Orchid mycorrhizal and endophytic fungal diversity of three co-occurring terrestrial orchids in the large African genus *Disa* (Orchidaceae)

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by

Nondumiso Venessa Khambule

(g17k3828**)**

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Supervision By

Prof Joanna F. Dames

Department of Biochemistry and Microbiology

and

Prof Craig Peter

Department of Botany

Abstract

Orchids (in the family Orchidaceous) are one of the richest plant families and approximately 500 species are found in South Africa. A number of orchid species are found on disturbed areas and many of the terrestrial species grow in poor soils with low mineral nutrient availability. Most orchid species are thought to be associated with mycorrhizal fungi for germination and mycorrhiza provides nutrients for the survival of adult plants. The aim of this study was to select Orchidaceous plant species and to isolate, identify and characterize the orchid endophytes and assess these isolates for potential antimicrobial and enzymatic activities Isa is the largest genus in South Africa and three *Disa* species co-occurring in a small geographical area were selected. These included *Disa bracteata, D. cornuta* and *D. polygonoides* which span three sections of the genus.

Roots were stained to confirm the mycorrhizal status of the *Disa* species. Mycorrhizal pelotons structures were microscopically observed inside root cells. The presence of pelotons is indictive of mycorrhizal fungal interactions within the orchid roots and areas associated with the site of nutrient exchange between plant and fungus. The presence of pelotons, however, does not give n indication of the fungal species involved. The endophytes were successfully isolated in pure cultures on potato dextrose agar (PDA). All slow growing isolates were selected, and further molecular identification undertaken; DNA was extracted, and PCR amplified using internal transcribed spacer (ITS1F and ITS4) fungal primers. The amplified products were then sequenced and analysed by comparison to sequences in the GenBank database. *Trichoderma, Penicillium, Metapochonia, Talaromyces, Oidiodendron Neopestalotiopsis,* and *Chaetomium* were identified from these sequences. The presence of other fungal root endophytes was suspected despite the rigorous surface sterilization procedure used. The primers used to amplify the ITS region are the universal barcoding primers which are specific to fungi. ITS1F is one of the primers designed to amplify a broad range of fungi.

DNA was extracted from orchid roots and amplicons were cloned into a pGEMT plasmid vector. Individual clones were sequenced and aligned with Mega software and compared to sequences in the GenBank and UNITE database. Based on percentage sequence identity, unidentified *Tulasnella* species*, Tullasnela colaspora,*

and various Ascomycota endophytes were identified as contributing to the endophytic root fungal diversity of the selected *Disa* species.

The *Disa* species investigated in this study were associated with several soil endophytes. *D. bracteata*, *D. polygonoides* were collected from the same site along the road verge which is regarded as being disturbed. Based on both culture – dependent and independent techniques employed *Oidiodendron* was found associated with both species.

Antimicrobial activity was determined using a well diffusion method using extracts from the isolated fungi against the bacterial isolates *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas puptida.* Most of the isolated fungi showed at least one potential inhibition effect against one of the bacterial isolates. The extracts that showed potential antimicrobial activity could be further screened to determine the compounds produced as secondary metabolites using techniques such as LC-MS

Enzymatic activities of protease, cellulose and amylase were determined using solid media amended with milk protein, carboxymethylcellulose (CMC) and starch. The majority of fungal isolates tested positive with amylase and cellulose with only a few fungal isolates testing positive for protease activity. Broth cultures containing CMC and starch were shown to enhance biomass production in approximately 40 % of the fungal isolates. Degradation of the substrates is required in order to provide carbon to the fungus under test in order to optimize fungal growth as well as to gain insight into their ecological role. Enzyme activity was evident particularly when cellulose and starch were provided as substrates. All the fungal isolates tested grew on the amended medium, with 40% of the isolates preferring to utilize CMC and/or starch, indicating the ability of these fungi to utilize various resources for carbon acquisitions.

Declaration

I, Nondumiso Venessia Khambule (g17k3828), declare that this thesis is my original work. It is being submitted for the degree of Master of Science in Microbiology in the Department of Biochemistry and Microbiology, Faculty of Science, Rhodes University, South Africa. It has not been previously submitted for any degree for examination in any other university.

Date:

Signature

Nondumiso Venessia Khambule

Dedication

This thesis is dedicated to the Almighty God, my parents and my sisters who have been my pillar of strength, encouragement, and always believing in me. Your resilience made it possible.

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Firstly, I thank God, for making it possible for me to complete this study and thesis. I would like to extend my deep sincere gratitude to my Supervisor, Professor Joanna Dames and co-supervisor Professor Craig Peter for their patience, guidance and support throughout the course of the project.

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List of abbreviations

^{0}C degrees Celsius

µl microlitre

AM Arbuscular mycorrhizal

ANOVA Analysis of Variance

BLAST Basic Local Alignment Search Tool

Bp base pairs

C carbon

Ca calcium

CEC Cation exchange capacity

DNA Deoxyribose Nucleic Acid

dNTP Deoxyribose Nucleotide Phosphate

DSE Dark septate endophytes

EC electrical conductivity

EcM Ectomycorrhizal

e.g. for example

ER Ericoid mycorrhizal

g gram

hr hour

IPGT Isopropyl-β-D-thiogalactoside

ITS Internal transcribe sequence

Kb kilobyte

LA Luria Agar

LB Luria broth

MEA Malt Extract Agar

Mg magnesium

MH Mycoheterotrophic

Min minute

MMN Modified Melin-Norkrans

N nitrogen

NA Nutrient Agar

NB Nutrient broth

Na sodium

NCBI National Centre for Biotechnology Information

OM Orchid mycorrhizal

P phosphorus

PCR Polymerase Chain Reaction

PDA Potato Dextrose Agar

rpm revolutions per minute

sec seconds

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1: Root Fungal Associates of Orchids

Introduction

The majority of plants form a mycorrhizal relationship with selected soil fungi. This association is beneficial to both partners as the fungus can enhance the uptake of nutrients and water from the soil and transfer these to their host plants. In return, the fungi obtain carbon from photosynthesis provided by the host plant. The relationship plays a very significant role in the growth and survival of host plants. The fungal symbionts, primarily recognized for their beneficial impact on plant growth and plant protection, are integral components of soil ecosystems and as such, play an active role in improving plant productivity and diversity as well as soil microflora and microfauna diversity (Dalpé 2003). Plants, in general, are photoautotrophic organisms using resources such as sunlight, water, CO_2 and mineral ions, which are acquired from the abiotic environment (Rasmussen and Rasmussen 2009). Most of the flowering plants rely on the mycorrhizal fungi to obtain abiotic resources particularly if they are in complex and unavailable forms which plants are unable to retrieve (Smith and Read 2008).

Mycorrhizal fungi are present in almost all ecosystems, from deserts to tropical forests and in arable land forming associations with a diverse range of plants (Read 1991; Brundreth 2009; Van der Heijden et al 2015). Mycorrhizal fungi also provide a wide range of other ecosystem functions and have a large impact on the establishment of seedlings (Van der Heijden & Horton 2009), and resistance to abiotic and biotic stress that the plant faces such as drought (Augé 2001), heavy metals and pathogens (Newsham et al 1995). Several studies have shown that mycorrhizal fungi modify competitive interactions between plants (Wagg et al 2011). The plant community structure and diversity are altered depending on the presence (Hartnett & Wilson 1999; O'Connor et al 2002) and/or composition of mycorrhizal fungal communities (van der Heijden et al 1998; Vogelsang et al., 2006). Under reduced availability of soil nutrients, mycorrhizal fungi provide adequate amounts of nitrogen (N) and phosphate (P) to their hosts (Leake et al 2004). Studies have proven that arbuscular mycorrhizal fungi contribute up to 60% of plant P requirements, while ectomycorrhizal and ericoid fungi can supply up to 80% of plant

N and P (Simard and Durall 2004; Read and Perez-Moreno 2003; Hobbie and Hobbie 2006) mainly from organic nitrogen and phosphate sources (Leake et al 2004; Smith and Smith 2011). Mycorrhizal fungi reduce the risks of nutrient deficiencies which are essential to support the growth of host plants (Asghari et al 2005; Asghari and Cavagnora 2012; Bender et al 2015). Prevention of nutrient losses is a result of the ability of mycorrhizal fungi to enhance nutrient use efficiency and ecosystem sustainability (Van der Heijden 2010). Ecosystems services like these are important in nutrient-poor ecosystems where plant productivity is limited by nutrient availability (Veresoglou et al 2012).

There are four major types of mycorrhizal associations that have been described based on their structure, function, and interaction with the host plants. Namely, arbuscular mycorrhizal (AM), ectomycorrhizal (EcM), orchid mycorrhizal (OM) and ericoid mycorrhizal (EM) types (Van der Heijden et al 2015). Mycorrhizas are known to reside inside the root cortex of plants, on the surface of roots, or around the epidermal cells within the root (van der Heijden et al 2015). The hyphae of these fungi grow out into the soil and extract nutrients that assist with plant growth (Read and Perez-Moreno 2003). In exchange, the fungus obtains their carbohydrates from host plants (Smith and Read 2008). Mycorrhizal symbionts play a major role in the cycling of carbon (C), nitrogen (N), and phosphate (P) in the ecosystem (van der Heijden et al 2015). Most plants (approximately 74%) have been estimated to form AM associations with Glomeromycotan fungi (Smith and Read 2008; Brundrett 2009). Approximately 2% of plants form EcM associations, these include *Pinus* and *Eucalyptus* plant species, while all orchidaceous plants form associations with OM fungi (Brundrett 2009). EcM fungi are found to be associated with a variety of hosts some being more generalist across plant genera while others are more specific only colonizing certain host species or host genera (van der Heijden et al 2015).

1.2 Orchid mycorrhizal associations

Orchid mycorrhizas are mutualistic interactions between fungi and members of the Orchidaceae (Dearnaley 2007), the world's largest plant family. The family of Orchidaceae has approximately 25 000 species. During the early stages of development many orchid species are non-photosynthetic and are nourished by

endophytic fungal associations such as OM fungi (Smith and Read 2008; Bougoure et al 2014). Mycorrhizas in the Orchidaceae have a unique physiology, anatomy, and identity (Taylor and Bruns 1997). Fungi from the phylum Basidiomycota mostly interact with orchids, with the majority belonging to the *Rhizoctonia* complex, within the Heterobasidiomycetes. Unlike other mycorrhizal interactions, these fungi not only provide water and nutrients needed by the plant but also supply carbon (Rasmussen 2002; Smith and Read 2008; Bougoure et al 2014). These fungi typically live as soil saprotrophs or in an endophytic/ECM association with neighbouring trees or shrubs (Dearnaley et al 2012). This relationship is particularly important during seedling development and in non-photosynthesising host orchid species (i.e. achlorophyllous orchids) (Selosse et al 2002a; Smith and Read 2008; Hynson et al 2013; Bougoure et al 2014). Orchids have extremely small seeds weighing approximately 0.3-14 µg and lack an endosperm (Arditti and Ghani 2000). The seedlings are therefore completely dependent on colonization by these fungi to supply nutrients and water in their early development (van der Heijden et al 2015) and further into mature plants through the assistance of a nutritional model known as mycoheterotrophy (Leake 1994). Mycoheterotrophic dependency can be prolonged into adulthood in many orchid species highlighting the impact that these fungal associates have on plant fitness and growth (Rasmussen 2002).

Orchid seeds may sometimes remain viable in the soil bank for several years (Whigham et al 2006; Rasmussen and Rasmussen 2009) avoiding germination in the absence of a suitable fungal partner. Studies indicate that the presence of fungi may increase or accelerate germination of seeds, but this requires live hyphae that are in physical contact (Rasmussen and Rasmussen 2009).

Seeds are produced from all flowering plants and orchids produce seeds in profusion (Martino et al 2018). It is reported that Darwin estimated the number of seeds of *Cephalanthera longifolia* to be many thousands produced by a single flower and orchid progeny could cover a large area (Bidartondo and Read 2008). The absence of these fungi in an environment has a major impact that prevents the unlimited multiplication of seeds.

Most mycorrhizal fungi have different nutritional strategies and these differ from one environment to the next. Many of EcM and OM fungi are partly saprotrophic and can

be grown on artificial agar media without host plants (Agerer et al 2012). The majority of EcM fungi have a wide range of hosts while some are more specific and colonise certain hosts or host genera (Molina et al 1992). OM fungi are typically saprotrophs in soils and may form endophytic or EcM associations with neighbouring trees (Dearnaley et al 2012). The advance of molecular techniques has shown orchids to be host-specific with some of the fungal partners (Martos et al 2012; Jacquemyn et al 2015). ErM are also soil saprotrophs and recent studies show that some ericoid mycorrhizal fungi could act as plant endophytes to other plants and some Basidiomycetes are thought to form ericoid mycorrhizal associations (Villarreal-Ruiz et al 2004; Grelet et al 2009). Fungal root endophytes are also associated with plants such as orchids and ericoids (Jumpponen and Trappe 1998; Waller et al 2005; Weiss et al 2011; Shakya et al 2013). Endophytes are important as they improve plant growth and provide resistance to stress and pathogens (Rodriguez et al 2009). However, endophytes do not form any specialised plant-fungal characteristics for resource exchange (van der Heijden et al 2015).

1.3 Colonisation

Most plant roots are colonised by more than one fungus and many mycorrhizal fungi are not host specific and can colonise various host plants at the same time (van der Heijden et al 2015). Orchids are colonised by mycorrhizal fungi that form specialised structures called pelotons with the root cortex (Smith and Read 2008). Pelotons in the roots are detected visually and are the most reliable characteristic indicating orchid mycorrhizal colonization.

These special structures are only viable for a certain period after which they collapse and often degraded especially in epiphytic orchids allowing endophytes to enter (Otero et al 2002; Perole et al 2018). Colonisation by OM fungi is important for the germination stage as orchids produce seeds that lack endosperm and cannot germinate on their own without the presence of these fungi. After germination, all orchids form protocorms, an undifferentiated mass of cells that become photosynthetic and then differentiate into roots, stems and leaves of the plantlet (Smith and Read 2008). For development to occur there must be exogenous nutrients or colonisation by a compatible mycorrhizal fungus (Dearnaley 2007).

Mycorrhizal fungi are likely to be important throughout the rest of the orchid life cycle as it provides nutrients required for their growth and establishment. Orchid plants are colonised by OM fungi can also habour other endophytic fungi which do not cause any harm and may provide certain benefits to the plant. Plants within the same environment end up being interconnected by mycorrhizal mycelial networks (Simard et al 1997). A study by van der Heijden et al (2015) reported that some plant communities show that EM and OM plants form these mycorrhizal networks (Villarreal-Ruiz et al 2004; Bougoure et al 2007). The presence of these mycelial networks assists in transferring nutrients from one plant to another (Bougoure et al 2007). Carbon and nutrients can be transferred from one plant to another through the fungal hyphae contributing to the mycorrhizal networks (Simard et al 1997). Nutrients such as N and C, are translocated from one plant to another through these hyphal networks (Selosse et al 2006).

1.4 Life cycle of Orchids

Orchids, in general, have a complex life cycle with relatively low germination, recruitment, and establishment success (Swarts and Dixon 2009; McCormick and Jacquemyn 2014; Rasmussen et al 2015). Orchids produce very small seeds that are referred to as “dust-like” consisting of minute embryos that lack food reserve (Smith and Read 2008). Orchids depend on pollinators and mycorrhizal fungi to complete their life cycles. Terrestrial orchid seed is difficult to germinate *in vitro* and *ex vitro* because they require specific nutrients and environmental conditions (Vujanovic et al 2000). Research has focused on providing nutrients under culture conditions that are suitable for optimal germination and early seedling development *in vitro* (Thompson 2005). However, pollinator and mycorrhizal interactions play a significant role in the success of orchid populations (Remy et al 1994) and increase biodiversity (Thompson 2005). These interactions have influential roles in structuring plant communities and increasing their biodiversity (Hartnett and Wilson 2002; Fontaine et al 2005). Competition among these plants is avoided by being selective in their interactions (van de Heijden et al 2003; Peakall et al 2010).

In later stages of the orchid life cycle its dependence on mycorrhizal fungi is highly variable and some tropical epiphytic orchids are less dependent on mycorrhizas

when adults, while many terrestrial orchids remain mycorrhizal (Gebauer and Meyer 2003; Smith and Read 2008). In adult orchids, the mycorrhizal association is assumed to be important for mineral nutrition because the root systems of many terrestrial orchids are poorly developed (Gebaur and Meyer 2003; Smith and Read 2008; Brundrett 2009). Terrestrial orchids that are autotrophic are partially mycoheterotrophic depending on the mycorrhizal fungi to provide additional carbon and nitrogen (Smith and Read 2008). Achlorophyllous orchids are fully mycoheterotrophic depending solely on the orchid mycorrhizas to supply carbon and nitrogen throughout their life cycle (Leake 1994).

It has generally been assumed that mycoheterotrophic (MH) plants obtain their food and nutrients for growth from organic matter in soil through the activity of saprotrophs (Leake 2005). However, Merckx et al 2009 suggested that MH plants were epiparasites involved in a triangle symbiosis through shared mycorrhizal fungi with adjacent autotrophic plants. Commonly known mycorrhizal symbionts are ectomycorrhizas and arbuscular mycorrhizas that are highly exploited by MH plants. The MH families of Aneuraceae, Orchidaceae and Ericaceae can exploit ectomycorrhizal networks while Burmanniaceae, Corsiaceae, Gentianaceae, Thismiaceae and Triuridaceae exploit arbuscular mycorrhizal networks (Leake 2005). Some MH orchids are associated with litter- and wood-decay fungi (Ogura-Tsujita et al. 2009).

1.5 Specificity

Specificity or specialisation is defined as an association of orchids with a small number of fungal partners (Irwin et al 2007; Dearnaley et al 2012). Narrow specificity is where an orchid associates with only a single mycobiont (Dearnaley et al 2012). Associations differ between *in vitro* conditions and natural conditions (Rasmussen 2002). Narrow specificity in orchids can be the result of plant species vulnerability and rarity (Rasmussen 2002). This was evident in a rare orchid species, *Corallorhiza mertensiana* (Freudenstein 1997), that was found to be associated with few mycobionts as compared to the more wide-spread *C. maculata* (Taylor and Bruns 1999). Narrow specificity was also detected in other rare orchids. Bougoure et al (2009) found that the rare underground orchid *Rhizanthella gardneri* associated with

limited related fungal taxa while Taylor et al (2004) found that *C. maculata* associated with Russulaceae species only in the western United States. Orchids investigated by Bonnardeaux et al (2007) displayed less fungal specificity, whereas the Australian grassland species, *Microtis media,* was found to be associated with members of Sebacinales and the Ceratobasidiaceae.

Adult plants may become a host to a variety of fungi (Rasmussen and Whigman 1998). Orchids in some cases employ a variety of fungal species to avoid mutual competition for food as was demonstrated for *Pterostylis 7cuminate* when compared to co-occurring orchid species (Perkins and McGee 1995; Rasmussen 2002). Specificity in orchid plants is influenced by the presence of mycobionts, geography and habitat conditions (Taylor and Bruns 1999, Rasmussen 2002). Specificity is more prevalent in orchids during adulthood where they either become autotrophic or MH.

Mycorrhizal specificity is important to MH plants because they will not germinate or develop in the absence of their targeted fungal symbiont (Bruns and Read 2000). They will not survive the germination stage even if stimulated by a close relative of the partner fungus (Bidartondo and Read 2008). There is low fungal specificity between fungi and plants forming arbuscular and ectomycorrhizal relationships. Autotrophic plants are generally associated with closely related mycorrhizal fungi and conversely these related fungi can simultaneously associated with multiple non-related plants, indicating a more generalisation approach from the fungi side (Giovannetti et al. 2004; Lian et al. 2006). Fully MH plants have been proven to be highly specific to the fungi they associate with even though the fungi remain generalists (Bidartondo et al. 2002). In Thailand, MH *Aphyllorchis* species were found to be associated with members of Thelephoraceae, Russulaceae and Sebacinales (Roy et al 2009).

Specificity in fungal partners is influenced by distribution, habitat and genetics. In the study conducted by Taylor et al. (2002) the data showed that unlike most autotrophic plants, some of fully MH species in the Orchidaceae and Monotropoideae (Ericaceae) are fungal specialists.

Germination in orchids may be stimulated by several fungi, without forming a relationship with the plant in nature (Rasmussen 2002). Fungal specificity enhances

germination rates in orchids (Otero et al 2004; Bonnardeaux et al 2007). Associations between plants and specific fungi, has led to improved efficiency in nutrient exchange (Bonnardeaux et al 2007). This is evident particularly with carbon uptake by fully MH orchids in low-light habitats that are highly dependent on carbon provided by fungus (Otero et al 2004). Nutrient exchange is one of the main drivers for fungal specificity in orchids (McCormick et al 2006) and has been linked with speciation in the Orchidaceae (Otero and Flanagan 2006; Shefferson et al 2007; Waterman and Bidartondo 2008).

Two approaches have been employed to study fungal specificity in orchids; firstly, the analysis of seed germination and growth paired with fungal strains under laboratory conditions and secondly, morphological description and isolation of fungi from adult plants (Taylor et al 2002). Several studies have been successful in finding a high degree of fungal specificity in some partially or fully MH orchids using seed germination and growth *in vitro* (Taylor et al 2002). Other studies such as those conducted by Umata (1995, 1997 and 1998), have indicated low specificity. Studies conducted on green autotrophic orchids have shown inconsistent results leading to the conclusion that they generally lack fungal specificity (Taylor et al 2002).

The distribution of fungi also plays an important role in influencing specificity among orchids. A variety of fungal species can be associated with the roots of plants in a specific site (Taylor et al 2002; Selosse et al 2004; Selosse et al 2007; Stark et al 2009). Roots of trees growing in close proximity to *Cephalanthera austiniae* was found to be colonized by a variety of ectomycorrhizal fungi, including species from Russulaceae, their presence being confirmed in orchid roots (Taylor and Bruns 1997). Taylor et al (2002) argued that the host plant may have a stronger influence on the occurrence of fungi than habitat suggesting that plant genotype is associated with distinct fungal lineages, regardless of the presence of other plant genotypes and other fungal species at the site. The patterns of fungal distribution across plant communities impacts upon the observed patterns of specificity. These patterns can be uncovered by molecular analysis (Taylor et al 2002).

Studies conducted by Dearnaley et al (2012), Pandey et al (2013) and van der Heijden et al (2015) showed that orchids can display a vast variation in mycorrhizal specificity. *Disa* species have also been found to be associated with diverse fungi

(Bonnardeaux et al 2007). In contrast, wide spread orchids tend not to be limited in their associations (Shefferson et al 2007). The orchid, *Anacamptis morio*, which occurs across Europe and Asia appeared to be less specific in their mycorrhizal associations (Bailarote et al 2012). Waud et al (2017) found that *Liparis loeselii* was associated with many orchid mycorrhizal fungi such as Caratobasidiceae, Tulasnellaceae, Thelephoraceae and Sebacinaceae; and hypothesised that site differences in orchid distribution mostly influence fungal communities.

