy and obtain sustainable control of the red palm weevil. *Insects*, *13*(1), p.103.
65. Rumbos, C.I. and Athanassiou, C.G., 2017. Use of entomopathogenic fungi for the control of stored-product insects: can fungi protect durable commodities?. *Journal of pest science*, 90, pp.839-854.
66. Takahashi, K. and Nei, M., 2000. Efficiencies of fast algorithms of phylogenetic inference under the criteria of maximum parsimony, minimum evolution, and maximum likelihood when a large number of sequences are used. *Molecular biology and evolution*, *17*(8), pp.1251-1258.

67. Tkaczuk, C., Król, A., Majchrowska-Safaryan, A. and Nicewicz, Ł., 2014. The occurrence of entomopathogenic fungi in soils from fields cultivated in a conventional and organic system. *Journal of Ecological Engineering*, *15*(4), pp.137-142.
68. Tkaczuk, C., Tipping, C., Majchrowska-Safaryan, A., Krol, A., Wyrzykowska, M. and Wegensteiner, R., 2019. The occurrence of entomopathogenic fungi in soils from apple orchards protected in different farming systems. *Fresen. Environ. Bull*, *28*, pp.7906-7914.
69. Tuininga, A.R., Miller, J.L., Morath, S.U., Daniels, T.J., Falco, R.C., Marchese, M., Sahabi, S., Rosa, D. and Stafford, K.C., 2014. Isolation of entomopathogenic fungi from soils and *Ixodes scapularis* (Acari: Ixodidae) ticks: prevalence and methods. *Journal of Medical Entomology*, *46*(3), pp.557-565.
70. Wakil, W., Ghazanfar, M.U., Riasat, T., Kwon, Y.J., Qayyum, M.A. and Yasin, M., 2013. Occurrence and diversity of entomopathogenic fungi in cultivated and uncultivated soils in Pakistan. *Entomological Research*, *43*(1), pp.70-78.
71. White TJ, Barns SM, Lee S, et al. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis NA, Gelfand DH, Sninsky JJ et al. (eds), PCR protocols: a guide to methods and applications: 315-322/ Academic Press, Inc., New York, USA.
72. Woudenberg, J.H.C., Aveskamp, M.M., De Gruyter, J., Spiers, A.G. and Crous, P.W., 2009. Multiple Didymella teleomorphs are linked to the *Phoma clematidina* morphotype. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, *22*(1), pp.56-62.
73. Xia, Y., Sahib, M.R., Amna, A., Opiyo, S.O., Zhao, Z. and Gao, Y.G., 2019. Culturable endophytic fungal communities associated with plants in organic and conventional farming systems and their effects on plant growth. *Scientific Reports*, *9*(1), pp.1-10.
74. Yang, T., Lupwayi, N., Marc, S.A., Siddique, K.H. and Bainard, L.D., 2021. Anthropogenic drivers of soil microbial communities and impacts on soil biological functions in agroecosystems. *Global Ecology and Conservation*, *27*, p.e01521.
75. Yang, X., Zhu, K., Loik, M.E. and Sun, W., 2021. Differential responses of soil bacteria and fungi to altered precipitation in a meadow steppe. *Geoderma*, *384*, p.114812.

Chapter 3

Does insect-based oil formulation enhance the pathogenicity of indigenous entomopathogenic fungal isolates from the Cape Peninsula region against *Planococcus ficus*, grapevine mealybug?

Abstract

Fungal entomopathogens play a fundamental ecological role in regulating insect populations in nature, controlling destructive insect pest populations and, are increasingly exploited as biocontrol agents worldwide. Synthetic insecticides are often toxic to the environment and more susceptible to insect resistance. Consequently, there is a huge interest in finding sustainable alternatives. Entomopathogenic fungi are attractive alternatives to chemical-based active ingredients, but their widespread inclusion in IPM is curtailed by ineffective formulation. This study aimed to screen indigenous fungal strains against the grapevine mealybug and assess the prospect of using insect-based oil for formulating EPF against the mealybug *in vitro*. The study focused on screening 96 fungal isolates collected from soils in the Cape Peninsula region against grapevine mealybug using a dipping method. The results showed that insect mortalities caused by the fungal strains, varied significantly ($\chi 2 = 204.8$, $p < 0.01$, dF = 380). *Metarhizium isolates* induced the highest mortalities (ranging from 50% - 80%) within three days. Based on high insect mortality, two fungal isolates (Isolate 71 and 74) were selected and formulated in oil (Ento-oil®) obtained from black soldier fly at varying conidial concentrations of 1×10^7, 1×10^5, and 1×10^3 conidia mL^{-1}. *Metarhizium brunneum* isolate (Isolate 74), tested at 1×10^7 conidia/mL and formulated in 20% Ento-oil (20% oil and 0.5% aqueous Tween 80 mixture) yielded the highest mealybug mortality (83%) within 3 days. The addition of oil obtained from black soldier fly larvae to the formulation increased the virulence of the *M. brunneum* isolate.

3.1 Introduction

Fungal entomopathogens play a fundamental ecological role in regulating insect populations in nature (Mantzoukas & Eliopoulos, 2020). Because they are ubiquitous, safe and capable of regulating destructive insect pest populations, they are increasingly being exploited as biocontrol agents worldwide. The increasing popularity of EPF also correlates with the increasing demand for organically cultivated crops, as consumers become more conversant with the health and environmental risks associated with conventional agriculture (Altinok *et al*., 2019; Deka *et al*., 2021).

