Cape Peninsula University of Technology

Bioefficacy of selected entomopathogenic fungal endophytes (Ascomycota) against grapevine mealybug (*Planococcus ficus*)

by

SIPHOKAZI MOLOINYANE

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Supervisor: Prof. Felix Nchu

Co-supervisor: Dr. Pia Addison

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Title page

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DECLARATION

I, Siphokazi Moloinyane, 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.

Signed **Date**

DEDICATION

I dedicate this thesis to:

- My mother Neliwe Moloinyane; you will always be my greatest teacher, a teacher of love, compassion and fearlessness. Iintsikelelo zemithandazo yakho ziyakuhlala nam ubomi bam bonke Mamfene, Lisa, Jambase, Hlathi, Buswayo, Canzi, Sanzanza.
- My daughter Kentse, may this be a reminder that “Success is no accident, it is hard work, perseverance, learning, sacrifice and most of all love for whatever you’re doing”-Pele.

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CONTENT TABLE

LIST OF ACRONYMS

ANOVA	Analysis of Variance
B	Boron
C	Carbon
C/N RATIO	Carbon Nitrogen ratio
Ca	Calcium
CA	Correspondence Analysis
CAF	Central Analysis Facilities
CYA	Czapek Yeast Agar
EPF	Entomopathogenic fungi
GC-MS	Gas Chromatography – Mass Spectrometry
IPW	Intergrated Production of Wine
ITS	Internal Transcribed Spacer
K	Potassium
MEA	Malt Extract Agar
Mg	Magnesium
N	Nitrogen
OA	Oatmeal Agar
ºC	Degrees Celsius
P	Phosphorus
PDA	Potato Dextrose Agar
S	Sulphur
SAWIS	South African Wine Industry & Systems

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ABSTRACT

Global demand for environmentally-friendly grapevine cultivation and pest control has necessitated an improved understanding of the relationship between soil properties and beneficial naturally occurring antagonists like entomopathogenic fungi (EPF). This group of fungi presents a viable alternative for the control of destructive pests such as the grapevine mealybug.

Sixty-six soil samples were collected from 22 vineyards in the Western Cape, South Africa. The association between soil nutrient status and EPF prevalence was then examined. Fungi were isolated with methods of insect baiting and selective media. Fungal strains were identified and characterized using light microscopy and DNA analysis (ITS and BTub). In addition, fungal isolates were tested against a key grapevine pest, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) using an immersion bioassay at a concentration of 1 x 10^8 conidia ml^{-1}. Twenty-three fungal strains were isolated and correspondence analysis (CA) of data indicated a positive association between fungal occurrence and moderate to high levels of soil-based macronutrients. Binomial logistic regression analysis revealed that soil N, K, Ca, Mg and S concentrations and C/N ratio were correlated with at least one EPF species. This study showed that some soil nutrient properties correspond to greater occurrence of EPF in grapevine soils. Strains of *Beauvaria bassiana* (Hypocreales: Clavicipitaceae) caused the highest mortalities (82% to 87%).

In chapter three, I examined the effect of *B. bassiana* inoculation of grape plants on the infestation level of *P. ficus*, and the growth and volatile constituents of potted grape plants. The grapevines were inoculated with 1 x 10^8 conidia ml^{-1} of *B. bassiana* by drenching before experimentally infesting them with thirty *P. ficus* adult females. At four weeks post treatments, the fungus was re-isolated from leaves of 50% of the fungus exposed plants. No significant difference ($P > 0.05$) was observed in all the plant growth parameters measured in the fungus treated and control plants. Plant tissue analysis revealed markedly higher contents of Ca and Mg in leaf tissue of plants exposed to the *B. bassiana* relative to the control. GC-MS analyses showed that a significantly ($X^2=5.1$; $P<0.02$) higher number of known anti-insect volatile compounds (9) including napthtalene were present among fungus treated plants compared to the control plants (5). However, *B. bassiana* did not have any significant

effect on total polyphenol, alkaloid and flavonoids. Overall, treatment with fungus did not offer any protection against infestation of *P. ficus*.

In conclusion, this is the first study to report on the isolation of indigenous entomopathogenic fungal (EPF) strains within vineyards of the Western Cape. The study revealed that inoculating grapevine plants during cultivation had a net positive effect on the production of volatile compounds in grapevines. These findings shed light on the mechanisms involved in endophytic fungus-plant-insect interactions. This study contributes valuable information to future development of ecological approaches involving EPF for insect control in vineyards and in general, agricultural settings.

Keywords: biological control; entomopathogenic fungi; endophytic fungi; grapevine; *Planococcus ficus*; soil nutrient content; volatile compounds

CHAPTER ONE

General introduction and literature review

1.1 Introduction

The damage caused by pest insects is one of the primary factors leading to the reduced production of major agricultural crops. Many insects are herbivores and dependant on crops for their food. However, heavy insect infestations can cause major economic losses (Oliveira et al., 2014). A dramatic increase in economic losses due to mealybug has been observed with more than seven species of mealybug attacking grapevine. In extreme cases mealybug damage in India has been recorded up to 90% (Azam, 1983; Rajagopal et al., 1997; Batra et al., 1987). In order to mitigate the losses due to insect infestations, synthetic insecticides are used widely and rampantly for control of insects in farming systems (Wilson & Tisdell, 2001). Unfortunately, rapid development of resistance by insects, environmental contamination and high costs characterise the excessive and large-scale usage of synthetic insecticide (Pimentel et al., 1992). It is, therefore, imperative to find more long-term sustainable insect control methods.

Grapevine farming is one agricultural sector that is susceptible to severe insect infestations. One such pest is the grapevine mealybug, *Planococcus ficus* (Signoret); it infects various plant species, but it is most abundant in grapevine (Walton & Pringle, 2017). It occurs in all grape growing regions and causes productivity and quality reductions in wine and table grapes (Ben-Dov, 1994). The mealybug family Psuedoccidae consists of approximately 2 240 species, and 68 of those species are indigenous to South Africa (Millar, 2002). According to Walton et al. (2004) and Walton (2003), *Planococcus ficus* (Signoret) is the most abundant mealybug species in South African vineyards. It is a soft bodied wingless insect that often appears as white cotton masses on the leaves, stems and fruits of grapevine. It is a phloem feeder and can cause direct damage by sucking on plant fluids from the phloem sieve tubes using long sucking mouthparts called stylets. This can result in stunting, wilting, leaf yellowing and possibly plant death (McKenzie, 1967; Geiger & Daane, 2001; Walton & Pringle, 2017; Blumberg & Mendel, 2015). Besides being a vector for grapevine leafroll virus, grapevine mealybug can cause indirect damage through the secretion of honey mildew, which acts as a medium for sooty mould disease, resulting in the reduction of photosynthetic rate (Engelbrecht & Kasdorf, 1990; Geiger & Daane, 2001; Tsai et al., 2008).

For decades' viticulturists have relied on chemical control as the main means of controlling this pest. With organophosphate insecticides such as cypermethrin and fipronil constituting some of the main products used to control mealybugs populations. In 2017, the IPW (Intergrated Production of Wine) indicated that it was illegal for wine growers to use the chemical Fipronil to control ants as part of their control strategy linked to the grapevine mealybug. IPW has recommended the use of products with alpha-cypermethrin or chlorpyrifos as an active ingredient for control of grapevine mealybug; however, these chemicals are both harmful to bees, birds and aquatic life; for example, alpha- cypermethrin are cytotoxic and genotoxic (Kocaman & Topaktas, 2008). Despite demonstrated efficacies of synthetic insecticides, these environmental hazards and other well-known setbacks, such as cryptic habitats of mealybug and acquired insect resistance to these chemicals make it difficult to effectively control this pest using synthetic pesticides. Hence, alternatives, which are environmentally benign, are being explored. There has been a noticeable shift toward bio rational control agents. The use of fungal endophytes would allow the control of this pest systematically, thereby overcoming the challenges associated with its cryptic habits while presenting an environmentally friendlier alternative. Fungal endophytes have the ability to live asymptomatically within plant tissues (Carroll, 1988). This makes them potentially efficient in controlling insects with sucking mouthparts such as the mealybug.

The function and stability of terrestrial ecosystems is determined by factors, such as species diversity and composition. However, the ecological mechanisms and factors that may govern the occurrence of microbes such as EPF are not well understood (Rodriguez et al., 2009). Quesada-Moraga et al. (2007) evaluated the occurrence of EPF in cultivated and natural habitats in Spain. Thier study found no significant effect between habitat and the oocurrence of *B. bassiana* and found a strong correlation between *Metarhizhium anisopliae* (Hypocreales) and cultivated habitats. The findings from the current research project shed light on some of the occurrence of indigenous EPF in vineyard soils in the Western Cape. It also helps to identify indigenous EPF strains that may be pathogenic to *Planococcus ficus* (Signoret) (grapevine mealybug). Importantly, the study throws more light on the mechanisms through which the fungal inoculum influence insect infestations by assessing effects of fungal inoculum on the tissue nutrient content and volatile organic compounds of *Vitis vinifera*.

1.2 Hypotheses of the study

- Some soil nutrients influence the occurrence of EPF in vineyard soils.
- Some indigenous EPF, which are pathogenic to grapevine mealybug occur in the soils of vineyards of the Cape Winelands.
- The EPF ioculation will affect secondary metabolites produced by grapevine plants, quantitatively and qualitatively.
- The inoculation of plants with entomopathogenic fungus will improve the growth and leaf number of grapevine plants.
- Inoculating plants with entomopathogen conidia will reduce *P. ficus* infestation on grapevine under greenhouse and laboratory conditions.

1.3 Overall aim of the study was:

To evaluate bio-efficacy of selected South African entomopathogenic fungi against the grapevine mealybug.

Specific objectives of the study were:

- To determine the relationship between soil nutrients and the occurrence of EPF in vineyard soils.
- To assess the pathogenicity of some indigenous entomopathogenic fungal isolates *in vitro*.
- To assess the protective effect of EPF by looking at insect attack symptoms, infestation levels and fungal colonization of plant tissues.
- To evaluate the effect of entomopathogenic fungi on plant growth and tissue nutrient content.
- To assess chemical profile of secondary metabolites of plants exposed to EPF.

1.4 Structure of thesis

This study is a compilation of four succinctly written chapters.

Chapter One: This chapter comprises of introduction, background to the research problem and literature review.

Chapter Two: This chapter focuses on the occurrence of EPF in vineyard soils in the Western Cape and the nutrient factors that could affect their occurrence.

Chapter Three: This chapter focuses on the effect of EPF on the plant physiology of *Vitis vinifera*, specifically its influence on plant secondary metabolites.

Chapter Four: This chapter comprises of a general discussion the results, implications of these results in this area of study as well as recommendations from the author.

1.5 Literature Review

1.5.1 The Cape Winelands

The first vineyard in South Africa was established in 1655 and the first wine was made from the vineyard in 1659 by Jan van Riebeeck (Saayman, 2009). Unfortunately, farmers of the time were inexperienced when it comes to viticulture, so this industry only started to succeed in 1679 under the leadership of Simon van der Stel. This industry has been booming ever since. The Cape wine lands now stretch from the coastal regions of the Western Cape to the Klein Karoo (Breslin, 2011) and are spread into six regions. These six regions include Breede River Valley, Cape South Coast, Coastal Region, Klein Karoo, Olifants River and Boberg (Anonymous, 2015) and can be further split into the 12 districts (Figure: 1.1).

Figure 1.1: Geographical map of the Cape Winelands, Western Cape, South Africa (adapted from: https://www.wine-searcher.com/m/2012/09/south-african-winemakers-call-for-regional-shake-up).

1.5.2 South African Wine Industry

South Africa is one of the world's top ten wine producers, holding the 9th position in 2011. The industry has now grown and holds the 7th position accounting for 3% of wine produced worldwide (Anonymous, 2014). The industry has contributed R36 145 million to the annual GDP in 2013 alone and R19 287 million of that stayed within the Western Cape. According

to SAWIS (2015) the wine industry is responsible for employment amounting to R167 494 in the Western Cape. SAWIS (2015) also states that R5 972 million was generated through wine tourism worldwide and 43% of all tourists who come to the Western Cape go through the Cape Wine lands. Apart from wine the edible fruit is also used for making products such as raisins, currants and sultanas (Brady et al., 2010),

1.5.3 Wine Production

Wine is produced using the plant *V. vinifera*, which belongs to the family, Vitaceae. It is the most economically important plant in this family (Hickey & King, 1981). It originated in Europe and the use of grapes dates back to the 5th century and documentation of its use is seen in ancient Sumerian text as well as ancient Egyptian hieroglyphics (Vivier & Pretorius, 2001; South Africa, Department of Agriculture, 2012). It is estimated that there are between 5000-8000 varieties of *V. vinifera.* Hickey and King (1981) describe *V. vinifera* characteristics as: "A deciduous, woody climber, with forked tendrils occurring opposite 2 out of 3 of the palmate, 3 to 5 lobed, coarsely toothed leaves." It can grow up to 20 m if it is not pruned and uses its tendrils to climb (Shapiro, 2006). They form clusters of either male or female flowers.

1.5.4 Viticulture

The main objective of viticulture is to obtain a balance between grapevine vegetative growth and yield. Climate, slope & soil play important roles in overall quality of grapevines. Ideal summer temperatures for grapevine start at an average of 22 ºC and these temperatures are important for ripening the grape's fruit. Winter temperatures are necessary for the plant to enter dormancy but if the temperatures are too low it may damage the plant. Grapevines are often planted on slopes to maximize their exposure to the sun's rays.

Quality soil is essential for healthy root growth and poor soils can lessen vine growth and fruit yields. In addition, various nutrients influence vegetative growth and reproductive health in different ways. Nitrogen is required for most metabolic functions while potassium plays a role in protein synthesis and is an important component of cell vacuoles.

1.5.5 Pests of grapevine

The grapevine is host to several pests (Gonzalez, 1983; Viss, 1996) which have been documented to cause losses in grapevine production; for example, *Lobesia botrana* causes yield losses of up to 2767 kg/ha for the first carpophagous generation and 5685 kg/ha for the

second carpophagous generation (Thiery & Moreau, 2005). The occurrence of phylloxera in the Western Cape also caused significant damage and economic loss of grapevines (Annecke & Moran, 1982), consequently resistant rootstocks are being used for grapevine cultivation (Koundouras et al., 2008). Grapevine hawk moths, grapevine mealy bug, grape rust mite, vine leaf hopper and other insects have also been documented to cause significant damages to the leaves of *V. vinifera* (Nel, 1983; Cho et al., 2013). In South Africa, *P. ficus* has emerged as a major pest causing severe damage to the vine, directly and indirectly through the spread of the grapevine leaf roll virus (Walton & Pringle, 2017).

1.5.6 Biology & life cycle of mealybug

Figure 1.2: The Grapevine mealybug (adapted from: http://www.chemtica.com/site/?p=3014).

