-ERICOID MYCORRHIZAL FUNGI AND POTENTIAL FOR INOCULATION OF COMMERCIAL BERRY SPECIES (*Vaccinium corymbosium* L.)

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ABSTRACT

Ericaceous plants are the richest growth form of the fynbos vegetation of South Africa. The fynbos is characterized by highly leached acidic soils, low mineral nutrients and climatically it is a winter rainfall and dry summer region. Ericoid mycorrhizal fungi associate with *Erica* species enhancing their ability to access essential nutrients for survival under unfavourable growth conditions. The aim of this study was to select local Ericaceae plant species and to isolate, identify and characterize the ericoid endophytes and assess these isolates as potential inocula for commercial berry species. Two ericaceous plants *Erica cerinthoides* L. and *Erica demmissa* Klotzsch ex Benth. were identified from the Mountain Drive area of Grahamstown, Eastern Cape.

Root staining was used to confirm the mycorrhizal status of both plants. Hyphal coils typical of ericoid association were observed within the epidermal cells of the hair roots under a light microscope. The endophytes were successfully isolated in pure culture on 2% malt extract agar (MEA) and modified Fontana medium. Cultural morphology and microscopy were used for initial identification. Two slow growing isolates were selected. These isolates were further subjected to molecular identification; extracted DNA was amplified using ITS1 and ITS4 fungal primers. The rDNA gene internal transcriber spacer (ITS) was then sequenced and analyzed by comparison to sequences in the GenBank. On the basis of percentage sequence identity *Lachnum* Retz. species and *Cadophora* Lagerb. & Melin species were identified as the ericoid endophytes of *E. cerinthoides* and *E. demmissa* respectively.

The optimum growth parameters of the fungal isolates were determined in 2% MEA incubated at varying temperatures and pH. It was established that both species had optimum growth at 27^{o}C and pH 5. The Ericaceae species are sometimes found in metal contaminated sites were ericoid fungi have been proved to alleviate toxicity of their host. The fungal isolates were grown in increasing concentration of Cu^{2+} and Zn^{2+} in 2% MEA. The growth of *Lachnum* species decreased with increasing Zn^{2+} ions above 2.7 mM while *Cadophora* species showed a change in morphology and also decreased in growth with increased ion concentration. However there were no significant differences recorded in the growth of *Cadophora* and *Lachnum* species on increasing Cu^{2+} concentration.

Lachnum and *Cadophora* isolates were formulated into a semi solid inoculum and inoculated onto micropropagated *Vaccinni corymbosum* L. plantlets of 5 different varieties. Colonization was low for all varieties, Elliott and Brightwell varieties recorded the highest colonization of 35% and 31% respectively. *Lachnum* species infected roots showed potential ericoid structures while the *Cadophora* inoculated plantlets had hyphal coils within the cortical cells typical of ericoid mycorrhizas. Inoculation significantly enhanced the shoot growth of Brightwell and Elliott varieties. The Chandler variety inoculated with *Lachnum* species showed improved shoot dry weight. The Bluecrop and Elliott varieties inoculated with *Cadophora* and *Lachnum* accumulated more root biomass. All inoculated Bluecrop plantlets had an improved canopy growth index. Brightwell plantlets inoculated with *Lachnum* species also had an enhanced canopy growth index. The growth responses were variable within varieties and between varieties. Treatments with the *Cadophora* and *Lachnum* have shown potential in the promotion of growth of the Blueberry species. The findings indicate the need to conduct trials under conditions which simulate the commercial growth conditions so as explore the optimum potential of the isolates.

This thesis is dedicated to my husband and my parents who have been a source of inspiration and have shown great faith in me throughout my studies.

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LIST OF ABBREVIATIONS

°C	degrees celcius
µl	microlitre
Al	aluminium
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
Bp	base pairs
Cd	cadmiun
cm	centimeter
Cu	copper
DGGE	Denaturating Gradient Gel Electrophoresis
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribose Nucleotide Phosphate
e.g.	for example
ERM	ericoid mycorrhizal
Fe	iron
g	gram
GMO	genetically modified organisms
h	hour
LSU	Large sub unit
Mn	manganese
mM	millimolar

N	nitrogen
NCBI	National Centre for Biotechnology Information
P	phosphorus
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
s	seconds
SABPA	South African Berry Producers Association
SD	standard deviation
Tm	melting temperature
Zn	zinc

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Mycorrhizal associations are a symbiotic relationship that occurs between plant roots and soil fungi (Smith and Read, 2009). This relationship generally entails improved uptake of nutrients mediated by the fungi in exchange for photosynthetically fixed carbon, proteins and other organic nutrients from the plant roots, essential for the completion of the fungal life cycle. The fungal mycelium extends through and beyond the nutrition depletion zone area that surrounds the root system in search of nutrients (Goldbold 2004; Novero et al., 2009). Approximately 90% of all terrestrial plant species are involved in mycorrhizal associations (Brundrett, 2004).