The Cape Peninsula region forms part of the Cape Floral Kingdom of South Africa, and is well-known for its rich floristic diversity. The region is host to various reserves, and farmlands, producing large amounts of food, consumed by locals (Pryke & Samways, 2009). A few studies have successfully isolated EPF from the soils, among which were highly pathogenic strains against well-known insect pests, such as mealybug and codling moths (Abaajeh & Nchu, 2015; Moloinyane *et al*., 2020; Mathulwe *et al*., 2022).

While the use of EPF as a biological control is becoming more popular because of their better sustainability attributes than synthetic insecticides, which are often toxic to the environment, are increasingly becoming more susceptible to insect resistance, they are unstable, producing inconsistent efficacies under field conditions (FitzGerald *et al*., 2016). Fungal formulation is crucial in maintaining high efficacy of EPF. Several EPF have been successfully formulated into commercial products for controlling insect pests of economic importance (Maina *et al*., 2018). The major focus of microbial formulation is the preservation of the microorganism and enhancing their antagonism against the target pests and diseases. A microorganism in a formulation requires nutritional and physical support to

survive and proliferate under field conditions and to enhance, efficacy, handling, application, and shelf life (Keswani *et al*., 2016).

Previous studies have demonstrated that oil formulations enhance the adhesion of conidia to insect cuticle, protect conidia from ultraviolet radiation and prolong shelf-life and stability (Lei *et al*., 2022; Umaru & Simarani., 2022). The insect epicuticle is lipid-based, lipophilic, compatible with oil carriers, and a primary site of the establishment of fungal conidia (Bandani & Esmailpour, 2006). Oil-based sprays of EPF are more efficacious than aqueous sprays (Inglis *et al*., 2002). Oil-based formulation is a superior spray carrier compared to water-based formulation because it is capable of spreading lipophilic conidia (Sedighi *et al*., 2013). Oil-based formulations can overcome some of the constraints on EPF in the field.

Insects are an important source of protein and renewable oil. Insect oils have been extracted from many edible species and the oils are considered as valuable food ingredients (Womeni *et al*., 2009; Tzompa-Sosa *et al*., 2019; Jayanegara *et al*., 2020). Insect oil is reported to have high oxidative stability and rich in fatty acids and high Omega-3 content (Mariod, 2011). Many previous studies have focused on isolating sterols for nutraceutical applications and as ingredients for functional foods and biodiesel (Mariod, 2011). However, because insect oil may contain contents that may enhance EPF virulence, it is interesting to study the prospect of using insect oil as a carrier of EPF conidia in pathogenicity bioassays.

Grapevines are prone to grapevine mealybug (*Planococcus ficus*) [Hemiptera: Pseudoccoccidae]) infestations. This pest is common in grape growing areas, causing quality and productivity reductions in table and wine grapes (Moloinyane, 2018). In South Africa, it is the main pest of grapevine, causing damages like rolling and discoloration of vine leaves (Platt *et al*., 2019). These pests feed on the phloem, which diminishes the flow of sap to the fruit, causing damage to vines and reducing yield.

The objectives of this study were to screen indigenous fungal strains against the grapevine mealybug and assess the prospect of using insect oil for formulating EPF against mealybug, *in vitro*.

3.2 Methods and Materials

3.2.1 Research Design

This quantitative research was carried out in two parts. The first part, focused on the screening of fungal isolates collected from soils in the Cape Peninsula region, against grapevine mealybug. In the second part, conidia of selected fungal strains, based on insect mortality, were formulated in oil, obtained from black soldier fly (Ento-Oil®) and supplied by Entomon Pty Ltd (Philippi, Cape Town, South Africa), at varying conidial concentrations 1×10^7, 1×10^5, 1×10^3 and the pathogenicity against female mealybug assessed.

3.2.2 Fungal isolates

96 fungal stains isolated from soil samples collected from various sites during the winter and summer seasons, using soil plating and insect bait methods were used in the study. The full description of the soil sampling, isolation and identification of the fungal strains are described in Chapter 2.

3.2.3 Rearing of P. ficus

Adult female grapevine mealybugs were reared on butternut in a darkroom at 25 ± 2 ℃ and 60 % RH. They were maintained in the darkness to prevent the insects from crawling off the butternut because light attracts them. The mealybugs were reared at the ARC (Agricultural Research Council) Infruitec-Nietvoorbij, Stellenbosch, South Africa by Dr. K.A. Achiano (Moloinyane *et al*., 2018).

3.2.4 Assessing pathogenicity and virulence against P. ficus

Prior to assessing pathogenicity, the viability of conidia was determined by spread-plating 0.1ml of the conidial suspension, titrated to 1×10^7conidia ml^{-1} on PDA plates. Two replicated sterile microscope cover slips were placed on each plate and incubated at 26 ± 2 °C. Plates were examined after 24h and germination percentage was obtained by using a haemocytometer. Generally, the percentage of germination was above 90%.

3.2.5 Immersion bioassay

An immersion bioassay was used to evaluate the pathogenic effect of the 96 EPF fungal strains belonging to nine species, see Chapter 2) collected from soil samples against adult female *P. ficus*.