Mycorrhizal specificity thus appears to be complex and involves a diversity of mycorrhizal symbionts that are phylogenetically diverse both in plant and fungal taxa (Hibbett and Matheny 2009; Terdersoo et al 2010; van der Heijden et al 2015). Some orchids lose their ability to photosynthesize and become entirely MH during adulthood (Waterman and Bidartondo 2008). This has led many MH orchids to be highly specific in their fungal partners often forming ectomycorrhizal relationships with trees rather than with free-living *Rhizoctonia*-forming fungi, which are anamorphic fungi in the order Cantharellales (Ogura-Tsujita and Yukawa 2008, Waterman and Bidartondo 2008). Photosynthetic orchids exploit ectomycorrhizal mutualists for carbon supply (Selosse et al 2004). Studies found that photosynthetic orchids associated with a few fungal partners over a large geographic area, indicating narrow specificity (Shefferson et al 2005; 2007; McCormick et al 2006; Bonnardeaux et al 2007; Irwin et al 2007). Epiphytic orchids have been shown to have highly specialised mycorrhizal interactions (Otero et al 2002; Ma et al 2003; Suarez et al 2006). Mycorrhizal specificity can be used as drivers of diversity indirectly by studying the patterns of orchid distribution (Otero et al 2005; Otero and Elanagan 2006; Waterman and Bidartondo 2008).

1.6 Endophytes

Endophytes are microbes commonly known to colonise internal host tissues but remain inconspicuous and symptomless (Stone et al 2000; Brundrett et al 2006). These endophytes may be fungi or bacteria living within healthy plant tissue (leaves, stems, roots) at least for part of their life cycle (Malloch et al. 1980; Petrini 1991; Wilson 1995; Stone et al; 2000; Evans et al. 2003). Fungal endophytes are both

associated with aboveground and belowground (roots, bulbs, rhizomes, tubers) plant parts (Clay 1988; Petrini 1991; Kobayashi and Palumbo 2000).

Symbiosis is defined as two different organisms living together (de Bary 1879). The partner in the symbiosis may have originated from eukaryotes, prokaryotes, archaea, and viruses (Wegley et al 2004; Moran et al 2005; Márquez et al 2007; Rodriguez et al 2009; Roossinck 2010). Microbe-plant relationships may sometimes be mutualistic, antagonistic or parasitic. Recent studies have suggested that sometimes parasitic symbioses are a result of mutualistic breakdown (Sachs and Simms 2006). Plants are host to fungal endophytes which are described as mutualistic plant-microbe interactions (Bao and Roossinck 2013). These endophytes are grouped into four classes based on their life histories (Rodriguez et al 2009). Endophytes are very host specific and initially were thought to be mostly associated with the grass family Poaceae, their metabolism contributing to enhanced plant resistance to biotic and abiotic stresses (Kuldau and Bacon 2008).

Fungal endophytes have been found from roots of almost all plants, including orchids, studied to date (Stone et al 2000; Kobayashi and Palumbo 2000; Sieber 2002). Colonisation by assemblages of endophytes varies with host and habitat; aquatic fungi are adapted to a specialized habitat to colonize submerged roots (Brundrett 2006). Some of the endophyte's communities may be host specific (Stone et al 2000; Berg et al 2002; Cohen 2004) or have a host preference (Carroll 1999) or show host-exclusivity (Zhou and Hyde 2001) but this is an adaptation that has occurred between host and endophyte (Brundrett 2006). The adaptation may not always be to a host, but to endophytic growth in one plant organ such as in the roots (Sieber 2002; Schulz and Boyle 2005). Endophytic associations may be mutualism, commensalism, latent pathogenic and exploitation (Brundrett et al 2006). The interactions depend on their genetic dispositions, developmental stage and nutritional status as well as environmental factors (Kobayashi and Palumbo 2000). Endophytic fungi do not solely interact with the plant host but with other organisms, including mycorrhizal fungi (Bayman and Otero 2006). Nematophagous fungi which are found in all soil types, switch from a saprotrophic to a parasitic stage to kill and

digest living nematodes but can also grow endophytically in plant roots (Lopez-Llorca et al 2006).

Class 1 endophytes are well studied and have been used to represent the entire endophytic group (Bao and Roossinck 2013). These fungi are from the family Hypocreales (Ascomycota) that include free living and symbiotic species associated with insects, grasses, rushes and sedges (Bacon and White 2000). The Hypocreales include many well-known plant pathogens, saprotrophs and endophytes, many of which secrete bioactive compounds (Rodriguez et al 2009). They are also soil-inhabiting species that include some of the most geographically widespread taxa such as *Cordyceps japonica, Verticillium epiphytum* and *Sphaerostilbella berkeyana* (Spatafora et al 2007). Ancestral insects and host plants did not possess enzymes, toxins or defense mechanisms to protect themselves from harm, and therefore did not inhibit colonisation (Rodriguez et al 2009). Among class 1 endophytes, there are also three different types namely Type I, Type II and Type III based on *Epichloe* species (anamorphs: *Neotyphodium*) that are endophytic symbionts of cool-season grasses (White et al 1996; Moy et al 2000; Dugan et al 2002; Tadych et al 2007). During flowering of grasses, the fungus grows over the developing inflorescence to form a stroma (Rodriguez et al 2009) which may form on all or most of the tillers and no endophytic infection is found on any tillers that have escaped the fungus (Clay and Schardl 2002). Type 2 produce stromata only in a proportion of the tillers, allowing seed production and vertical transmission within seeds (Clay and Schardl 2002). Type 3 are asexual endophytes and are classified as a species of *Neotyphodium* (Clay and Schardl 2002; Rodriguez et al 2009). Class 1 endophytes do not provide resistance to host plants against herbivory (Faeth et al 2006). They do, however, enhance the ecophysiology of host plants and enable plants to strive under abiotic stresses such as drought and metal contamination (Malinowski and Belesky 2000). They also stimulate longer root hair development for the efficient absorption of nutrients such as soil phosphorus (Malinowski and Belesky 2000).

Compared to class 1, endophytes from class 2 are highly diverse, consisting of species from Ascomycota and Basidiomycota including the Agaricomycotina (Rodriguez et al 2009). They colonize whole plant and plant tissues such as roots,

stems, and leaves (Bao and Roossinck 2013). Class two endophytes are found to be predominant species in plants under high-stress conditions (Redman et al 2002; Rodriguez et al 2004; Rodriguez et al 2009), they increase plant root and shoot biomass (Ernst et al 2003; Mucciarelli et al 2003; Maciá-Vicente et al 2008) and provide tolerance against biotic stress such as disease (Narisawa et al 2002; Campanile et al 2007) and abiotic stress such as drought, desiccation, heat and salinity (Rodman et al 2001; 2002; Marquez et al 2007; Rodriguez et al 2008). Colonization is through infection structures such as direct penetration of plant tissue via hyphae or appressoria (Ernst et al 2003). These endophytes are culturable on various simple media (Rodman et al 2002) such as malt extract agar. Some of these endophytes have been confirmed to be mutualistic, providing nutrition for the host via symbiosis (Rodriguez et al 2009). Class 2 endophytes are known to increase plant biomass under stressful conditions (Rodriguez et al 2009).

Class 3 endophytes are distinct based on their occurrence primarily or exclusively in above- ground tissues (Arnold et al 2000; Gamboa and Bayman 2001). Endophytes from class 3 include hyper diverse endophytic fungi associated with leaves of tropical trees (Arnold et al 2000) as well as non-vascular, vascular plants, seedless vascular, conifers and woody and herbaceous angiosperms (Davis et al 2003; Higgins et al 2007; Murali et al 2007; Davis and Shaw 2008). Endophytes from class 3 are found to occur in flowers and fruits, as well as in asymptomatic wood and inner barks (Barengo et al 200OR0; Kumar and Hyde 2004; Tejesvi et al 2005). Class 3 endophytes have a high diversity within individual host tissues, plants and populations (Arnold and Herre 2003; Arnold et al 2003). These endophytes are often different from pathogens associated with the same host species (Ganley et al 2004). Unlike class 2 endophytes, they are hardly isolated from seeds (Arnold et al 2003; Ganley et al 2004). Most endophytes in this class are members of Ascomycota and Basidiomycota (Rodriguez et al 2009). Ascomycota is well represented especially by members of the Pezizomycotina (Higgins et al 2007). Members of Basidiomycota belonging to the Agaricomycotina, Pucciniomycotina (rust fungi) and Ustilaginomycotina (smut fungi) also form part of class 3 endophytes although less is reported on them (Rodriguez et al 2009).

Class 4 endophytes include fungi that are referred to as ‘dark septate endophytes’ (DSE) (Rodriguez et al 2009). These endophytes are primarily ascomycetous fungi

that are conidial or sterile and form melanized structures such as inter- and intracellular hyphae and microsclerotia in the roots (Rodriguez et al 2009). Dark septate endophytic fungi exhibit little host or habitat specificity and are in association with approximately 600 plants including plants that are non-mycorrhizal occurring in Antarctic, Arctic, alpine, sub-alpine, and temperate zones as well as from African coastal plains and lowland ecosystems (Jumpponen and Trappe 1998; Jumpponen 2001). DSE still require intensive study to determine their diversity and plant host ranges (Mandyam and Jumpponen 2005). They are associated with fine roots of trees and shrubs, especially of conifers found in boreal and tem perate forests (Rodriguez et al 2009). DSE fungi are not pathogenic because they are observed on healthy fine roots and they colonize living plant organs without any apparent, negative effects (Jumpponen and Trappe 1998; Rodriguez et al 2009). DSE are widespread, found in high-stress environments and are common in occurrence and abundant across various ecosystems (Rodriguez et al 2009). They play an important role in the ecophysiology of plants (Rodriguez et al 2008). Colonization is superficial and forms a loose network of hyphae on the root surface. Individual hyphae grow along the main axis of the root and can enter between cortical cells and within the depression between epidermal cells (Rodriguez et al 2008). It is proposed that DSE colonization may play a role in deterring pathogens by minimizing available carbon in the rhizosphere (Mandyam and Jumpponen 2005). However, the role of these class 4 endophytes is still largely unknown.

Members of the Orchidaceae family have a symbiotic relationship with Class 2 endophytic fungi (Rasmussen 2002) forming orchid mycorrhizal associations as is evident by the intracellular coils called pelotons (Smith and Read 2008), enhancing the uptake of nutrients such as N and P (Rasmussen 1995) and providing C for the early phase of germination, protocorm development and seedling growth (Dearnaley 2007). Extensive growth of endophytic fungi is commonly found within roots (Stone et al 2000; Schulz and Boyle 2005). Root colonisation can be both inter-and intra-cellular, hyphae often forming intracellular coils (Stone et al 2000; Sieber 2002). A basidiomycete *Piriformospora indica* (Varma et al 1999) is an endophyte that forms intracellular coils within roots. Many orchid roots are colonized by fungi from the species complexes of (Ma and Zeng 2003) and *Leptodontidium* (Bidartondo et al 2004) while other orchids are found to be colonized by endophytes such as

Talaromyces rotundus; Eupenicillium inusitatum; Hymenogaster bulliardii and Sarcosomatuceous fungi (Selosse et al 2004).

The relationship between orchids and endophytes is over-shadowed by interest in orchid mycorrhizas (Brundrett et al 2006). Most of studies to date have focused on OM fungi in orchid roots and have ignored all fungi not thought to be mycorrhizal (Bayman and Otero 2006). In some cases, a fungus has been presumed to be mycorrhizal when they may be endophytes (Brundrett et al 2006). Diversity of orchid root endophytes remains largely unexplored (Bayman and Otero 2006). Terrestrial orchids are found worldwide and remain the most studied orchid-fungal associations (Brundrett et al 2006). Terrestrial orchids have non-photosynthetic roots that often show marked seasonal differences in growth and composition (Rasmussen 1995). Roots of terrestrial orchids have two distinct morphological types, one is mycorrhizal (Rasmussen 1995) and non-mycorrhizal which tends to have more xylem and amyloplasts for starch storage compared to the mycorrhizal roots (Brundrett et al 2006). The concept of endophytes living inside plants without any possible benefits for either party (Bronstein et al 2003) is difficult to comprehend and requires further investigation. In contrast to other mycorrhizal relationships orchids provide little obvious direct benefit to their fungal partner, particularly in early stages of development, seedling germination and protocorm formation indirectly these benefits may include a constant mesic environment, constant moisture and low competition (Andersen and Rasmussen 1996; Taylor et al 2002). The relationship tends to be parasitic rather than mutualistic (Rasmussen 2002). Fungal endophytes that are non-mycorrhizal in the field may stimulate orchid seed growth in culture and this is referred to functional specificity as opposed to ecological specificity as defined by Masuhara and Katsuya 1994. Studies to date have focused on *Rhizoctonia*-like fungi with the assumption that the relationship is mycorrhizal without adequately demonstrating functional benefits to the plant (Bayman and Otero 2006). *Rhizoctonia*-like fungi can be plant pathogens, endophytes or saprotrophs (Carling et al 1999; Rasmussen 2002). Sebacinales are basidiomycetes that belong to the *Rhizoctonia*-like fungi and a known orchid mycorrhizal group (Rasmussen 1995). Selosse et al 2009 found that Sebacinales colonise both mycorrhizal and non-mycorrhizal host roots. Sebacinales belong to Class 4 endophytes, the DSE, (Rodriguez et al 2009) and are divided into two clades (Weiss et al 2004); clade A

whose species form ectomycorrhizas with heterotrophic orchids (Selosse et al 2002b) and clade B whose species are either endomycorrhizal with autotrophic orchids and ericas or endophytic with the more primitive liverworts (Kottke et al 2003; Selosse et al 2007; 2009).

Taxa from Clade A form ectomycorrhizas while some taxa in clade B have evolved to form orchid or ericoid mycorrhizas (Selosse et al 2007). Although ecology restricts Sebacinales to ectomycorrhizal, ericoid or orchid mycorrhizal roots molecular analysis has found the Sebacinales to be associated with many different plant roots indicating a more flexible nature (Selosse et al 2009).

Three problems are detected when studying endophytic fungi, including orchid root endophytes (Bayman and Otero 2006). Firstly, most endophytic fungi do not sporulate in pure culture and become very difficult to identify (Brundrett et al 2006). Secondly, the majority of endophytic fungi are not described and do not fit well into previously described taxa (Hawksworth 2000). Arnold et al (2001) suggested that endophytes may represent a largely unstudied reservoir of fungal biodiversity. Lastly, some endophytes are unculturable (Brundrett et al 2006). Molecular approaches that amplify DNA directly from orchid roots using fungal-specific primers have provided a means for the detection and identification of these unculturable fungi (Brundrett et al 2006). This approach was used by the study done by Vandenkoornhuyse et al (2002) and revealed that some endophytes of grass roots belong to previously unknown major taxa of fungi. Bayman and Otero (2006) also isolated non-mycorrhizal endophytes from various terrestrial photosynthetic orchids. One of them was a common endophyte, *Fusarium* which is apparently capable of forming orchid mycorrhizas (Vujanovic et al 2000). Sometimes isolation attempts of mycorrhizal fungi from orchids produce cultures of bacteria, actinomycetes and common endophytes, as well as ectomycorrhizal fungi and ericoid fungi (Kristiansen et al 2001; Otero et al 2002; Bidartondo et al 2004). A decrease in non-*Rhizoctonia* fungi has been shown when isolations are taken from a single peloton as compared to surface-sterilized tissue blocks, which provides evidence that fungi isolated using culture methods are endophytes (Brundrett et al 2006). It is recommended that only fungi that assist with germination and development of orchids from seedling stage to green leaf should be designated as being orchid mycorrhizal fungi (Batty et al 2002).

Endophytic fungi associating with orchids are different from other mycorrhizal fungi, as they do not belong to distinct evolutionary or taxonomically groups (Brundrett 2002). These fungi are simply efficient plant colonizers where each has multiple roles as endophytes, parasites, ectomycorrhizal fungi and/or saprophytes (Brundrett et al 2006).

1.7 Orchidaceae

1.7.1 Orchid family

The Orchidaceae is the most highly and uniquely modified of all angiosperm families and is still undergoing fast diversification and speciation (Smith and Read 2008; Chase et al 2015). Terrestrial orchids represent one-third of species in the orchid family, many occupy cooler temperate zones that are undergoing warming due to climate change (Zettler et al 2017). Terrestrial orchids maintain a lifelong association with fungi, utilizing both autotrophic and mycotrophic strategies (McCormick et al 2012). Terrestrial orchids are more likely to undergo extinction because of many threatening processes, particularly under current climatic change conditions (Swarts and Dixon 2009). The presence and efficiency of mycorrhiza in soils have impacted most of terrestrial orchids allowing them to tolerate abiotic and biotic factors. The abundance of terrestrial orchids is dependent on factors involving the underground and above-ground life history phases of species (McCormick et al 2012).

The family consists of five recognised subfamilies; the Apostasioideae (basal lineage), Cypripediodeae, Vanillioideae, Orchidioideae and (Smith and Read 2008). Most of the orchid species are autotrophic in their adult stages of their life cycle but may still rely on the mycorrhizal fungi. The mycorrhizal relationships are extremely important especially to orchids that are achlorophyllous as adults (Leake 2004). The fully autotrophic orchids are associated with fungi belonging to saprotrophic forms of the genus *Rhizoctonia* (Smith and Read 2008). Characteristic features of orchid mycorrhizal relationships are the formation of pelotons within root cells. The association facilitates the transfer of nutrients from the fungi to the host plant. The

potential pathways used are 1) biotrophic (uni- or bi-directional) transfer of nutrients across the active interface formed between plant and fungus, 2) necrotrophic transfer of nutrients (fungus to plant) after lysis of fungal pelotons or 3) a combination of both (Smith and Read, 2008; Bougoure et al 2014).

1.7.2 Orchid distribution

Orchidaceae is one of the largest angiosperm families, with between 25000 to 28000 species in 796 genera (Chase et al 2003; Chinsamy et al 2011). Orchids occur in almost every part of the world with a distribution from the tropics to the subarctic zone (Linder et al 2005). The richest places where orchids are found are generally the tropics. The largest numbers of orchids are known from the tropical cloud or mist forests. Australia is known to have rich orchid flora with over 1300 species, around 90% which are endemic (Govaerts et al 2016). Brazil has a high diversity of seed plants and Orchidaceae is the second richest family with about 2500 species, of which 60% occur in the Atlantic forest (Forzza et al 2012; Barberana et al 2018). Mexico has a great diversity of orchids with around 1260 species and 170 genera (Hagstar et al 2005; Soto et al 2007; Salazar-Cereze et al 2018). In India orchids are comprised of 32 genera with 106 taxa with 41 endemic orchid species spread over 13 genera (Jalal et al 2018).

Orchids are well represented on the African continent although the African orchid flora is rather poor when compared to that of tropical America or Asia (Linder et al., 2005). The overall distribution of orchids in Africa is approximately 1500 species (Brummit 2001; McCormick et al 2008). In southern Africa orchids are also well represented with 479 species in 54 genera, 65% being endemic (Chinsamy 2011; Gebauer and Meyer 2003). The southern African orchid flora is made of South Africa, Namibia, Botswana, Lesotho, and Swaziland (Linder et al 2005) and includes species from the tribe Diseae in the subfamily Orchidioideae (Linder and Kurzweil 1999; Linder et al 2005). The orchids of southern Africa are distributed among the six of the seven biomes which include; fynbos, succulent karoo, grassland, savannah, forest, thicket (Linder et al 2005). The orchids that belong to Diseae, subtribe Disinae, are the focus of this study.

Disinae includes 170 species in the genus *Disa*, of which 137 are native (Linder et al 2005). Their distinct morphology includes guleate and usually spurred dorsal sepal and reduced but patent lip (Linder and Kurzweil 1999; Bellstedt et al 2001). They occupy a wide range of habitats that include full sun habitats to partial shade, from semi-arid habitats to perennially wet stream banks (Linder et al 2005). Fynbos consists of great diversity of mycorrhizal types that are well distributed. Stock (1993) sampled 332 species that were growing in the Cape lowland vegetation. The recods show that 62% of arbuscular mycorrhiza, 23% of non-mycorrhiza, 8% of ericoid mycorrhiza, 4% of unknown mycorrhiza and 2% of orchid mycorrhiza found on plants growing in the region (Allsopp and Stock 1993; Bazibane 2012). Disinae is mostly distributed in the fynbos biomes, consisting of 102 species (Linder et al 2005). *Disa* species inhabit a wide range of habitats and are particularly common in disturbed areas such as road side verges and rehabilitated mine sites (Hoffman and Brown 1992; Grand and Koch 2003; Bonnardeaux et al 2007). A South African, *Disa bracteata,* is widespread across South Africa and found to be invasive in Australia (Bonnardeaux et al 2007).

Mycorrhizal interactions with indigenous orchid species are poorly studied in South Africa. The root tubers of southern African orchids may not contain hyphae, and the plants must therefore be re-infected each growing season (Linder et al 2005). The dependency on the mycorrhizal fungi contributes to why many orchid seeds prove difficult to germinate (Gebauer and Meyer 2003).

Symbionts play an important role in influencing plant population dynamics and structuring ecological communities (Stachowicz et al 2009; McCormick et al 2018). Mycorrhizal fungi and pollinators affect the distribution of orchids; patchy distribution of mycorrhizal fungi can limit the size of the plant population which will affect seed germination, growth and survival (McCormick et al 2018). In contrast, patchy fungi and plants can contribute to diversification because of limited gene flow between populations and small size populations may lead to speciation (Tremblay et al 2005).

1.7.3 Pollination of orchids

Diversity in the Orchidaceae family is driven by specific pollination syndromes (Fenster et al 2004; Peakall 2007) and mycorrhizal interactions (Smith and Read, 2008). Pollination interactions also play a role in the success of flowering plants, structuring plant communities and in increasing floral biodiversity (Harrtnett and Wilson 2002; Fontaine et al 2005; Thompson 2005). Plant species avoid competition by displaying selective preferences for pollinators and mycorrhizal fungi (van der Heijden 2003; Peakall et al 2000). Most orchids depend on specialized pollination systems for reproduction (Waterman and Bidartondo 2008). It is speculated that one-third of orchid species are pollinated through food-deception and may resemble a specific co-occurring rewarding species (Johnson 2000). Sexual deception strategies are used by some orchids to deceive the male insects to copulate with the flower (Cozzolino et al 2005; Schiestl 2005). About 400 orchid species from several genera, including *Ophrys* in Europe (Borg-Karlson et al 1993), *Disa* in southern Africa (Steiner et al 1994); are known to be sexually deceptive (Waterman and Bidartondo 2008).