The mycorrhizal status of the plants can be microscopically determined and some mycorrhizal fungi can be isolated and cultivated in a nutrient medium and re-inoculated on to the host plant under semi-natural conditions. Establishment of the relationship can then be microscopically confirmed (Norris et al., 1991). Mycorrhizal plants are classified into two distinct groups, facultative and obligate with regard to their dependency on their mycorrhizal relations. Facultative mycorrhizal plants do not sorely rely on the association for nutrient supply and survival. They are able to absorb essential nutrients when available in abundance in the soil. Hence, the strength of the association is highly determined by the water and nutrient status of the soil. Obligate mycorrhizal plants are entirely dependent on the mycorrhizal association for their mineral nutrient supply and survival as these plants usually occur in regions with harsh edaphic and environmental conditions which do not favour plant growth and establishment. The plant species in the order Ericales are an example of obligate mycorrhizal plants (Brundrett, 2004).

The minority of the universal plant population exhibits poor or no mycorrhizal colonization at all and are referred to as non-mycorrhizal plants. These plant species have roots that may contain, or secrete alkaloids, cyanogenic glucosinolates and other antifungal compounds into the soil which may inhibit fungal activity. These families include Chenopodiaceae, Cruciferaceae and Rosaceae (Brundrett, 2004; Mitchell and Gibson, 2006). Some non mycorrhizal plants have a range of adaptive features which enable them to grow and reproduce successfully in the absence of the mycorrhiza. An example is the production of organic acids by the plant root system that aids solubilization of phosphate while other plants have developed long fine roots with even longer hair roots, which exploit immobile soil nutrients in a similar manner as external hyphae in mycorrhizal plants. Although not very common mycorrhizal relations are not always exclusively mutually beneficial, host responses ranging from positive to negative can be observed. The fungal partner can act as commensals, necrotrophs or antagonists of the host depending on the lifespan of the association. On the other hand, the fungi can also be exploited by the host without any apparent benefit in return. An example is mycoheterotrophy which occurs in orchid mycorrhizas were the mutualistic symbiosis shifts towards the exploitative use of the mycorrhizal fungi by plants that have lost their photosynthetic nature (Smith and Read, 2009). Since the discovery of mycorrhizal associations in 1855, extensive research has been conducted including the inclusion of mycorrhizal technology in agriculture, horticulture and agro-forestry in-order to improve plant welfare and productivity.

1.2 Classification of mycorrhizal associations

Mycorrhizal associations are differentiated and classified on the basis of the structural characteristics of the symbiotic boundaries and taxonomic identity of the symbionts. Different plant species associate with certain soil fungi in a particular manner which results in specific mycorrhizal associations (Johansson, 2001). According to Straker (1996) mycorrhizal relations vary mainly due to the biochemical foundation of specificity and the unique biology of the fungal partner under particular ecological conditions. Classifications based on functional criteria are not ideal as it can only successfully distinguish between balanced and exploitative mycorrhizal association.

There are two main classes of mycorrhizal associations namely ectomycorrhiza and endomycorrhiza. These are differentiated by the interaction of the plant root and the external hyphal structures. In ectomycorrhizas the fungal hyphae surrounds the roots with a hyphal sheathing structure and grow between root cortical cells to form the ‘Hartig net’ while endomycorrhizal relations are characterized by hyphal penetration of the cell wall and invagination of the cell membrane to form intracellular structures such as arbuscules and coils (Raven et al., 1999; Smith and Read, 2009). Endomycorrhizas have further been divided into arbuscular, ericoid, monotropoid, arbutoid and ectendomycorrhizas. Arbutoid, ericoid and monotropoid are also classified as ericaceous mycorrhizas as they exclusively occur in the Ericaceae host family (Wang and Qui, 2006).

There is however great controversy regarding the classification of the ectendomycorrhizas as some researchers classify it under ectomycorrhiza or endomycorrhiza both on the basis of highly identical characteristic features they share (Setaro et al., 2006). In a review by Wang and Qui (2006) ectoendomycorrhizas are described as an intermediary mycorrhizal behavior between ectomycorrhizas and the ericaceous mycorrhizas. This is because both the ectomycorrhizas and ectendomycorrhizas are specifically found in the basal linkages of Ericaceae, Monotropoideae and Arbutoideae family. The former is characterized by a sheathing structure, a simple 'Hartig net' and intracellular structures resembling pegs while the latter has coils (Smith and Read, 2009). Fig. 1.1 shows the distribution of all the mycorrhizal types among the known terrestrial vegetation according to a survey conducted by Brundrett (2009).