Conidia were harvested from 3 – 4-week-old fungal cultures by scraping the conidia, suspending them in 50 ml centrifuge tubes containing 25 ml of sterile water mixed with 0.05% Tween80. Each suspension was mixed vigorously using a vortex shaker for 5 minutes to homogenize it. The conidial concentration of 1×10^7 conidia ml^{-1} was determined using a Neubauer haemocytometer. The control solution consisted of sterile water mixed with 0.05% Tween80. Ten insects were immersed into 5 ml conidial suspensions or control solution for 10 s, ensuring the suspensions can stick to the insect bodies. Thereafter, the ten insects were placed in a 9 cm diameter Petri dish containing a sterilized grapevine (*Vitis vinifera*) leaf of 3 cm in diameter, replicated five times. The Petri dishes were kept in a growth chamber at 25 °C and 60% RH. Mortality data was recorded after 3-days post-treatment. Insect cadavers were surface sterilized in 70% ethanol for 5 s, rinsed for 1 min in sterilized distilled water and incubated at 25 ± 2 °C and 90 ± 5% RH. Mycosis was observed under a dissecting microscope. Only insect cadaver with mycosis were considered to have been killed by a fungal strain. High incidence of mycosis was observed on the fungus-treated insects (Figure 6). No mycosis was perceived on the control insects.

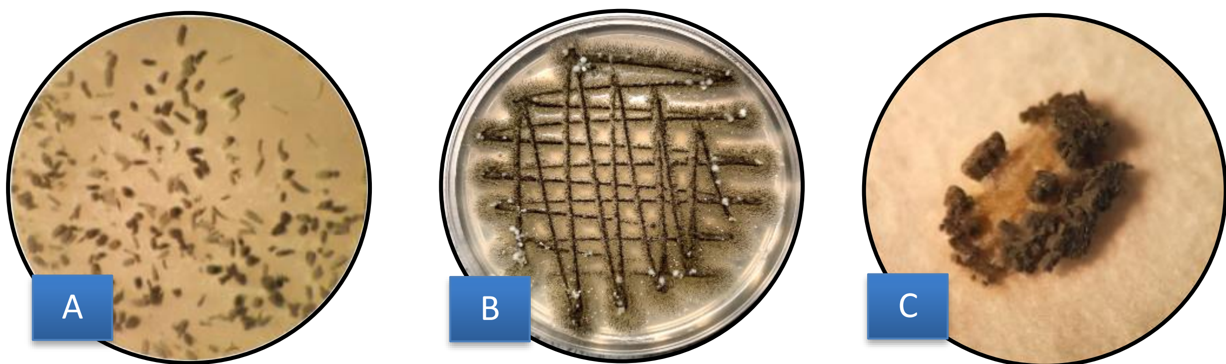

Figure 6: A – Metarhizium brunneum* (Isolate 74) *spores observed under a light microscope, B – 3 week old M. brunneum streaked on growth media, C- M. brunneum*, *mycelial outgrowth from a mealybug cadaver.

In the second bioassay, only the two most virulent fungal strains were used. Their conidia were suspended in oil-water emulsion and 100% oil formulations at varying concentrations (1×10^7, 1×10^5, and 1×10^3 conidia/mL). The emulsifiable formulation consisted of a mixture of 20 % Ento-oil® and 80 % aqueous and Tween80. The conidia of the fungal strains were suspended in 99.5% Ento-oil® and 0.05% Tween 80. The respective control treatments did not have conidia.

3.2.6 Statistical Analysis

Data of insect mortality in the immersion bioassay were Abbott-corrected, (1925). The non-parametric Kruskall-Wallis test was used to assess the effects of fungal treatments on insect

mortality in the immersion bioassay using PAST. The statistical level of significance was fixed at $p \leq 0.05$.

3.3 Results

A total of 96 fungal isolates were screened against the mealybug for mortality (Table 7A and B). Mealybug mortalities caused by the fungal strains varied significantly ($X^2 = 204.8$, $p < 0.01$, dF = 380). From the isolated species, *Metarhizium* spp. strains were the most virulent, inducing higher mortalities of mealybugs. Meanwhile species like *Fusarium* spp. and *Penicillium* spp. were the least active against mealybugs.

Table 7A: Screening winter-collected indigenous fungal isolates against P. ficus.

No. of Isolate	Source of Isolation	Location	GPS Co-ordinates	Fungal Species Identification	Abbott Corrected Mortality ± SE
1	Soil	Site 1	-32.084874 18.423375	*Penicillium simplicissimum*	5.78 ± 8.82
2	Soil	Site 1	-32.084874 18.423376	*Talaromyces cellulolyticus*	45.56 ± 19.20
3	Soil	Site 1	-34.073828 18.423021	*Clonostachys rosea*	28.00 ± 14.97
4	Soil	Site 1	-34.073828 18.423021	*Clonostachys rosea*	14.67 ± 2.67
5	Soil	Site 1	-34.073663 18.424859	*Metarhizium anisopliae*	12.44 ± 10.15
6	Soil	Site 1	-34.074971 18.424566	*Penicillium restrictum*	0.00 ± 0.00
7	Soil	Site 1	-34.074971 18.424566	*Fusarium* spp.	5.00 ± 5.00
8	Soil	Site 1	-34.074971 18.424566	*Penicillium restrictum*	0.00 ± 0.00
9	Insect	Site 2	-34.040717 18.580460	*Metarhizium anisopliae*	0.00 ± 0.00
10	Soil	Site 2	-34.039208 18.580647	*Metarhizium anisopliae*	0.00 ± 0.00
11	Soil	Site 2	-34.039208 18.580648	*Fusarium oxysporum*	14.00 ± 6.00
12	Soil	Site 2	-34.039208 18.580649	*Pochonia chlamydosporia*	5.78 ± 6.15
13	Insect	Site 2	-34.039208 18.580650	*Metarhizium guizhouense*	8.00 ± 8.00
14	Insect	Site 2	-34.039208 18.580651	*Metarhizium anisopliae*	16.44 ± 7.56
15	Soil	Site 2	34.039934 18.581148	*Metarhizium* spp.	3.78 ± 5.25
16	Soil	Site 2	34.039934 18.581148	*Talaromyces cellulolyticus*	4.00 ± 5.32
17	Insect	Site 2	34.039934 18.581148	*Metarhizium guizhouense*	10.22 ± 5.48
18	Insect	Site 2	-34.041544 18.582249	*Metarhizium brunneum*	12.22 ± 6.97
19	Insect	Site 3	-34.018103 18.557428	*Metarhizium robertsii*	19.39 ± 3.94
20	Insect	Site 3	-34.018103 18.557428	*Metarhizium anisopliae*	20.00 ± 7.07