Life cycle of P. ficus

Adult female mealybugs lay eggs, which hatch from their wax thread covered egg-sacs after 7-10 days at an average temperature of 25 °C. These emergent crawlers cast their skins to become 2nd instar larvae and third instar larvae become adult females (Figure 1.2). After mating, the egg-sac develops and the female starts laying eggs at a rate of approximately 750 eggs at a time (Daane et al., 2012). The difference between the male and the female mealybug is that in the 2nd instar the male spins a cocoon in which the 3rd instar, pre-pupae and pupae develop. Females emit a sex pheromone to attract male counterparts and they are able to mate multiple times. The number of times they mate can also influence the number of eggs that the female lays (Waterworth et al., 2011). The damage caused to grapevine is mostly caused by the female due to its sucking mouth part, which males do not possess (Walton, 2001). The grapevine mealybugs complete their life cycle in 3-4 weeks during

summer. In winter they hide beneath loose bark, roots and cracks on the trunk of vines, where they feed and lay eggs but at a much slower rate because of low temperatures. In spring when temperatures start to increase the crawlers emerge and go to feed on new growth which has higher nutrient content. In late summer to autumn they start to migrate to their overwintering/hibernation areas (Le Vieux & Malan, 2016). Their populations are highest in January and February at which point the move towards grape bunches to feed high infestation can result in the desiccation of grape bunches and render them unmarketable (Goussard, 2013).

1.5 2 Morphology

The close resemblance and lack of morphological description of the different mealybug species can make it difficult to distinguish between species as demonstrated by the misidentification of *P. ficus* as *P. citri* (Cox, 1981; Walton, 2003; Walton et al., 2017). There have been advances in the identification of mealybug, such as the provision of a key to help identify South African Pseudococcidae genera by Millar (2002) and the development of diagnostic key that includes morphometric characters to distinguish mealybug species in South Africa (Wakgari & Giliomee, 2005).

1.5.7 Control of grapevine mealybug

1.5.7.1 Chemical Control

Chemical control has been the primary means of control for grapevine pests for decades. Control of this pest in South Africa rests on the application of chlorpyrifos or prothiofos. These chemicals are usually applied before budburst, after which, a supplementary application using a chemical with a short residual period is applied (Noubar et al., 2012). Yet, this pest is not easily controlled by these treatments due to the colonies being covered by waxy secretions and its cryptic habitats (Cloyd, 2017). These insects tend to spend most of their life cycle in inaccessible areas such as leaf junctures, which are difficult to reach with spray/contact insecticides, especially when plants have complex plant structures. Stringent legislation on pesticide use, increased concerns over negative environmental consequences, quality control, and consumer demands have encouraged the search for more environmentally friendly alternatives (Pertot et al., 2017).

1.5.7.2 Monitoring

If a vineyard is continuously plagued by mealybug infestation, it is important to monitor it early in the season. Monitoring only serves to predict the problem in order to plan for possible control strategies. The presence of ants is used as an early indicator due to the relation between the presence of ants and mealybug (Ripa & Rojas, 1990). At least 20 vines evenly distributed over a block of 1 hectare must be monitored. Later in the season, the presence of honeydew and sooty mold is used as an indicator because even if the pest is hidden under the bark it will secrete honeydew, which will make the infected area to look darker. Leaf yellowing/browning is also used as an indicator of the presence of mealybug and a mealybug count can be made at harvest time using highly infested berry clusters (Geiger & Daane, 2001). A more time efficient method is using sticky traps baited with sex pheromone (Hinkens et al., 2001). Infested vines are marked throughout the season to make it easier to control the infestation accurately and effectively. Once monitoring is done, a suitable control strategy is chosen. There is no method which is completely effective for the visual monitoring of grapevine mealybug. In addition, these methods require too much labour and time to be efficient (Geiger & Daane, 2001).

1.5.8 Biological Control

1.5.8.1 Predators and Parasitoids

DeBach (1964) defines biological control as "the actions of parasites, predator and pathogens in maintaining another organism's density at a lower average than would occur in their absence". According to Walton (2001), there is insect resistance to the use chemical pesticide globally, and grapevine growers in South Africa are advised to keep chemical pesticide usage minimal. The most common natural enemies of mealybug include the parasitic wasps; *Anagyrus pseudococci*, *Leptomastix dactylopii* and *Coccidoxenoides peregrines*. These wasps lay eggs in the mealybug; the eggs then develop in the body cavity of the mealybug. When the parasite has engulfed the body cavity and hatched it is able to kill the mealybug. The presence of these wasps is hard to track once they have been released and it is difficult to establish whether mealybugs have been controlled by these wasps because they are difficult to see with the naked eye. A challenge presented by this technique is that these predators have a low tolerance to winter temperatures. The most abundant mealybug predator is the lady beetle. They are attracted to large mealybug infestation along with the honeydew produced. More research is needed on the effectiveness of lady beetle as mealybug predators (DeBach, 1949). Lacewings have also been shown to suppress mealybug populations by

killing small mealybugs. Yet, lacewigs have difficulty feeding on eggs which are protected by wax secretions of the mealybug (Danee et al., 2008).

1.5.8.2 Entomopathogenic Fungi

Webber (1981) demonstrated how the presence of fungi in their hosts can cause a reduction in insect attacks. Other documented cases of EPF include that of Funk et al. (1983) who showed its protection of rye grass against the sod web worm. Studies by Barker et al. (1984) and Prestidge et al. (1984) demonstrate that plants without the presence of EPF were extremely affected by insect attacks. Strains of the fungi *B. bassiana* and *M. anisopliae* have been used to control the tsetse fly (Maniania & Ekesi, 2013). The control of potato psyllid the vector for the potato chip disease using *M. anisopliae* and *Isaria fumosorosea* (Lacey et al., 2010) and the control of redbay ambrosia beetle using *I. fumosorosea* and strains of *B. bassiana* have been demosntrated.

Fungal endophytes are common and are diverse organisms that live asymptomatically and sometimes systematically within plant tissues (Fahey, 1991; Wilson, 1995; Gómez-Vidal, 2006). They usually inhabit the above ground organs of plants, which is what differentiates them from mycorrhiza (Remy et al., 1994; Carroll, 1988). The host and endophytes relationship in natural populations is poorly studied. Endophytes are considered mutualists mainly through reducing pathogens and herbivores via production of mycotoxins (Vidal & Jaber, 2015). Some agronomic grasses infected with endophytes exhibit toxic and noxious effects on insect pests and increased competitive abilities (Clay, 1988). This can be as a result of the production of alkaloids such as peramine by fungal endophytes (Muller & Krauss, 2005).

Plant secondary metabolites do not play an important role in basic plant functions, but they are crucial to plant adaptation and defences. Among these defences/adaptations is the production of phytoalexin antimicrobial molecules (Gao et al., 2010). It is suggested that the production of secondary metabolites induced by fungal endophytes is one of their main reasons for their role in plant protection (Rohlfs & Churchill, 2011). Many researchers have focused on these secondary metabolites. Yong et al. (2009) showed how endophytic fungi (*Fusarium* spp.) could promote the terpenoid content of *Euphorbia pekinensis* (Gaom et al., 2011). A similar result was observed for the suspension cultures of *Taxus cuspidate* with the addition of endophytes, which led to the increase yield of paclitaxel (Li & Tao, 2009).

Although EPF have gained interest in their defensive mutualism, they may also have an effect on the physiological growth of plants as well as secondary metabolite production within the plants. A few studies have looked into their ability to promote plant growth. Raya-Diaz et al. (2017) observed an increased plant height in seed treated sorghum plants coupled with increased root length and finer root hairs; this study suggests that this increase in growth may be linked to how EPF are able to increase the bioavailability of nutrients. The study also demonstrates improved health of sorghum plants due to Fe acquisition systems of EPF inoculated into plants. EPF is known to release organic acids which facilitate an increase in Fe, Cu, Ag, Zn and P (Crespo et al., 2008). Additionally, *Metarhizium robertsii* has been shown to promote nitrogen absorption in switchgrass and haricot bean (Sasan & Bidochka, 2012).

1.5.8.3 EPF mode of action

EPF infect insects via penetration on the host's cuticle. During this process the fungus produces specialized infective structures that include penetration pegs/appressoria which enable the growing hyphae to penetrate the host integument (Dar, 2017; Mora, 2017). When inside the insect they can kill the host via hyphal growth. Some entomopathogenic fungi, such as *Beauveria* spp. can produce insecticidal peptides and toxic metabolites to kill their host (Inglis et al., 2001; Ortiz-Urquiza & Keyhani, 2013). These bioactive compounds which include efrapeptins, cordycepin and destruxins secreted by *Tolypocladium sp., Cordyceps militaris* Link and *M. anisopliae* (Metsch) have insecticidal and antifeedant properties (Amiri et al., 1999; Bandani & Butt, 1999; Bandani et al., 2000; Kim et al., 2002). Extracts of the protein bassiacridin originating from the EPF *B. bassiana* were proved to be toxic to *Locusta migratoria* (Quesada-Moraga et al., 2004). *M. anisopliae* is known to produce destruxins A&B while beauvericin one of the most prominent mycotoxins is produced by *B. bassiana*, *Fusarium* spp and *Peacilomyces fumosoroseus* (Wang & Xu, 2012; Golo et al., 2014). Yet, according to Quesada-Moraga et al. (2006), these insecticidal proteins have not been adequately exploited for sustainable insect management.

The anti-insect activity of EPF is not only limited to direct contact/hyphal growth within insects. EPF are known to repel insects, induce weight loss, cause growth and development reduction of insects as well as increase the pests' death rate (Azevedo et al., 2000). The anti-

insect activities have been linked to the production of toxins by endophytic EPF as well as the ability of the fungi to make the plant unpalatable to insect pests (Carroll, 1988; Clay, 1988a; 1988b). A study by Bacon et al. (1977) further supported this by looking into the toxins produced by EPF *Epichloe typhina* which rendered its host toxic to herbivores. Fungal endophyte *Acremonium coenophialum* also produces lolines, which alter the feeding behaviour and weight of insect pests feeding on *Festuca arundinacea* (Reidell et al., 1991). Bacon and Hill (1996) also observed that alkaloids produced by entomopathogenic fungi are toxic to insects. Other toxins that have been found in EPF that control insects include heptelidic acid, rugulosne and N-formilonine (Miles et al., 2012).

Despite the evidences of the protective effects of EPF against harmful parasites of plants (phytophagous insects and phytopathogens) the toxins they produce can have a deleterious effect on humans and livestock. For examples, entomopthoralean fungus *Conidiabolus coronatus* causes chronic granulomatous infections of the nasal submucosa and *Basidiobolus ranarum* have been linked to chronic subcutaneous infections. Therefore, while it is important to understand the effects of entomopathogenic fungi on the production of secondary metabolites with anti-insecticidal properties, it is equally imperative to establish the effects of fungi in the accumulation of metabolites which are potentially toxic to humans (Costa et al., 1991; French & Ashworth, 1994; Gugnani, 1999; Pang, 2004). However, Glare (2004) argues that any development of the hazardous strains into biocontrol agents is unlikely because mammalian toxicity packages must be submitted during registration processes to demonstrate safety.

1.5.9 Inoculation of EPF into plants

M. anisopliae and *B. bassiana* are EPF that have been used to effectively control insect pests on plants endophytically (Tiago, 2014). The fungal inocula are taken up by plant tissues. Artificial inoculation techniques can be used to expose plant tissues to these fungi. According to Azevedo et al. (2000), research in this area is also scarce. Bing & Lewis (1992a; 1992b; 1991) successfully placed *B. bassiana* inside corn tissues by inoculating it via injection and aspersion; they achieved 98.3% and 95% tissue colonization by aspersion and injection methods, respectively.

Farmers including grapevine farmers apply systematic chemical insecticides to the soil to reduce mealybug populations. They are taken up by the roots and translocated through the plant's vascular system. While they may be effective in control, long term application of the insecticides threaten ecosystem services provided by terrestrial and aquatic ecosystems including soil and freshwater functions (Edwards, 1966; Changnon et al., 2015). Fungal endophytes, which are generally considered as being environmentally benign, have prospects to substitute some of the toxic synthetic insecticides.

Since entomopathogenic fungi present an environmentally friendly alternative to these pesticides, they can be applied systematically by drench and be taken up by plant roots and translocated through the vascular system; xylem vessel elements or phloem sieve tubes. This will ensure insects with piercing mouthparts such as aphids, whiteflies and potentially *P. ficus* are exposed to infective fungal inocula at lethal doses. The fact that EPF are translocated via the vascular systems means that they may protect cultivated plants better than contact insecticides.

Entomopathogenic fungi can be employed under 3 biological control strategies.

1. Classic biological control

   This refers to the use of natural enemies on an insect host which is exotic in an area.

2. Augmentation

   Natural enemies are present in indigenous pest populations but they are either too few or are active too late to limit pest populations. In these instances, natural enemies can be augmented by inoculation or inundation.

3. Conservation

   Conservation is the modification of farming practices to enhance the activity of naturally occurring EPF conservation and to identify effective indigenous natural enemies and use practices which enhance these.

1.5.10 Fungal ecology in soil

Fungi fulfil a range of ecological functions, particularly those associated with nutrients and carbon cycling processes in the soil (Behie & Bidochka, 2013). Some fungi are widely distributed in soils while others are limited to certain habitats. The occurrence and distribution of these organisms is influenced by factors, such as soil and climatic conditions, surface vegetation and soil management practices (Lauber et al., 2008; Boddy et al., 2014).

Current understanding of fungal biodiversity in soil is limited. Studies show that fungi are most abundant in the surface of soil and decrease as you go deeper into the soil profile. Although Frey (2007) points out the lack of thorough research on the distribution of organisms throughout the soil profile. According to Taylor (2014) molecular studies have so far failed to reach comprehensive estimates of fungal diversity. A variety of approaches have been used to estimate how many fungal species exist (Hawksworth, 2001; Schmit & Mueller, 2007; Hoshino & Morimoto, 2008).

Little is known about the small-scale distribution of fungi in soil and how that may link to particular soil conditions. Although fungi have been shown to be more abundant in spring and autumn because of higher nutrient availability during these seasons (Voříšková et al., 2014), the abundance of fungi, particularly EPF, may be hindered by certain agricultural practices, such as the use of pesticides. In a study by Clifton et al. (2015) organic fields and their margins had significantly more EPF species than conventional fields and their accompanying margins. Klingen et al. (2002) argues that organic fields are more suitable environments for EPF due to lack of synthetic inputs. This is backed by multiple studies stressing concerns over the adverse effects of synthetic chemicals on the abundance of EPF in soils. The feasibility of fungi as biocontrol agents depends on numerous constraints including soil conditions and nutrients which may affect growth and occurrence. Jabbour and Babercheck (2009) found a positive correlation/association between EPF abundance and metallic ions, gravimetric water content and organic matter content in soils. Although little has been studied with regards to the link between soil nutrients and EPF occurrence, Vega (2003) measured the *in vitro* spore yield of EPF at six C/N ratios. The highest spore yields were observed at 38g L^{-1} carbon concentration and a C/N ratio of 10:1.