Arbuscular mycorrhizas are the most common as they occur in an estimated 72.6% of all terrestrial plants and are of great economic importance. There is however 7.9% of angiosperms which could not be statistically classified as non-mycorrhizal or arbuscular mycorrhizas with certainty (Brundrett, 2009). This is because some plant species develop a "Paris" type of arbuscular mycorrhiza which lacks well developed systems of intracellular hyphae and do not always form arbuscles (Read, 2000). Non-mycorrhizal plants are believed to have evolved from plants families that engaged in arbuscular mycorrhiza association. The ability of these plants to acquire adequate nutrients through their hair roots led to genetic changes which hindered mycorrhizal formation (Wang and Qui, 2006). Ericoid mycorrhizas represent an association which evolved as a means of countering edaphic stress encountered by the Ericaceae plants in their habitats. Ericoid mycorrhizas occur in about 1.4% of all terrestrial vegetation and are the third most important mycorrhizal association.

1.3 INTRODUCTION

1.3.1 Ericoid mycorrhizal associations

Ericoid mycorrhizas are a symbiotic relationship between ericaceous plant roots and soil fungi principally of the ascomycetous family (Midgely et al., 2004; Perotto et al., 2000 Smith and Read, 2009).

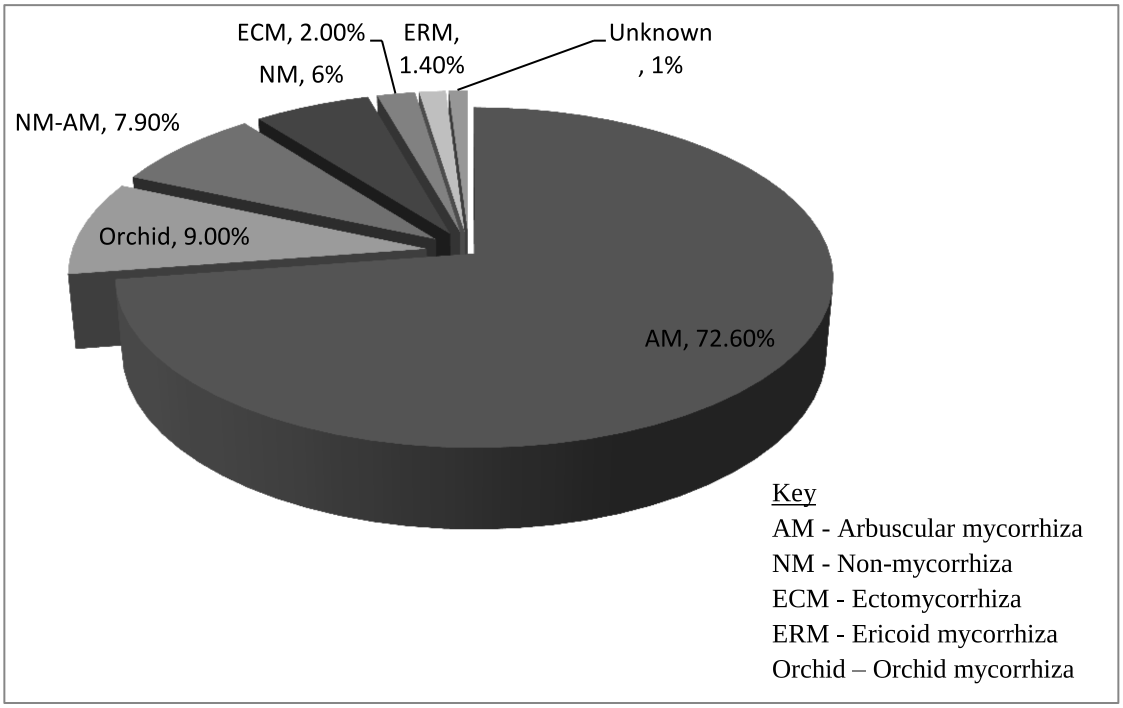

Figure 1.1 Percentage distribution of mycorrhizal typologies in terrestrial plants as reported in literature. Adapted from Brundrett (2009).