Food-deceptive orchids are known to be pollinated by more than one insect species and share substantial pollinators (Waterman and Bidartondo 2008). While sexually-deceptive orchids are found to be pollinated by a single insect that does not pollinate any other species (Waterman and Bidartondo 2008). Orhcids sharing the same pollinators avoid inter-species crosses by placement of pollinia on different parts of the pollinating insects' body (Pauw 2006; Waterman and Bidartondo 2008). Pollination deception has become a highly successful evolutionary strategy in the Orchidaceae family (Johnson et al 1998; Kores et al 2001; Bateman et al 2003). Due to fruiting failure in deceptive orchids, the pollinator often immediately leaves the patch after discovering the lack of reward (Ayasse et al 2000; Johnson 2000; Tremblay et al 2005). The exact behavior is also found in sexually-deceptive orchids (Peakall and Beattie 1996; Peakall and Schiestl 2004). The overall outcome has been outcrossing and long-distance pollen flow transferred to another orchid in a different patch (Waterman and Bidartondo 2008). Shifts in pollinator distribution has had an impact on speciation that differently affects patterns of gene flow as well as

by exerting divergent selection pressure on populations (Grant 1992; Johnson et al 1998; Schluter 2009; Stökl et al 2009; Vereecken et al 2010).

1.8 Conservation of orchids

Many factors play an important role in the genetic diversity in plant populations and species (Chung et al 2017). Orchidaceae consists of 30% of terrestrial species and 70% are epiphytic or lithophytic species (Gravendeel et al 2004). Orchids are known to be one of the most endangered plant taxa (Pillion and Chase 2007; Swarts and Dixon 2009; Vogt-Schilb et al 2015; Zhang et al 2015). The orchid family is one of the largest plant families and therefore they are an important group in conservation biology (Swarts and Dixon 2009). There are numerous factors that threatened orchids such as habitat loss; especially as many are epiphytes in forest canopies and terrestrial species underperform in improved soils (Pillion and Chase 2007). Collection of orchids for botanical and horticultural interest has become one of the significant threats (Gribb 2005). Orchid distribution and abundance are concentrated in the tropics and differs between continents and within regions and species richness (Meyer et al 2011). Terrestrial orchids can persist efficiently in mycorrhizal soil which also enables survival for just a few years after loss of the endophyte (Swarts and Dixon 2009) and this allow them to be conserved under laboratory conditions. Over the past 50 years, organisms and ecosystems have become vulnerable to extinction and orchids represent approximately 10% of all named plants that are subject to risk (Koopowitz et al 2003; Swarts and Dixon 2009). Conservation approaches that better preserve species include seed and germplasm banks, *in vitro* propagation, *in situ* conservation and conservation via assisted migration (Keel 2007; Swarts and Dixon 2009). Orchid conservation should holistically consider genetics, mycorrhizal associations, pollinator interactions, and *in situ* and *ex situ* conservation (Swarts and Dixon 2009).

Conservation of genetics provides frameworks and practical tools to conserve diversity and shapes the genetic diversity in natural populations (Qamaruz-Zuman et al 1998). With the advancing of molecular tools, it is possible to maintain and restore genetic diversity and this is mostly used to conserve rare and threatened species (Hopper 2000). Molecular methods and phylogenetic studies are now used to design

and determine application of conservation strategies (Hopper 2000; Mattner et al 2002). Techniques such as PCR have improved the narrative of orchid conservation studies, such as DNA sequencing of a range of loci (Selosse et al 2002b; Otero et al 2004), amplified fragment length polymorphisms (Hedrén et al 2001; Smith et al 2004) and plastid microsatellites (Fay and Cowan 2001; Fay et al 2009).

Orchid mycorrhizal endophytes can be difficult to identify in soil and an efficient method is the direct isolation from orchid tissues (Zettler et al 2013). Over the years, researchers have developed simple and effective methods for obtaining pure endophyte cultures (Swarts and Dixon 2009; Zettler et al 2013). Morphological characteristics were used to identify mycobionts associated with host orchids and included teleomorphic stages (Rasmussen 1995), hyphal branching patterns and the presence of cell clusters (Sneh et al 1991). Orchid endophytes have been identified mainly through the morphological descriptions of pure colony appearance (Swarts and Dixon 2009). More recently the use of molecular techniques to identify fungal partners has dominated research of the orchid-fungal relationship and assist in the conservation of orchids (Kristiansen et al 2001). Genetic databases like GenBank and UNITE are used for analysis of DNA sequences that permits rapid conclusion of taxonomic understanding of orchid endophytes (Swarts and Dixon 2009). Nuclear ribosomal internal transcribed spacer (nr ITS) region is mostly used for DNA sequencing of fungi (e.g. Kristiansen et al 2001; Pope and Carter 2001; Otero et al 2002; Bougoure et al 2005; Bonnardeaux et al 2007; Swarts and Dixon 2009). For implementation of recovery and restoration programmes, mycorrhizal associations of orchids are important and genetic studies used to identify the diversity of fungi associated with some orchids are demonstrating a marked degree of specialization (Fay and Krauss 2003).

An orchids' dependence on narrow specific interactions with fungi and pollinators may contribute to it becoming rare (Bonnardeaux et al 2007; Dearnaley 2007; Swarts et al 2010). However, this is not always the case in all orchids; studies conducted by Phillips et al 2011 suggested that the west Australian *Drakeae spp.* associated with a specific fungus but did not become rare. Human interaction remains the main driver leading to the decline of many rare orchid taxa (Brundrett 2007). This happens through direct harvesting for horticultural purposes, vegetation clearing, altered fire regimes, weed introduction and climate change (Dearnaley et al 2012). Conservation

of orchids includes site protection of existing populations, *ex situ* storage of tissues and restoration procedures (Swarts and Dixon 2009).

Molecular identification of fungi is used as a tool for conservation of orchids as it provides insight to the fungal ecological status (Dearnaley et al 2012). Organic soils are important to the conservation of most autotrophic orchids as this is the favoured habitat of their *Rhizoctonia* associates (Brundrett et al 2003). Preservation of orchids means preservation of their fungal partners and protection is provided for both and not only those taxa involved in mycorrhizal associations but the surrounding soil fungi and endophytes as well (Dearnaley et al 2012).

Ex situ symbiotic conservation is a common approach used to conserve threatened orchids (Batty et al 2006; Stewart and Kane 2007; Zettler et al 2007). Mycorrhizal fungi isolated from adult plants are used to assist seed germination (Batty et al 2001; Dearnaley et al 2012). Liquid nitrogen is used for long term maintenance of OM fungi (Batty et al 2001) alternatively both seed and fungi can be encapsulated in alginate beads with low-temperature storage (Sonmerville et al 2008). Conservation of orchids requires conserving a life history dependent not only on symbiotic fungi buy also on sympatric flora, and pollinator guilds (Bernhardt et al 2017). For full restoration of orchids that have suffered regional extinction, a pollinator is required (Dixon 2009). It requires an understanding of the biology of each species first before the employment of conservation methods (Dearnaley 2007).

Motivation and Objective

Orchid mycorrhizal interactions are one of the important interactions in the plant kingdom as the fungi provides the plant with water, nutrients and a supply of carbon, unlike other mycorrhizal interactions. In many countries such as Australia and the America's orchid mycorrhiza has been relatively well researched. In African countries particularly South Africa, less research has been conducted on the interactions between orchids and mycorrhizal fungi. This study will assist in expanding the knowledge base of OM and other fungal endophytic associations of indigenous orchids in South Africa. The expansion of this knowledge is necessary and important in terms of knowing how these terrestrial orchids survive in disturbed areas. To know

the role that these fungi play in assisting these plants to survive throughout their life cycles. Biological activities of endophytes are important antimicrobial strategies for biodiversity and conservation of rchid plants. This will provide new information for the assessment of fungal diversity, distribution and discovery of new species and bioactive compounds.

Therefore, the overall objectives of this investigation were to:

1) assess mycorrhizal and root endophytic fungal diversity between and within three co-occurring *Disa* species; and

2) determine biological activity of isolated fungi.

This was achieved through the:

collection of root material and soil from the selected *Disa* species
assessment of diversity using both a culture dependent and independent approach (cloning) and
determination of biological properties of fungal isolates

Chapter 2: Root Endophytic Fungal Diversity Of Three *Disa* Species

2.1 Introduction

Fungi play an important role in most ecosystems and have significant functions in the environment (Salazar-Cerezo et al 2018). Microbiota associated with plants contribute to maintaining their biological diversity in terrestrial ecosystems through different biological processes in response to biotic and abiotic stress; stimulating plant defense mechanisms against pathogens and production of secondary metabolites with antimicrobial activity (Tsavkelova et al 2008; Hajiboland et al 2010; Contreras-Cornejo et al 2011; Khan Pathan et al 2012; Khan et al 2015). Microorganisms thus contribute to the conditions required for plant health (Andrews and Harris 2000; Santamaría and Bayman 2005; Rodriguez et al 2009) and maintaining microbial biodiversity (Huang et al 2008; Kharwar et al 2010).

It is well known that orchids have a mutualistic partnership with mycorrhizal fungi throughout their life cycle due to poorly developed root systems. Orchids serve as host plants to mycorrhizal fungi which provide nutrients from the soil in exchange for organic carbon assimilated by photosynthesis (Smith and Read, 2008). Most orchids are entirely dependent on mycorrhizal fungi (Brundrett et al., 2003; Ferreira et al., 2015) and orchid mycorrhizas are considered unique when compared with other mycorrhizal relationships (Yuan et al., 2009). Orchid mycorrhizal (OM) fungi are recognized by being able to colonize orchid tissues forming characteristic features such as pelotons. These are intracellular hyphal coils within root cells (Rasmussen and Rasmussen 2014; Rasmussen et al 2015). Orchid species are dependent on OM fungi for germination and establishment of seedlings because they generally produce seeds with limited nutrient and energy reserves which are provided by the OM fungi (Valaderes et al., 2014). The OM relationship is maintained in adult orchid plants and may receive carbon either via photosynthesis or through mycorrhizal transfer depending on their mode of nutrition (Rasmussen and Rasmussen, 2009). The survival, composition, and distribution of orchids are highly influenced by the diversity of compatible fungi (Bonnardeaux et al., 2007; Rasmussen, 1995; Currah et al., 1997; Batty et al., 2002).

The Orchidaceae are associated with basidiomycetous fungi, with the majority belonging to the *Rhizoctonia complex*, which contains three now taxonomically disparate Agaricomycetes (=Hymenomycetes) taxa: Sebacinales, Ceratobasidiaceae, and Tulasnellaceae (Dearnaley et al. 2012) within the Heterobasidiomycetes (Rasmussen 2002). None of them have the primitive traits that fit the description of the asexual genus *Rhizoctonia* by De Candolle (1815). *Rhizoctonia* was one of the first fungus to be partnered with orchids and is very difficult to identify due to the fungus rarely exhibiting any reproductive stage (Bougoure et al. 2005).

The fungi involved in establishing OM relationships are generally thought to have originated from the *Rhizoctonia* genus. This is a diverse polyphyletic fungal group comprising of pathogens, endophytes, saprophytes and mycorrhizal fungi (Warcup 1981; Sivasithamparam 1993; Rasmussen 1995; Currah et al., 1997). Molecular methods have become the standard means of evaluating orchid fungi within the *Rhizoctonia* complex (Taylor et al., 2003; McCormick et al., 2004; Weiss et al. 2004; Shetterson et al., 2005). These fungi do not form asexual spores (Nogueira et al., 2013) and have distinctive morphological characteristics such as the right-angled hyphal branching and a constriction at the branch origin (Otero et al 2002; Sneh et al 1991). In the *Rhizoctonia*-complex, orchids utilize a great diversity of fungi with different nutritional strategies (Rasmussen 2002).

Many fungi associating with orchid seedlings and roots of adult plants can be cultured *in vitro* on media containing complex carbohydrates, this is useful as it enables researchers to demonstrate physiological interactions (Látalová and Baláž 2010; Guimarães et al 2013). Studies conducted on OM fungal interactions have focused on *in vitro* symbiotic techniques using fungal isolates obtained from adult roots (Chutima et al., 2011). Thus, indicating the need to isolate and identify compatible fungi. Sequencing of fungal DNA from orchid tissues has also been used to identify orchid root fungal endophytes (Rasmussen et al 2015).

Orchid mycorrhizal associations are possibly known as the easiest symbiotic systems to be manipulated under laboratory conditions as both partners can generally be cultured (Dearnaley et al. 2014). Even though OM fungi are easy to manipulate it is still problematic to accurately identify the fungal mycobiont; as

isolates obtained are mainly of saprotrophic contaminants or endophytes (Wilson 1995). With advanced technology new molecular approaches have been developed enabling the identification of mycorrhizal fungi associated with orchids. Fungal partners of orchids are now identified not only through culture-dependent methods but also directly from protocorms, tubers, rhizomes and roots using a culture-independent strategy (Bougoure et al. 2005; Martos et al. 2009; Swart et al. 2010).

Methods such as *in vitro* propagation and seed germination are now mostly used to determine fungal partners associated with orchids, hence the need for targeted isolations. One of the commonly used approaches is the Polymerase Chain Reaction (PCR) amplification of colonised orchid tissues using fungus-specific primers (Dearnaley and Le Brocque 2006; Dearnaley and Bougoure 2010). Over the past decade, the method of choice for identifying orchid mycorrhizal taxon has been sequencing the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (White et al. 1990; Gardes and Bruns 1993). The ITS1F and ITS4 are the universal primers that are broadly used to identify fungi from cultures or from plant tissues. ITS1-F is the fungal-selective primer that efficiently amplifies ascomycetous and basidiomycetous fungi (Gardes and Bruns 1993). New primer sequences have been developed to specifically target all the Basidiomycota (Taylor and McCormick 2008). These primers have been helpful in not selecting non-mycorrhizal and non-Basidiomycota fungi associated with orchid roots as the majority of orchid mycobionts are known to be Basidiomycetes (Rasmussen 2002).

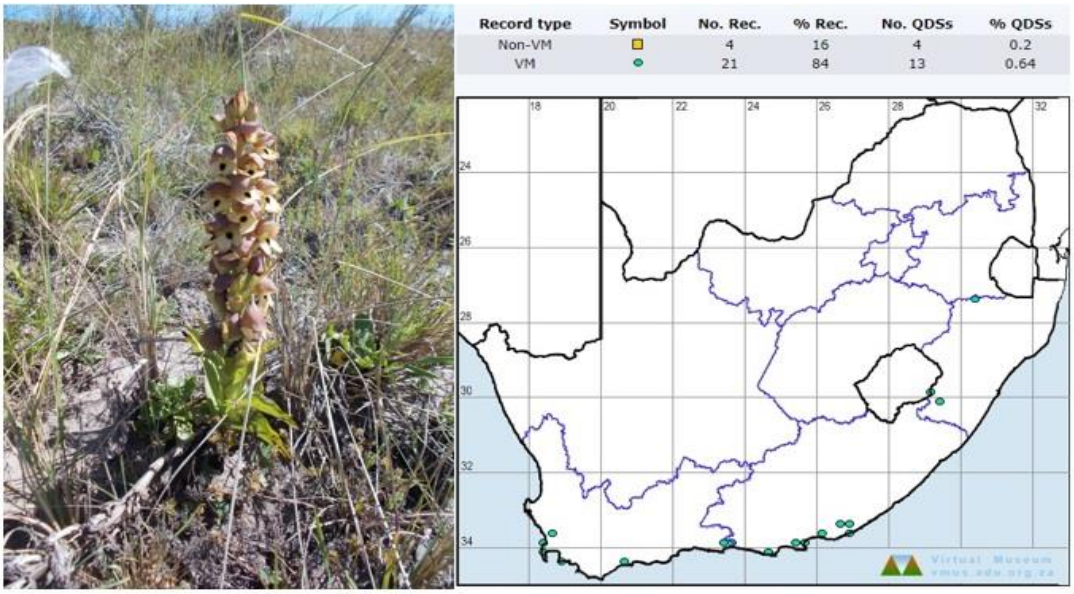
Orchid mycorrhizal associations have not been well studied in South Africa and the objective of this study was to isolate and identify fungi associating with the roots of three *Disa* species namely *Disa cornuta* (L.) Sw., *Disa bracteata* Sw. and *Disa polygonoides* Lindl.

2.2 Materials and Methods

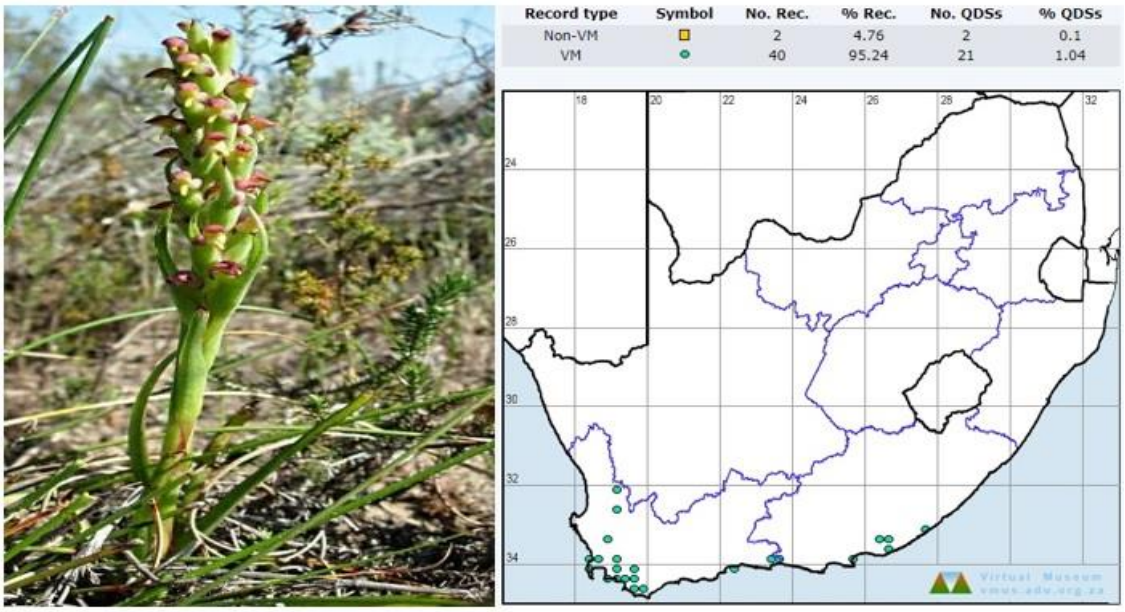
2.2.1 Collection of plant roots and soil samples

Orchid species were collected from around Grahamstown, Eastern Cape, South Africa during the flowering season of 2017. *Disa polygonoides* and *Disa bracteata* were collected in May (-33º32’19.8” S -26º53’36.8” E) and *Disa cornuta* (-33º32’59.2” S, -26º50'71.5" E) in November (Fig. 2.1). Species were identified with the assistance of Prof C. Peter, Botany Department, Rhodes University. Five whole plants were carefully excavated with soil to ensure that roots remained intact. Plants were returned to the laboratory where roots were removed and washed with water and brushed to remove any adhering soil particles. Roots were placed on filter paper to remove excess moisture and divided for the following analysis: A portion of roots was placed in 50% ethanol for staining, a second portion was reserved for fungal isolations and lastly, roots for molecular analysis were placed in RNA later (Sigma, R0901-100ML-PW) and subsequently frozen at -20^{0}C.

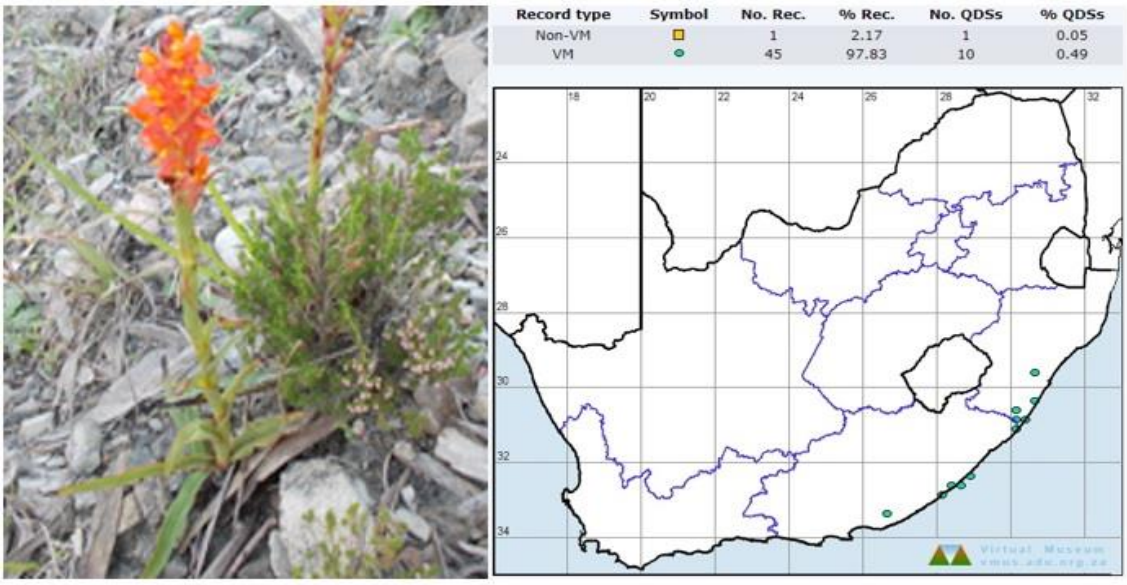
Soil samples were collected from around the orchid roots and sent to Eco Analytica Laboratories, Potchefstroom, South Africa for nutrient analysis. Nutrients analyzed include phosphate (P), nitrogen (N), calcium (Ca), magnesium (Mg), sodium (Na), pH, and cation exchange capacity (CEC).



Disa cornuta (L.) Sw.



Disa bracteata Sw.



Disa polygonoides Lindl.

Figure 2.1 Distribution of *Disa cornuta*, *D. bracteata*, and *D. polygonoides* orchids in South Africa (Distribution maps downloaded from Orchid Map: http://orchidmap.adu.org.za/).

2.2.2 Colonization of roots

Root pieces 2-4 cm in length were removed from the ethanol storage solution and rinsed with water. Roots were covered with 5% KOH and placed in a water bath at 90^0C for 30 min. The KOH treatment disrupted cell membranes and assisted with the removal of cellular content. After the KOH treatment, the solution was discarded, and roots were rinsed with water. Roots were then bleached to remove pigments with freshly prepared alkaline H_2O_2 solution for 30 min. After roots were rinsed with water and acidified using a 0.1 M HCl solution overnight they were stained with lactoglycerol (lactic acid: glycerol: water, 13:12:16) solution that contained 0.05 % Trypan Blue (Sigma, Merck 1.11732.0025) for 30 min in a 90^0C water bath. The stain was discarded, and roots were covered with lactoglycerol (without stain) and allowed to stand for at least 24 hours (Koske and Gemma, 1989; Smith and Dickson 1998).