Fungi are a major component of the functioning of all terrestrial ecosystems. Successful survival and growth of plants are highly dependent on soil abiotic factors as well as the activity of microbial populations. The improved understanding of the ecology of indigenous populations of these fungi is essential to pest control in agro-ecosystems. Although these fungi have been vastly researched for their biocontrol properties, in contrast there is a lack of reaserch into the fundamental ecology of these fungi in terrestrial ecosystems (Meyling & Eilenberg, 2007)

1.5.11 *Beauvaria bassiana*

B. bassiana has garnered vast interest into its potential as an insect biocontrol agent. It has the ability to form intimate asymptomatic relationships with its host plant and has a wide plant host range (Quesada-Moraga et al., 2009; Rehner, 2005). It has aubiquitous distribution and is used against agricultural, veterinary and medical insect pests. It has been researched and developed commercially for control of soil borne insects and foliar feeding insects (Lord, 2005). It causes white muscadine diseases in insects and its current commercial formulations are Mycotrol®, Mycotrol 0®, BotaniGard®ES, BotaniGard®22WP and Naturalis®L (Groden, 1999). These are registered for use against the mealybug and other pest insects. *Beauvaria* products are considered reduced risk pesticides and are generally nontoxic to beneficial insects although care should be taken in applying to areas where bees are actively foraging (Vandenbergi, 1990; Zimmermann, 2007; Thungrabeab, 2007). *B. bassiana* has been isolated from soils of countries all over the world including Burkina Faso, Benin, Congo, Togo, Kenya and more recently South Africa (Fargues et al., 1997; Ekesi et al., 2002).

1.5.12 *Metarhizium* spp.

M. robertsii and *M. anisopliae* are among the most commonly isolated insect pathogenic fungi (Bischoff et al., 2009). *Metarhizium* spp. has over 200 insect hosts and establishes mutualistic interactions with plants as a rhizospheric fungus (Hu & St. Leger, 2002; Prior, 1992; Roberts & St. Leger, 2004). Sasan & Bidochka (2012) showed that plant roots treated with *M. robertsii* grew faster and had increased density of root hairs compared to control plants. *Metarhizium* spp. are commonly isolated from soils in Brazil, most of which are in forested areas (Rocha et al., 2013; Rezende et al., 2015).

1.5.13 *Clonostachys rosea*

C. rosea is an endophyte, which is parasitic to insects, has inhibiting effects against nematodes and is a mycoparasite against other fungi (Toledo et al., 2006). It produces a range of volatile organic compounds which are toxic to insects and, thus, have attracted interest in its use as a biocontrol agent (Rodríguez et al., 2011; Stewart et al., 2012). It has been suggested that this fungus may induce systemic defense in plants which could lead to inhibitory effects on nematodes (Zhang et al., 2008; Zhai et al., 2016). *Clonostachys rosea f catenula* is currently used to control *Fusarium* root and stem rot as well as Pythium damping-

off disease (Rahman & Punja, 2007; Chatterton & Punja, 2010). *C. rosae f catenula* strain J1446 (Prestop®) is currently available in Finland as an antagonist against root and foliar greenhouse pathogens. In spite of existing research into the use of this fungus in biocontrol, information on its mechanisms of action is still scanty.

CHAPTER TWO

Association between chemical properties of vineyard soils and occurrence of entomopathogenic fungi with varying virulence against *Planococcus ficus*

2.1 Introduction

Conventional control of arthropod pests in agricultural production including vineyards has depended on synthetic chemicals, which have been liable contributors to environmental degradation (Huber et al., 2000; Gaskin et al., 2002). This has necessitated the quest for alternative and more environmentally benign control strategies, which has led to the recognition of biological control agents such as entomopathogenic fungi (EPF) (Strasser et al., 2000; Fernandez-Cornejo 1998; Epstein & Bassein 2003).

EPF are generally ubiquitous (Goettel, 1984). However, farming and farming system influence species distribution (Schuster & Schroder, 1990; Chandler et al., 1997; Goble, 2009). For example, Goble (2009) recovered significantly more isolates of the EPF *Beauveria bassiana* (Hypocreales: Clavicipitaceae) from organically farmed soils than conventionally farmed soils of citrus orchards in the Eastern Cape Province of South Africa. Farming activities such as grapevine cultivation disrupt natural biotic and abiotic conditions, causing ecological disequilibrium, which can affect insect occurrences as well as those of their natural enemies, including EPF (Pell et al., 2010; Bruck, 2010; Crous et al., 2017).

The abundance of soil microorganisms is affected by soil nutrient content in space and time (Koorem et al., 2014). Therefore, in order to achieve optimum agronomic practices that simultaneously ensure optimum grapevine yield and EPF occurrence, it is important to understand the ecological relationship between soil nutrient status and EPF (Safavi et al., 2007). Studies such as those by Quesada-Moraga et al. (2007) and Klingen et al. (2002) have already found a positive correlation between the level of soil organic matter content and the presence of EPF species, *Beauveria bassiana* and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae), which they attributed to the release of nitrogen during the mineralization of soil organic matter.

The Cape Winelands of South Africa cover 99,680 hectares and 80% of grapevine cultivation in the country occurs in this area (WOSA, 2016). It is characterized by a Mediterranean climate with intense sunlight, dry heat and cold, wet winters (Directorate Plant Production, 2012). Most of the vineyards in this area use conventional cultivation approaches to achieve

high yields, control insects and phytopathogens. Nevertheless, insect pests, such as the grapevine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) remain serious obstacles to achieving optimum crop production. To the best of our knowledge, this is the first study that examined the occurrence of EPF in the Cape Winelands of southern Africa and the relationships between EPF presence and soil nutrient content.

The objectives of this chapter were to (i) survey the occurrence of EPF in vineyard soil, (ii) explore the relationship between soil properties and presence of EPF, and (iii) ascertain their pathogenicity against the grapevine mealybug.

2.2 Materials and Methods

2.2 1 Soil sampling

A total of 66 soil samples were collected from 22 vineyards in the Western Cape, South Africa in the spring (October – November) of 2016, when minimum temperatures were between 12-16 ºC and maximum temperatures 22-26 ºC. These selected vineyards are located in five of the Western Cape's major wine production areas, namely Stellenbosch, Constantia, Franschoek, Paarl and Worcester (Tables 2.1 & 2.2, Figure 2.1). At each vineyard, three blocks of grapevine were randomly selected, 200 m apart. Soil samples were collected with a garden spade at a depth of 15 – 20 cm after removal of surface debris. Soil samples, 300 g and 1 kg in weight, were put into transparent plastic containers and paper bags, respectively. The 1 kg bags of soil were sent to Bemlab PTY. LTD (Somerset West, Western Cape, South Africa) for soil analysis and the 300-g soil was used in the process of fungal isolation within 24h of collection.

Figure 2.1: Soil collection (A) at vineyards (B).

2.2.2 Isolation of entomopathogenic fungi

Fungi were isolated from the soil samples by baiting with 5th instar larvae of *Cydia pomonella* (Lepidoptera: Tortricidae) obtained from a continuous laboratory culture held at Entomon PTY. LTD. (Stellenbosch, Western Cape, South Africa) and fed on an artificial diet mixture described by Stenekamp (2011). 5th instar larvae had been previously used to isolate 39 EPF isolates from soils in various locations of the Western Cape Province of South Africa (Abaajeh & Nchu, 2015). Soils in the transparent plastic containers were sieved until fine and sprayed with sterile distilled water (2.5 mL) until moist. Fifty grams of each sample was passed through a metal sieve with a mesh size of 4 mm and transferred to a plastic container (Figure 2.2), and then sprayed with sterile distilled water (2.5 mL) until moist. Fifteen fifth instar *C. pomonella* larvae were placed on the surface of each soil sample in the plastic container and incubated in the dark at 25 °C. Containers were briefly inverted and returned to upright position once per day for the first week to increase contact between insects and soil particles and checked 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 1 min, and then placed on moistened filter paper and incubated at 25 ºC. Sporulating larvae were placed on a selective media of half strength PDA (Potato dextrose agar) (Sigma-Aldrich PTY. LTD., South Africa) (19.5 g/L) supplemented with 0.02 g/L penicillin and 0.04 g/L streptomycin. From each soil sample a suspension was made using soil (100 mg) and sterile 0.05% Tween 80 (Sigma-Aldrich Pty. Ltd., South Africa). The mixture was agitated for 10 min to form a suspension. The suspension (50 µL) was plated on medium in Petri dishes (9

cm diameter) with half strength PDA (19.5 g/L) supplemented with 0.02 g/L penicillin and 0.04 g/L streptomycin per soil sample. Each soil sample had 5 replicates.

Figure 2.2: Isolation of fungi using codling moth.

2.2.3 Fungal identification

To obtain single spore isolates the method described by Ho & Ko (1997) was followed. Conidia from the single-conidia cultures of each fungal isolate were harvested and surface cultured on half strength PDA at 25 ± 2 °C; 70 ± 2% RH for three to four weeks. Twenty-three fungal cultures were transferred to the Molecular Biology Laboratory in the Department of Microbiology, Stellenbosch University for a more precise morphological identification. All strains were plated on Malt Extract agar (MEA), Czapek Yeast agar (CYA) and Oatmeal agar (OA) for initial grouping. The morphology was determined from structures mounted in lactic acid, using a Nikon Eclipse E800 light microscope. For molecular characterization, DNA was extracted from fresh cells using the ZR fungal/bacterial DNA kit (Zymo Research, California, USA) and the presence of genomic DNA was checked on a 1% agarose gel, stained with ethidium bromide. PCR reactions were done using a GeneAmp PCR System 9700 (Applied Biosystems, USA). The reaction mixture contained 0.5 µl (±50 ng/ul) of the purified genomic DNA, 500 nM of each primer and 5 µl of 2X Kapa Taq Ready mix (Kapa Biosystems, South Africa) in a total volume of 10 µl. Gene regions used for the comparisons included the internal transcribed spacer region (ITS) (ITS1 [forward] ITS4 [reverse]) (White et al., 1990), (Bt2a [forward] and Bt2b [reverse]) (Glass & Donaldson 1995). The PCR conditions consisted of initial denaturing step at 94 °C for 5 min, followed by 30 cycles (ITS)/35 (BTub) denaturing at 94 °C for 30 s (ITS)/45 s(BTub), annealing at 56 °C (ITS)/56°C (BTub) for 30 s (ITS)/45 s (BTub) and elongation at 72°C for 45 s(ITS)/ 1 min (BTub). The reaction was

completed with a final elongation for 7 min at 72°C, and then cooled and held at 4°C. PCR samples were separated on a 1% agarose gel, stained with ethidium bromide and visualized using ultraviolet light. The amplicons from the PCR reactions were run on an ABI 3010xl Genetic Analyser. Sequences were compared using BLAST on the NCBI Genbank database (www.ncbi.nlm.nih.gov) and trees were compiled using ClustalX for the alignment and PAUP for the analysis. Distance analyses using the neighbour joining were performed and the strength of the branches was calculated with a 1000 bootstrap repetition. Fungal cultures are kept in the germplasm of CPUT and Department of Microbiology, Stellenbosch University. Conidia from fungal monocultures of each of the fungal strains were harvested and surfaced cultured on PDA (Potato dextrose agar) in 9 cm diameter Petri dishes at 25 ± 2°C; 70 ± 2% RH for 3-4 weeks.

2.2.4 Soil Analysis

Soil was air dried and sieved (2 mm sieve) prior to tests. Total P, K, Ca, Mg were determined as described in Campbell & Plank, (1998) with slight modifications, and the pH was measured as described by The Non-affiliated Soil Analyses Work Committee (1990). Optical Total C and N were determined using total combustion using a LecoTruspec® C/ N analyzer. A guide containing the recommended nutrient levels of each nutrient needed for optimum cultivation of grapevine was used to categorize nutrient levels in soil into Low, Optimum and High. This guide was obtained from Bemlab Pty Ltd, a commercial testing laboratory. Bemlab is accredited by the South African National Accreditation System (SANAS) and complies with ISO/IEC 17025 standards of SANAS. Optimum C/N ratio levels were adapted from Cooke (1967).

2.2.5 Assessing pathogenicity against *Planococcus ficus*

Prior to assessing pathogenicity, the viability of conidia was determined by spread-plating 0.1 ml of conidia suspension, titrated to 1 x 10^6 conidia ml^{-1} on PDA plates. Two replicated sterile microscope cover slips were placed on each plate and incubated at 26 ± 2 °C. Plates were then examined after 24 h and percentage germination determined from 100-spore counts under each cover slip. The germination percentage was over 90%.

An immersion bioassay was used to assess the virulence of 15 EPF isolates (belonging to four species selected based on of their reported pathogenicity on insect) collected from the sampled soils against *P. ficus*. Adult female of *P. ficus* were obtained from the ARC

Infruitec-Nietvoorbij (Agricultural Research Council) Stellenbosch, South Africa courtesy of Dr K.A. Achiano. The mealybugs were reared on butternut squash in a darkroom at 25 ºC and 60% RH (Figure 2.3). Three to four-week-old aerial conidia of the different fungal isolates were harvested from the single spore cultures by scraping, and then suspended separately in 50 ml centrifuge tubes containing sterile 0.05% Tween 80 and glass beads. Each suspension was mixed vigorously using a vortex shaker for 5 min to homogenize it. Conidial concentration was determined using an improved Neubauer haemocytometer; the fungal isolates were tested at a standardized conidial concentration of $1x10^8$ conidiaml^{-1}. The control solution had sterile 0.05% Tween 80. Insects were dipped individually into 3 ml conidial suspensions or control solution for 30 seconds. For each treatment, ten adult female insects were placed in a 9-cm diameter Petri-dish containing a disk (6 cm in diameter) of sterilized leaves of *V. vinifera* 'Cabernet Sauvignon', replicated five times. The Petri-dishes were kept at 25 ºC, 60 % RH and 12:12 L:D in a growth chamber and mortality data was recorded on the 4^{th} day. Insect cadavers were surface sterilised in 70% ethanol for 5 s, rinsed in sterile distilled water for 1 min and incubated at 25 ± 2 °C and 90 ± 5% RH, and then checked for mycosis under a dissecting microscope.

Figure 2.3: Mealybugs rearing on butternuts

Statistical analysis

Data on insect mortality in the immersion bioassay was Abbott-corrected (Abbott, 1925). Mortality data was arcsine square-root transformed, and then analyzed using one-way analysis of variance (ANOVA) in PAST (Hammer et al., 2001). Means were separated by Tukey's pairwise comparisons test. The statistical level of significance was fixed at P = 0.05. The relationship between soil nutrient content level and EPF presence (*B. bassiana*, *M. robertsii* and *C. rosae*) was determined with a correspondence analysis (CA) using the

statistical software PAST (Hammer et al., 2001). This was followed by binomial logistic regression analysis to establish the association between the concentrations of the different nutrients (independent and continuous variables) and the occurrence of fungi (presence or absence) isolated from soil samples using Statistical Package for Social Sciences (SPSS) software (version 16, SPSS, Inc, Chicago, IL, USA).

2.3 Results

2.3.1 Isolation and identification of entomopathogenic fungi

A total of 23 fungal isolates were obtained from 63% of sampled vineyards comprising of 11 different species with some sites yielding multiple isolates. (Table 2.1) The isolated fungal species were: *Metarhizium robertsii* (Hypocreales:Clavicipitaceae), *Fusarium inflexum*, *Fusarium falciforme*, *Fusarium solani* (Hypocreales:Nectriaceae) and *Beauvaria bassiana* (Hypocreales:Clavicipitaceae), *Clonostachys rosea* cf *catenula* (Hypocreales: Bionectriaceae) *Talaromyces pinophilus, Talaromyces ruber, Talaromyces sayulitensis* (Eurotiales), *Aspergillus oryzae* and *Aspergillus laciniosus* (Eurotiales: Trichocomaceae.). The predominant species isolated from vineyard soil was *C. rosea catenula*, which recorded 26% (6 of 23 isolates) and its isolates were mostly obtained by soil dilution method (Table 2.2). On the other hand, isolates of the two well-known EPF species (*M. robertsii* and *B. bassiana*) were frequently collected using the insect bait method (Tables 2.1 & 2.2). The BLAST results based on ITS and Beta-tubulin (BTub) primers are presented in Table 2.1. Neighbour-joining trees (based on ITS and BTub sequences) showing relationships in the different genera are presented in Figure 2.4.