21	Insect	Site 3	-34.017793 18.557612	*Fusarium oxysporum*	12.44 ± 5.99
22	Insect	Site 3	-34.0173701 18.557090	*Metarhizium guizhouense*	14.89 ± 4.35
23	Insect	Site 3	-34.016975 18.558143	*Metarhizium robertsii*	12.28 ± 6.80
24	Insect	Site 3	-34.016975 18.558143	*Metarhizium anisopliae*	8.22 ± 3.78
25	Insect	Site 3	-34.017303 18.558631	*Metarhizium anisopliae*	12.00 ± 5.83
26	Insect	Site 3	-34.017303 18.558631	*Talaromyces aculeatus*	8.00 ± 3.74
27	Insect	Site 3	-34.017410 18.558708	*Metarhizium robertsii*	14.22 ± 6.75
28	Insect	Site 4	-34.037853 18.532446	*Metarhizium anisopliae*	13.50 ± 11.50
29	Insect	Site 4	-34.037853 18.532447	*Fusarium foetens*	6.00 ± 4.00
30	Insect	Site 4	-34.037836 18.532918	*Metarhizium guizhouense*	16.22 ± 8.37
31	Insect	Site 4	-34.037807 18.533138	*Metarhizium brunneum*	1.78 ± 5.04
32	Insect	Site 4	-34.037947 18.532915	*Metarhizium guizhouense*	0.00 ± 0.00
33	Insect	Site 4	-34.037967 18.533076	*Metarhizium brunneum*	5.78 ± 6.91
34	Insect	Site 4	-34.037967 18.533077	*Talaromyces cellulolyticus*	0.00 ± 0.00
35	Insect	Site 4	-34.038129 18.533305	*Metarhizium guizhouense*	18.67 ± 6.20
36	Insect	Site 4	-34.038129 18.533306	*Metarhizium robertsii*	8.00 ± 3.74
37	Insect	Site 4	-34.038104 18.532756	*Metarhizium robertsii*	12.22 ± 3.72
38	Insect	Site 4	-34.038167 18.532897	*Metarhizium anisopliae*	2.00 ± 2.00
39	Insect	Site 4	-34.038167 18.532898	*Trichoderma virens*	4.00 ± 5.32
40	Soil	Site 5	-33.938023 18.393001	*Aspergillus* sp.	0.00 ± 0.00
41	Soil	Site 5	-33.936861 18.394758	*Penicillium chalabudae*	0.00 ± 0.00
42	Soil	Site 5	-33.936861 18.394759	*Penicillium philippinense*	0.00 ± 0.00
43	Insect	Site 6	-33.955736 18.41610	*Fusarium oxysporum*	6.00 ± 4.00
44	Soil	Site 6	-33.954718 18.415303	*Fusarium oxysporum*	3.50 ± 5.45
45	Soil	Site 6	-33.954718 18.415304	*Fusarium oxysporum*	1.78 ± 3.92
46	Insect	Site 7	-33.97595 18.447537	*Metarhizium* spp.	16.00 ± 8.12
47	Soil	Site 7	-33.970514 18.447378	*Talaromyces stipitatus*	0.00 ± 0.00
48	Soil	Site 7	-33.970514 18.447379	*Talaromyces stipitatus*	0.00 ± 0.00
49	Soil	Site 7	-33.970201 18.447417	*Fusarium oxysporum*	0.00 ± 0.00

50	Soil	Site 8	-34.057600 18.434654	*Umbelopsis ramanniana*	0.00 ± 0.00
51	Soil	Site 8	-34.057600 18.434655	*Micromucor* spp.	0.00 ± 0.00
52	Insect	Site 9	-34.074747 18.399280	*Metarhizium guizhouense*	8.22 ± 3.78
53	Insect	Site 9	-34.074747 18.399281	*Penicillium sanguifluum*	0.00 ± 0.00
54	Insect	Site 9	-34.074747 18.399282	*Metarhizium pinghaense*	0.00 ± 0.00

Table 7B: Screening summer-collected indigenous fungal isolates against P. ficus.