Stained root segments were placed onto microscope slides using lactoglycerol as the mountant. On placing the coverslip roots were gently squashed using a flat eraser. Roots were microscopically examined for typical OM fungal structures i.e. pelotons. Images were captured using a Light Microscope Olympus BX series at the Rhodes University Microscopy Unit.

2.2.3 Isolation and culture of root fungi

Root pieces reserved for isolations were surface sterilized by immersing in 3 % (w/w) H_2O_2 solution for 10 min, this was followed by two rinses in sterile distilled water. Roots were sectioned into smaller segments and transferred onto Potato dextrose agar (PDA) and Malt extract agar (MEA) medium supplemented with filter sterilized chloramphenicol (50 mg/l) to reduce bacterial growth (Yau et al., 2008). Additional roots were macerated in sterile water, using a micropestle, in order to release pelotons. Aliquots of 100 µl were spread plated onto PDA and MEA plates and were incubated at 25^0C and regularly observed for fungal growth and contamination. The fungicide, Benomyl (5 g into 50 ml of sterile water) was also added to some PDA medium to reduce the growth of fast-growing fungal species. Fungal colonies from actively growing isolates were sub-cultured and maintained on fresh PDA and

incubated at 25^{o}C. Microscopic observations were made using a tape mount technique (Koske and Gemma 1989; Smith and Dickson 1997), stained with lactoglycerol Trypan Blue and microscopically examined using light microscope. Isolates were examined for right-angled hyphal branching pattern indicative of *Rhizoctonia* and the presence of sporulation indicative of other root endophytes.

2.2.4 Molecular Characterization of orchid roots

2.2.4.1 DNA extraction

DNA was extracted using the ZR Fungal/Bacterial DNA Miniprep kit (Zymogen Catalog No. D6005). Two hundred microliters of sample were placed into the ZR Bashing Bead™ Lysis Tubes. An aliquot of 750 µl of Lysis solution was added to the tube to break the cell wall and cellular membrane. The tubes were then secured on bead beater (Labnet) and processed at maximum speed for 5 minutes to disrupt cells. The ZR Bashing Bead™ Lysis tubes were then centrifuged (MiniSpin variable speed microcentrifuge) at 10 000 ×g for 1 min. Up to 400 µl supernatant was transferred to a Zymo-Spin™ IV Spin filter and collection tube and centrifuged again at 7 000 ×g for 1 min. To the filtrate obtained in the collection tube 1,200 µl of fungal DNA binding buffer was added to bind to the DNA. An aliquot of 800 µl of the mixture was transferred to a Zymo-Spin™ IIC column and centrifuged at 10 000 ×g for 1 min. The filtrate was then discarded, and this step was repeated for the remaining 800 µl.

An aliquot of 200 µl DNA Pre-Wash buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10 000 ×g for 1 min to remove an excess of proteins and pigments. An aliquot (500 µl) of fungal and bacterial DNA Wash buffer was then added to the Zymo-Spin™ IIC column and centrifuged at 10 000 ×g for 1 min to remove impurities. The Zymo-Spin™ IIC column was transferred to a clean new tube and centrifuge at 10 000 ×g for 1 min; this step was essential to produce a clean DNA sample as the presence of ethanol inhibits downstream processing. The IIC column was then transferred into a microcentrifuge tube and heated at 50^{o}C open for 2 min as to encourage additional ethanol evaporation. Twenty-five µl of sterile water was then added to the column matrix and incubated at

room temperature for 2 min followed by centrifugation 10, 000 ×g for 1 min. This step was repeated to obtain a total volume of 50 µl of DNA which was stored at -20ºC.

DNA extraction directly from roots

The Quick-DNA™ Plant/Seed Miniprep kit (D6020) was used to extract DNA from the roots of *D. cornuta, D polygonoides,* and *D. bracteata*. DNA extraction was performed as above with a few modifications. The roots were removed from RNA later and macerated using liquid nitrogen and sterile waster. The Zymo-Spin™ III-HRC filter was placed in a clean collection tube and 600 µl Prep solution was added, this was followed by centrifugation at 13 400 ×g for 3 min. The eluted DNA was then transferred to the prepared Zymo-Spin™ III-HRC Spin filter and centrifugation was repeated. This process removes polyphenolics making it ideal for downstream molecules-based applications.

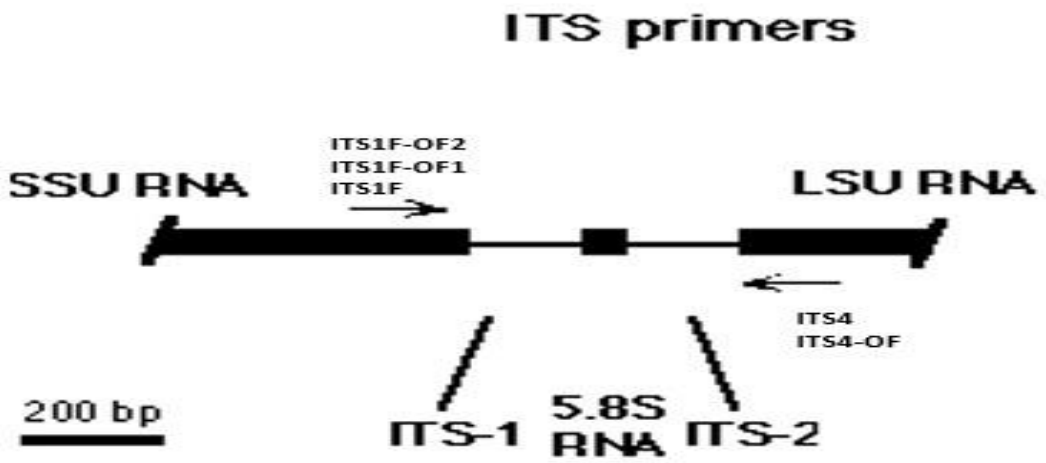


Figure 2.2 Internal transcribed (ITS) region primers. Target region amplified by the universal primers ITS1F and ITS4 and by orchid specific primers ITS1F-OF and ITS4-OF during PCR (Adapted from http://home.psu.ac.th/~4823002/Molecular _ITS.htm).

2.2.4.2 Polymerase Chain Reaction amplification

The ITS region of fungal isolates was amplified and sequenced with the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (Fig 2.2) (White et al., 1990).

Table 2.1 PCR mixture components (25 ul) used for amplification of fungal cultures.

Materials	Volume of Sample(µl)	Volume of negative control (µl)
DNA template	5.00	_
Ready-mix Kapa SYBR FAST	12.5	12.5
10 µM ITS1-F	1.00	1.00
10 µM ITS4	1.00	1.00
Sterile distilled water	5.5	10.5

PCR cycling included an initial denaturation step at 94ºC for 4 minutes. This was followed by 25 cycles of the following; 94ºC for 30 seconds, primer annealing at 47ºC for 45 seconds, extension of 3' end of primers was conducted at 72ºC, to allow for nucleotide insertion complementary to the template DNA for 60 seconds and a final extension step at 72ºC for 7 minutes.

PCR for cloning

The primers used were for cloning of DNA extracted from orchid roots included ITS1F (Gardes and Bruns 1993) and ITS4 (White et al 1990). Additionally, the orchid specific primers were also used: ITS1F-OF1; 1 ul, ITS1F-OF2, and ITS4-OF (Fig 2.2). (Taylor and McCormick 2008) (Table 2.3).

Table 2.2 PCR mixture components (25 ul) used for amplification of DNA extracted from orchid roots.

Materials	Volume of Sample (µl)	Volume of negative control (µl)
DNA template	5.00	_
0.5mM dNTPs	0.4	0.4
10× Ammonium Buffer	2.5	2.5
AccuPOL DNA polymerase (AMPLIQON, A211199)	0.2	0.2
10 µM Forward Primer	1.00	1.00
10 µM Reverse Primer	1.00	1.00
Sterile water	14.9	19.9

PCR cycles for orchid specific primers

An initial 2 min denaturation at 95^{o}C was followed by 23 cycles of the following; 30 sec denaturation at 96^{o}C, 40 sec annealing at 47^{o}C, extension and elongation at 72^{o}C for 1 min. The AccuPOL DNA polymerase is slower than *Taq* DNA polymerase, allowing 1 min for the amplification of 1 kb. A final elongation at 72^{o}C for 10 min ensured that any remaining single-stranded DNA was fully extended.

Table 2.3 Primers used in the fungal and root DNA amplification and sequencing of the ITS region.

Primer name	Sequence (5'->3')
ITS1-F	CTTGGTCATTTAGAGGAAGTAA
ITS4	TCCTCCGCTTATTGATATGC
ITS1-OF	AACTCGGCCATTTAGAGGAAGT
	AACTTGGTCATTTAGAGGAAGT
ITS4-OF	GTTACTAGGGGAATCCTTGTT

2.2.4.3 Purification of PCR product

The PCR products were purified using Wizard ® SV Gel and PCR Clean-Up system kit (Promega, A9281).

The extraction of gel was done following electrophoresis, DNA bands were cut, and the gel slice placed in a microcentrifuge tube. A volume of 10 µl Membrane Binding Solution was added per 100 mg of gel slice. The mixture was vortexed and incubated at 60^0C until the gel was completely dissolved. For PCR product, an equal volume of Membrane Binding Solution was added to the PCR sample for amplification. For the binding of DNA, SV mini-columns were inserted into collection tubes and the prepared PCR product/band was transferred to the mini-column assembly. This was incubated at room temperature for 1 min and centrifuged at 13, 400 ×g, the flow-through was discarded and the mini-column was inserted back into the collection tube. Washing was then performed by adding 700 µl membrane wash solution (ethanol added) followed by centrifugation at 13, 400x g for 1 min. The flow-through was once again discarded and the wash repeated with 500 µl membrane wash solution and further centrifugation for 5 min. The column assembly was centrifuged again for 1 min with the lip left open to allow for evaporation of any residual ethanol. The elution step was done by carefully transferring the mini-column to a clean 1,5 microcentrifuge tube (allowed to sit for an additional 2 min open to allow for extra ethanol evaporation). An aliquot of 50 µl Nuclease-free water was then added to the mini-column membrane directly, incubated at room temperature for 1 minute and centrifuged at 13, 400x g for 1 minute. The cleaned product was stored at 4^0C or -20^0C until required.

2.2.4.4 Cloning of orchid root PCR product

Competent cell protocol

The bacterial strain *Escherichia coli* DH5 alpha was used to make competent cells. A single colony was inoculated into 5 ml of Luria broth (LB) and incubated overnight to increase the growth rate of bacteria. One milliliter of bacterial culture was added into 100 ml LB and shaken for 1.5 to 3 hrs. The culture was carefully monitored for active

growth by continually measuring optical density at 600 nm until a density of 0.6 was reached. The bacterial cells were then stored on ice for 10 min and recovered by centrifugation at 6 000 rpm for 3 min. The pellet was re-suspended in 10 ml cold 0.1M $CaCl_2$ on ice for 30 min to produce holes in the membrane and make the cells competent. The cells were then re-centrifuged at 6 000 rpm for 3 min and re-suspended in 5 ml cold 0.1M $CaCl_2$ 15% glycerol to protect the cells from forming ice crystals. The suspension was dispensed into cold microcentrifuge tubes to improve cell viability and maintain transformation efficiency. These were stored immediately at -80^{o}C.

All purified PCR product obtained from the root extraction were cloned using the pGEM®-T Easy Vector Systems (Promega, A1360) as per manufacturer's instruction. The ligation reaction (10 µl) was prepared as per table 2.4. The reaction was incubated overnight at 4^{o}C to increase the number of ligations.

Table 2.4 Ligation reaction (10 µl) used for cloning into pGEM-T Vector.

Reagents	**Standard Reaction**	**Positive Control**
2X Rapid Ligation Buffer	5 µl	5 µl
pGEM®-T Easy Vector (50ng)	1 µl	1 µl
PCR product	2 µl	_
Control Insert DNA	_	2 µl
Deionized water	1 µl	1 µl

During transformation, the DNA is introduced into the competent bacterial strain, so that the bacteria may then replicate the sequence of interest in amounts suitable for analysis. To begin the transformation process, 60 µl of competent cells was added to the 10 µl ligation solution in in microcentrifuge tubes and placed on ice for 30 min to allow the bacterial membrane to stabilize and to increase the interaction between the calcium cation and the negatively charged components (Selosse et al 2004). Heat shock was then performed by exposing the bacterial suspension to 42^{o}C for 45 secs to change the fluidity of the membrane and allow the plasmids to enter the bacteria

at an efficient rate. The cells were then returned to the ice for 2 min to allow the cell membrane to re-stabilize. To allow for cell replication 500 µl LB was added and incubated while shaking at 37^{o}C for 45 min. The new cell growth was then re-suspended via centrifugation at 6 000 rpm for 45 secs and 100 µl of resuspended cells was spread plated onto X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Luria Agar) LA plates. The plates had been prepared by spread plating X-gal (20 mg/ml) and IPTG (Isopropyl β-D-1-thiogalactopyranoside) (20 mg/ml) and ampicillin (100 mg/ml) onto the LA plates and then allowing these to dry. After spread plating the cell suspension plates were allowed to dry and incubated for 16 hr at 37^{o}C. Five white colonies with plasmid containing an insert and two blue colonies without insert were picked using sterile technique and grown in 5 ml LB with ampicillin at 37^{o}C for 16 hrs.

The vector plasmids were then extracted using the Thermo™ Scientific GeneJET™ Plasmid Miniprep kit (K0502) following the manufacturer's instructions. All centrifugations were conducted at 12 000 ×g. An aliquot of 3 ml of each colony broth culture was placed into microcentrifuge tubes and centrifuged to obtain a cell pellet. The pelleted cells were re-suspended in 250 µl of Resuspension solution and transferred into a microcentrifuge tube. The bacteria were re-suspended completely by pipetting up and down until no clumps remained. A volume of 250 µl of the Lysis solution was added and mixed thoroughly by inverting the tube slowly 6 times until the solution became viscous and slightly clear. A volume of 350 µl of the Neutralization solution was added and mixed by inverting the tube 6 times and centrifugation for 5 min. The supernatant was then transferred to a GeneJET™ spin column by pipetting and centrifugation for 1 min, after which the flow-through was discarded. The column was washed by adding 500 µl of wash solution to GeneJET™ spin column, to remove impurities. The buffer was then removed by centrifugation for 45 secs and the flow-through discarded. This wash step was repeated. The GeneJET™ spin column was then transferred into a sterile 1.5 ml microcentrifuge tube, and 50 µl of the elution buffer was added (to wash away unbounded proteins and release the desired protein) at the center of the column membrane to elute the plasmid DNA. Before centrifugation the spin column was incubated for 2 min at room temperature and then centrifuged for 2 min, to elute the plasmid which was stored at -20^{o}C.

Thermo™ Scientific FastDigest Restriction Enzymes was used to check for inserts in the plasmid (Fig 2.3). In a 1.5 ml microcentrifuge tube the following was combined: 1 µl DNA, 15 µl nuclease-free water, 2 µl of 10× FastDigest Green Buffer (B72), 1 µl FastDigest enzyme (Thermo Fisher, FD1014) making the complete reaction of 20 µl. The solution was then incubated at 37ºC in a heat block for 15 min. To confirm an insert the digested plasmids were then visualized using electrophoresis to confirm an insert of the desired size.

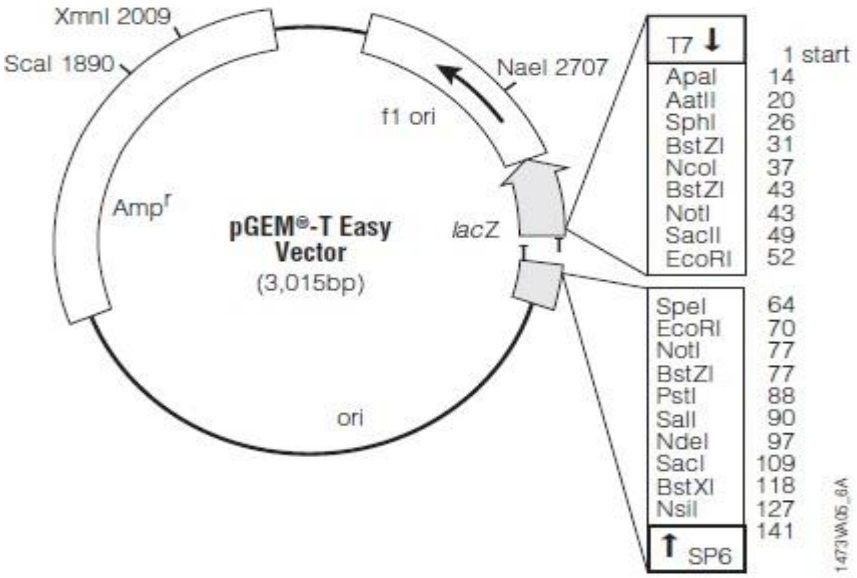


Figure 2.3 pGEM®-T Easy Vector Map and Sequence Reference points for inserts cloned into the pGEM®-T Easy Vector (Adapted from pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual). Digest with EcoRI to release inserts cloned into the pGEM®-T Easy Vector.

2.2.4.5 Electrophoresis

Visualization of DNA in electrophoretic gels typically requires UV radiation and the fluorescent dye ethidium bromide (Adkins and Burmeister 1996). An electrophoretic gel was prepared with 1% of agarose (Promega, V4121) stained with ethidium bromide (Merck 1.11608.0030). Tris-buffer was used to prepare the 1x buffer solution used in the preparation of agarose gel and 100 base pair (bp) ladder (Promega, G2101) was used for comparison of size. Blue/Orange 6x loading dye (Promega, G1881) was used to load 5 µl aliquot of DNA or PCR products into gel

wells. The gel electrophoresis apparatus containing 1x buffer allowed the flow of an electric current with 80 voltage (V). Gel were visualized and photographed using a UV Transilluminator (Bio-Rad ChemiDoc XRS systems, Universal Hood II).

2.2.4.6 Molecular Analysis

The purified PCR products from both fungal cultures and cloned plasmid DNA were sent to Inqaba Biotechnologies, Pretoria, South Africa for Sanger Sequencing. Nucleotide sequence chromatograms were analyzed and edited using Fitch TV software. For fungal DNA sequencing in only one direction was performed using one of the ITS primers. For plasmid DNA the primers M13F (50 - CGCCAGGGTTTTCCCAGTCACGAC-30) and M13R (50 - TCACACAGGAAACAGCTATGAC-30) (Yun et al., 2000) were used to produce both forward and reverse sequences which were aligned using MEGA Software version 7.0.2.6. Sequence results were compared to sequences in the National Centre for Biotechnology Information (NCBI) database GenBank (http://www.ncbi.nlm.nih.gov/) and UNITE (https://unite.ut.ee/analysis.php) database. Sequence comparisons were carried out using BLAST analysis which aligns two or more homologs to detect for the presence of one or more ambiguous region within the segments under comparison.

2.3 Results

2.3.1 Soil nutrient analysis

Chemical properties of soils sampled are shown in Table 2.5 and 2.6 respectively. Analysis of soil samples from each site sampled showed variation from each other. Soil from site 1 (*D. polygonoides* and *D. bracteata)* had a relatively low magnesium, potassium, and calcium concentration as compared to the sample site 2 (*D. cornuta*). Phosphorus was higher in sample site 1 compared to sample site 2. The electrical conductivity (EC) values of the soils were found to be 38 mS/m in site 1 and 16 mS/m in site 2. The pH as measured in water varied from 4.57 to 5.95 and the pH for salts (KCL) varied from 3.72 to 4.74. Both C and N were present at low levels with an increase of 6 and 4 times, respectively at site 2.

Table 2.5 Nutrient analysis for soil collected from the verge of a national road (*Disa polygonoides*, *D. bracteata* – Site 1) and Mountain Drive (*D. cornuta* - Site 2) Grahamstown.

Soil Sample	**Ca**	**Mg**	**K**	**Na**	**P**	**pH (H_2O)**	**pH (KCl)**	**EC**	**LECO**	**LECO**
			(mg/kg)					**(mS/m)**	**%N**	**%C**
Site 1:										
D. polygonoides* and *D. bracteata	104.0	48.5	31.5	33.0	7.2	4.57	3.72	38	0.02	0.51
Site 2:										
D. cornuta	787.0	180.5	101.0	23.0	6.0	5.95	4.74	16	0.12	2.29

Table 2.6 Soil exchangeable cations for soil collected from the verge of a National road (*Disa polygonoides*, *D*. *bracteata* – Site 1) and Mountain Drive (*D. cornuta* - Site 2) Grahamstown**.**

Sample	Ca	Mg	K	Na	CEC	S-value	Base satu-	pH (H_2O)	pH (KCl)
			(cmol(+)/kg)				ration (%)		
Site 1:									
D. polygonoides* and *D. bracteata	0.52	0.40	0.08	0.14	16.11	1.14	7.09	4.57	3.72
Site 2:									
D. cornuta	3.93	1.49	0.26	0.10	23.07	5.77	25.02	5.95	4.74

2.3.2 Assessment of mycorrhizal colonization

All roots examined for each of the *Disa* species displayed characteristic hyphal coiling referred to as pelotons within cortical root cells. The coils of mycorrhizal fungi were shown to be crossing each other and were composed of what appeared to be a single hyphal type (Fig. 2.4).