The association is particularly confined to acidic, nutrient poor soils and areas rich in recalcitrant organic matter (Smith and Read, 2009). These unfavourable soil conditions have arisen due to very low rates of evapotranspiration which hinder decomposition of plant matter (Cairney and Burke, 1998). These stressful growing conditions both climatic and edaphic has led to the development of sclerophylly in ericaceous plants, a condition were plants develop tough foliages consisting of tissue rich in lignin and cellulose but deficient in N and P. The ericoid mycorrhizal system is believed to have evolved monophyletically as a means of conserving minerals in its sclerophyllous host associates (Brundrett, 2002; Setaro et al., 2006; Straker 1996,).

Ericoid mycorrhizal associations are characterized by distinctive coiling of fungal hyphae in the individual cortical cells of thin roots which lack cortical parenchyma and are referred to as 'hair roots' of the host plant (Bergero et al., 2000). These distinctive fungal coils are crucial for the identification of ericoid mycorrhiza and the mycorrhizal roots formed by Ericales are highly identical throughout the family (Norris et al., 1991; Smith and Read, 2009). The intercellular hyphal connections between independently colonized cells enable each hair root to accommodate several fungi (Perotto et al., 1996). The roots of the Ericaceae host plants grow vertically in the soil giving rise to secondary roots, these further branch into finer roots in which primary colonization by the fungi takes place. This occurs in the upper 10 cm of the soil profile as the host plants are shallow rooted. The hair roots are made up of an inner stele which is surrounded by a cortex of layers namely the inner endodermis and outer hypodermis and only a single layer of epidermal cells forms the outer surface of hair roots having a diameter of 40-100 µl, Fig. 1.2 (BassiriRad, 2005; Read, 1997; Smith and Read, 2009).

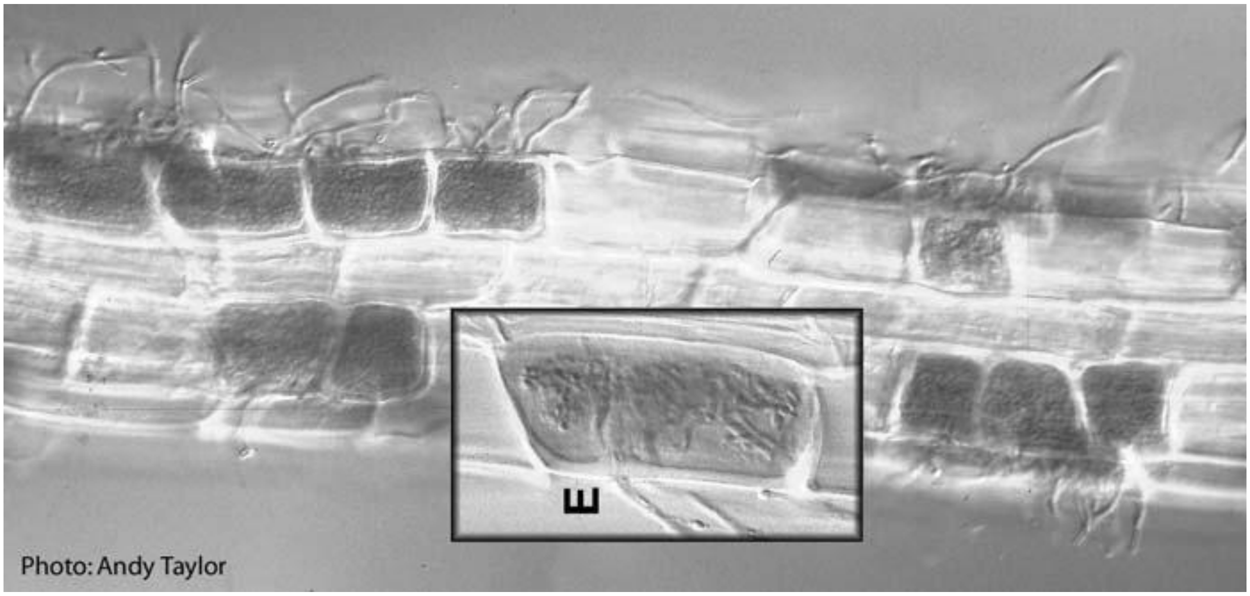

Figure 1.2 The structure of the hair roots of *Vaccinium vitis-idea* colonized by ericoid fungi *Hymenoscyphus erica*. Hyphal coils occupying most of the epidermal cells. The enlarged insert highlights hyphal penetration through the cell wall. (Photo by Andy Taylor. Adapted from BassiriRad, 2005).