No. of Isolate	Source of Isolation	Location	GPS Co-ordinates	Fungal Species Identification	Abbott Corrected Mortality ± SE
55	Soil	Site 1	-32.074404 18.423000	*Fusarium oxysporum*	14.67 ± 9.70
56	Soil	Site 1	-34.073828 18.423021	*Clonostachys rosea*	17.50 ± 9.01
57	Soil	Site 1	-34.073487 18.423816	*Clonostachys rosea*	20.00 ± 3.16
58	Soil	Site 1	-34.074971 18.424566	*Fusarium foetens*	1.56 ± 6.06
59	Soil	Site 2	34.039934 18.581148	*Fusarium solani*	5.50 ± 6.34
60	Insect	Site 2	34.039934 18.581148	*Metarhizium guizhouense*	16.67 ± 9.14
61	Soil	Site 2	-34.040337 18.581483	*Clonostachys rosea*	24.73 ± 16.05
62	Insect	Site 2	-34.041544 18.582249	*Metarhizium guizhouense*	48.06 ± 6.85
63	Insect	Site 2	-34.042082 18.582677	*Fusarium oxsporum*	0.00 ± 0.0
64	Soil	Site 2	-34.042526 18.582969	*Fusarium oxysporum*	10.00 ± 4.47
65	Soil	Site 2	-34.042997 18.583101	*Clonostachys rosea*	12.44 ± 4.86
66	Insect	Site 2	-34.042997 18.583102	*Metarhizium brunneum*	12.44 ± 3.99
67	Soil	Site 2	-34.04337 18.583209	*Fusarium solani*	6.00 ± 4.00
68	Insect	Site 2	-34.04337 18.583210	*Metarhizium anisopliae*	12.44 ± 3.99
69	Insect	Site 3	34.018296 18.557834	*Metarhizium guizhouense*	60.67 ± 10.97
70	Soil	Site 3	34.018103 18.557645	*Fusarium solani*	0.00 ± 0.00
71	Soil	Site 3	34.018103 18.557646	*Fusarium solani*	34.44 ± 7.29

72	Insect	Site 3	-34.017793 18.557612	*Metarhizium guizhouense*	28.00 ± 3.74
73	Insect	Site 3	-34.017680 18.557096	*Metarhizium guizhouense*	30.00 ± 7.07
74	Insect	Site 3	-34.017301 18.557090	*Metarhizium brunneum*	53.44 ± 12.32
75	Soil	Site 3	-34.016975 18.558143	*Fusarium oxysporum*	12.00 ± 6.81
76	Insect	Site 3	-34.016975 18.558144	*Metarhizium anisopliae*	38.17 ± 3.42
77	Insect	Site 3	-34.016980 18.558523	*Metarhizium robertsii*	52.44 ± 10.96
78	Soil	Site 3	-34.01735 18.558631	*Fusarium oxysporum*	0.00 ± 0.00
79	Soil	Site 4	-34.037990 18.523520	*Fusarium solani*	8.22 ± 3.78
80	Soil	Site 4	-34.037990 18.523521	*Fusarium oxysporum*	3.72 ± 5.52
81	Insect	Site 4	-34.037990 18.523522	*Metarhizium robertsii*	51.11 ± 4.61
82	Soil	Site 4	-34.037817 18.533344	*Fusarium oxysporum*	10.00 ± 4.47
83	Insect	Site 4	-34.037817 18.533345	*Metarhizium anisopliae*	22.00 ± 5.83
84	Soil	Site 5	-33.939982 18.393157	*Fusarium oxysporum*	6.00 ± 4.00
85	Soil	Site 5	-33.938023 18.393001	*Trichoderma simplex*	3.56 ± 7.01
86	Soil	Site 6	-33.955941 18.416378	*Fusarium oxysporum*	3.78 ± 5.25
87	Soil	Site 6	-34.955899 18.416378	*Fusarium oxysporum*	2.00 ± 2.00
88	Soil	Site 6	-34.955899 18.416379	*Trichoderma simplex*	15.67 ± 6.74
89	Soil	Site 7	-34.979203 18.44781	*Fusarium oxysporum*	7.78 ± 6.79
90	Soil	Site 7	-34.971349 18.447938	*Fusarium oxysporum*	3.00 ± 8.31
91	Insect	Site 7	-34.971349 18.447939	*Metarhizium brunneum*	42.89 ± 14.96
92	Soil	Site 7	-34.971526 18.447749	*Fusarium oxysporum*	0.00 ± 0.00
93	Soil	Site 8	-34.057600 18.434655	*Fusarium oxysporum*	3.22 ± 7.73
94	Soil	Site 8	-33.57436 18.434972	*Fusarium oxysporum*	8.00 ± 3.74
95	Soil	Site 8	-33.056362 18.433446	*Fusarium oxysporum*	6.00 ± 4.00

96	Soil	Site 9	-34.073296 18.398715	*Fusarium oxysporum*	0.00 ± 0.00

The formulations of Isolate 71 (*F. solani*) did not significantly influence mortalities; 20% Ento-oil ($\chi2$= 6.69, p = 0.07, dF = 3) and 100% Ento-oil® ($\chi2$ = 6.86, p = 0.07, dF = 3). However, *M. breunneum* (Isolate 74) had a significant difference for 20% Ento-oil ($\chi2$ = 7.73, p = 0.04, dF = 3) and 100% ($\chi2$ = 8.39, p = 0.03, dF = 3). *M. breunneum* performed better than *F. solani*, inducing mortalities ranging just above 80% in 3 days.

Table 8: Mortalities (Abbott-corrected ± SE) induced by conidial suspensions of Fusarium solani and Metharizium brenneum isolates, formulated in 20% and 100% insect oil (Ento-oil®), against P. ficus.