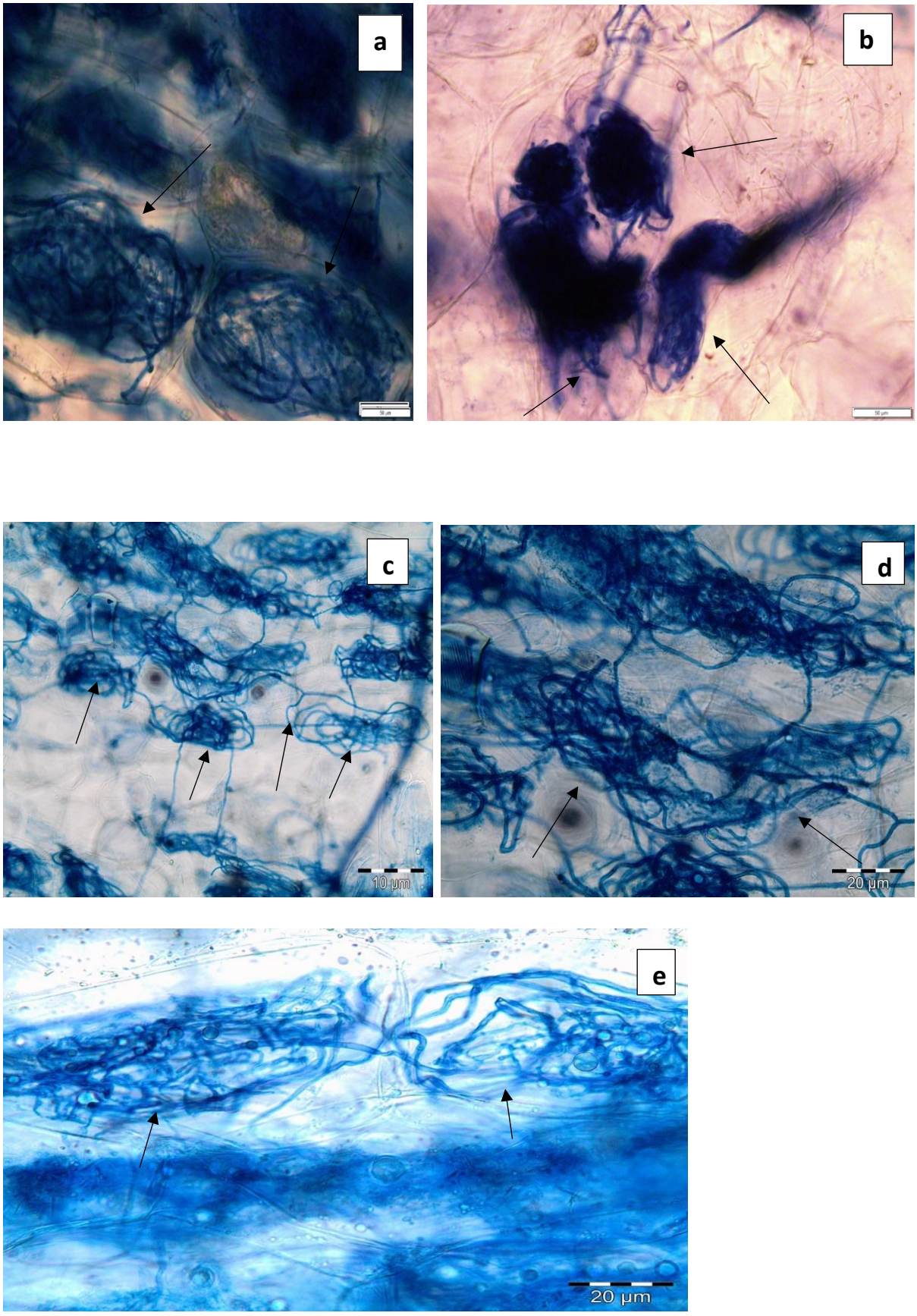


Figure 2.4 Orchid mycorrhizal colonization of Disa polygonoides (a, b); D. bracteata (c, d) and D. cornuta (e) roots fungal pelotons indicated by arrows

2.3.3 Fungal Isolation

Fungi were successfully isolated from *D. polygonoides*, *D. bracteata*, and *D. cornuta* and grouped according to similar colony morphological traits resulting in 24 fungal isolates. Successful isolation of fungi was achieved by selection of media (PDA and MEA) with chloramphenicol and benomyl. PDA with chloramphenicol supported good fungal growth of all isolates and thus was used for maintaining subcultures. The fungal isolates showed distinct morphologies (Fig 2.5) such as **1).** Yellow-orange mycelium with brownish underside (flower like appearance); **2).** White-greyish powdery mycelia; **3).** White mycelium with yellow-green concentric circles; **4).** Hard mycelial surface accompanied by a change in media color to yellow; **5).** Hard, white-brownish mycelial flat surface. Fungal isolates which were slower growing were selected for further identification.

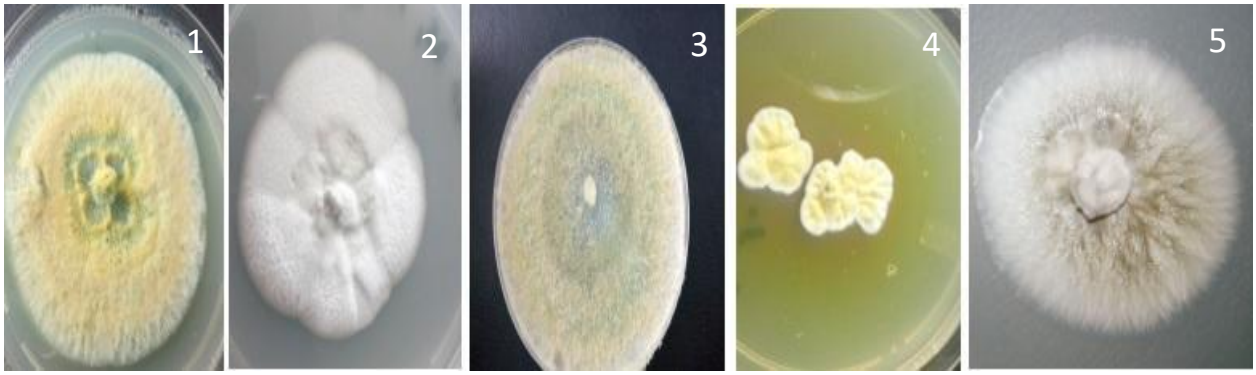


Figure 2.5 A selection of fungal morphotypes growing on PDA. **1**. D.P 8.1.3; **2**. D.B 1.2; **3**. D.P 4.4; **4**. D.P 7.2.1; **5**. D.P 8.3.

2.3.4 Molecular identification of the fungal isolates

Fungal isolates obtained from *D. polygonoides*, *D. bracteata*, and *D. cornuta* were ITS amplified resulting in PCR product of approximately 600 bp (Fig 2.6).

The fungal DNA sequences generated with ITS1F and ITS4 were compared against a sequence database. BLAST searches with ≥ 80% query coverage and ≥ 96%-

100% sequence similarity were considered best for assigning a species name. The best matching sequence for the isolates obtained through the GenBank database was between 96% and 99% identical to a species name (Table 2.7). All the sequences of the isolates were deposited in the GenBank and assigned accession numbers.

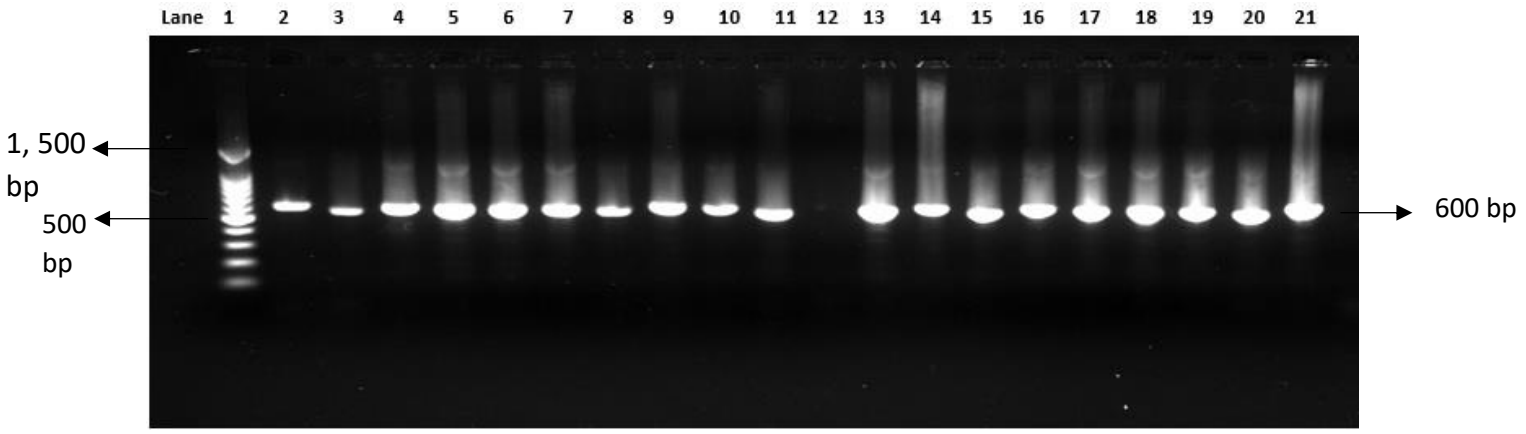


Figure 2.6 PCR products amplified using ITS1F and ITS4 primers. Lane 1 is the 100 bp ladder; Lane 2-21; are ITS region of the fungal isolates that are approximately 600 pb in size. Confirmed by visualization on a 1% agarose gel stained with ethidium bromide.

Table 2.7 Molecular identification of fungal isolates from *Disa polygonoides*, *D. bracteata* and *D. cornuta*. BLAST results of ITS sequences were obtained through either the GenBank or UNITE databases.

Orchid species	Fungal Isolates	Accession Number	Description/Accession number of closest match	Percentage (%) coverage	E-value	Percentage (%) Identity
Disa bracteata	D.B 1.3	MK239058	*Penicillium sp.*/ KY073422. 1	98	0.0	99
	D.B 1.2	MK239054	*Penicillium sp.*/ KF973213.1	99	0.0	99
	D.B 1	MK239056	*Penicillium sp.*/ KU365879.1	97	0.0	99
Disa polygonoides	DB3		*Metapochonia*/KY977566.1	100	4e-123	100
	D.P 5.2	MK239053	*Pencillium spinulosum*/ DQ132828. 1	95	0.0	97
	D.P 8.1	MK239057	*Pencillium sp.*/ HM461909.1	99	0.0	99
	D.P 8.3	MK239062	*Oidiodendron sp.*/ HM208747.1	98	0.0	99
	D.P 5.1.1	MK239051	*Talaromyces proteolyticus*/ NR_103685.2	99	0.0	98
	D.P 5.2.6		*Oidiodendron sp.*/ HM208747.1	99	0.0	99
	D.P 8.1.1	MK239059	*Penicillium sp.*/ HM469417.1	99	0.0	99
	D.P 4.3	MK239049	*Trichoderma sp.*/ HM771017.1	93	0.0	100
	D.P 5.2.1	MK239050	*Trichoderma koninquiopsis*/ JQ278015.1	98	0.0	99
	D.P 5.2.3	MK239055	*Pencillium sp.*/ KF973212.1	99	0.0	99
	D.P 5.2.5	MK239061	*Oidiodendron sp.*/ JQ272359.1	98	0.0	99
	D.P 6.2.2	MK239047	*Trichoderma harzianum*/ KJ028794.1	99	0.0	99

	D.P 7.2.1	MK239048	*Talaromyces radicus*/ AB457007.1	98	0.0	96
	D.P 8.2.2	MK239060	*Penicillium chalabudae*/ NR 144845.1	99	0.0	99
	D.P 9.1		*Neopestalotiopsis sp.*/ MF136539.1	98	0.0	99
	D.P 4.5	MK239052	*Trichoderma hamatum strain*/ KC576720.1	99	0.0	99
	D.P 5		*Oidiodendron sp.*/ HM208722.1	99	0.0	99
	D.P 8.1.3		*Chaetomium aureum strain*/ GU966501.1	98	0.0	99
Disa cornuta	1DC2.1	MK239063	*Trichoderma sp.*/ MH745146.1	91	0.0	99

All fungal isolates belonged to the Ascomycota, with percentage identities allowing for species matches where available.

2.3.5 Molecular identification of plasmid DNA

A total of 13 fungal clones were isolated after restriction digest (Fig 2.7) and sent for sequencing. On comparison to the databases clones were divided into Basidiomycota with 2 different amplicons and Ascomycota with 11 difference amplicons belonging to different genera (Table 2.8). All cloned sequences were deposited in GenBank and assigned accession numbers. The 300 bp means that most of these fungi have an EcoRI restriction (Fig 2.3) site with the PCR product, therefore the bands are possibly double, as it is visible in lane 4. Fungi from lane 1, 9 and 15 looks more between 500-600 bp indicating no EcoRI restriction site.

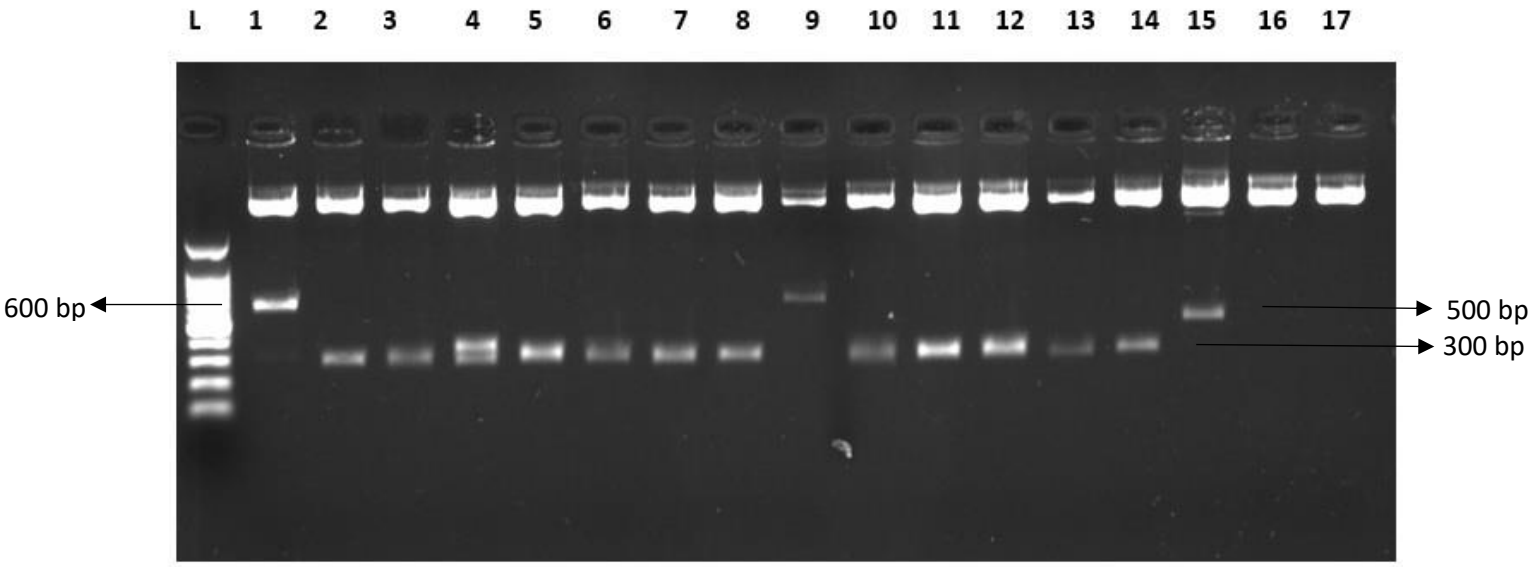


Figure 2.7 Amplification products obtained from plasmid DNA isolated from *D. polygonoides*, *D. cornuta*, and *D. bracteata* cloned DNA. Lane L; is the 100 bp ladder; Lane 1-15; plasmid DNA; Lane 16-17; negative control from Insert control DNA. Visualized on 1% agarose gel stained with ethidium bromide.

Table 2.8 Clones obtained from roots of *Disa cornuta*; *D. polygonoides and D. bracteata*, and sequence comparisons with the GenBank database using ITS1F and ITS4 primers.

Orchid root	Clone code	Description/ Accession number of closest match	Query coverage (%)	Identity (%)	E-value	Accession Number
Disa cornuta	DC1	*Epicoccum nigrum*/ MH290364.1	93	99	0.0	MK239038
	DC2	*Tulasnella sp.*/JX514389.1	78	96	0.0	MK239039
	DC3	Fungal sp. Strain/KU839098.1	91	99	0.0	MK239040

	DC4	*Helotiales sp.*/KX440158.1	88	100	0.0	MK239041
	DC5	Uncultured fungus/KT957785.1	89	98	0.0	MK239042
Disa polygonoides	DP1	*Terfezia boudieri*/LT718229.1	29	100	2e-79	Not submitted due to poor match
	DP2	Uncultured fungus/HQ850140.1	93	99	0.0	MK239043
	DP3	Uncultured *Ascomycota*/JX998699.1	92	95	0.0	MK239044
	DP4	*Tulasnella calospora*/GU166421.1	92	98	0.0	MK239045
	DP5	Uncultured *Helotiales*/JX317118.1	92	99	0.0	MK239046
Disa brecteata	DB1	*Sordariales sp.*/KY228640.1	89	99	3e-130	MK239034
	DB3	Uncultured ectomycorrhizal fungus/FR731633.1	71	80	8e-74	MK239035
	DB4	Uncultured *Agaricales*/FJ553698.1	91	99	0.0	MK239036
	DB5	Uncultured fungus/LC271287.1	92	99	0.0	MK239037

Disa cornuta and *D. polygonoides* associated with an orchid mycorrhizal fungi *Tulasnella* (Basidiomycota) and endophytes from Ascomycota, while *D. bracteata* associated with ectomycorrhizal fungus (Basidiomycota) and an uncultured fungus.

Table 2.9 Clones obtained from roots of *Disa cornuta* and *D. bracteata* using orchid specific primers; ITS1F-OF and ITS-OF and sequence comparisons obtained from the UNITE database.

Orchid species	**Clone**	**Accession number**	**Description/accession**	**Score (Bits)**	**E-value**	**Identity percentage (%)**
D. cornuta	DCC7	UDB00954	*Tomentella lateritia*	1050	0.0	100
D. bracteata	DBC6	HM451775	*Cantharellales*	860	2e-15	85
	DBC7	AB831844	*Atheliceae*	145	5e-33	91
	DBC7	AB568453	*Thelephoraceae*	136	3e-30	92

Disa cornuta and *D. bracteata* were cloned using orchid specific primers ITS1-OF and ITS4-OF and found to be associated with members of ectomycorrhizal families. Clones were not successfully obtained from *D. polygonoides* using these primers.

2.4 Discussion and Conclusion

The orchid plants sampled in this study were growing in soil with a reduced availability of macro and micro-nutrients. The general optimum pH for orchids is around 6.5 with a pH of between 5.5 and 6.5 considered adequate for most orchids as most nutrients are freely available without being toxic (Skinner 2001). *D. polygonoides and D. bracteata* were growing in more acidic soils having a pH of 4.57. *D. cornuta* was growing in soil which was less acidic (pH 5.95). There was a distinct difference between site 1 and site 2 with regards to soil nutrients. At site 1 *D. polygonoides* and *D. bracteata* were able to grow in soils with low organic C% and N%. The soil around Grahamstown is a weakly developed acidic lithosols with low mineral nutrients and with dark grey topsoil (Bizabani 2011). This was observed at both collection sites. *D. polygonoides* and *D. bracteata* were growing on a rocky outcrop with partially weathered rocks and sparse vegetation on the verge of a national road. Erica plants also grow alongside the road, particularly *Erica caffra* (Bizabani 2011). *D. cornuta* was growing on the mountain top with deeper, less disturbed soil covered with grass and shrubs, this vegetation is predominantly arbuscular mycorrhizal (Skinner 2001; Hawley and Dames 2004; Bizabani 2011).

Disa species inhabit a wide range of habitats and are particularly common in disturbed areas such as roadside verges and disturbed sites (Hoffman and Brown 1992; Grant and Koch 2003; Bonnardeaux et al 2007). This is certainly the case for both *D. polygonoides* and *D. bracteata, D. cornuta* was growing in less disturbed soils with increased soil nutrients indicative of enhanced nutrient cycling in this environment.

Microscopy is used to confirm the presence of pelotons and thus mycorrhizal associations (Kottke et al 2009). *D. polygonoides*, *D. cornuta* and *D. bracteata* were all colonized by characteristic orchid mycorrhizal structures (Fig 2.4) The presence of pelotons is indicative of mycorrhizal fungal interactions within the orchid roots and areas associated with the site of nutrient exchange between the plant and fungus (Dearnaley et al. 2012). The presence of both active and slightly degraded hyphae was observed. Pelotons are known to degrade or collapse because of plant digestion (Dearnaley et al 2012). The presence of pelotons, however, does not give an indication of the fungal species involved (Cameron et al 2006; 2008; Valadares

2014). Two important factors that are thought to influence root colonization by endophytic fungi are the growing season and the growth stage of the plant (Swarts and Dixon 2017).

The culture-dependent approach was successful in obtaining pure fungal isolates from surface sterilized root material. Several fast-growing isolates were discarded as they were unlikely to be mycorrhizal. It was apparent that not all isolates were mycorrhizal fungi, as these are notoriously slow growers. The presence of other fungal root endophytes was suspected despite the rigorous surface sterilization procedure used. Chloramphenicol was used as an antibiotic to prevent the growth of bacteria, which may also be plant endophytes (Bidartondo et al 2004). A fungicide was also incorporated into media and this did limit the growth of some faster growing saprotrophic fungi. Pelotons were difficult to excise from roots, but this approach has been used in several other studies (Dearnaley et al 2012). Maceration of root material provided an alternative approach allowing for growth that was more sporadic due to the presence of pelotons. These cultures however proved difficult to maintain in subculture due to their very slow growth. Plant roots form multiple interactions with different fungal partners belonging to different taxa (Bergero et al 2000). Root-associated fungi are distributed across all habitats and a range of hosts representing over 100 families including species from arbuscular, ericoid, orchid, ectomycorrhizal or non-mycorrhizal fungi (Bergero et al 2000). The success of isolation in many orchids varies with season and prior disturbance (Ramsay et al 1986).

PCR amplification using primers is commonly used for the identification of fungi associated with colonized tissues of orchids (Dearnaley and Le Brocque 2006; Dearnaley and Bougoure 2010). The sequences obtained were submitted and compared to the closest matching sequences on GenBank. BLAST searches revealed (Table 2.7) that these orchid roots were associated with endophytic fungi. The primers used to amplify the ITS region are the universal barcoding primers which are specific to fungi. ITS1F is one of the primers designed to amplify a broad range of fungi (Waud et al. 2014). However, Taylor and McCormick (2008) stated that these universal primers cannot amplify certain groups of fungi. The ITS region although accepted as the fungal barcoding region has been shown to be less

effective for the identification of environmental fungi at the genus and species levels (Bruns 2001; Seifer et al. 2007).

Studies have documented orchid mycorrhizal fungal associates, while non-mycorrhizal fungal endophytes have not been well recorded and recognized (Otero 2006). Root associated-fungal endophytes provide beneficial effects on host plants (Herre et al. 2007), such as protecting plants from being harmed by pathogens (Arnold et al. 2003). The presence of pelotons in roots does not necessarily mean that isolated fungi are mycorrhizal and responsible for the formation of these structures as multiple fungi can colonize the root (Kristiansen et al. 2001; Suárez et al. 2006). Over time the pelotons degrade in the cells and the contaminants enter the cells, non-mycorrhizal fungal endophytes are therefore commonly present in orchid roots (Bayman 2016).

Only six fungal genera were identified from the isolates obtained from the roots of *D. polygonoides*, *D. cornuta*, and *D. bracteata* namely *Trichoderma*, *Penicillium*, *Oidiodendron*, *Talaromyces*, *Chaetomium*, *Neopestalotiopsis*, and *Metapochonia.*

Trichoderma (D.P 6.2.2) was identified as an endophyte and the genus belongs to the Hypocreaceae (Ascomycota) (Jaklitsch and Voglmayr 2015). Most of the *Trichoderma* phenotypes produce powdery green conidia on fast-growing colonies (Samuel 2006) that are typically known to produce asexual spores (Bissett 1991; Chaverri et al 2001). *T. harzianium* (D.P 4.5) is known to be a biological control agent with several strains being used in commercial products (Samuel 2006). *Trichoderma* is present in soils and organic matter at all latitudes and is well-known soil fungus (Widden 1979; 1980; Roiger 1991; Hagn et al 2003; Wuczkowski et al 2003). These species are also referred to as opportunistic, avirulent plant symbionts that do not cause measurable damage to the host (Harman et al 2004). They induce resistance to fungal parasites in crop plants by the addition of small amounts of fungus applied as a seed treatment (Chet 1998; Harman et al 2004) and produce enzymes such as cellulase which degrades cellulose (Samuels 2005). The seed treatment with *Trichoderma* results in improved yields and enhance plant growth (Harman et al 2004), its presence in association with orchid roots could indicate a similar protective function in this harsh environment.