Table 2.1: Sequence identification of extracted DNA from entomopathogenic fungal isolates collected from vineyards in the Cape Winelands.

Location		Fungal Isolate	Primer	GenBank accession no.	GB ID	Blast results – fungal species identification
Stellenbosch	Site 1	SM 3	BTub	MH598822	BankIt2131828 seq11	*Beauveria bassiana*
			ITS	MH595805	SUB4279761 seq34	
		CPUT 15	BTub	MH598813	BankIt2131828 seq2	*Metarhizium robertsii*
			ITS	MH595796	SUB4279761 seq25	
		SM 4A	BTub	MH598823	BankIt2131828 seq12	*Clonostachys rosea cf catenula*
			ITS	MH595806	SUB4279761 seq35	
	Site 2	SM 6	BTub	MH598826	BankIt2131828 seq15	*Clonostachys rosea cf catenula*
		SM 2	ITS	MH595809	SUB4279761 seq38	
		SM A	BTub	MH598821	BankIt2131828 seq10	*Fusarium falciforme*
			ITS	MH595804	SUB4279761 seq33	
			BTub	MH598833	BankIt2131828 seq22	*Beauveria bassiana*
			ITS	MH595816	SUB4279761 seq45	
	Site 4	SM 7	BTub	MH598827	BankIt2131828 seq16	*Fusarium inflexum*
		SM 5	ITS	MH595810	SUB4279761 seq39	
			BTub	MH598825	BankIt2131828 seq14	*Talaromyces sayulitensis*
			ITS	MH595808	SUB4279761 seq37	
	Site 5	CPUT 14	BTub	MH598812	BankIt2131828 seq1	*Talaromyces pinophilus*

		CPUT 17	ITS	MH595795	SUB4279761 seq24	
			BTub	MH598816	BankIt2131828 seq5	*Talaromyces ruber*
			ITS	MH595799	SUB4279761 seq28	
Constantia	Site 7	CPUT 16	BTub	MH598814	BankIt2131828 seq3	*Metarhizium robertsii*
		CPUT 20	ITS	MH595797	SUB4279761 seq26	
			BTub	MH598818	BankIt2131828 seq7	*Talaromyces pinophilus*
			ITS	MH595801	SUB4279761 seq30	
	Site 9	CPUT 19	BTub	MH598817	BankIt2131828 seq6	*Metarhizium robertsii*
		SM 8	ITS	MH595800	SUB4279761 seq29	
			BTub	MH598828	BankIt2131828 seq17	*Clonostachys rosea cf catenula*
			ITS	MH595811	SUB4279761 seq40	
	Site 10	SM B	BTub	MH598834	BankIt2131828 seq23	*Fusarium solani*
			ITS	MH595817	SUB4279761 seq46	
	Site 11	SM 9	BTub	MH598829	BankIt2131828 seq18	*Clonostachys rosea cf catenula*
			ITS	MH595812	SUB4279761 seq41	
Paarl	Site 14	SM 10	BTub	MH598830	BankIt2131828 seq19	*Clonostachys rosea cf catenula*
			ITS	MH595813	SUB4279761 seq42	
		SM 13	BTub	MH598832	BankIt2131828 seq21	*Beauveria bassiana*
			ITS	MH595815	SUB4279761 seq44	
Malmesbury	Site 15	CPUT 18	BTub	MH598816	BankIt2131828 seq5	*Clonostachys rosea ct catenula*
			ITS	MH595799	SUB4279761 seq28	
	Site 17	SM 1	BTub	MH598820	BankIt2131828 seq9	*Fusarium solani*

			ITS	MH595803	SUB4279761 seq32	
Franschoek	Site 20	SM 5	BTub	MH598824	BankIt2131828 seq13	*Aspergillus oryzae*
			ITS	MH595807	SUB4279761 seq36	
	Site 22	CPUT 22	BTub	MH598819	BankIt2131828 seq8	*Metarhizium robertsii*
			ITS	MH595802	SUB4279761 seq31	
		SM 12	BTub	MH598831	BankIt2131828 seq20	*Aspergillus laciniosus*
			ITS	MH595814	SUB4279761 seq43	

Table 2.2: Locations within the Western Cape Winelands, South Africa where soils were sampled, fungal species isolated and Abbott-corrected insect mortality means ±SE (%) caused by fungal isolates (1 x 10^8 spores ml-^{1}) against adult females of *Planococcus ficus* at four days post treatment.

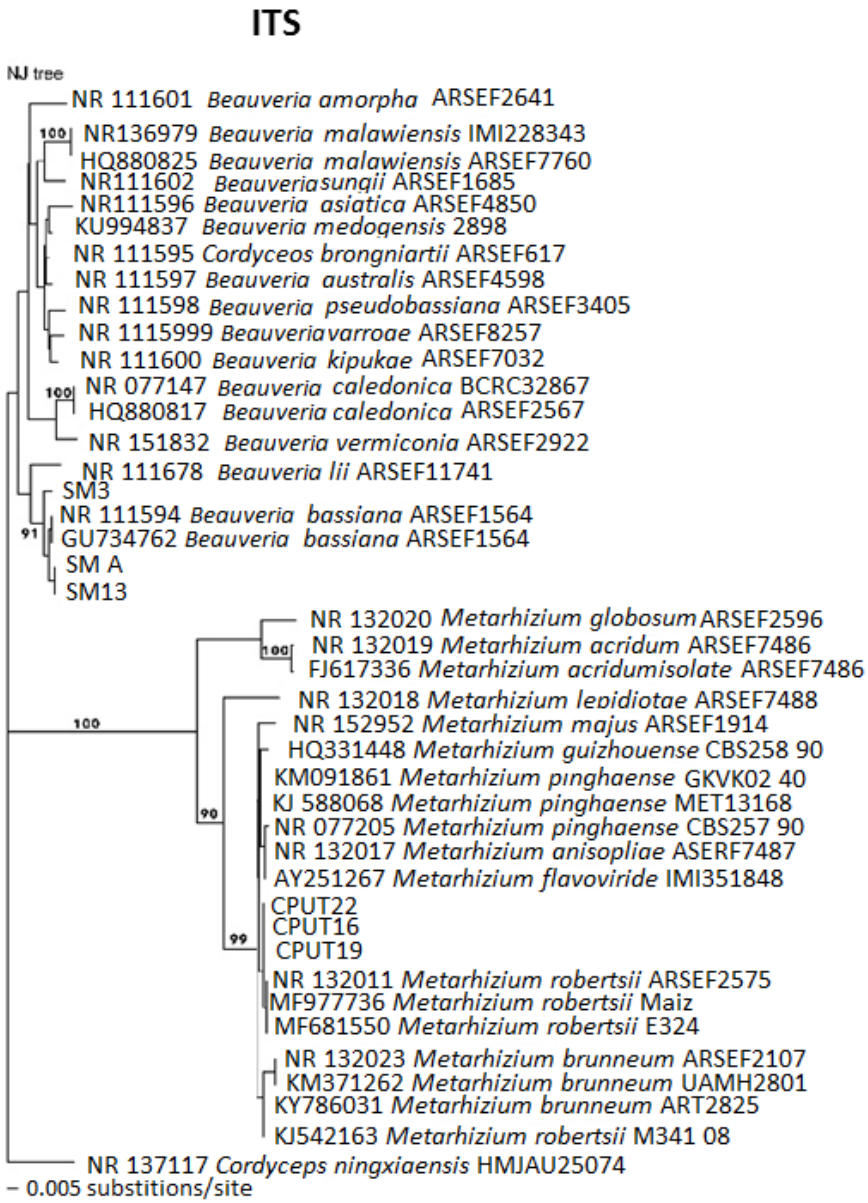
Location	Site	GPS coordinates	Fungal Isolate	Isolation method I (Insect bait) SD (Soil dilution)	Fungal Species	Abbott corrected mortality ±SE
Stellenbosch	Site 1	18.492800°-33.532048°	SM3	I	*Beauveria bassiana*	87±3ab
			CPUT15	SD	*Metarhizium robertsii*	44±5efgh
				SD		
			SM4A		*Clonostachys rosea cf catenula*	46±1.0efg
	Site 2	18.749890°-34.015281°	SM6	SD	*Clonostachys rosea cf catenula*	69±10abcde
			SMA	I	*Beauveria bassiana*	77±2abcd
			SMA	I	*Fusarium falciforme*	-
	Site 3	18.873310°-33.923300°	-	-	-	-
	Site 4	19.280972°-34.236746°	SM7	I	*Fusarium inflexum*	-
			SM5	SD	*Talaromyces sayulitensis*	
	Site 5	18.767340°-33.957130°	CPUT14	SD	*Talaromyces pinophilus*	-
			CPUT17	SD	*Talaromyces ruber*	

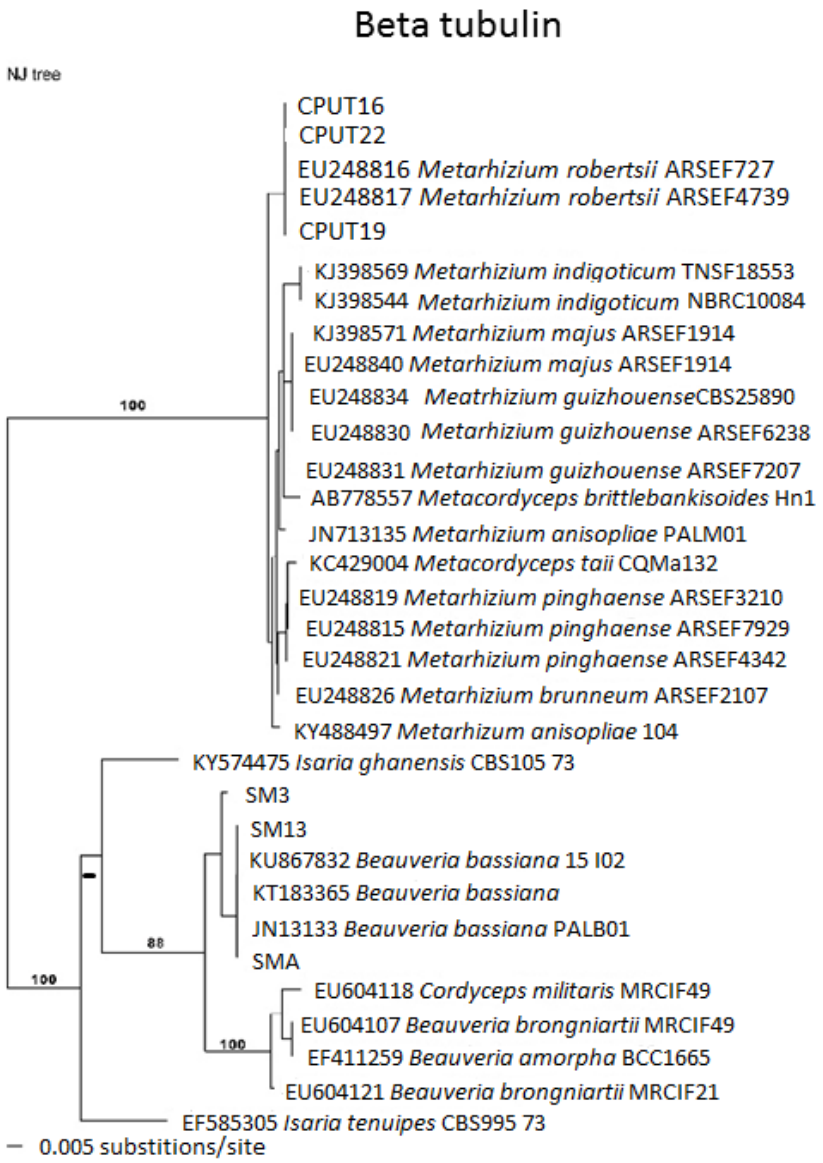
	Site 6	18.913177°-34.009773°	-	-	-	-
Constantia	Site 7	18.426574°-34.043625°	CPUT16	I	*Metarhizium robertsii*	23±6ij
			CPUT20	SD	*Talaromyces pinophilus*	-
	Site 8	18.411317°-34.045568°	-	-	-	-
	Site 9	18.404093°-34.034385°	CPUT19	I	*Metarhizium robertsii*	46±1efg
			SM8	SD	*Clonostachys rosea cf catenula*	52±1cdef
	Site 10	19.320406°-34.059153°	SMB	I	*Fusarium solani*	44±1efgh
	Site 11	19.320306°- 34.062953°	SM9	SD	*Clonostachys rosea cf catenula*	18±1.1j

Mean values followed by the same letter in the column do not show significance at P > 0.05 following comparison using the Tukey test.

Table 2 continues:

Location	Site	GPS coordinates	Fungal Isolate	Isolation method I (Insect bait) SD (Soil dilution)	Fungal Species	Abbott corrected mortality ±SE
Paarl	Site 12	19.002860°-33.720840°	-	-	-	-
	Site 13	19.117831°-33.918309°	-	-	-	-
	Site 14	18.958800°-33.766000°	SM10	I	*Clonostachys rosea cf catenula*	50±2defg
			SM13	I	*Beauveria bassiana*	82±2abc
Malmesbury	Site 15	20.014027°-33.988441°	CPUT18	SD	*Clonostachys rosea act catenula*	32±2efghi
	Site 16	19.987705°-33.991239°	-	-	-	-
	Site 17	19.987805°-33.991239°	SM1	SD	*Fusarium solani*	56±1.0bcdef
	Site 18	20.127851° -33.887736°	-	-	-	-
Franschoek	Site 19	20.127851° 33.887636°	-		-	-
	Site 20	19.117514° -33.907261°	SM5	SD	*Aspergillus oryzae*	-
	Site 21	18.413525°-34.015845°	SM12	I	*Aspergillus laciniosus*	-
	Site 22	19.167682°-33.551830°	CPUT22	I	*Metarhizium robertsii*	31±0.6e