Concentration	**Fungal Species Identified**			
	Fusarium solani (Isolate 71)	*Metarhizium brenneum* (Isolate 74)	*Fusarium solani* (Isolate 71)	*Metarhizium brenneum* (Isolate 74)
	Abbott Corrected Mortality ± SE (Concentration 20% Ento-oil)		Abbott Corrected Mortality ± SE (Concentration 100% Ento-oil)	
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1×10^7	43.33 ± 3.33	80 ± 5.77	33.33 ± 6.67	83.33 ± 3.33
1×10^5	36.67 ± 6.67	70 ± 5.77	35.56 ± 6.79	70 ± 5.77
1×10^3	40 ± 5.77	83.33 ± 3.33	20.22 ± 5.38	80.00 ± 5.77

3.4 Discussion

The 96 isolates induced varied mortalities against mealybug females: however, the mortality was generally lower compared to a previous study by Moilonyane *et al.* (2020), which recorded 18 to 87 % mealybug mortalities (Table 5A and B). Mealybug are difficult to kill because of different defense mechanisms. Their epidermal wax glands secrete a waxy substance that is transferred to rest of the body surface via, pores, ducts and protects the insects. The wax cover is hydrophobic preventing the mealybugs from water penetration or drowning (Franco *et al.,* 2009). The protective wax can prevent conidial suspension from sticking to the insect pest and preventing EPF from colonizing the mealybug.

Metarhizium spp. induced the highest mortalities (ranging from 50% - 80% kill rate) within 3-days. Similar studies by Ujjan *et al.* (2015) and Pacheco da Silva *et al.* (2021) revealed that indigenous *Metarhizium* spp. are pathogenic against mealybugs. Numerous studies have proven the effectiveness of *Metarhizium* spp. and how they can control a wide range of insect pests (Stone & Bidochka *et* al., 2020; Sheng *et al*., 2022). These species are quite adaptable and are compatible with various formulations, making them fairly versatile (FitzGerald *et al*., 2016). In a previous study Mathulwe *et al*. (2022), local *Metarhizium* spp. isolates showed great potential in reducing mealybug populations.

According to da Silva Santos *et al.* (2020) some *Fusarium* spp are effective in controlling insects. Anwar *et al.* (2017) stated some *Fusarium* species causes moderate to high infection, primarily against the insect orders, Hemiptera and Diptera. In this study, strain 71 (*F. solani*) was pathogenic against grapevine mealybugs.

Understanding how EPF propagules interact with their host insect is important when developing formulations. In this case, the targeted insect organ would be the mealybug's cuticle. Hence, using an oil-based formulation would enable the conidia to stick to the insect body, making it easier for the fungal entomopathogens to penetrate and colonize the insect host (Carrillo *et al*., 2015; Mascarin & Jaronski,

2016). However, in formulations containing 100% oil, water might not be readily available for conidia to germinate, but oil-based formulation can offer the necessary moisture for germination. Previous studies by Akbar *et al.* (2005) and Sedighi *et al.* (2013) suggest that oil-based formulations enhance conidia spread and optimize the attachment of conidia to hydrophobic insect cuticle. A study done by Sedighi *et al.* (2013) revealed that water and oil formulation was more effective.

Isolate 74 (*M. brunneum*) tested at 1×10^7 conidia/mL and formulated 20% Ento-oil (20% oil and 0.5% aqueous Tween 80 mixture) yielded the highest mealybug mortality (83%) at 3 days post-treatment. In contrast, strain 71 (*Fusarium solani*), formulated in 20 % Ento-oil® mixture (20% oil and 0.5% aqueous Tween 80 mixture) and yielded a low mortality of 43% (Table 6). This result suggests that Ento-oil could have a negative effect on the pathogenicity of the *F. solani* fungal strain.

For future studies, different formulations could be tested to see variations in the results to improve the final formulations. Field trials should be carried out to assess the prospects of using Ento-oil® in formulating indigenous EPF isolates. Furthermore, looking into other factors like shelf-life, conidia viability over time is necessary.

3.5 Conclusion

In conclusion the grapevine mealybug was successfully controlled in this study and with the addition of the Ento-oil to the formulation, it increased the effectiveness of the *M. brunneum* isolate. The findings of this study could open a new channel of biological control mixtures in the Cape Peninsula region.