The orchid *D. polygonoides* was found in this study to be associated with an ericoid mycorrhizal fungus, *Oidiodendron sp*. This EM fungus has also been isolated from roots of other non-ericoid host plants (Bergero et al 2000; Kernaghan and Patriquin 2011). The fungus occurs in soils and organic matter in temperate ecosystems (Bergero et al 2000; Rice and Currah 2006; Tedersoo et al 2009; Grelet et al 2010; Kernaghan and Patriquin 2011; Vohník et al 2013) and colonize co-occurring ectomycorrhizal and neighbouring non-ectomycorrhizal plants (Chambers et al 2008).

The genus *Talaromyces* belongs to the family Trichomaceae (Ascomycota) (Yilmaz et al 2014). This genus was first characterized by soft ascocarps with interwoven and typically yellow ascomata (Benjamin 1995). *Talaromyces* has species that are medically important (Yilmaz et al 2014). *Talaromyces radicus* (D.P 7.2.1) produce rugulosin and Skyrim; rugulosin is a pigment with specific antibacterial effect against *Staphylococcus aureus* (Yamazaki et al 2010a, b, c). Species of *Talaromyces* generally produce a yellow, orange or red pigment in the mycelium, which may diffuse into the medium (Mapari et al 2009). They produce enzymes such as endoglucanase and phosphatases and soluble pigments that make this genus important for biotechnological purposes (Reyes et al 1999; Narikawa et al 2000). Association of *Talaromyces* with orchid roots could impart antibacterial activity against bacterial endophytes.

The genus *Penicillium* belongs to the same family as the genus *Talaromyces*, the family Trichomaceae being very diverse (Visagie et al 2014). *Penicillium* is the most common fungus occurring in all habitats, from soil to vegetation to air, indoor environments and some food products (Visagie et al 2014). Its main function is as a decomposer of organic materials (Frisvad and Samson 2004; Pitt and Hocking 2009; Samson et al 2010), but they also produce diverse mycotoxins that are secondary metabolites and can be produced in foods as a result of fungal growth (Frisvad et al 2004). *Penicillium* mycotoxins are ochratoxin A (OTA), patulin and citrinin and are important as they are involved with the human food chain (Michael et al 1998). *Penicillium* species are screened for production of novel enzymes because of its degradative abilities (Li et al 2007; Adsul et al 2007; Tarrasan et al 2010). Given this degradative ability these fungi could enhance nutrient access to orchids.

Chaetomium is a member of the Pyrenomycetes, Chaetomiaceae (Ascomycota) (Aly et al 2010) and they are a rich source of bioactive secondary metabolites such as cytoglobins that are intracellular proteins endowed with hexa-coordinated heme-Fe atoms and have a protective function during conditions of oxidative stress (Li et al 2006). They are found in soils and sub-tropical areas (Aly et al 2011) and are true saprotrophs. Junior et al 2018 found that media containing lead nitrate support good growth of *Chaetomium aureum* (D.P 8.1.3). These fungi have enzymatic surface machinery that can break down complex molecules such as cellulose (Cragg et al 2015). *Chaetomium* can also control a variety of plant diseases through the action of antibiosis, mycoparasitism and nutrition competition as well as promote plant growth by producing ergosterol that maintains membrane fluidity, permeability and structure (Park et al 2005; Zhang et al 2011).

Neopestalotiopsis belongs to the order Xylariales, the family of Sporocadaceae and is generally known as a pestalotioid fungus (Maharachchikumbura et al 2014). It is a significant plant pathogen causing postharvest fruit rot and trunk diseases in grapevines in many countries (Arzanlou et al 2013; Jayawardene et al 2015). They are commonly distributed in tropical and temperate ecosystems (Maharachchikumbura et al 2011; 2013). *Neopestalotiopsis* produces a variety of bioactive secondary metabolites (Hu et al 2007; Xu et al 2010; Debbab et al 2012; Xu et al 2014) and is saprotrophic (Maharachchikumbura et al 2014).

Sequence analysis of the ITS region of fungal root associates obtained from cloning identified several clones as being non-mycorrhizal fungi (Table 2.6 and 2.7). These results add to the growing evidence that fungal root endophytes are associated with many terrestrial plants (Rasmussen 1995; McCormick et al 2004; Selosse et al 2004; Abadie et al 2006; Brundrett 2006 Selosse et al 2007; Shefferson et al 2009; Dearnaley et al 2012). Some of these root fungal endophytes may grow into the surrounding soil environment (Abadie et al 2006) contributing to nutrient mobilization and plant uptake (Shefferson et al 2005).

Previous studies suggest that green terrestrial orchids from temperate regions habour non-mycorrhizal endophytic fungal communities dominated by helotialean species, for example, *Leptodontidium orchidicota*, *Tetracladium sp*. and many uncultured Helotiales taxa (Stark et al 2009; Kohout et al 2013). *D. cornuta* and *D.*

polygonoides were also found to be associated with Helotiales taxa. Members of this fungal order have a heterogeneous ecology, acting as pathogens (Queloz et al 2011) or mutualistic symbionts (Grelet et al 2009). They are found in various environments including, soils and freshwaters (Piercey et al 2002; Shearer et al 2007). Helotiales is described as an ascomycetous order, with many undescribed species that interact as mycorrhizas (Jalou et al 2005). Helotiales are a diverse order including plant pathogens, different types of saprobes, plant endophytes and both ericoid and ectomycorrhizal fungi (Vralstad et al 2002; Wang et al 2006). It is also a well-known ericoid fungus (van der Heijden 2015) that was recently placed as a new fungal lineage of Ericaceae (Selosse et al 2009; Bizabani 2015).

Other fungal associates of the *Disa* species included known basidiomycetous orchid mycorrhizal (OM) fungi belonging to the Talasnellaceae, a group commonly found in most photosynthetic orchids (Selosse et al 2002a). Tulasnelloid fungi are well-studied endomycorrhizal fungi associated with many green orchids and promote seed germination, especially species of *Tulasnella* (Suarez et al 2006). *D. cornuta* and *D. polygonoides* were shown to be colonized by *Tulasnella* fungi which form part of the *Rhizoctonia* complex. A study conducted by Bonnardeaux et al (2007) on the South African orchid, *D. bracteata,* found that it was mostly associated with *Epulorhiza* clade with the Tulasnellales and EM species that were not identified to species level.

The presence of ectomycorrhizal taxa indicates that *D. bracteat*a can associate with other Basidiomycetes (Stark et al 2009). Associating with ectomycorrhizal fungi may provide a more stable carbon and nutrient resource and could contribute to orchid growth in diverse habitats but this would require further investigation (Stark et al 2009). Uncultured ectomycorrhizal fungi were found to be associated with *D. bracteata*. This was also evident in a study conducted by Stark et al (2009) where *Gynmadenia conpsea,* a terrestrial orchid, was found to be associated with endophytes from ectomycorrhizal taxa such as *Helotiales*, *Cadophora*, *Terfezia*, *Peziza*, and *Tetracladium* indicating that the plant utilizes these as their mycorrhizal partners. The study also showed less specificity to a fungal clade meaning that most identified taxa were shown to be either orchid-mycorrhizal or ectomycorrhizas. These ectomycorrhizal fungal partners may have benefits for the survival of terrestrial

orchids. Selosse et al (2004) suggested that *Neottia* may use ectomycorrhizal fungi to have access to fungal carbohydrates where *Rhizoctonia* fungi are not available.

The *Disa* orchids grow alongside other ericoid plants (Bizabani, 2011). Vrålstad et al (2000) suggested that EM fungi can act as ectomycorrhizal fungi under mixed communities. It is accepted that extraradical hyphae produced by ectomycorrhizal plants may contact neighboring roots resulting in colonisation (Simard et al 1997) particularly if the associations have low specificity for host plants (Newman 1988; Simard et al 1997). The ecological consequences of these interconnections are not well understood but may result in the interplant transfer of carbon or nutrients when different plants species form mycorrhizas with the same fungal species (Hamel and Smith 1992; Newman and Eason 1993; Arnebrant et al 1993). Mycorrhizas play a role in the plant to plant interactions of mixed species by exchange of nutrients through interconnecting hyphae as well as improving plants health through improved nutrition, improved water status and protection against pathogens (Simard 1997). Ericoid mycorrhizal fungi also form coiled structures within the hair roots of Ericaceae host plants. It is highly likely that ericas and orchid plants might be interlinked by common endophytic hyphae, and compounds might have been transferred between the two host plants (Graves et al 1997; Simard et al 1997). In many studies, ericoid endophytes have been found to associate with other plant roots (Perrotto et al 1996; Cairney and Meharg 2003; Williams et al 2004). Ericoid mycorrhizal endophytes also associate with ectomycorrhizal root tips (Bergo et al 2000; Vralstad et al 2000); they are widespread and occur on several taxa (Cairney and Meharg 2003). Bergero et al (2000) used random amplified polymorphic DNA to show that endophytic mycelia isolated from hair roots of *Erica arborea* and from ectomycorrhizal roots of neighboring *Querus ilex* were genetically identical. This observation proved the existence of a mycelial link between the two plant taxa (Cairnery and Meharg 2003).

The ascomycetes found to be associated with these terrestrial orchids could be intercellular endophytes or rhizoplane colonizers or even decaying fungi such as *Sordariales* (Bidartondo et al 2004). Members of the Ascomycetes have been used to promote plant establishment in environments plagued by mining activities, terrestrial oil spills or other soils contaminants (Regvar et al 2010).

The objective of this study was to isolate and molecularly identify root fungi associated with *D. polygonoides*, *D. cornuta*, and *D. bracteata*. Mycorrhizal colonization was confirmed microscopically for all the *Disa* species as indicated by the dense coiled hyphae structures that are the main characteristics of orchid colonization by orchid mycorrhizal fungi.

Isolation of fungal isolates was obtained successfully with distinct morphological characteristics. Pure cultures were then molecularly identified using ITS1F and ITS4 universal primers for identifying fungi. BLAST search results revealed that *D. polygonoides*, *D. cornuta*, and *D. bracteata* were associated with mostly endophytes. Root DNA of *D. bractaeta*, *D. polygonoides*, and *D. cornuta* was cloned using the pGEM® -T vector systems and clones were successfully obtained and identified. The aligned sequences identities belonged to both Basidiomycetes and Ascomycetes. *D. bracteata* was found to associate with EcM, uncultured fungus and ascomycetous fungi. *D. cornuta* and *D. polygonoides* were associated with species from Tulasnellaceae family (orchid mycorrhizal fungi).

The fungal isolation step is one of the major problems in orchid mycorrhizal research (Taylor and McCormick 2008). Although mycobionts can be routinely isolated (Rasmussen 1995), there is a decline in vitality within hours of collection in some orchids (Suarez et al 2006) and mycobionts from non-photosynthetic hosts are difficult to isolate (Taylor and Bruns 1997; Taylor et al 2003). Molecular identification is important because not all fungi isolated from pelotons are members of the *Rhizoctonia* complex (Bannardeaux et al 2007). This study highlighted the difficulty in obtaining pure cultures of OM fungi, possibly due to their very slow growth. Bannardeaux et al (2007) in their study used a three-stage confirmation process to identify orchid mycorrhizal fungi; direct isolation from mycorrhizal structures such as pelotons; symbiotic germination assays using a culture-dependent approach and molecular confirmation that fungi belonged to groups within the *Rhizoctonia* complex known to contain orchid mycorrhizal fungi. Although a similar approach was taken symbiotic germination was not conducted on any of the fungal isolates or *Disa* species. These steps are important in determining an accurate orchid mycorrhizal association and some of these steps could be further investigated in the future studies of South African orchids.

Accurate identification of orchid mycorrhizal fungi using molecular techniques is necessary to know the diversity of fungi compatible with orchids (Hollick et al 2005). Despite all attempts to isolate OM fungi non-*Rhizoctonia* root endopytic fungi are still more commonly isolated from orchid roots (Bayman and Otero 2006; Brundrett 2006). The question arises as to whether some of these fungi form mycorrhizal associations; this can only be answered unequivocally if re-synthesis between orchid and a fungal isolate is successful as is evident by the formation of pelotons.

In conclusion, the *Disa* species investigated in this study were associated with several soil endophytes. *D. bracteata*, *D. polygonoides* were collected from the same site along the road verge which is regarded as being disturbed. Based on both culture – dependent and independent techniques employed *Oidiodendron* was found associated with both species. Erica plants are found in the same environment and may facilitate mycorrhizal connections between roots to allow for the exchange nutrients especially carbon. Other associates of *D. bracteata* included ECM fungal groups, Cantherellales and Thelephoraceae while *D. polygonoides* was associated with *Tulasnella* (OM) and Heliotiales (EM, EcM) fungi. *D. cornuta* on the other hand was collected from the less disturbed mountain drive area and was found to be associated with *Tulasnella*, Heliotiales and *Tomentella* (EcM) fungi. Jacquemyn et al (2012) showed that different fungal lineages can exploit different resources and leads to plants having multiple partners at the same time, allowing them to maximize their nutrient uptake under poor nutrient conditions. Under the soil nutrient limiting conditions the *Disa* species investigated were shown to associate with multiple potential mycorrhizal partners as well as other root endophytes that have saprotrophic abilities.

Chapter 3: Biological Properties Of Orchid Associated Fungi

3.1 Introduction

Endophytic fungi in the phyla Ascomycota, Basidiomycota, and Zygomycota that are associated with different plants around the world represent an important reservoir of worldwide fungal diversity (Ferriera et al 2015). Endophytes may produce substances of potential use to modern medicine, agriculture, and industry, such as novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds (Strobel and Daisy 2003; Mitchell et al 2008). Endophytic fungi possess unique structures and diverse bioactivities, that represent a large reservoir which offers untapped potential for exploitation (Tan and Zou 2001; Zhang et al 2006).

Fungi associated with medicinal plants produce antibacterial molecules and pharmacologically active substances with potential to act as antifungal agents (Katoch et al 2014). Endophytes produce enzymes, and novel enzyme systems employed to assist in host tissue colonization ability (Strobel 2001). Enzymes isolated from endophytes can be used commercially in food processing, medical therapy, and in the field of molecular biology (Falch 1991; Katoch et al 2014). The screening of antibacterial plant and fungal extracts represents an approach to finding new compounds with potential to act against multidrug-resistant bacteria (Suffredini et al 2004). Over the past 15-20 years, there has been an increased interest in antimicrobial substances that could be produced by endophytic fungi (Stinson et al 2003; Weber et al 2007; Qin et al 2009; Christina et al 2010). This has given rise to screening endophytic fungi for antimicrobial properties.

Antimicrobial metabolites are important strategies for biodiversity and conservation of orchid plants. Fungal endophytes are diverse and abundant (Huang et al 2008; Naik et al 2008; Zimmerman and Vitousek 2012), producing bioactive compounds which could be developed into novel antimicrobial drugs (Wang et al. 2011; Chandra 2012; Gutierrez et al. 2012). Novel antimicrobial compounds from endophytic fungi may be an important resource to overcome insufficiency of current antibiotics against human pathogens (Strobel et al 2001; Marston et al 2016).

Research conducted on endophytes of plants is important as it provides information for the assessment of global fungal diversity and distribution, as well as for the

discovery of new species (Bezerra et al 2012). Enzymatic production by endophytes can be used to elucidate their function within the plant tissues (Bhagobaty and Joshi 2011). Endophytes colonising tissues of economically important plants have drawn interest for example, the discovering of the new anti-cancer drug Taxol produced from *Taxomyces sp.* growing on the cortical tissue of *Taxus baccata*. Endophytes produce hydrolytic extracellular enzymes as a mechanism of resistance protection of hosts against microbial invasion (Tan and Zou 2001, Bezerra 2012). These enzymes include pectinases, esterases, cellulases, lipases, proteases and xylanases (Suto et al 2002; Silva et al 2006; Bezerra 2012).

Amylase is one of the most important enzyme groups within the field of biotechnology (Corrêa 2014). This enzyme group is employed to convert starch into different sugar molecules (Corrêa 2014). Several types of enzymes are involved in the degradation of starch, mainly β-amylase (1, 4 α-glucan maltohydrolase), α-amylase (1, 4 α-glucanohydrolase) and glucoamylase (1, 4 α-glucan glucohydrolase) (Pandey et al 2000).

Protease refers to a group of enzymes with catalytic function to hydrolyze peptide bonds of proteins (Corrêa 2014). They are also referred to as proteolytic enzymes or proteinases (Barrett et al 2003). Proteases belong to the class of hydrolases and are ubiquitous in nature (Mahajan and Badgujar 2010; Li et al 2013; Corrêa 2014). Proteases are economically important as they are largely used in detergents and in the leather, food and pharmaceutical industries and in bioremediation processes (Barrett et al 20003).

Endophytes were isolated from roots of *D. polygonoides, D. bracteata* and *D. cornuta* and successfully identified (Ch 2). The objective of this study was to determine the metabolic potential of these endophytes. This was achieved by screening for enzymes activities of amylase, cellulase and protease and antimicrobial bioactivities against two Gram-positive and two Gram-negative bacterial species. A second objective was to assess growth potential of the endophytic fungi on starch and cellulose substrates.

3.2 Materials and Methods

3.2.1 Screening for antimicrobial activity

Twenty-four fungal isolates obtained from *D. polygonoides*, *D. cornuta*, and *D. bracteata* roots were screened for antimicrobial activity. A plug of 5 mm fungal isolate, obtained from an actively growing isolate, was centrally inoculated onto petri dishes containing 20 ml of basal Modified Melin-Norkrans (MMN) broth (Table 3.1; Choi et al 2005) and incubated at 25^{o}C for two weeks. The liquid medium was removed from each of the isolates and filter sterilized using a 0.20 µm disposal syringe filter and stored in sterile microcentrifuge tubes at 4^{o}C.

Table 3.1 Composition of Modified Melin-Norkrans medium (MMN).

Components	**g L^{-1}**
Glucose	20 g
Potassium dihydrogen orthophosphate	1 g
Ammonium nitrate	0.5 g
1% (1g in100ml) Ferric citrate	0.5 ml
1% (1g in 100ml) Zinc solution	0.5 ml
Bacteriological agar*	15 g
Thiamine (50 mg in sterile H_2O) filter sterilized	10 ml
pH	5.00

*agar was added for solid medium only

Microbial preparation

The bacterial isolates were obtained from the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa. Four bacterial isolates were used: *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), *Pseudomonas putida* (*P. putida*), and *Staphylococcus aureus* (*Staph. aureus*). The bacteria were discontinuously streaked onto nutrient agar (NA, Sigma-Aldrich, 70148) and grown overnight at 37^{o}C for purity determination. A loop full of a single colony was used to inoculate 5 ml sterile nutrient broth (NB, Sigma-Aldrich, 70122).

Fresh NA plates were excavated with a 5 mm corer to create 5 wells, four well for fungal extracts and a central well as a control (un-inoculated MMN broth). Onto each NA plate, 100 µl of the selected bacterial culture was spread plated onto the surface, and allowed to dry, the wells were filled with 75 µl of the fungal extract and the control medium. Plates were incubated at 25^{O}C for 24-48 h. Zones of clearance around the extract wells indicated a positive response, showing bacterial inhibition.

3.2.2 Screening for enzyme activity

To assess the enzymatic activity of the fungal isolates, pure cultures were grown on basal medium, MMN. Initially a qualitative assay was conducted as this is an important tool in screening fungi for detecting the presence of starch, cellulose and protein degrading enzymes (Choi et al 2005).

3.2.2.1 Protein degradation

Protease activity was conducted on MMN agar media (Table 3.1) enriched with fat-free milk. After autoclaving 250 ml of skim milk was mixed with the medium (ratio 1:4) and approximately 20 ml was poured to sterile petri dishes. A plug of fungal mycelium from the selected isolates was inoculated onto the solid medium and incubated at 25^{o}C for 3 weeks. An un-inoculated plate constituted a control. Three replicates of each fungal isolate were tested. Observations of a zone of clearance indicated positive protease activity.

3.2.2.2 Cellulose degradation

Determination of cellulase activity was conducted according to the method of Pointing, (1999) with amendments of 1% w/v carboxymethylcellulose (CMC, Sigma-Aldrich, 419338) and 5 g of glucose to the basal medium (MMN). After autoclaving and cooling, approximately 20 ml of the medium was poured into petri dishes. The plug of the fungus mycelium was inoculated onto the media once set and incubated at 25^{o}C for 3 weeks. An un-inoculated plate constituted a control. Three replicates of each fungal isolate were tested. After observation of growth, the plates were flooded with Congo red (0.1% w/v, Sigma-Aldrich, 573-58-0EC) for 15 min and fungal

colonies that appeared yellow against red were indicative of the presence of cellulase activity. The diameter of the zone of clearance was recorded.

3.2.2.3 Starch degradation

To determine the presence of amylase activity a method modified from Behal et al (2006) and Rele (2004) was used. The MMN agar medium was enriched with 1% soluble starch (Merck, 101252) reduced glucose (5 g) was supplied and approximately 20 ml of the medium was poured into petri dishes. The plug of the fungal isolate was inoculated onto the medium and incubated at 25^{o}C for 3 weeks. An un-inoculated plate constituted a control. Three replicates of each of the fungal isolates was tested. To observe zones of clearance around mycelial growth, the plates were flooded with an iodine solution (1% w/v) and diameter of clearance was recorded.

3.2.3 Determination of fungal biomass

The isolates were grown in liquid basal MMN with 2.5 g glucose as the control medium. To this medium 1% CMC or 1% starch was added as the test substrates. The fungi were inoculated as previously described into petri dishes containing 20 ml of MMN control, CMC and starch media. The fungal isolates were inoculated in triplicates and incubated for 3 weeks at 25^{o}C. Mycelium was filtered onto pre-weighed and dried Whatman No.1 filter paper using a Buchner funnel and vacuum. The biomass was washed with distilled water and dried at 45^{0}C to constant weight. Dry weights were corrected for the filter paper weight and recorded as dry biomass.

3.2.3.1 Statistics analysis

All results presented are the means of the three independent replicates for each of the fungal isolates. Fungal biomass was analysed using one-way analysis of variance (ANOVA). Substrate comparisons were further assessed using Tukey HSD test at $P \leq 0.05$. All statistical analysis was conducted using R software.