ITS
NJ tree
NR 111601 Beauveria amorpha ARSEF2641
NR136979 Beauveria malawiensis IMI228343
HQ880825 Beauveria malawiensis ARSEF7760
NR111602 Beauveria sungii ARSEF1685
NR111596 Beauveria asiatica ARSEF4850
KU994837 Beauveria medogensis 2898
NR 111595 Cordyceos brongniartii ARSEF617
NR 111597 Beauveria australis ARSEF4598
NR 111598 Beauveria pseudobassiana ARSEF3405
NR 1115999 Beauveria varroae ARSEF8257
NR 111600 Beauveria kipukae ARSEF7032
NR 077147 Beauveria caledonica BCRC32867
HQ880817 Beauveria caledonica ARSEF2567
NR 151832 Beauveria vermiconia ARSEF2922
NR 111678 Beauveria lii ARSEF11741
SM3
NR 111594 Beauveria bassiana ARSEF1564
GU734762 Beauveria bassiana ARSEF1564
SM A
SM13
NR 132020 Metarhizium globosum ARSEF2596
NR 132019 Metarhizium acridum ARSEF7486
FJ617336 Metarhizium acridumisolate ARSEF7486
NR 132018 Metarhizium lepidiotae ARSEF7488
NR 152952 Metarhizium majus ARSEF1914
HQ331448 Metarhizium guizhouense CBS258 90
KM091861 Metarhizium pınghaense GKVK02 40
KJ 588068 Metarhizium pinghaense MET13168
NR 077205 Metarhizium pinghaense CBS257 90
NR 132017 Metarhizium anisopliae ASERF7487
AY251267 Metarhizium flavoviride IMI351848
CPUT22
CPUT16
CPUT19
NR 132011 Metarhizium robertsii ARSEF2575
MF977736 Metarhizium robertsii Maiz
MF681550 Metarhizium robertsii E324
NR 132023 Metarhizium brunneum ARSEF2107
KM371262 Metarhizium brunneum UAMH2801
KY786031 Metarhizium brunneum ART2825
KJ542163 Metarhizium robertsii M341 08
NR 137117 Cordyceps ningxiaensis HMJAU25074
0.005 substitions/site
100
100
91
100
100
90
99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JN713135 Metarhizium anisopliae PALM01
KC429004 Metacordyceps taii CQMa132
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KY488497 Metarhizum anisopliae 104
KY574475 Isaria ghanensis CBS105 73
SM3
SM13
KU867832 Beauveria bassiana 15 I02
KT183365 Beauveria bassiana
JN13133 Beauveria bassiana PALB01
SMA
EU604118 Cordyceps militaris MRCIF49
EU604107 Beauveria brongniartii MRCIF49
EF411259 Beauveria amorpha BCC1665
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88
100
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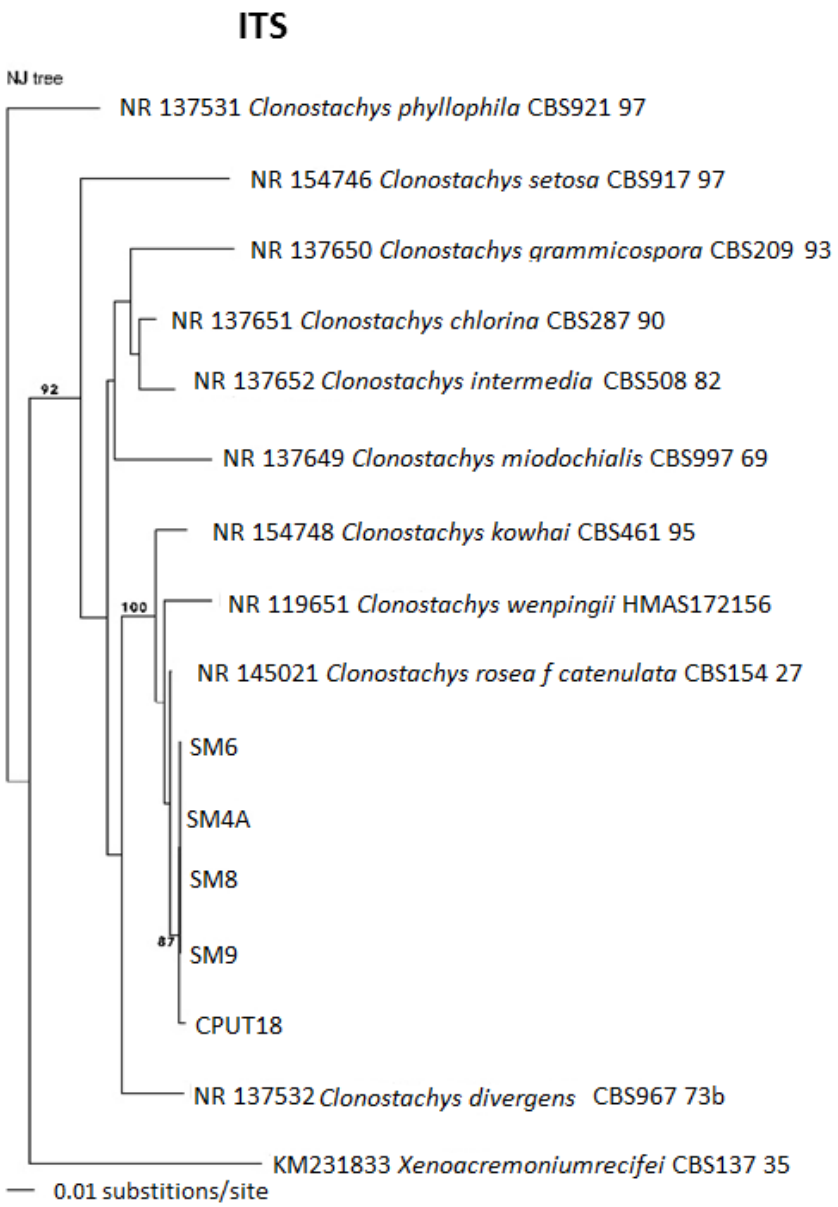
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NR 145021 Clonostachys rosea f catenulata CBS154 27
SM6
SM4A
SM8
SM9
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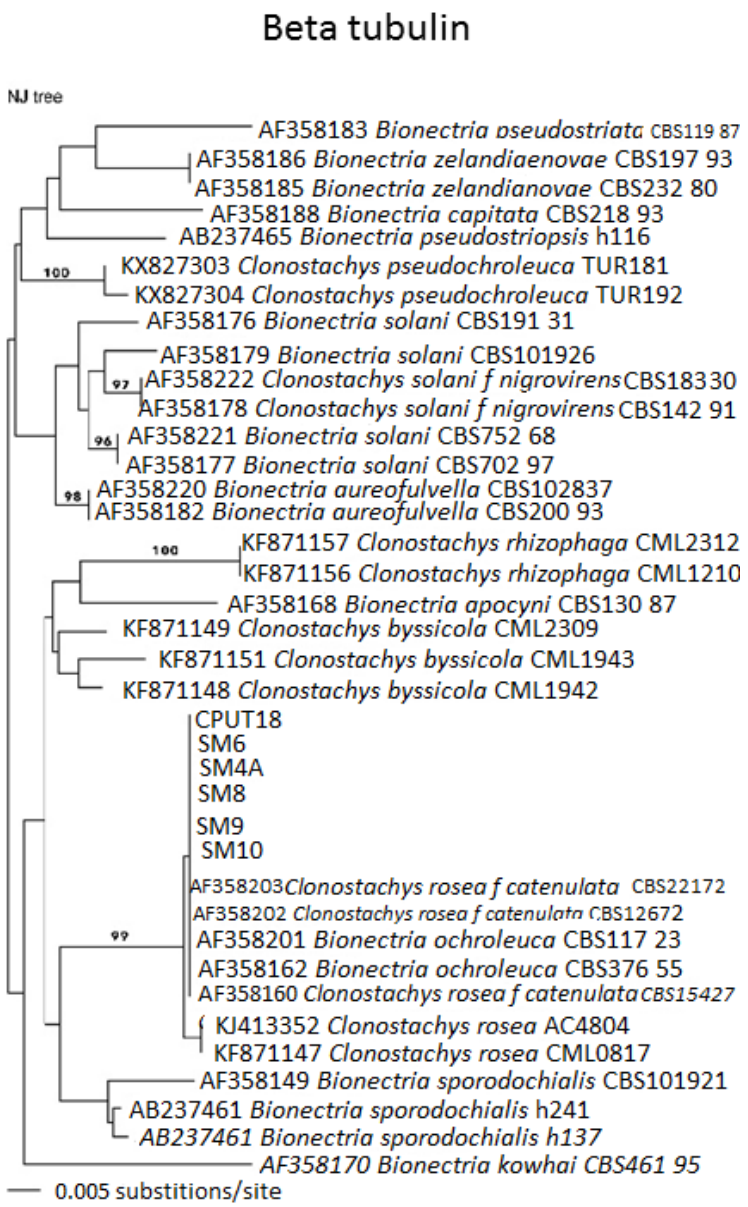
92
100
87
0.01 substitions/site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AF358188 Bionectria capitata CBS218 93
AB237465 Bionectria pseudostriopsis h116
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AF358176 Bionectria solani CBS191 31
AF358179 Bionectria solani CBS101926
AF358222 Clonostachys solani f nigrovirens CBS18330
AF358178 Clonostachys solani f nigrovirens CBS142 91
AF358221 Bionectria solani CBS752 68
AF358177 Bionectria solani CBS702 97
AF358220 Bionectria aureofulvella CBS102837
AF358182 Bionectria aureofulvella CBS200 93
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KF871156 Clonostachys rhizophaga CML1210
AF358168 Bionectria apocyni CBS130 87
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KF871148 Clonostachys byssicola CML1942
CPUT18
SM6
SM4A
SM8
SM9
SM10
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AF358201 Bionectria ochroleuca CBS117 23
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100
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0.005 substitions/site

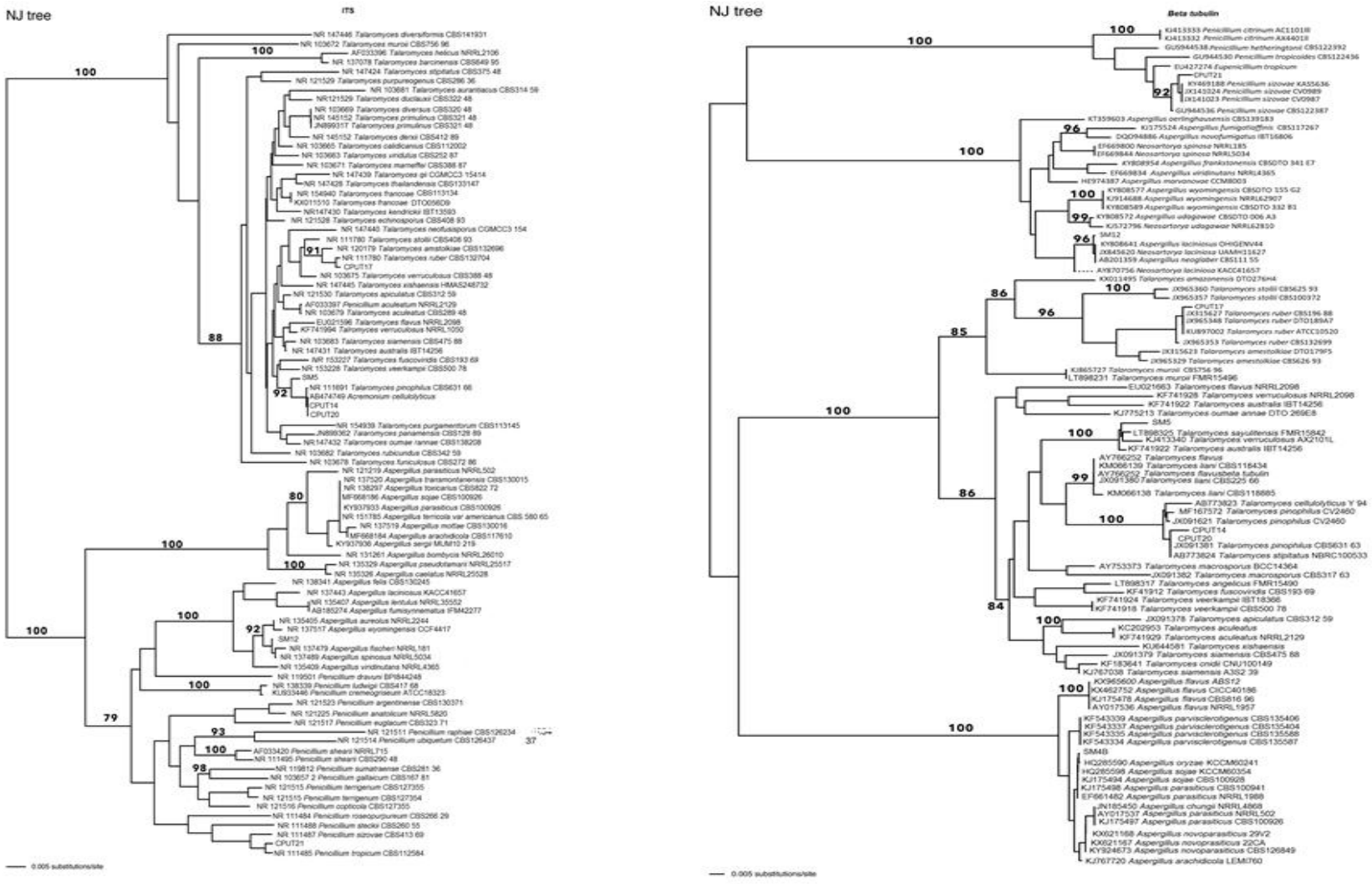

Figure 2.4: Neighbour-joining trees (based on ITS and BTub sequences) showing affinity with ITS and BTub sequences, as well as relationships in the different genera and corresponding bootstrap values.

2.3.2 Influence of soil nutrient status on the occurrence of entomopathogenic fungi

Soil nutrient levels and pH for all sites are presented in Table 2.3. The nutrient-data were categorized into high,optimum and low levels, and these were used in a correspondent analysis (CA) to establish the relationship between soil nutrient level and fungal occurrence in the different sites. The first two axes of the CA explained 45% of the variation of the data (Figure 2.5). Based on the correspondence analysis loadings, there was positive association between optimum to high soil nutrient contents and fungal occurrence (*B. bassiana* and *C. rosea*), while low K, N and Optimum Ca, and high C/N ratio were associated (Figure 2.5). Binomial logistic regression analysis revealed that soil K, Ca, S levels, as well as C/N ratio level had significant associations with the occurrence of *M. robertsii* ($df = 1$; $P < 0.01$) (Table 2.4). Meanwhile, Mg had a significant association with the occurrence of *B. bassiana* and *F. solani*. Logistic regression models were significant for the relationship between N, P or K and occurrence of *C. rosea catenula*. However, no significant association ($df = 1$; $P > 0.05$) was detected between any level of the soil nutrient and all the fungi combined.

Table 2.3: Soil nutrient and pH levels (mean ± standard error [SE]) in soil samples obtained from selected sites in the Cape Winelands, South Africa, and recommended critical values of nutrients.