3.6 References

1. Abaajeh, A.R. and Nchu, F., 2015. Isolation and pathogenicity of some South African entomopathogenic fungi (Ascomycota) against eggs and larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). *Biocontrol Science and Technology*, *25*(7), pp.828-842.
2. Abbott, W.S., 1925. Formula for correction of observed mortality due to natural mortality. *Econ. Entomol*, *18*, pp.265-267.
3. Akbar, W., Lord, J.C., Nechols, J.R. and Loughin, T.M., 2005. Efficacy of *Beauveria bassiana* for red flour beetle when applied with plant essential oils or in mineral oil and organosilicone carriers. *Journal of Economic Entomology*, 98(3), pp.683-688.
4. Altinok, H.H., Altinok, M.A. and Koca, A.S., 2019. Modes of action of entomopathogenic fungi. *Curr. Trends Nat. Sci*, *8*(16), pp.117-124.
5. Anwar, W., Haider, M.S., Shahid, A.A., Mushtaq, H., Hameed, U., Rehman, M.Z.U. and Iqbal, M.J., 2017. Genetic diversity of *Fusarium* isolated from members of Sternorrhyncha (Hemiptera): Entomopathogens against *Bemisia tabaci*. *Pakistan Journal of Zoology*, *49*(2).
6. Bandani, A.R. and Esmailpour, N., 2006. Oil formulation of entomopathogenic fungus, *Beauveria bassiana*, against Sunn pest, *Eurygaster integriceps* puton (Heteroptera: Scutelleridae). *Communications in agricultural and applied biological sciences*, *71*(2 Pt B), pp.443-448.
7. Carrillo, D., Dunlap, C.A., Avery, P.B., Navarrete, J., Duncan, R.E., Jackson, M.A., Behle, R.W., Cave, R.D., Crane, J., Rooney, A.P. and Peña, J.E., 2015. Entomopathogenic fungi as biological control agents for the vector of the laurel wilt disease, the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae). *Biological Control*, *81*, pp.44-50.
8. da Silva Santos, A.C., Diniz, A.G., Tiago, P.V. and de Oliveira, N.T., 2020. Entomopathogenic *Fusarium* species: a review of their potential for the biological control of insects, implications and prospects. *Fungal biology reviews*, *34*(1), pp.41-57.
9. Deka, B., Baruah, C. and Babu, A., 2021. Entomopathogenic microorganisms: their role in insect pest management. *Egyptian Journal of Biological Pest Control*, *31*(1), pp.1-8.

10. *FitzGerald, V.C., 2014. Screening of entomopathogenic fungi against citrus mealybug (Planococcus citri (Risso)) and citrus thrips (Scirtothrips aurantii (Faure)) (Doctoral dissertation, Rhodes University).*
11. Franco, J.C., Zada, A. and Mendel, Z., 2009. Novel approaches for the management of mealybug pests. *Biorational control of arthropod pests: application and resistance management*, pp.233-278.
12. Indriyanti, D.R., Widiyaningrum, P., Slamet, M. and Maretta, Y.A., 2017. Effectiveness of *Metarhizium anisopliae* and Entomopathogenic Nematodes to Control *Oryctes rhinoceros* Larvae in the Rainy Season. *Pakistan journal of biological sciences: PJBS*, *20*(7), pp.320-327.
13. Inglis, D.G., Jaronski, S.T., Wraight, S.P., Beattie, A., Watson, D., Stevens, M. and Rae, D., 2002. Use of spray oils with entomopathogens. *Spray Oils Beyond 2000: Sustainable Pest and Disease Management*, pp.302-312.
14. Jayanegara, A., Gustanti, R., Ridwan, R. and Widyastuti, Y., 2020. Fatty acid profiles of some insect oils and their effects on *in vitro* bovine rumen fermentation and methanogenesis. *Italian Journal of Animal Science*, *19*(1), pp.1310-1317.
15. Keswani, C., Bisen, K., Singh, V., Sarma, B.K. and Singh, H.B., 2016. Formulation technology of biocontrol agents: present status and future prospects. *Bioformulations: for sustainable agriculture*, pp.35-52.
16. Lei, C.J., Halim, N.A., Asib, N., Zakaria, A. and Azmi, W.A., 2022. Conidial Emulsion Formulation and Thermal Storability of *Metarhizium anisopliae* against Red Palm Weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Dryophthoridae). *Microorganisms*, *10*(7), p.1460.
17. Maina, U.M., Galadima, I.B., Gambo, F.M. and Zakaria, D., 2018. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *J. Entomol. Zool. Stud*, *6*(1), pp.27-32.
18. Mantzoukas, S. and Eliopoulos, P.A., 2020. Endophytic entomopathogenic fungi: A valuable biological control tool against plant pests. *Applied Sciences*, *10*(1), p.360.
19. Mariod, A.A., 2011. Insect oils: Nutritional and industrial applications. *Int. News Fats, Oils Rel. Mat*, *22*, pp.266-268.
20. Mathulwe, L.L., Malan, A.P. and Stokwe, N.F., 2022. Mass Production of Entomopathogenic Fungi, *Metarhizium robertsii* and *Metarhizium pinghaense*, for Commercial Application against Insect Pests. *J. Vis. Exp*, *181*, p.e63246.
21. Mathulwe, L.L., Malan, A.P. and 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), pp.397-417.
22. Moloinyane, S., 2018. *Bioefficacy of selected entomopathogenic fungal endophytes (Ascomycota) against grapevine mealybug (Planococcus ficus)* (Doctoral dissertation, Cape Peninsula University of Technology).
23. Moloinyane, S., Addison, P., Achiano, K.A. and Nchu, F., 2020. Association between chemical properties of vineyard soils and occurrence of entomopathogenic fungi causing different levels of mortality in *Planococcus ficus*. *BioControl*, *65*(2), pp.197-209.
24. Mora, M.A.E., Castilho, A.M.C. and Fraga, M.E., 2018. Classification and infection mechanism of entomopathogenic fungi. *Arquivos do Instituto Biológico*, 84.
25. Pacheco da Silva, V.C., Cocco, A., Benelli, G., Botton, M., Lucchi, A. and Lentini, A., 2021. Sustainable management of the vine mealybug in organic vineyards. *Journal of Pest Science, 2021, 94 (2): 153-185.*
26. Platt, T., Stokwe, N.F. and Malan, A.P., 2019. Foliar application of *Steinernema yirgalemense* to control *Planococcus ficus*: Assessing adjuvants to improve efficacy. *South African Journal of Enology and Viticulture*, *40*(1), pp.1-7.