3.3 Results

3.3.1 Antimicrobial activity

A total of 24 fungal isolates were tested for antimicrobial activity. A visual qualitative assessment of any bacterial growth inhibition was recorded with a + or - response. The majority (70%) of fungal extracts showed some degree of inhibition of bacterial growth of *B. subtilis* and *S. aureus* represented by "+ or ++''. None of the fungal extracts showed inhibition against *P. putida* and only extracts from the fungus D. P 8.1.1 and D.P. 5.2.2 showed some degree of inhibition against *E. coli* (Table 3.2)

Table 3.2 Response of the bacterial isolates *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas putida* and *Escherichia coli* to selected fungal extracts.

Fungal Isolate	*Bacillus subtilis**	*Staphylococcus aureus**	*Pseudomonas putida**	*Escherichia coli**
D.P 8.2.1	+	+	-	-
D.P 5.2.6	+	+	-	-
D.P 4.4	+	+	-	-
D.P 5.2.4	+	+	-	-
D.P 7.2.1	+	+	-	-
D.P 13.2.2	+	+	-	-
D.P 8.1.3	+	+	-	-
D.B 1.2	+	+	-	-
D.P 10.2	+	+	-	-
D.P 6.2.2	+	+	-	-
D.P 5.2.5	+	+	-	-
D.P 5.3.1	+	+	-	-
D.P 8.1.1	+	+	-	++
D.P 5.2.2	+	+	-	+
D.P 8.1	-	+	-	-
D.P 4.6	-	-	-	-
D.P 8.1.2	+	+	-	-
DB1A1	-	+	-	-
D.P 9.1	-	-	-	-
DBA1AS2	-	-	-	-
D.P 5.1.1.2	-	-	-	-
1DB 3.1	+	-	-	-
D.P 5.2.2 Tric	+	+	-	-
DB2	+	-	-	-
D.P 8.3	+	+	-	-
1DC 2.1	-	+	-	-
DB 2.2 DB	-	-	-	-

* **++** = strong reaction showing a clear zone of clearance; **+** = weak reaction showing a slight clear zone; **-** = no reaction with no clear zone forming.

3.3.2 Enzyme activity

3.3.2.1 Enzyme production

Of the 27 fungal isolates screened, 24 were negative for the ability to degraded milk protein. Three of the isolates showed protease activity indicating positive casein degradation. This was clearly observed as zones of clearance. The DB1AS isolate exhibited the most visibly clear zone, while D.P 7.2 showed clearance and medium discoloration, isolate D.P 7.2.1 changed the color of the media to yellow (Figure 3.1).

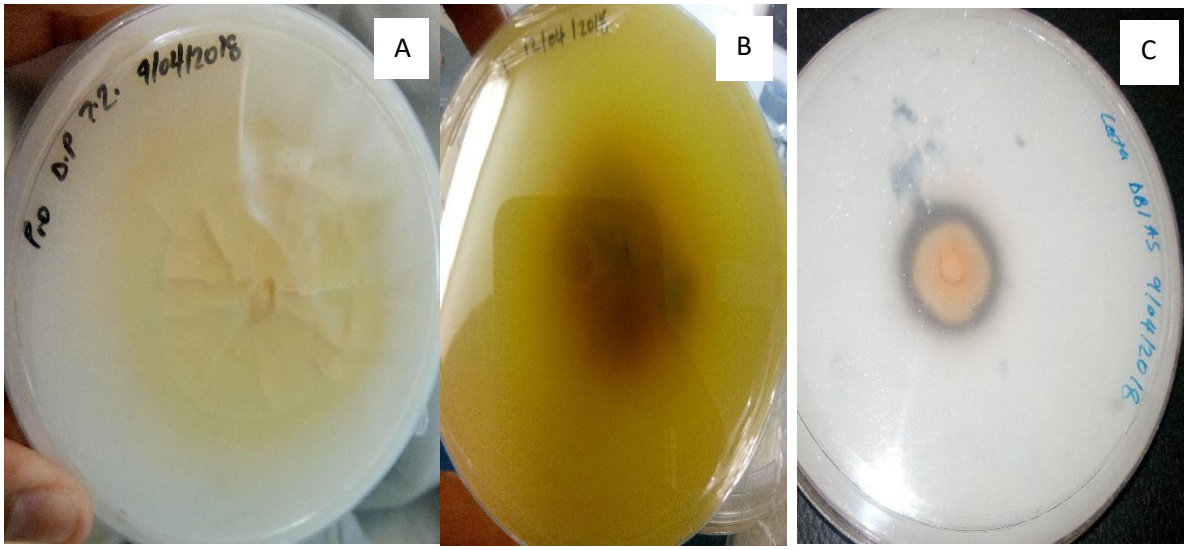


Figure 3.1 Response of selected fungal isolates grown on skim milk amended MMN media **A**) D.P 7.2 clearance and medium discoloration, **B**) D.P 7.2.1 yellow discoloration of medium, and **C**) DB1AS strong clearance zone. Approximately 70% of the fungal isolates screened were positive for either amylase or cellulase activity or both. Clear zones were visible after being flooded with iodine and Congo red, respectively (Table 3.3 and Fig 3.2 & 3.3).

Table 3.3 Production of extracellular enzymes by fungal isolates as measured by width of clearing or colour reaction zone in mm.

Isolate	Protease	Cleared zone diameter (mm)	Cellulase	Cleared zone diameter (mm)	Amylase	Cleared zone diameter (mm)
D.P 8.2.1	-	0	-	0	-	0
D.P 5.2.6	-	0	-	0	-	0
D.P 4.4	-	0	-	0	-	0
D.P 5.2.4	-	0	-	0	-	0
D.P 7.2.1	++	25	+	0	+	0
D.P 13.2.2	-	0	+	0	+	0
D.P 8.1.3	-	0	++	27	+	0
D.B 1.2	-	0	++	64	++	33
D.P 10.2	-	0	-	0	-	0
D.P 6.2.2	-	0	+	0	+	0
D.P 5.2.5	-	0	+	0	+	0
D.P 5.3.1	-	0	++	41	++	51
D.P 8.1.1	-	0	+	0	+	0
D.P 5.2.2	-	0	++	60	++	48
D.P 8.1	-	0	++	47	++	62
D.P 8.1.2		0	+	0	+	0
DB1AS	++	15	-	0	++	45
D.P 9.1	-	0	-	0	-	0
DBA1AS2	-	0	++	11	+	0
D.P 5.1.	-	0	++	25	++	20
DB2	-	0	++	35	++	11
D.P 8.3	-	0	++	21	++	20
1DC 2.1	-	0	++	70	++	10
D.P 7.2	++	40	+	0	+	0

Values represent measured diameters. - = no reaction/ negative result; + = minimum activity (1-9 mm); ++ = maximum activity (10-70 mm).

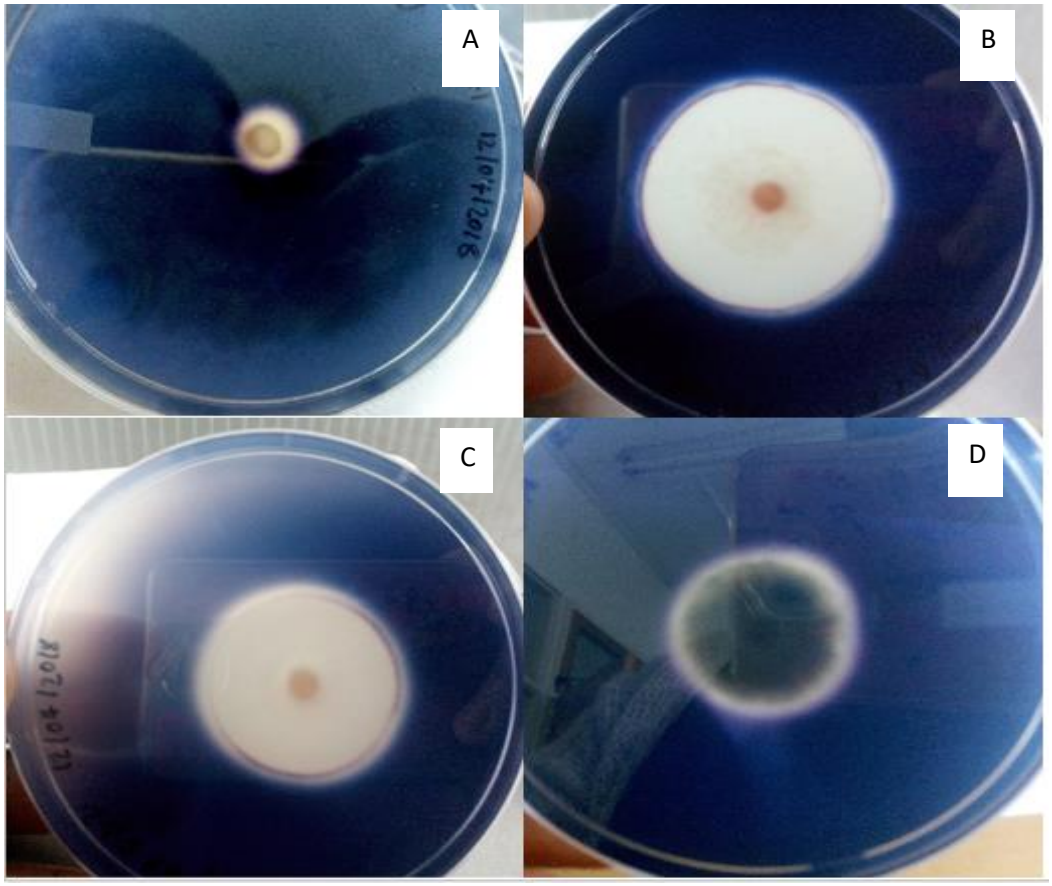


Figure 3.2 Assessment of amylase on solid MMN media supplemented with soluble starch shown by a clear zone **A**) Isolate D.P 8.3; **B**) Isolates D.P 8.1; **C**) Isolate D.P 5.3.1; **D**) Isolate D.P 5.2.

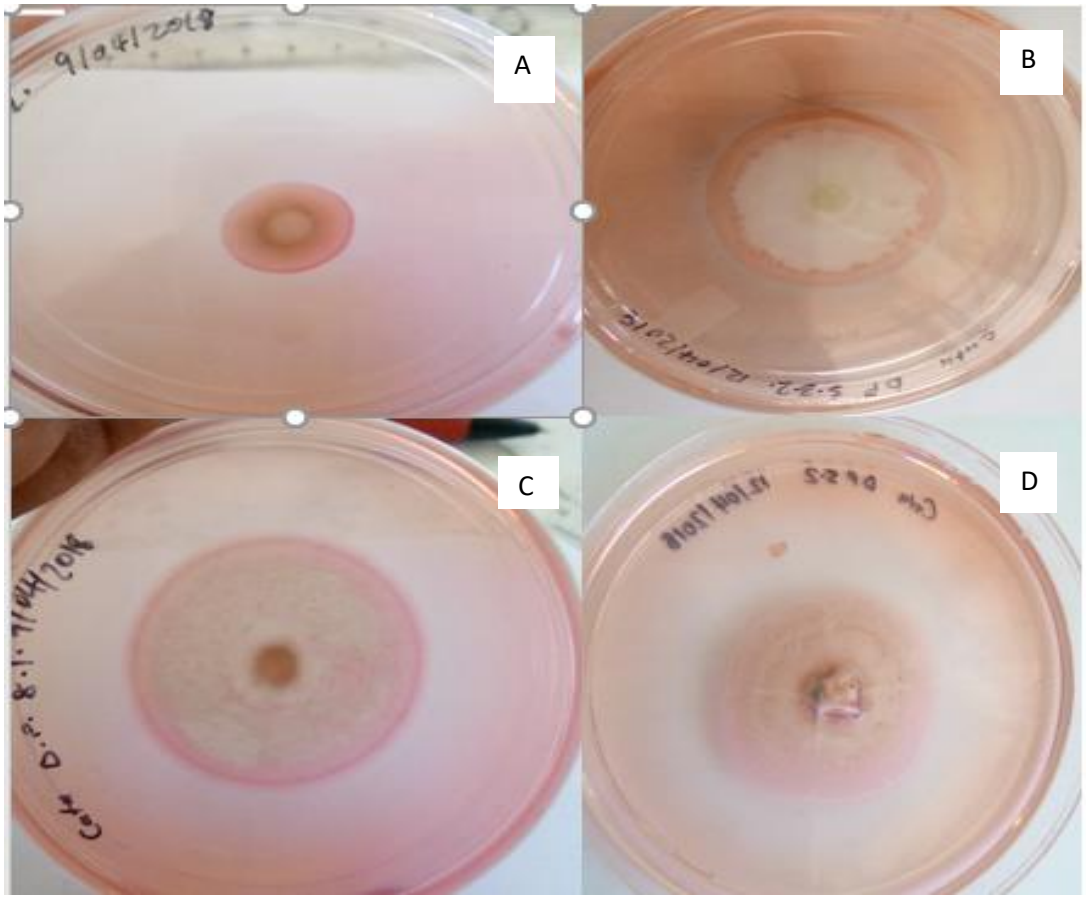


Figure 3.3 Assessment of cellulose on the solid MMN media supplemented with soluble CMC shown by a clear zone. **A**) Isolate IDC2.1; **B**) Isolate D.P 8.1; **C**) Isolate D.P 5.3.1; **D**) Isolate DB1.2.

3.3.3 Fungal biomass

All fungal isolates grew to some extent on the various amended media. Fungal biomass of 40% of the isolates was greater in medium amended with either CMC and/or starch (Figure 3.4) Significant differences between control and starch as well as control and CMC amended media were apparent (Table 3.4) indicating that some fungal isolates preferred either CMC and/or starch as a carbon substrate. Sixty percent of isolates had no significant preference for any of the media.

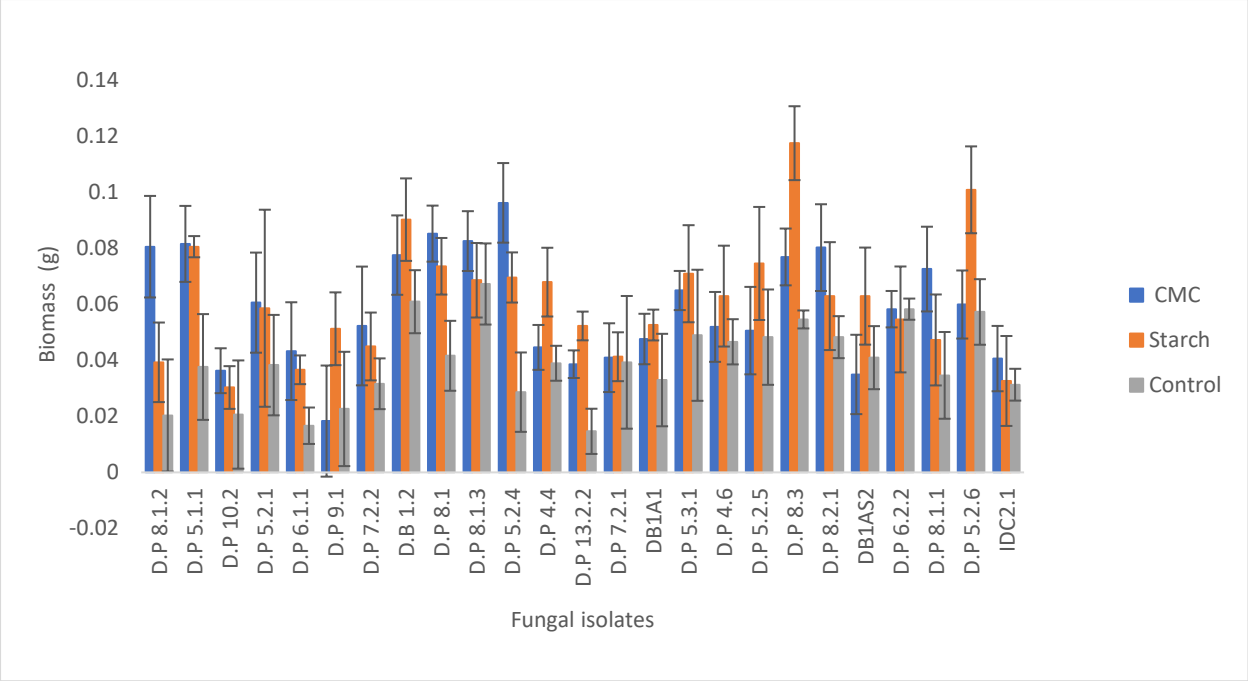


Figure 3.4 Biomass of endophytic fungi grown on liquid MMN amended with 1% carboxymethylcellulose (CMC) and starch as carbon sources. Columns represent means of three replicates; error bars represent ± standard deviations.

Table 3.4 Mycelial biomass of fungal isolates grown on MMN medium amended with 1% carboxymethylcellulose (CMC) and starch as compared to control medium.

Isolate	F-value (2.6)	P-value	Significant Treatment (control- cellulose/starch P < 0.05	
			Cellulose	Starch
DP812	9.179	0.05	Pr > F	NS
DB1A1	2.445	>0,05	NS	NS
DB1AS2	3.11	>0,05	NS	NS
DB12	3.57	>0,05	NS	Pr > F
DP102	1.131	>0,05	NS	NS
DP1322	28.21	0.001	Pr > F	Pr > F
DP44	8.36	0.05	NS	Pr > F
DP46	1.143	>0,05	NS	NS
DP511	10.21	0.05	Pr > F	Pr > F
DP521	0.73	>0,05	NS	NS
DP524	21.6	0.01	Pr > F	Pr > F
DP525	2.028	>0,05	NS	NS
DP526	10.24	0.05	NS	Pr > F
DP531	1.295	>0,05	NS	NS
DP611	4.635	0.1	Pr > F	Pr > F
DP622	0.097	>0,05	NS	NS
DP721	0.013	>0,05	NS	NS
DP722	1.454	>0,05	NS	NS
DP81	12.81	0.01	Pr > F	Pr > F
DP811	4.59	0.1	Pr > F	NS
DP813	1.293	>0,05	NS	NS
DP821	3.454	>0,05	NS	NS
DP83	31.92	0.001	Pr > F	Pr > F
DP91	2.95	>0,05	NS	NS
IDC21	0.537	>0,05	NS	NS

*NS = No significant difference; *= > 0.05; *Pr > F = significance probability value associated with the F Value.

3.3 Discussion and Conclusion

All endophytic fungi in this study were screened for their antibacterial potential on solid media (Table 3.2). Most of the fungal extracts showed potential inhibitory activity against the Gram positive, *Bacillus* and *Staphylococcus* strains. Two fungal isolates (D.P 8.1.1 and D.P 5.2.2) showed possible mode of action of inhibition activity against *E. coli*. Preliminary screening of fungal isolates allows for the detection of the microorganisms that possess interesting antimicrobial activity (Mefteh et al 2017).

Solid media enzyme assay is used to determine enzyme synthesis, released from the mycelium (Abdel-Raheem and Shearer 2002). The skim-milk agar plate technique is an easy and rapid way to screen for protease activity (Saran et al 2007). Only three fungal isolates showed some proteolytic activity (Figure 3.1). The highest protease activity as determined qualitatively was shown by two *Talaromyces sp.* (D.P 7.2 and D. P 7.2.1) isolated from *D. polygonoides* and one *Penicillium sp.* isolated from *D. bracteata* (DB1AS). Several studies conducted on endophytes such as *Penicillium*, *Trichoderma*, *Chaetomium* and *Talaromyces* confirm the ability of these fungi to produce and secrete protease (Reddy et al 1996; Maria et al 2005; Sunitha et al 2013). Sunitha et al (2013) and Reddy et al (1996) used different protein supplements in the media such as gelatin as well as skimmed milk.

Enzymatic hydrolysis of starch is facilitated by the enzyme amylase (Chi et al 1995; Farid et al 2002; Behal et al 2006). Seventy five percent of endophytes were able to produce amylase indicating the ability to degrade starch. This was clear in the following isolates: D.P 8.3, D.P 8.1; D.P 5.3.1 and D.P 5.2 (Figure 3.3). These fungi can utilize starch as a carbon source. Amylase enzymes are distributed in various bacteria, fungi, plants and animals playing a crucial role in the utilization of polysaccharides (Ribeiro 2000; Hagihara et al 2001; Zoltowska 2001; Bassinello et al 2002; Haq et al 2003). Amylase enzymes are produced by microorganisms with different specificities, properties and action patterns (Talamond et al 2002; Behal et al 2006) and are ultimately responsible for the release of glucose molecules that the microorganisms can use as a carbon source to support growth. Plants store starch which is one of the most easily digested food sources within plants tissues (Choi et al 2005). Once plant cells die, the starch becomes available through the action of

amylase enzymes allowing endophytes to utilize the starch in competition to new colonisers (Choi et al 2005).

Cellulolytic activity was found in 71% of the fungal isolates screened and the production of cellulase was evident from isolates obtained from all the *Disa* species (Table 3.3). Cellulase activity was mostly detected in the isolates 1DC2.1, D.P 8.1, D.P 5.3.1, DB1.2 (Figure 3.2). The isolates that showed high activity of cellulose activity are from *Trichordema* and *Penicillium* genera. The results agree with those of Mefteh et al 2017 who recorded that *Penicillium* species were very effective at degrading CMC. A solid media method was also used but the endophytic fungi were inoculated onto GYP agar supplemented with 0.5% CMC. *Penicillium* and *Trichordema* genera are well known to produce an extensive range of extracellular enzymes (Arikan 2008; Park et al 2016; Mefteh et al 2017).

Cellulose is composed of a linear polymer of D-glucose linked by β-1,4 bonds which can be degraded by a cellulose enzyme complex (Karnchanatat et al 2008). The enzyme complex is made of endoglucanases which cleave internal portions of the cellulose chain (Wood 1992; Youssef and Berekaa 2009). Fungal pathogens and endophytes utilize cellulase to break the cellulose plant cell wall for nutrient acquisition and colonization (Khan and Husaini 2006).

Some of the fungal isolates showed both amylase and cellulose activity showing the ability to grow in media with CMC or starch (Table 3.4). Few were also able to degrade both CMC and starch, those fungal isolates include: 1DC2.1; D.P 8.3; D.P 5.1; D.P 5.2.2; D.P 8.1 and DB 1.2.

The production of extracellular enzymes by fungal endophytes may provide a resistance mechanism to the host against pathogenic invasion by secretion of secondary compounds and improve plant nutritional status (Saikkonen et al 2004; Choi et al 2005; Fouda et al 2015). Isolate DB1AS (*Penicillium)* had the greatest ability to produce all the tested extracellular enzymes and this is supported by many studies such as Mefteh et al 2017.Extracellular enzymatic activity of endophytes assists in degradation of polysaccharides and proteins during plant senesce contributing to the cycling of nutrients (Amirita et al 2012; Fouda et al 2015).

A negative result i.e. lack a clearance zone does not indicate the fungal isolates inability to produce an enzyme as it may be produced and not released from the mycelium (Pointing 1999). This study focused on extracellular enzyme production. All fungal isolates grew on all the tested substrates, given the reduced glucose as a carbon source in the media this growth may well be due to the release of glucose from the substrate under investigation (Abdel-Raheer and Shearer 2002).