Location	Site	pH	Phosphorus mg/kg	Potassium mg/kg	Magnesium mg/kg	Calcium cmol(+)/kg	Sulphur mg/kg	Nitrogen cmol(+)/kg	Carbon cmol(+)/kg	C/N ratio
Stellenbosch	Site 1	5.7±0.4	52.±42	189±25	207±13	1094±209	7±2	1180±69	13266±1159	11:1
	Site 2	5.7±0.4	87±21	152±31	173±32	879±109	9±3	1253±30	14866±1795	11:1
	Site 3	5.7±0.2	25±4	132±30	216±17	1026±136	8±2	1176±200	14533±763	12:1
	Site 4	6.4±0.2	116±21	23±6.7	109±34	729±101	10±1.7	600±81.6	12400±100	17:1
	Site 5	5.6±0.09	86±30	29±12	123±68	596±372	14±2	700±360	9866±5216	12:1
	Site 6	6.2±0.1	139±36	37±15	162±51	918±207	13±5	733±305	15300±8056	20:1
Constantia	Site 7	6.2±0.9	58±44	75±24	57±20	900±462	7±2	1080±405	10733±1975	9:1
	Site 8	6.2±0.4	75±86	85±21	119±17	926±325	13±9	1346±180	12533±4479	9:1
	Site 9	6±0.6	41±49	127±41	95±42	2214±1445	21±8	2370±708	29600±9778	12:1
	Site 10	5.8±0.2	138.±93	26±22	156±58	793±137	11±3	566±57	15100±3736	26:1
	Site 11	5.5±0.0	17±12	45±35	237±34	1056±245	15±13	2800±339	23300±3104	23:1
Paarl	Site 12	5.7±0.4	144.±32	29±11	98±23	1234±292	13±4	1166±550	17600±6055	14:1
	Site 13	5.4± 0.3	101±36	26±21	38±9	535±99	7±5	1423±635	17000±5670	11:1
	Site 14	5.7±0.5	90±56	20.8±12	24±20	274±352	9±2	1690±673	15933±4129	9:1

Table 2.3 continues

Location	Site	pH	Phosphorus mg/kg	Potassium mg/kg	Magnesium mg/kg	Calcium cmol(+)/kg	Sulphur mg/kg	Nitrogen cmol(+)/kg	Carbon cmol(+)/kg	C/N ratio
Malmesbury	Site 15	5.8±0.1	62±6	16.9±1.8	74±8	388±76	9.2±0.9	333±152	7866±1184	22:1
	Site 16	6.2±0.5	29±5	20±2	79±20	578±211	10±0.4	466±57	7433±1643	16:1
	Site 17	5.6±0.2	98±20	20±5	99±67	476±359	13±2	566±288	9266±6615	15:1
	Site 18	5.6±0.6	58±4	31±10	203±75	694±251	11±3	633±152	13700±2773	21:1
Franschoek	Site 19	6.2±0.3	114±27	27±3	171±62	1140±298	12.5±5.1	666±208	16400±7049	24:1
	Site 20	5.4±0.4	116±102	29±18	137±89	626±344	12±6	733±450	12400±8510	17:1
	Site 21	5.7±0.4	84±52	180±9	213±83	169±795	6±2	1530±496	23500±8286	15:1
	Site 22	5.9±0.3	158±31	227±15	139±21	2066±345	7±3	1773±105	20100±1135	11:1
[a]Optimum ranges		5.5-7.5	25-170	70-120	60-240	400-1200	20-200	600-1500	8000-15000	10:1

[a]Ranges are published by Bemlab Pty. Ltd. (http://www.bemlab.co.za/uploads/GENERIC%20SOIL%20ANALYSIS%20NORMS_a.pdf)

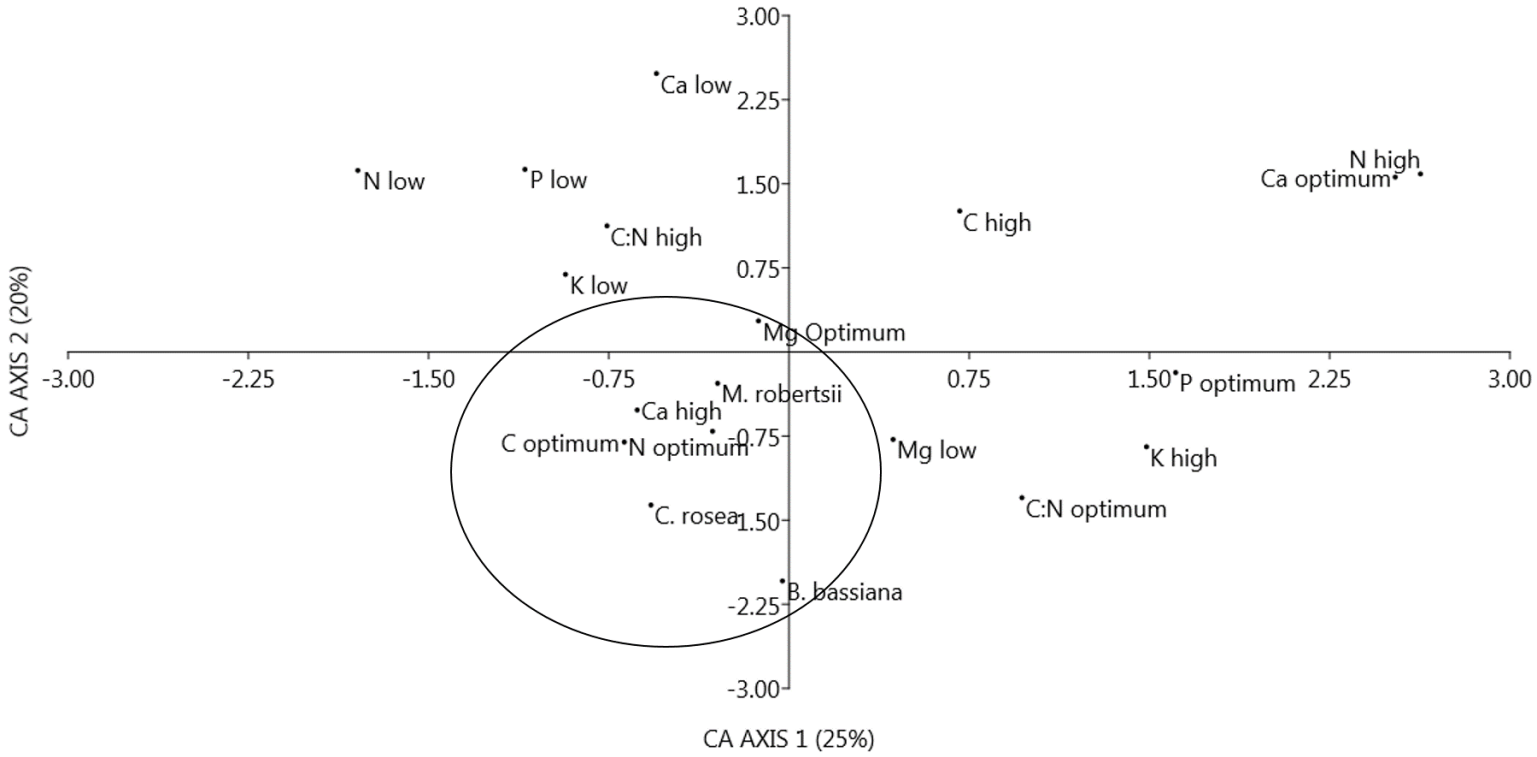

Figure 2.5: Association between fungal occurence and soil nutrients (N, C/N ratio, K, Mg and Ca) of sampled soils of grapevines in the Western Cape Winelands following correspondence analysis (CA).

Table 2.4 Binary logistic regression analysis of the association between the independent variable (nutrient) and the dependent variable (occurrence of fungi) from soils sampled from selected vineyards in the Cape Winelands, South Africa.

Independent variable (nutrient)	Binomial regression analysis (overall model fit) Dependent variables (fungi)				
	Beauveria bassiana	*Metarhizium robertsii*	*Clonostachys rosea*	*Fusarium solani*	All EPF combined
Nitrogen	X^2 = 0.5149; df = 1; P = 0.4730	X^2 = 1.4081; df = 1; P = 0.2354	X^2 = 4.9260; df = 1; P = 0.0265	X^2 = 3.3039; df = 1; P = 0.0691	X^2 =2.8225; df = 1; P = 0.0930
Potassium	X^2 = 1.8292; df = 1; P = 0.1762	X^2 = 7.6356; df = 1; P = 0.0057	X^2 = 8.6871; df=1; P = 0.0032	X^2 = 2.4310 ; df=1; P = 0.1190	X^2 = 2.3115; df = 1; P = 0.1284
Calcium	X^2 = 0.2752; df=1; P = 0.5999	X^2 = 9.6557; df = 1; P = 0.0019	X^2 = 0.3799 ; df = 1; P = 0.5377	X^2 = 0.7202; df=1; P = 0.3961	X^2 = 1.4946; df = 1; P = 0.2215
Carbon	X^2 = 0.0244; df = 1; P = 0.8758	X^2 = 1.7888; df = 1; P = 0.1811	X^2 = 1.6010; df = 1; P = 0.2058	X^2 = 0.8563; df = 1; P = 0.3548	X^2 = 0.5211; df = 1; P = 0.4704
Sulphur	X^2 = 2.4011; df = 1; P = 0.1213	X^2 = 0.0322; df = 1; P = 0.8575	X^2 = 0.5522; df = 1; P = 0.4574	X^2 = 0.2621; df = 1; P = 0.6087	X^2 = 0.0012; df = 1; P = 0.9726
Magnesium	X^2 = 0.0023 ; df = 1; P = 0.9615	X^2 = 0.1048; df = 1; P = 0.7461	X^2 = 0.0082; df = 1; P = 0.9278	X^2 = 0.0201; df = 1; P = 0.8874	X^2 = 0.2613; df = 1; P = 0.6092
Phosphorus	X^2 = 0.2025; df = 1; P = 0.6527	X^2 = 0.2324 ; df = 1; P = 0.6297	X^2 = 4.4492; df = 1; P = 0.0349	X^2 = 1.5655; df = 1; P = 0.2109	X^2 = 0.3879; df = 1; P = 0.5334
C/N ratio	X^2 = 5.2693; df = 1; P = 0.0217	X^2 = 5.6700; df = 1; P = 0.0173	X^2 = 0.1346; df = 1; P = 0.7137	X^2 = 2.1221; df = 1; P = 0.1452	X^2 = 0.1216; df = 1; P = 0.7273

2.3.3 Pathogenicity of EPF against mealybug

All the 15 selected fungal isolates proved to be pathogenic against adult females of *P. ficus*, with 4-day mortalities ranging from 18-87% (df = 14, 60; F=49.6; P < 0.01) (Table 2.2). Strains of *B. bassiana* (SM1, SM2) and *C. rosea* catenula (SM6) were the most pathogenic strains inducing 87 ± 3%, 77 ± 2% and 82 ± 2% *P. ficus* mortalities, respectively. The lowest levels of mortality were observed primarily among isolates of *M. robertsii* and *C. rosea catenula* (Table 2.2).

2.4 Discussion

A range of fungi, 23 strains in total, were isolated from vineyard soils in the Cape Winelands, and among these are well-known EPF species, *B. bassiana*, *M. robertsii* and *C. rosea catenula*, which caused varying mealybug mortality levels when tested in an *in vitro* bioassay. The study further revealed, remarkably, that N, K, Ca, Mg and S concentrations and C/N ratio were correlated with at least one entomopathogenic fungal species in soils of the sampled vineyards.

Of the 23 fungal isolates collected from the sampled vineyards, 26% belonged to the species *C. rosea catenula*, closely followed by *M. robertsii* (22%) and *B. bassiana* (17%). On the basis of this study and a previous study by Abaajeh and Nchu (2015), it appears *M. robertsii* is the predominant *Metarhizium* species in the Cape Peninsula region. In an earlier study, 62 EPF strains representing 21% occurrence were isolated from soils in the Sunday's River Valley citrus producing region (Eastern Cape Province, South Africa) and *B. bassiana* and *Metarhizium anisopliae* var. *anisopliae* were the most frequently isolated (Goble, 2009). In countrywide surveys carried out throughout South Africa, 1506 soil samples collected and baited with *G. mellonela* yielded 441 isolates of EPF, 81% of which were *B. bassiana* and 13% *M. anisopliae* (Hatting et al., 2004). *C. rosea* was the most encountered species (30.05 ± 3.38%), followed by *B. bassiana* (12.57 ± 2.37%) in Portuguese Douro vineyards (cultivated habitat) and adjacent hedgerows (semi-natural habitat) (Sharma et al., 2018). In this study, while the majority of the isolated *M. robertsii* and *B. bassian*a strains were obtained using the insect bait method most of the isolates of *C. rosea catenula* were collected by soil dilution method. These results are in agreement with the argument that EPF isolation methods are not equivalent for the determination of the occurrence and distribution of EPF (Medo & Cagáň, 2011). Sharma et al. (2018) demonstrated that the isolation of *M. robertsii* was associated both with *Tenebrio molitor* (Coleoptera: Tenebrionidae) baiting and cultivated habitats, while *B. bassiana* was linked with *G. mellonella* baiting only. A combination of methods is, therefore, necessary for optimum isolation of EPF from the environment.

Fifteen strains of four well-known EPF *B. bassiana, M. anisopliae, C. rosea and Fusarium solani* were selected and assessed for pathogenicity against the grapevine mealybug, *P. ficus*. As expected, the results obtained in this study suggest that the pathogenicity of an isolate is not only species dependent but also varies between strains of the same species. *C. rosea* isolates yielded mortality ranging from 18% to 82% when used in bio-assays against *P. ficus*. *C. rosea* is known as a mycoparasitic fungus, which is usually used as a biocontrol agent for

plant pathogens, and it has also been observed as a facultative pathogen of nematodes (Sutton et al. 2002; Zhang et al. 2008). In the present study, *B. bassiana*, a widely researched species, showed high insect mortality (77-87%). *B. bassiana* is used as an active ingredient for numerous mycopesticides worldwide (Feng et al., 1994; Ownley et al., 2004; Pu et al., 2005). Kreutz et al. (2004) recorded 100% mortality in the bark beetle, *Ips typographus* after 7 days inoculation at 1 x 108 conidia/ ml-1. Generally, *M. roberstii* strains induced lower mortalities in the current study. While the *Metarhizium isolates* we collected may not be effective against grapevine mealybug, *B. bassiana* should be considered as a potential biocontrol agent for the pest because of its higher virulence that was observed in the bioassay.

The occurrence of *M. robertsii* isolates was mainly at the range of 11:1 - 15:1, while *C. rosea catenula* occurred at all C/N ranges including 9:1. The appropriate C/N ratio for growth or virulence of fungi may also differ from species to species as well as between strains, but a ratio of at least 10:1 is recommended (Shah et al., 2005), which somewhat concurs with the results obtained in this study. Vega et al. (2003) found that the highest spore yield in EPF under varying C/N ratios occurred at 10:1. In a study by Safavi et al., (2007), an isolate of *M. anisopliae* produced its highest amount of conidia at 10:1(C/N), and increase of this ratio led to reduced conidial growth. All vineyards in the current study had pH levels at optimum range for grapevine production. Rousk et al. (2009) suggested that as soil pH decreases fungal growth increases and only decreases at pH levels of 4.5 or less. This implies that all sites in this study had appropriate pH levels for fungal growth.

We demonstrated using binary logistic regression analysis that macronutrients, including potassium, nitrogen and calcium are significantly associated with the presence of some EPF. The sites with fungi also showed optimum levels of phosphorus and high levels of nitrogen. The results in this study contrast the findings of (Jabbour & Barbercheck, 2009) that showed no relationship between *Metarhizium* detection and soil nutrients like Ca, K, and P. However, in the same study, they also found that *M. anisopliae* detection was negatively associated with soil moisture, organic matter, zinc, sulfur and copper concentrations in the soil. Previous studies have mainly focused on nutrient source use in *in vitro* culturing of fungi (Zhu et al., 2008; Mishra & Malik, 2012) and little information is available on specific nutrient levels required for fungal growth under field conditions. Although nutritional requirements and tolerances may vary with species/strains, generally, microorganisms require

significant quantities of nitrogen, phosphorus and sulfur, which are the essential ingredients of growth factors viz., amino acids, purines and pyrimidines and vitamins. In a study by Tsvuura et al. (2017), mycelial biomass more than tripled with the addition of nitrogen into grassland plots.