27. Pryke, J.S. and Samways, M.J., 2009. Conservation of the insect assemblages of the Cape Peninsula biodiversity hotspot. *Journal of Insect Conservation*, *13*(6), pp.627-641.
28. Sedighi, N., Abbasipour, H., Askary, H. and Sheikhi Gorjan, A., 2013. Effect of oil suspended conidia of *Metarhizium anisopliae* var. major on mortality of the sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). *Archives of Phytopathology and Plant Protection*, *46*(2), pp.128-140.
29. Sheng, H., McNamara, P.J. and Leger, R.J.S., 2022. *Metarhizium*: an opportunistic middleman for multitrophic lifestyles. *Current Opinion in Microbiology*, *69*, p.102176.
30. Stone, L.B. and Bidochka, M.J., 2020. The multifunctional lifestyles of *Metarhizium*: Evolution and applications. *Applied microbiology and biotechnology*, *104*, pp.9935-9945.
31. Tzompa-Sosa, D.A., Yi, L., Van Valenberg, H.J.F. and Lakemond, C.M.M., 2019. Four insect oils as food ingredient: physical and chemical characterisation of insect oils obtained by an aqueous oil extraction. *Journal of Insects as Food and Feed*, *5*(4), pp.279-292.
32. Ujjan, A.A., Khanzada, M.A., Mahar, A.Q. and Shahzad, S., 2015. Efficiency of *Metarhizium* spp.(Sorokin) strains and insecticides against cotton mealybug Phenacoccus solenopsis (Tinsley). *Pakistan Journal of Zoology*, 47(2).
33. Umaru, F.F. and Simarani, K., 2022. Efficacy of entomopathogenic fungal formulations against *Elasmolomus pallens* (Dallas)(Hemiptera: Rhyparochromidae) and their extracellular enzymatic activities. *Toxins*, *14*(9), p.584.
34. Womeni, H.M., Linder, M., Tiencheu, B., Mbiapo, F.T., Villeneuve, P., Fanni, J. and Parmentier, M., 2009. Oils of insects and larvae consumed in Africa: potential sources of polyunsaturated fatty acids. *Oléagineux, Corps gras, Lipides*, *16*(4-5-6), pp.230-235.

Chapter 4

4.1 General Discussion

Entomopathogenic fungi are quickly becoming the future of pest management and soil rehabilitation globally, mainly becausethey are environmentally friendly and effective. The poineering study examines the complex relationship between, how land-use and season influence ecology and the occurrence of EPF and general fungal populations in the Cape Peninsula Region. A total of 96 fungal strains were isolated, which includes the following species: *Aspergillus* spp. *Clonostachys* spp., *Fusarium* spp., *Metarhizium* spp., *Micromucor* spp., *Penicillium* spp., *Talaromyces* spp., *Trichoderma* spp., and *Umbelopsis* spp.

This study also revealed that fynbos-rich reserves yielded a lower number of isolated EPF strains compared to agricultural land. There are two explanations for this finding. Firstly, the increased fire incidences in reserves, mainly driven by the highly flammable fynbos spp. and dry summers, heat the soil, suppressing fungal growth and reducing occurrence (Cilliers *et al.,* 2005). Secondly, adding agricultural inputs, including fertilizers, increases the level and availability of nutrients in agricultural sites for EPF.

The study also screened the pathogenicity of the isolated EPF strains on grapevine mealybug (*P. ficus*) and tested the effect of using insect oil as a carrier under laboratory conditions. The EPF strains induced varying numbers of insect mortality. The mortality bioassay revealed that some indigenous strains of *M. robertsii* caused high mortality and have the potential as biological agents against grapevine mealybug. Furthermore, mixing EPF conidia and ento-oil, produced promising results that warrant further testing. These results showed that insect oil obtained from black soldier fly larvae could be used in formulating EPF. The use of insect oil as a carrier opens up new research opportunities. The insect oil enhances the fungal activity, improving contact with the insect cuticle and the stability of the formulation. Oil adjuvants contribute to sustaining conidial spores, making them last longer in storage (Batta, 2016; Sedighi *et al*., 2012). These are all encouraging indications of moving towards a more sustainable approach to integrated pest management (IPM) strategies.

4.2 Recommendations

The following recommendations are advocated, based on the findings of this study.

1. The occurrence of EPF is most abundant in winter; therefore, collection of soil samples should be done during the winter months, when the fungi is thriving and diverse.
2. Isolation and screening of indigenous fungal strains could facilitate the identification and development of more efficient biological control agents.
3. Further testing insect oil as it could lead to improved EPF formulations.
4. Encourage the establishment of University-Industry partnerships to improve the quality of research, innovation and commercialization of EPF-based insectidicides.

4.3 References

1. Batta, Y.A., 2016. Recent advances in formulation and application of entomopathogenic fungi for biocontrol of stored-grain insects. *Biocontrol Science and Technology*, *26*(9), pp.1171-1183.
2. Cilliers, C.D., Botha, A., Esler, K.J. and Boucher, C., 2005. Effects of alien plant management, fire and soil chemistry on selected soil microbial populations in the Table Mountain National Park, South Africa. *South African Journal of Botany*, *71*(2), pp.211-220.
3. Sedighi, N., Abbasipour, H., Askary, H. and Sheikhi Gorjan, A., 2013. Effect of oil suspended conidia of *Metarhizium anisopliae* var. major on mortality of the sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). *Archives of Phytopathology and Plant Protection*, *46*(2), pp.128-140.

Supplementary attachments

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