All isolates of endophytic fungi produced measurable biomass on CMC and starch amended media (Figure 3.2). Fungal biomass yield on un-amended (control) MMN was decreased for all the endophytic fungal isolates. Isolate D.P 8.3 (*Oidiodendron*), produced significantly more biomass on starch than on any other substrate. Isolate IDC2.1 (*Trichoderma*) produced the least biomass on all the substrate. Differences in biomass between fungal isolates may reflect differing strategies for storing and or recycling carbon sequestered within either the hyphae or the agar plug (Midgley et al 2003).

Fungal isolates that were not significantly affected by the source of C in the medium this suggests an ability to use varied sources that may be available in the environment without reducing fungal growth. The fungal isolates that showed significant difference at $p< 0.05$ on both CMC and starch were also shown to degrade CMC or starch on the solid screening media. These fungal isolates included D.P 5.1.1 (*Taloromyces proteolyticus)*, and D.P 8.1 (*Penicillium)*. Most of the fungal isolates showing enzymatic activity also showed potential antibacterial activity. This is supported by stuidies conducted on *Trichoderma, Penicillium,* and *Taloromyces* (Choi et al 2005; Arikan 2008; Park et al 2016).

Chapter 4: General Discussion and Conclusion

4.1 Mycorrhizal and root fungal endophytic diversity in three *Disa* species

Analysis of soil samples from each site showed differences in the levels of soil nutrients with Site 1 being poorer than Site 2. Both sampling sites had poor nutrient soils with low levels of N, C and P nutrients. The availability of these nutrients can limit the growth of plants on the sites. On closer examination of the orchid distribution maps, sampling could in future be extended to other areas to determine the effect of soil nutritional status on the mycorrhizal interaction and fungal diversity. Fungal DNA could also be extracted from soil and analysed using Next generation sequencing techniques in order to give a more holistic understanding of the environmental fungal diversity. This has been an approach of some studies (Bonnardeaux et al 2008; Waud et al 2014; Waud et al 2017)

Colonization was successfully done and showed that the roots were colonized by OM fungi as was evident by the presence of pelotons. This was useful in order to confirm the mycorrhizal interaction of the adult plants that were sampled. The assessment of percentage colonization was not conducted in this study as comparisons were not being made between the different *Disa* species; however, this would be useful if differences in sampling area or life stages were being investigated (Deguchi et al 2017). The intensity of mycorrhizal colonisation in the roots could also be useful to quantify but would require an estimation of the number of root cortical cells colonized, and perhaps taking into account root length and diameter (Bertolini et al 2014). Overtime the OM colonisation degenerate and lose vitality which is highly influenced by the maturity of the plant. Once the OM degenerate environmental endophytes enters the roots of the plants.

The culture dependent method was successful in isolating fungi from orchid roots. The fungal isolates grew wells on the PDA media. However, the major disadvantage of this method was the poor ability to detect slow-growing fungi such as *Rhizoctonia* or un-culturable fungi, which constitute an important and diverse part of the root fungal community (Kohout et al 2013). Slower growing fungal isolates were often plagued by contamination despite the incorporation of a fungicide in the medium. Culturing directly from pelotons has been used in some studies (Bonnardeaux et al

2008), the *Disa* roots were not very fleshy and pelotons were not easily removed from cortical cells even when employing a dissecting microscope. Spread plating of macerate roots did release pelotons and slower growing fungal isolates were observed but were plagued by contamination despite the incorporation of a fungicide in the medium Accurate isolation and identification of OM fungi is important and necessary to determine the diversity of fungi compatible with a selected orchid species which can be tested through the symbiotic germination of seeds (Bonnardeaux et al 2008).

An ericoid mycorrhizal fungus, *Oidiodendron*, was isolated from several *D. polygonoides* root samples*,* whether this EM fungus is able to associate with *Disa* species would require further testing. *Oidiodendron maius* and *Heteroconium chaetospira* (Usuki and Narisawa 2005) form characteristic ericoid mycorrhizal infections after colonisation (Brundrett et al 2006). Bonnardeaux et al (2007) conducted a study on the South Africa orchid, *Disa bracteata,* that is widespread across Australia, and found these to be associated with mostly orchid and ericoid mycorrhizal fungi. A symbiotic germination approach would be a suitable method to determine the compatibility of the ericoid fungus. It was interesting to observe that many *Erica* host plants occur in the same area alongside the orchids, and this could suggest the orchids use this ericoid fungus as their mycorrhizal partner. It is known that EM fungi can also associate with the roots of EcM trees (van Heijden et al 2015) and therefore the interaction with orchids growing in the same vicinity is not unlikely.

All the fungi isolated and identified were fungal endophytes, similar results have been shown in other studies by endophytes such as *Talaromyces rotundus* (Selosse et al 2004). The ecological or functional role of these fungal endophytes is not clear but given their saprotrophic abilities (Selosse et al 2009) mineralization and increased availability to nutrients may be their primary role particularly in nutrient poor soils.

The advance in molecular technique and the use of DNA barcoding has allowed researchers to investigate the biodiversity of fungal communities in many ecosystems (Kristiansen et al 2001; Kohout et al 2013). The culture independent approach using a cloning vector was successfully achieved, and OM fungi was identified from two of the *Disa* species namely *D. cornuta* and *D. polygonoides,*

indicating that a member of the Tulasnellaceae family were associating with these terrestrial orchids, this is supported by research conducted by Selosse et al (2007). The fungi isolated from the three *Disa* species belong to class 1 and class 2 endophytes based on their characteristics. Most of the fungus isolated secretes bioactive compounds such as amylase, protease and degrade starch belong to class 2 endophytes. Orchid plants are known to have relationship with class 2 endophytic fungi that forms orchid mycorrhizal associations. The majority of fungi identified belonged to the Ascomycetes and are known endophytic fungi (Selosse at al 2007).

The universal fungal primers ITS1F and ITS4 were used in this investigation and identified a wide spectrum of root endophytes. These primers are commonly used in OM interaction studies (Waud et al 2014). ITS1-OF and ITS4-OF are basidiomycete-specific primers and were used to clone root DNA. These primers are broad-spectrum basidiomycete-specific primers that are recommended for characterization of orchid fungal partners (Taylor and McCormick 2008; Waud et al 2014). These primers however did not identify known OM fungi in *D. cornuta* or *D. bracteata* but did identify known EcM fungal taxa such as *Tomentella.* The transformation rate during cloning was very low which would have impacted on the success of obtaining OM fungal identities. The universal fungal primer pair, ITS1 and ITS4, is regarded as idea for isolated cultures and Sanger sequencing but do have limitation when profiling environmental fungal communities (Waud et al 2014).

Pairing of the orchid-specific primers with different primers has also been used successfully to identify mycorrhizal fungi (Waud et al 2014).

Disa species may be regarded as being mixotrophic using fungi to provide, nitrogen and other nutrients as well as additional carbon through mycoheterotophic strategies (Smith and Read 2008). Different associations between host and fungi may develop for example a fungus may form an ectomycorrhizal association in one host and appears as ericoid mycorrhizal in another host (Villarreal-Ruiz et al 2004) and a mycorrhizal fungus can grow as an endophyte in the roots of a non-host (Girlanda et al 2006). Several of the fungi identified to be associated with the *Disa* species are known to be EcM fungi. Mycoheterotrophs are commonly known to exploit EcM and AM fungi (Markox et al 2009).

Next generation Sequencing (NGS) is a powerful technique that enables the sequencing of thousands to millions of DNA molecules simultaneously (Sanger 1988) and provides higher sensitivity to detect low-frequency variants (Rivas et al 2011; Jamure et al 2014). Cloning only sequences a single DNA fragment at a time, NGS technologies such as Illumina and Iron Torrent platforms are favored because it allows for the reconstruction of longer barcodes (Liu et al 2013) and may be used to cover the entire ITS region (Waud et al 2014). Illumina sequencing was not part of this investigation but is being conducted on all sampled orchids including the *Disa* species used in this study.

Sequence identities for fungal isolates were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were also submitted to the UNITE database (https://unite.ut.ee/analysis.php) and gave similar matching identities. However, The UNITE database was more useful in identifying sequences obtained from clones, indicating that UNITE is a more suitable database to use for OM fungal detection.

All the plants were sampled at the flowering stage to ensure correct plant identification in the field and this may have impacted on the outcome of the results. It may become difficult to isolate OM fungi from adult plant roots as they become degraded allowing the entry of root endophytes (Dearnaley et al 2012). Different stages of development rom seed germination to flowering and seed set is recommended in order to establish changes in the fungal interactions.

4.2 Biological activity of isolated fungi

Extracts from fungal isolates obtained from orchid roots were assessed for antimicrobial activity using a well plate method. The extracts were tested against two Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas putida*).

Almost all isolates exhibited antimicrobial activity against at least one of the tested microorganisms, but most were more efficient in inhibiting for both Gram-positive

bacteria. This indicates action of inhibition may be targeted towards the disruption of the peptidoglycan layer which is a major component of Gram-positive cell walls (Bhagobaty and Joshi 2012). It was interesting that only one extract from D. P8.1.1 a *Penicillium* species showed strong inhibition of the gram-negative *E. coli.* the peptidoglycan layer is protected by an outer membrane (Mefteh et al 2017), this indicates a different mode of action. Alternative methods that could be used to improve the results is the determination of Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC). These methods are used to detect how active are fungal extracts against microorganisms (Corréa et al 2014).

The extracts that showed potential antimicrobial activity could be further screened to determine the compounds produced as secondary metabolites using techniques such as LC-MS (Mefteh et al 2017).

These fungal endophytes contribute to orchid growth and survival providing secondary metabolites that are beneficiary to the orchid plants (Bidartondo et al 2004). Endophytic fungi are known to elicit resistance to herbivore grazing through secretion of secondary metabolites (Bougoure et al 2007). The endophytes also assist in the degradation of complexed carbohydrates such as starch and cellulose, both important components of plant tissues (Pointing 1999). Fungal isolates were tested for the production of enzymes able to degrade protein, cellulose and starch substrates using amended medium. Degradation of the substrates is required in order to provide carbon to the fungus under test in order to optimize fungal growth as well as to gain insight into their ecological role. As such the amended medium contained reduced available sugar in the form of glucose. Enzyme activity was evident particularly when cellulose and starch were provided as substrates.

These substrates were also tested in broth culture to determine the effect on fungal biomass production. This approach can be used to produce extracts for further analysis. All the fungal isolates tested grew on the amended medium, with 40% of the isolates preferring to utilize CMC and/or starch, indicating the ability of these fungi to utilize various resources for carbon acquisitions.

In conclusion of the known mycorrhizal fungi isolated in culture and identified as *Oidiodendron* species were shown to inhibit both Gram-positive bacteria tested and extracellularly produced enzymes ensuring its ability to utilize cellulose and starch

substrates. This is important information required for future research in determining the interactions of *Oidiodendron* with orchid seeds and protocorms.

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Appendices

Appendix A

Roots clearing and staining solutions

A1. 5% KOH

100 g KOH

2 L distilled water

A2. Alkaline Peroxide H_2O_2

3 ml NH_4OH (Ammonia)

30 ml 10% H_2O_2

576 ml distilled water

A3. 0.1 M HCL (MW36.46)

22.79 ml HCL

2 L distilled water

A4. Lactoglycerol trypan blue stain

Lactic acid: Glycerol: Water (13: 12: 16)

520 ml Lactic acid

480 ml Glycerol

640 ml distilled water

Appendix B

Isolation and culture media

B1. 70% ethanol

700 ml ethanol

300 ml distilled water

Appendix C

C1. 1% agarose gel

1 g agarose powder

100 ml distilled water

C2. TE (Tris/ EDTA) Buffer pH 8

Tris/ HCI pH 8	10mM
EDTA pH 8	10mM

Preparation

To make 1-liter 5X TBE (Tris-EDTA) Buffer, mix following:

- 5.3 g of Tris base
- 27.5 g of boric acid
- 20 ml 500 mM EDTA pH 8.0
- 1000 ml distilled water

To make 1X TBE Buffer working solution

Add 200 ml of 5X TBE in 800 ml distilled water

Storage is at room temperature.

Appendix D

D1. R statistics data information

DP812

```
fta<-aov (Biomass~Treatment, data = ft)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq Mean Sq F value Pr(>F)
Treatment    2 0.005710 0.002855   9.179 0.0149 *
Residuals    6 0.001866 0.000311
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)
```

```
$`Treatment`
                          diff         lwr          upr     p adj
Control-Cellulose -0.06033333 -0.10451364 -0.016153023 0.0135779
Starch-Cellulose -0.04133333 -0.08551364 0.002846977 0.0639062
Starch-Control     0.01900000 -0.02518031 0.063180311 0.4359513
```

DB1A1

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DB1A1.csv", sep = ",", header = T)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0006269 0.0003134   2.445 0.167
Residuals    6 0.0007693 0.0001282
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff          lwr        upr     p adj
Control-Cellulose -0.01466667 -0.043034765 0.01370143 0.3211244
Starch-Cellulose   0.00500000 -0.023368098 0.03336810 0.8547559
Starch-Control     0.01966667 -0.008701431 0.04803476 0.1642413
```

DB1AS2

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DB1AS2.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001304 0.0006520    3.11 0.118
Residuals    6 0.001258 0.0002097
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                   diff          lwr        upr     p adj
Control-Cellulose 0.006 -0.030275488 0.04227549 0.8705378
Starch-Cellulose 0.028 -0.008275488 0.06427549 0.1207510
Starch-Control    0.022 -0.014275488 0.05827549 0.2297575
```

DB12

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DB12.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001299 0.0006493    3.57 0.0952.
```

```
Residuals    6 0.001091 0.0001819
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff          lwr        upr     p adj
Control-Cellulose -0.01666667 -0.050453824 0.01712049 0.3498849
Starch-Cellulose   0.01266667 -0.021120491 0.04645382 0.5213903
Starch-Control     0.02933333 -0.004453824 0.06312049 0.0829000
```

DP102

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP102.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0003749 0.0001874   1.131 0.383
Residuals    6 0.0009940 0.0001657
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose -0.015666667 -0.04791195 0.01657861 0.3592848
Starch-Cellulose  -0.006000000 -0.03824528 0.02624528 0.8399726
Starch-Control     0.009666667 -0.02257861 0.04191195 0.6487229
```

DP1322

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> fta<-aov (Biomass~Treatment, data = ft)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value   Pr(>F)
Treatment    2 0.002182 0.0010908   28.21 0.000888 ***
Residuals    6 0.000232 0.0000387
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1

> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff          lwr          upr     p adj
Control-Cellulose -0.02400000 -0.039578188 -0.008421812 0.0077190
Starch-Cellulose   0.01366667 -0.001911521 0.029244855 0.0800181
```

```
Starch-Control     0.03766667 0.022088479 0.053244855 0.0007541
```

DP44

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP44.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0014176 0.0007088    8.36 0.0184 *
Residuals    6 0.0005087 0.0000848
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff           lwr        upr     p adj
Control-Cellulose -0.005666667 -0.0287335854 0.01740025 0.7426468
Starch-Cellulose   0.023333333 0.0002664146 0.04640025 0.0478686
Starch-Control     0.029000000 0.0059330813 0.05206692 0.0196432
```

DP46

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP46.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0004162 0.0002081   1.143   0.38
Residuals    6 0.0010927 0.0001821
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose -0.005333333 -0.03914112 0.02847446 0.8812721
Starch-Cellulose   0.011000000 -0.02280779 0.04480779 0.6044226
Starch-Control     0.016333333 -0.01747446 0.05014112 0.3627965
```

DP511

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP511.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.003786 0.0018930   10.21 0.0117 *
Residuals    6 0.001112 0.0001853
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level
```

```
Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                     diff          lwr          upr     p adj
Control-Cellulose -0.044 -0.078105572 -0.009894428 0.0175328
Starch-Cellulose -0.001 -0.035105572 0.033105572 0.9955511
Starch-Control     0.043 0.008894428 0.077105572 0.0194005
```

DP521

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP521.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.000916 0.0004581    0.73   0.52
Residuals    6 0.003766 0.0006277
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                        diff         lwr        upr     p adj
Control-Cellulose -0.02233333 -0.08509769 0.04043103 0.5527892
Starch-Cellulose -0.00200000 -0.06476436 0.06076436 0.9947483
Starch-Control     0.02033333 -0.04243103 0.08309769 0.6068399
```

DP524

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP524.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq Mean Sq F value Pr(>F)
Treatment    2 0.006971 0.003485    21.6 0.00181 **
Residuals    6 0.000968 0.000161
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                        diff          lwr          upr     p adj
Control-Cellulose -0.06766667 -0.099487432 -0.035845901 0.0015047
Starch-Cellulose -0.02666667 -0.058487432 0.005154099 0.0932241
Starch-Control     0.04100000 0.009179234 0.072820766 0.0176321
```

DP525

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP525.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001275 0.0006374   2.028 0.212
Residuals    6 0.001886 0.0003143
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose -0.002333333 -0.04674978 0.04208311 0.9858127
Starch-Cellulose   0.024000000 -0.02041644 0.06841644 0.2947400
Starch-Control     0.026333333 -0.01808311 0.07074978 0.2419773
```

DP526

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP526.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.003595 0.0017974   10.24 0.0116 *
Residuals    6 0.001053 0.0001754
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff          lwr        upr     p adj
Control-Cellulose -0.002666667 -0.035849876 0.03051654 0.9672211
Starch-Cellulose   0.041000000 0.007816791 0.07418321 0.0211845
Starch-Control     0.043666667 0.010483458 0.07684988 0.0160523
```

DP531

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP531.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.000776 0.0003880   1.295 0.341
Residuals    6 0.001798 0.0002997
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                    diff         lwr        upr     p adj
```

```
Control-Cellulose -0.016 -0.05936784 0.02736784 0.5311137
Starch-Cellulose   0.006 -0.03736784 0.04936784 0.9069274
Starch-Control     0.022 -0.02136784 0.06536784 0.3326752
```

DP611

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP611.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001156 0.0005778   4.635 0.0607.
Residuals    6 0.000748 0.0001247
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff          lwr         upr     p adj
Control-Cellulose -0.026666667 -0.054638681 0.001305348 0.0597015
Starch-Cellulose -0.006666667 -0.034638681 0.021305348 0.7551336
Starch-Control     0.020000000 -0.007972015 0.047972015 0.1508730
```

DP622

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP622.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 2.69e-05 1.344e-05   0.097 0.909
Residuals    6 8.30e-04 1.383e-04
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose 0.000000000 -0.02946538 0.02946538 1.0000000
Starch-Cellulose -0.003666667 -0.03313204 0.02579871 0.9237762
Starch-Control    -0.003666667 -0.03313204 0.02579871 0.9237762
```

DP721

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP721.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0000069 3.440e-06   0.013 0.987
Residuals    6 0.0015793 2.632e-04
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level
```

```
Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose -0.0016666667 -0.04231192 0.03897859 0.9913237
Starch-Cellulose   0.0003333333 -0.04031192 0.04097859 0.9996510
Starch-Control     0.0020000000 -0.03864525 0.04264525 0.9875381
```

DP722

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP722.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0006587 0.0003293   1.454 0.306
Residuals    6 0.0013593 0.0002266
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff         lwr        upr     p adj
Control-Cellulose -0.020666667 -0.05837488 0.01704154 0.2864078
Starch-Cellulose -0.007333333 -0.04504154 0.03037488 0.8269784
Starch-Control     0.013333333 -0.02437488 0.05104154 0.5565230
```

DP81

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP81.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.003067 0.0015334   12.81 0.00683 **
Residuals    6 0.000718 0.0001197
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff          lwr         upr     p adj
Control-Cellulose -0.04366667 -0.071072005 -0.01626133 0.0065593
Starch-Cellulose -0.01166667 -0.039072005 0.01573867 0.4423575
Starch-Control     0.03200000 0.004594662 0.05940534 0.0269518
```

DP811

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP811.csv", sep = ",", header = T)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
```

```
Treatment    2 0.002246 0.0011231    4.59 0.0617.
Residuals    6 0.001468 0.0002447
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr         upr     p adj
Control-Cellulose -0.03800000 -0.07718645 0.001186455 0.0560838
Starch-Cellulose -0.02533333 -0.06451979 0.013853121 0.1969898
Starch-Control     0.01266667 -0.02651979 0.051853121 0.6080825
```

DP813

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP813.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0004329 0.0002164   1.293 0.341
Residuals    6 0.0010040 0.0001673
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                           diff         lwr        upr     p adj
Control-Cellulose -0.015333333 -0.04774041 0.01707374 0.3758199
Starch-Cellulose -0.014000000 -0.04640707 0.01840707 0.4330999
Starch-Control     0.001333333 -0.03107374 0.03374041 0.9912655
```

DP821

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP821.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001540 0.0007698   3.454    0.1
Residuals    6 0.001337 0.0002229
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr         upr     p adj
Control-Cellulose -0.03200000 -0.06940182 0.005401824 0.0870646
Starch-Cellulose -0.01733333 -0.05473516 0.020068491 0.3888277
Starch-Control     0.01466667 -0.02273516 0.052068491 0.4937587
```

DP83

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP83.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value   Pr(>F)
Treatment    2 0.006122 0.0030608   31.92 0.000634 ***
Residuals    6 0.000575 0.0000959
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff         lwr         upr     p adj
Control-Cellulose -0.02233333 -0.04686532 0.002198653 0.0704070
Starch-Cellulose   0.04066667 0.01613468 0.065198653 0.0054022
Starch-Control     0.06300000 0.03846801 0.087531986 0.0005421
```

DP91

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("DP91.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df   Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.001930 0.0009648    2.95 0.128
Residuals    6 0.001962 0.0003270
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                         diff         lwr        upr     p adj
Control-Cellulose 0.004333333 -0.04096920 0.04963586 0.9539858
Starch-Cellulose 0.033000000 -0.01230253 0.07830253 0.1431465
Starch-Control    0.028666667 -0.01663586 0.07396920 0.2076885
```

IDC21

```
> setwd ("C:\\Users\\Nonduh\\Desktop\\CHAPTER 4 STATISTICS")
> ft<-read.csv ("IDC21.csv", sep = ",", header = T)
> fta<-aov (Biomass~Treatment, data = ft)
> summary(fta)
            Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment    2 0.0001529 7.644e-05   0.537   0.61
Residuals    6 0.0008540 1.423e-04
> TukeyHSD (fta, conf. level = 0.95)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov (formula = Biomass ~ Treatment, data = ft)

$`Treatment`
                          diff         lwr        upr     p adj
Control-Cellulose -0.009333333 -0.03922168 0.02055501 0.6270274
```

```
Starch-Cellulose -0.008000000 -0.03788835 0.02188835 0.7046875
Starch-Control     0.001333333 -0.02855501 0.03122168 0.9897419
```