2.5 Conclusion

In conclusion, N, K, Ca, Mg and S concentrations and C/N ratio were correlated with the occurrence of at least one entomopathogenic fungal species and that optimal to higher grapevine growing nutrient levels in soils favor occurrence of EPF. This information is valuable for future development of ecological approaches involving EPF for insect control in grapevines. This study provides a basis from which to study their ecology and test EPF in further field scale trials in different agricultural settings.

CHAPTER THREE

The effects of endophytic *Beauveria bassiana* inoculation on infestation level of *Planococcus ficus*, growth and volatile constituents of potted greenhouse grapevine (*Vitis vinifera* L.)

3.1 Introduction

Numerous setbacks, including pollution, toxicity to animals and plants, and rampant insecticide resistance are associated with the use of synthetic insecticides (Aktar et al., 2009), and these have led to increased solicitation for alternative solutions to insect infestation problems on crops. Knowledge gained over the years on the influence of endophytic microbial symbionts on plant defense mechanisms vis-à-vis insect herbivory have opened up opportunities for management of insect pests using fungal endophytes. Endophytic fungi occur ubiquitously in plants and colonize them without adverse effects; meanwhile, plants serve as host and provide nutrient to these fungi. Through this mutualistic relationship with their plant hosts, endophytes enhance plants' tolerance to biotic and abiotic stresses (Lugtenberg et al., 2016). Also, endophytic fungi have been reported to induce increased growth in plants, such as grasses and strawberry (Dara et al., 2016; Clay, 1988). The growth enhancing effect can be attributed to the ability of fungi to mobilize valuable nutrients for plant growth; for example, *Metarhizium robertsii* (Hypocreales) promotes root growth and nitrogen absorption in switchgrass and haricot bean (Sasan & Bidochka, 2012). A study by Dara et al. (2016) demonstrated improved plant health coupled with an increase in shoot-root ratio for plants treated with the fungus *B. bassiana*. *M. robertsii* and *B. bassiana* are two well-known entomopathogenic fungal species, whose strains can cause natural epizootic deaths in many insects (Sandhu et al., 2012). Plant growth and productivity as well as defence could be enhanced by exploring the plant-fungus interaction.

There is evidence in literature proving that endophytic fungi can curb insect infestations on plants. For example, western tarnished plant bugs (*Lygus hesperus*) and southern green stink bugs (*Nezara viridula*) showed strong negative responses to flower buds (*L. hesperus*) and fruits (*N. viridula*) from plants that had been colonized by candidate endophytic fungi compared to control plants (Sword et al., 2017). A strain of the endophytic fungus *B. bassiana* reduced infestation rates and growth of *P. ficus* on potted grapevine plants (Rondot & Reineke, 2018). Interesting findings, such as the fore-mentioned have spurred researchers to study further the mechanisms through which fungal endophytes influence insect herbivory.

Surely, this knowledge will improve our understanding and exploitation of the fungus-plant-insect relationship for sustainable pest management.

Some endophytic fungi have been reported to produce metabolites that can reduce insect infestations on their host plants (Jaber & Ownley, 2017). It is believed that increased in quantity and diversity of secondary metabolites in endophyte-containing plants is somewhat responsible for the reduction of insect herbivory on plants (Hartley & Gange, 2009). In a study carried out by Jallow et al. (2008), *Acremonium strictum* systemically influenced the host selection of *Helicoverpa armigera* moths for oviposition, possibly, through changes in volatile emissions and some unknown biochemical parameters. Fungal endophytes can increase the production of antioxidant in plants as well as produce antioxidant compounds such as phenolics. They also produce plant growth hormones and enhance plant nutrient absorption favoring increased germination success and growth rate (Zandalinas, 2017; Hamilton et al., 2012).

The grapevine mealybug (*Planococcus ficus*[Homoptera]) is a sap-sucking insect that is difficult to control (Godfrey et al., 2003; Daane et al., 2006). It is among the most serious pests of vineyards causing substantial losses globally (Berlinger, 1977; Walton et al., 2004). The systemic colonization of plant tissues of young plants with endophytic *B. bassiana* can lead to sustainable control of *P. ficus*. The objectives of this study were to assess the effect of *B. bassiana* inoculation of grape plants on (i) the infestation level of *P. ficus,* and (ii) growth and volatile constituents of potted grapevines.

3.2 Materials and Methods

3.2.1 Plant Material

Fifty 2-year old *Vitis vinifera* L.cv Pinotage grafted onto Ramsey rootstock plants were obtained from Bosman Family vineyards, Wellington, South Africa. These plants were transported to the greenhouse at Cape Peninsula University of Technology (CPUT), Bellville, South Africa. The plants were debagged and the soil washed off the roots of each plant. All plants were measured to obtained baseline plant height, root length, leaf number and shoot number prior to their use in the green house experiment. Ten of these plants were used to obtained baseline data (dry weight & wet weight) and the remaining 40 plants were used for the greenhouse experiment.

3.2.2 Fungal culture

An indigenous *B. bassiana* (strain: SM3) that was originally isolated from a soil sample collected from a vineyard in the Cape Winelands was used in this study. This fungal strain and species was selected among others based on high virulence against *P. ficus in vitro* (Chapter 2) and reports of efficient plant tissue colonization of *B. bassiana*. This strain was identified using molecular and morphological techniques and is being maintained at Cape Peninsula University of Technology in Bellville, South Africa. Clean single-spore sub-cultures of the fungus were cultivated on half-strength Potato Dextrose Agar (PDA) containing 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin (Sigma-Aldrich) in 9 cm and 14 cm diameters Petri dishes and incubated in the laboratory at 25 °C and 12:12 h L:D. Four weeks (28 days) old *B. bassiana* conidia obtained from PDA plates were used for inoculation of the grapevine plants. Conidia were harvested by gently scraping the surface of the Agar using a spatula onto sterile aluminum foil. The conidia were suspended in 500 ml glass bottles containing sterile 0.01% Tween 80 in distilled water. Bottles were capped, mixed by agitating for 5 min by shaking and using a magnetic stirrer (at 20 °C and 300 rpm for 30 min) to homogenize the conidial suspensions. The conidia concentration was enumerated using a haemocytometer and observed with a light microscope at 40X magnification. In order to obtain the desired concentration (1×10^8 conidia mL^{-1}), the volume of sterile 0.01% Tween 80 was increased or conidia were added to the glass bottle. A conidial germination test to determine conidial viability was carried out according to the method described by Inglis et al. (2012) and high spore germination of more than 90% was obtained.

3.2.3 Greenhouse experiment

This experiment was conducted at the greenhouse of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), South Africa. The experiment was carried out under the following conditions: an average day temperature of 25 ± 5 ºC and average RH of 65±5% between March and April 2018. *V. vinifera* plants were transplanted separately into 40 glass bottles containing a plant growth medium consisting of a mixture of inert substrate materials; vermiculite and perlite in a ratio of 1:1. The bottles were covered with black cloth to prevent algal growth. Twenty potted plants were drenched with 200 ml suspension of *B. bassiana* at 1×10^8 conidia ml^{-1}. Twenty control plants were drenched with

the 200 ml of sterile distilled water containing 0.01% Tween 80 only. Throughout the experiment the plants were fertigated weekly with a hydroponic fertilizer, Nutrifeed (Starke Ayres, Cape Town) containing 65 g/kg N, 27 g/kg P, 130 g/kg K, 70 mg/kg Ca, 20 mg/kg Cu, 1500 mg/kg Fe, 10 mg/kg Mo, 22 mg/kg Mg, 240 mg/kg Mn, 75 mg/kg S, 240 mg/kg B and mg/kg Zn. Nutrient solutions were prepared by dissolving 60 g of fertilizer in 60 L reservoir with tap water, and each plant was hand fed with 500 ml every week. After two weeks, all experimental plants were infested with female adult mealybugs (30 per plant) by transferring insects onto plants using a bristle brush (Sempruch et al., 2014). Plants in test and control treatments were inoculated for a second time by drenching with a suspension of 200 ml *B. bassiana* at 1×10^8 conidial ml^{-1} in 0.01% Tween 80 and 0.01% Tween, respectively. The experiment ran for four weeks, after which plant growth parameters (shoot height, number leaves, number of shoots, root length, shoot and root fresh and dry weights) were recorded. Mealybug infestation levels were assessed by counting numbers of adult females and larvae on control and fungus-exposed plants. Six representative potted live plants; i.e, three from each treatment, were taken to the central analytical facilities, GC-MS Unit, Stellenbosch University for GC-MS analysis. Fresh leaf samples representing 6 control samples and 6 test samples were sent to Bemlab, a commercial testing laboratory in Somerset West, Western Cape for tissue analysis of nutrient content. The remaining plants were oven dried, separately, at 25 ºC for 7 days, and then ground with a Jankel and Kunkel Model A 10 mill into fine powder. One hundred milligrams (100 mg) of powdered material from each of 10 samples in control and 10 in the test groups was analyzed for secondary metabolites (total alkaloid, polyphenol and flavanol) at the Oxidative Stress Centre at CPUT.

3.2.4 Re-isolation of *B. bassiana*

Endophytic colonization of *B. bassiana* of leaf was assessed at 21 days by re-isolation following surface sterilization. One leaf was carefully excised from each plant and transferred to the laboratory on ice. From each leaf, rectangular leaf sections of 2 mm^2 were cut. These sections were individually surface sterilized with 0.5% sodium hypochlorite for 1 minute, followed by 70% Ethanol for 1 minute and rinsed twice in sterile distilled water and placed on the selective medium (19.5 g Potato Dextrose Agar [PDA], 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin [Sigma-Aldrich]). The leaf sections were examined visually on a daily basis for presence of any fungal growth. Fungal tissue was characterized as colonized by the fungus by observing *B. bassiana* white dense mycelia becoming creamy

at the edge (Humber, 1997), growing from the tissues of sterilized leaf sections. A total of 40 (20 control & 20 treatment) plants were sampled and 120 leaf sections were plated, equating to 3 leaf sections per plant. The presence of *B. bassiana* in at least one of the leaf sections was considered as an indication of successful colonization of a plant. The data was expressed as percentage colonization ([number of plant replicates colonized/ number of plant replicates excised] × 100).

3.2.5 Plant Growth

The effect of *B. bassiana* on the growth of *V. vinifera* L. was determined by measuring shoot height, number leaves, number of shoots, root length, shoot and root fresh and dry weights. Plant height was measured from the surface of the substrate to the tip of the stem.

3.2.6. Plant Tissue/Nutrient Analysis

Leaf samples were analysed for macro- and micro- at Bemlab (Pty) Ltd, a commercial laboratory in Somerset West, South Africa. Leaves were washed with Teepol solution, rinsed with de-ionised water and dried at 70 °C overnight in an oven. The dried leaves were then milled and ashed at 480 °C shaken up in a 50:50 HCl (50%) solution for extraction through filter paper (Campell & Plank, 1998; Miller, 1998). The Potassium (K), Phosphorus (P), Calcium (Ca), Magnesium (Mg), Sodium (Na), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Z) and Boron (B) content of the extracts were analysed using Ash method. Total Nitrogen (N) content of the leaves was determined through total combustion in a Leco N-analyser.

3.2.7 Analysis of secondary metabolites

Six representative potted live plants; i.e, three from each treatment, were used for this analysis. Only plants that showed evidence of successful fungal colonization were selected to represent the fungus treatment in the GC-MS analysis.

3.2.7.1 GC-MS Analysis (Head Space) and Secondary metabolite analysis

Sample Preparation

Whole leaves were cut off from fresh plants and freeze-dried at -80 ˚C (overnight). The leaves were then crushed using liquid nitrogen and 1 g was weighed into a solid phase microextraction (SPME) vial followed by 2 ml of 12% alcohol solution (v/v) at pH 3.5 and 3 ml of 20% NaCl solution. The samples were vortexed and the headspace of the sample was

analysed using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (gray).

3.2.7.1.1 Chromatographic separation

Separation of volatile compounds was performed with a gas chromatograph (6890N, Agilent Technologies Network) coupled to an inert XL EI/CI Mass Selective Detector (model 5975B, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler and the separation of volatiles present in the samples was achieved on a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. Helium gas was used as the carrier at a flow rate of 1 ml/min. The injector temperature was maintained at 250 °C and the split ratio was set at 5:1 split ratio. The oven temperature was programmed as follows: 35 °C for 6 min, at a rate of 3 °C/min to 70 °C for 5 mins, then at 4 °C/min to 120 C for 1 min and finally increased to 240 °C at a rate of 20 °C/min and held for 2.89 min. The Mass Selective Detector was operated in a full scan mode and the source and quad temperatures were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 250 °C. The mass spectrometer was operated under electron impact mode at ionization energy of 70eV, scanning from 35 to 500 m/z. Relative ratios were calculated using the expression (peak area/IS peak area) × IS concentration (IS = internal standard), and hence, are only approximate values. Only the organic volatile compounds with a match quality of at least 90% were identified and reported.

3.2.7.2 Determination of total flavonol, alkaloid and phenolic content.

A spectroscopic method was used to determine total alkaloids in the plant (Fadhil et al., 2007). Briefly, 100 mg of the powdered grapevine leaves were extracted with 10 mL of 60% ethanol for 2 h, centrifuged (4000 x g for 10 min) and the supernatant was used in the assay. Thereafter, 2 ml of the extract supernatant and atropine standard solutions were mixed with 5 mL sodium phosphate buffer and 12 mL bromocresol green solution. Twelve milliliters of chloroform was then added to the solution and the solution was mixed vigorously using a vortex mixer. The absorbance at 417 nm was determined and the concentration of the sample (mg/g) using a standard curve of atropine was calculated. The total polyphenol content of the various crude extracts were determined by the Folin-Ciocalteu method (Singleton et al., 1999; Swain & Hillis, 1959). Using a 96-well microplate, 25 µL of the sample was mixed with 125 µL Folin-Ciocalteu reagent (diluted 1:10 with distilled water) (Merck, South Africa). After 5 min, 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to each well. The method described by Daniels et al. (2015) was used to obtain the

absorbance reading of the solution in the microplates and results are expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW). The flavonol content was determined using quercetin, and the protocol was based on the method described by Daniels et al. (2011) was adopted for 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) as standard. In the sample wells, 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1% hydrochloric acid (HCl) (Merck, South Africa) in 95% ethanol, and incubated for 30 min at room temperature. The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

3.2.7.2 Statistical Analysis

The experimental data collected were analyzed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means at a level of significance, $P < 0.05$. These computations were performed using PAST version 3.21 (Hammer et al., 2001). The Pearson's Chi-sqaure test was used to compare the number of volatiles in the fungus and control plants at $P < 0.05$ level of significance.

3.3 Results

3.3.1 Re-isolation of fungus from grapevine tissues

At 28 days after inoculation *B. bassiana* was successfully re-isolated from 29 of 60 leaf sections (48%), representing 10 of the 20 fungus-treated plants (50%). No fungus was re-isolated from the control plants. Contamination was not observed on both control and treated plants.

3.3.2 Effect of fungus on plant growth parameters:

There was no significant difference (DF= 1, 38; F =0.829 P=0.3684) in plant heights between fungus exposed plants and control plants at four weeks post treatment. The plant heights ranged from 97 ± 9-101 ± 7cm (Table 3.1). In addition, experimental plants did not show significant variations ($P>0.05$) between fungus and control treatments for the other plant growth parameters, i.e. leaf number, number of shoots, and wet and dry weights (Table 3.1)

Table 3.1: Mean growth ± SE of *V. vinifera* exposed to *B. bassiana* inocula and control treatment for four weeks under greenhouse conditions.

Treatment	**Plant height (cm)**	**Leaf Count**	**Number of Shoots**	**Dry weight roots (g)**	**Dry weight shoots (g)**	**Wet weight roots (g)**	**Wet weight shoots (g)**
Inoculated plants	97±9a	30±3a	3±0.2a	8±0.6a	66±49a	35±3a	31±3a
Control plants	101±7a	25±4a	3±0.3a	8±0.9a	17±2a	33±2a	33±4a

Table 3.2: Tissue nutrients contents (Mean ± SE) of shoots of *V. vinifera* plants exposed to control and *B. bassiana* inocula for four weeks under greenhouse conditions.

Treatment	**Quantity(mg/kg)**										
	N	**P**	**K**	**Ca**	**Mg**	**Na**	**Mn**	**Fe**	**Cu**	**Zn**	**B**
Control	23433±912a	5900±717a	27583±1200a	13266±939b	2866±158d	6624±337a	47±4a	321±65a	4±0.2a	60±3a	26±1a
Fungus	23500±657a	4500±265a	27450±987a	16633±544c	3316±47e	8446±760a	49±3a	243±13a	5±0.1a	68±2a	27±0.5a

3.3.3 Effect of fungus on plant tissue nutrient content:

Generally, fungal inoculation of plants had no significant effect ($P > 0.05$) on the tissue macro nutrients' levels. However, Ca (16633 mg/kg) and Mg (3316 mg/kg), were significantly higher in the leaf tissues of fungus treated plants ($Df = 5,9$; $P < 0.05$) compared to the control plants, Ca (13266 mg/kg) and Mg (2866 mg/kg) (Table 3.2).

3.3.4 Effect of fungus on infestation level of grapevine mealybug:

Treatment with fungus did not have any significant ($P > 0.05$) beneficial affect against insect infestations numbers on plants over control; insect infestation numbers for the adult female and larvae ranged from 19 to 18 and 27 to 31, respectively (Table 3.3)

Table 3.3: Mean number of *V. vinifera* (immatures [larvae] and adult females) at four weeks following experimental inoculation of control and fungus-exposed under greenhouse conditions (6 replicates per treatment)

Treatment	**Mean no of insects**	
	Immature	**Adult**
Fungus	27±4	19±1.3
Control	31±5	18±2.2

3.3.5 Effect on secondary metabolite:

The fungus had no effect on the total polyphenol content. Alkaloids were not detected (Table 3.4). A wide range of volatile compounds were detected in the grapevine plants in both treatments (Table 3.5). Remarkably, a markedly higher number of volatile constituents that have been reported to have anti-insect activities were detected in fungus treated plants (9) compared to control (5) plants ($X^2=5.1$; $P = 0.02$). Some of the well-known insect repellents, which included Limonene, Beta pinene and Gamma terpinene (Tables 3.5 & 3.6). However no obvious trend in relative area ratios of these compounds between treatment and control was observed (Tables 3.6).

Table 3.4: Secondary metabolite contents in shoots of Grapevine following exposure to *B. bassiana* incocula and control treatment.

Treatment	Polyphenol (mg GAE/g)	Flavonols (mg QE/g)	Alkaloids (mg AE/g)
Control	8±0.5	3±0.3	N.D
Fungus	8±0.5	3±0.3	N.D

N.D.: Not detected.

Table 3.5: Volatile organic compounds with a match quality of at least 90% present in fungal treated and control shoots of grapevine.

	Control	Fungus
1	1,2-Benzenedicarboxylic acid *	1-Hexanol
2	1-Hexanol	2-Heptenal
3	1-Octadecene*	2-Hexen-1-ol
4	2-Furancarboxaldehyde*	2-Hexenoic acid*
5	2-Heptenal	3-Hexen-1-ol
6	2-Hexen-1-ol	3-Hexenoic acid
7	3-Hexen-1-ol	6-METHYL-5-HEPTEN-2-ONE
8	3-Hexenoic acid	alpha.-terpinolene*
9	6-METHYL-5-HEPTEN-2-ONE	Benzaldehyde
10	Benzaldehyde	Benzene*
11	Benzeneethanol	Benzeneethanol
12	Benzoic acid*	Benzofuranone*
13	Benzyl Alcohol*	Beta-Pinene
14	Beta.-Pinene	Butanoic acid*
15	CIS-3-Hexenol	CIS-3-Hexenol
16	CIS-3-Hexenyl Caproate*	CIS-3-Hexenyl Alpha. Methyl butyrate*
17	CIS-3-Hexenyl ISO-Butyrate*	CIS-3-hexenyl Valerate*
18	Citral	Citral
19	Cyclododecane*	Cyclohexasiloxane
20	Cyclohexadecane*	Cyclooctatetraene*

21	Cyclohexasiloxane	Cyclopentasiloxane*
22	Decanal*	Cyclotetrasiloxane*
23	dodecanoic acid	delta.-cadinene*
24	Farnesene*	E-3-hexenyl hexanoate*
25	Gamma.-Bisabolene*	Ethyl phthalate*
26	Gamma-Terpinene	Ethylidenecyclohexane*
27	Geraniol	Farnesyl Acetone*
28	Geranylacetone*	gamma.-terpinene
29	Heptadecanoic acid*	Geranial*
30	Hexadecanoic acid*	Geraniol
31	Hexanal	Geranyl Acetone*
32	Limonene	Heptadecene*
33	Linoleic acid*	Hexanal
34	Muskolactone*	Limonene
35	Myrcene	m-Cymene*
36	Myristic acid*	Myrcene
37	Octadecanoic acid*	Naphthalene*
38	Octanal	Nerolidol*
39	Oleic acid*	Octanal
40	p-Cymene*	Pentanoic acid*
41	Pentadecanoic acid*	Phenylethyl Alcohol*
42	Pentenal*	Styrene
43	Squalene*	**T**etradecamethylcycloheptasiloxane
44	Styrene	Trans 2-Hexenoic Acid*
45	Tetradecamethylcycloheptasiloxane	Trans,Trans-2,4-Heptadienal*
46	Thiosulfuric acid*	Trans-Beta-Ocimene*
47	Trans-2-Hexenal*	
48	Trans-Geraniol*	
49	Z-3-hexenyl 2-methylbutanoate*	

*Denotes compounds that are only present in only detected in at least 1 control or fungus treated plants.

Table 3.6: Selected and well-known insecticidal volatiles that were detected in *Vitis vinifera* and their relative area ratios following gas chromatography-linked mass spectrometry analysis of control and fungus treated plants.

Compound	**Reference**	**Area ratio**	
		Control	**Fungus**
Benzaldehyde	Paulraj et al. (2011)	0.23±0.1a	0.24±0.04a
Limonene	Hebeish et al. (2008)	4.5±1.2a	2.4±1.3a
Geraniol	Maia and Moore (2011) Chen and Viljoen (2010)	0.19±0.04a	0.29±0.05a
Geranyl acetone	Maia and Moore (2011)	-	0.52±0.1a
Gamma terpinene	Wang et al. (2006)	-	1.48±0.4a
Beta pinene	Dambolena et al. (2016)	0.94±0.21a	0.41±0.25a
Napthelene	Daisy et al. (2002)	-	0.23±0.02a
M-Cymene	Chang et al. (2012)	-	0.5±0.1a
Citral	Oyedele et al 2002 Abdel-Tawab and Mossa (2016)	0.03±0.03	0.06±0.06
No. of compounds		5	9*

* denotes significantly higher (DF = 1; X^2 = 5.1 and P = 0.02) number of compounds present following Pearson Chi square test.

3.4 Discussion

The re-isolation of the *B. bassiana* from the leaf tissue of 50% of fungus-inoculated plants demonstrated there was moderate colonization of the tissues of *V. vinifera* plants after experimental inoculation. The colonization of potted grapevine plants by endophytic fungi have been reported previously (Rondot & Reineke, 2018). It is worth mentioning that successful colonization is influenced by factors such as fungal species, fungal strain and host, etc. (Tefera & Vidal, 2009: Arnold & Herre, 2003; Gurulingappa, 2010). Vidal and Jaber (2015) found high variability in colonization efficiency of oilseed rape and faba bean with different strains of *B. bassiana*. They also pointed to the effect of different rhizosphere environments on establishment of *B. bassiana*.

Despite successful colonization, interestingly, the colonization by *B. bassiana* of the grapevine tissue did not translate to any noticeable increase in plant growth. Inconsistent effects of fungus on plant growth parameters have been reported. Akello et al. (2008) did not observe major difference in plant growth following inoculation of banana (*Musa* spp.) with endophtytic *B. bassiana* strain compared to the control treatment. However, in other similar studies, endophytic *B. bassiana* had induced higher growth in onion and tomatoes (Flori & Roberti, 1993; Bishop, 1999). The growth promoting effects of *B. bassiana* is probably dependent on the availability of nutrients in the growth medium. The absence of visible effects on the growth parameters assessed is seemingly consistent with the no effect of the fungus on the tissue macronutrients (N, P and K) contents observed in this study, plant growth is generally known to be correlated with these primary nutrients (Tripathi et al., 2014). Fungal microbes are generally known to promote plant growth by facilitating nutrient resource acquisition from the environment. In the current study, the plants were exposed to constant and adequate supply of the required nutrients, especially nitrogen, and this might have reduced any influence the fungus might have had on the supply of nutrients to plants. According to Tall & Meyling (2018) the mechanisms by which *B. bassiana* increases plant growth should be investigated further.

The two nutrients that were found to be significantly influenced by fungal inoculation were Calcium and Magnesium. These nutrients are important to plants for varying reasons. Calcium is an essential element for the development of new plant tissues, it strengthens cell walls and promotes cell elongation (Demarty et al., 1984). It is also known to help protect plants against fungi and bacteria. Magnesium is a building block for chlorophyll and important for the process of photosynthesis. It also activates certain plant enzymes needed for growth and contributes to protein synthesis (Wydrzynski & Gross, 1975). They can also influence secondary metabolite production. Supplementing plants with Ca has been proven to enhance total protein content as well phenol and flavonoid content (Ahmad, 2016). Although little is known about the role of Mg in plant secondary metabolism, Mg deficiency is known to increase phenolic compounds and putrescine accumulation in cells (Guo, 2016).

The key finding here is the clear association between the number of anti-insect volatile compounds detected and the fungus treatment (Table 5). There are many plausible

explanations for the higher anti-insect volatiles in fungus treated plants: perhaps this is mediated through calcium and magnesium uptake (Ahmad, 2016; Guo 2016), maybe it is due to direct production of secondary metabolites by fungi (Molnar, 2010) or better still it is as a result of plant defence reaction in the presence of the fungus in the tissue (Freeman, 2008). Interestingly, naphthalene was detected in fungus treated plants in this study. Naphtalene is a potent insecticide and insect repellent (Pajaro-Castro et al., 2017), and is the main active ingredient in mothballs. It has been demonstrated that *B. bassiana* releases volatile organic compounds including diisopropyl naphthalenes (Crespo et al., 2008). Earlier, Daisy et al. (2002) demonstrated that *Muscodor vitigenus*, an endophytic fungus can also produce naphtalene and further showed that the insect repellent activities of the fungus against adult stage of the wheat stem sawfly, *Cephus cinctus* was comparable to authentic naphthalene.

Plants have evolved several responses and defenses against biotic and abiotic stresses; the production of volatiles is an important and immediate response. These volatiles are known to be involved in communications with natural enemies of particular insect herbivores; they have the potential to enhance the effectiveness of host plant resistance to herbivory. Some of these volatiles are known to possess anti-insect properties (Table 6), for example, napthalene and limonene have been reported to cause up to 90-100% insect mortality in *Tribollium castaneum* Herbst and mealybugs, respectively (Hollingsworth, 2005; Pajaro-Castro et al. 2017). Nevertheless, no protective effect against *P. ficus* infestation (fungus treatment; immature (19 ± 1.3), adult (27 ± 4), and control treatment; immature (31 ± 5) and adult (18 ± 2.2)) was observed in this study despite the higher variety of volatile compounds in fungus treatment. It is worth remembering that insects have co-evolved mechanisms to overcome plant defense mechanisms (Da Costa et al., 1971),

3.5 Conclusion

In conclusion, this is the first report of the direct influence of *B. bassiana* on grapevine volatile production. This finding contributes to our understanding of mechanisms involved fungus-plant-insect relationship, which is relevant for management of insect pests using fungal endophytes.

CHAPTER FOUR

General Discussion and Recommendations

4.1 Discussion

Entomopathogenic fungi are increasingly being recognized as an important component in the future management of insect pests. This study sought to fill important knowledge gaps relating to the use of EPF against grapevine mealybug. This is the first study that established the link between occurrence of EPF in vineyard soils in Western Cape, South Africa and some soil nutrient contents as well as the link between fungal occurrence and optimum nutrient ranges for grapevine cultivation. A total of 23 fungal strains were isolated including well known species: *Clonostacys rosea*, *Beuvaria bassiana* and *Metarhizium robertsii*. Isolation of indigenous entomopathogeni fungi is essential to provide an insight into naturally occurring fungal biodiversity and provides a pool of potential biocontrol agents. This study successfully linked the presence/occurrence of EPF to specific soil nutrients, namely, Potassium, Nitrogen and Calcium. The study demonstrated the association of C/N ratio on the occurrence entomopathogenic fungal species in the field. Chemical pesticides can cause irreversible loss of soil fertility, loss of biodiversity and eco system services (Isenring, 2010). More recently this is evidenced by the diminishing population of honey bees in Cape Town, South Africa due to chemical control of ants which occur because of mealybug infestation (Pace, 2018). This research into indigenous EPF strains within intergrated pest management is a positive move towards sustainable agriculture.

Another important finding of this study is the influence of endophytic entomopathogen on the volatile compounds produced by grapevines. A markedly higher number of volatile compunds were detected among fungus-treated plants compared to control plants. This information can contribute to a deeper understanding of the endophytic fungus-plant-insect relationship and the role of volatile organic compounds in crop protection and mediation of insect herbivory. Several volatile organic compounds are emmited as part of natural defense against insects and research reports suggest some have repellent effects towards herbivorous insects as well as have the ability to attract predators of herbivorous insects (Paré & Tumlinson, 1999) .

4.2 Recommendations

Based on the findings of this study I, therefore, propose the following reccomendations;

1. Maintaining optimum nutrient levels during cultivation of grapevine favours occurrence of EPF.
2. The current study on EPF occurrence in vineyards was only limited to one season; however, sampling soils over time and in different seasons are recommended because these will enable a better understanding of the ecology of EPF in vineyards in the Cape Winelands.
3. Fungi produce mycotoxins, which are potentially toxic to humans and animals; these should be investigated to establish food safety risks that may be associated with fungal inoculation of food crops.
4. This study established the pathogenicity of some indigenous strains of EPF to *P. ficus*; their molecular basis of toxicity needs to be further analysed for improved exploitation.
5. We achieved successful inoculation of grapevine seedlings. Persistence of the conidia in tissue over time needs to be investigated. Also, different stages of plant growth need to be tested to determine the best stage or age for higher percentage colonization by fungal inocula.

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Appendix 1 Manuscript submissions