Hair roots are minute delicate structures confined to the upper layer of the soil profile which are highly susceptible to drying out. There is however uncertainty whether they are of seasonal occurrence or always present in the soil. A survey conducted on *Woollsia pungens* in South Wales, Australia established that the hair roots are always present in the soil regardless of the season. Abundance is however variable, 70% was observed in spring and summer while only 50% was observed in winter (Kemp et al., 2003). These research findings however differ from the observations made by Hutton et al. (1994) in Epacridaceae species of South West Australia, were greatest hair root abundance was observed in winter and spring with no hair roots occurring during the summer season. These observations are likely to be relevant to areas that experience a Mediterranean climate were the hair roots die back as the soil dries out during the dry summers (Smith and Read, 2009).

According to ultra-structural studies the sequence of events that occur during the life cycle of ericoid mycorrhizal associations are mainly initiation, establishment and degeneration (Duddridge and Read, 1982). The source of fungi which initiates colonization is not certain; some researchers believe that the soil contains enough fungal propagules essential for mycorrhizal initiation because most ascomycetous ascospores can withstand pasteurization. Ascospores can survive adverse soil conditions hence their continued presence in the soil. It has been established in *Woollsia pungens* occurring in the dry summer region of South West Australia that the colonized epidermal cells develop thick walls which protect the enclosed hyphae during the dry period, thus allowing the fungi to re-infect when the hair roots are formed again on the onset of autumn rains in the new season (Smith and Read, 2009). Ascospore germination and fungal re- growth can be stimulated by root exudates such as carbohydrates and organic acids (Huang et al., 2002).

The ericoid fungus comprises numerous micro-fibrils which contain polysaccharides on the cell wall; these attach the fungi to the plant roots forming a loose network around the hair root cells. This is regarded as the first step in colonization as the fungi and hair roots are in close proximity. The fungus then releases degrading hydrolytic enzymes such as cellulase and pectinase which breakdown the epidermal cell walls thus allowing the fungi to penetrate the hair root cells. The enzymatic activity plays a critical role in colonization regardless of the taxonomic position or geographic origin of the fungi. This is referred to as the initiation stage (Perotto et al., 1996).

The establishment stage is characterized by colonization of the epidermal cells by the fungal hyphae forming coils within individual cells (BassiriRad, 2005). It is the most important stage as it distinguishes the ericoid association from other types of mycorrhizas and promotes nutrient exchange. Approximately 90% of the root volume of the host plant can be fungal tissue and the external mycelium does not extend more than 1cm from the surface of the hair roots (BassiriRad, 2005). Colonization by ericoid fungi is strictly restricted to epidermal cells hence the apical region of the hair root behind the meristem remains uncolonized until cells differentiate and mature (Jackson and Mason, 1984). Colonization has been observed after 3 weeks in irradiated soil and 4 weeks in natural soil. The functional life of the colonized epidermal cell is short lasting not more than 11 weeks in natural soil and 8 weeks in irradiated soil. Nutrient transfer should therefore occur in the short period when both partners still have structural integrity (Duddridge and Read, 1982). The short life of the hair roots means that the ericoid fungi need to continuously re-establish the association as the root continues to grow. This is achieved through the degradation of older colonized cells as the root grows and subsequent colonization of the new meristem cells by the fungi.

After the functional life of the ericoid mycorrhiza has elapsed the degeneration process starts by disintegration of host organelles, cytoplasm and cells. The fungal structures then degenerate after the collapse of the host cytoplasm by becoming vacuolated before the contents become devoid. Subsequently, death of the host cell then occurs. The fungi will then establish on other new hair roots of the host. The fungus plays the role of a sapro-parasitic partner during a part of its life cycle, as it obtains nutrients from dead plant cell material and also depends upon the hair roots for its nutrient requirements for growth. The pattern of colonization indicates that most root epidermal cells when observed under a microscope are dead and devoid of contents (Fig. 1.3) (Janska and Vosatka, 2002).

Ericoid mycorrhizas have been regarded as a highly specific association because it is limited to the host plants in the order Ericales and a small group of ascomycetous fungi as mycobionts (Setaro et al., 2006; Straker, 1996). However cumulative data from continuous research over the years has revealed that ericoid endophytes have been isolated from plant roots of distant taxa while Ericales root systems can have several fungal taxa in their roots as well (Cairney and Meharg, 2003; Perrotto et al., 1996; Williams et al., 2004). This means that the taxonomic range of both host and fungal partner is not as restricted as previously understood (Brundrett 2004, Perotto et al., 2002; Straker, 1996).

It has also been postulated that in communities were ericoid mycorrhiza coexists with deeper root systems; different mycorrhizal taxa may offer different functional benefits to the host plant and are likely to influence functioning of the symbiosis on an individual plant basis (Midgely et al., 2004).

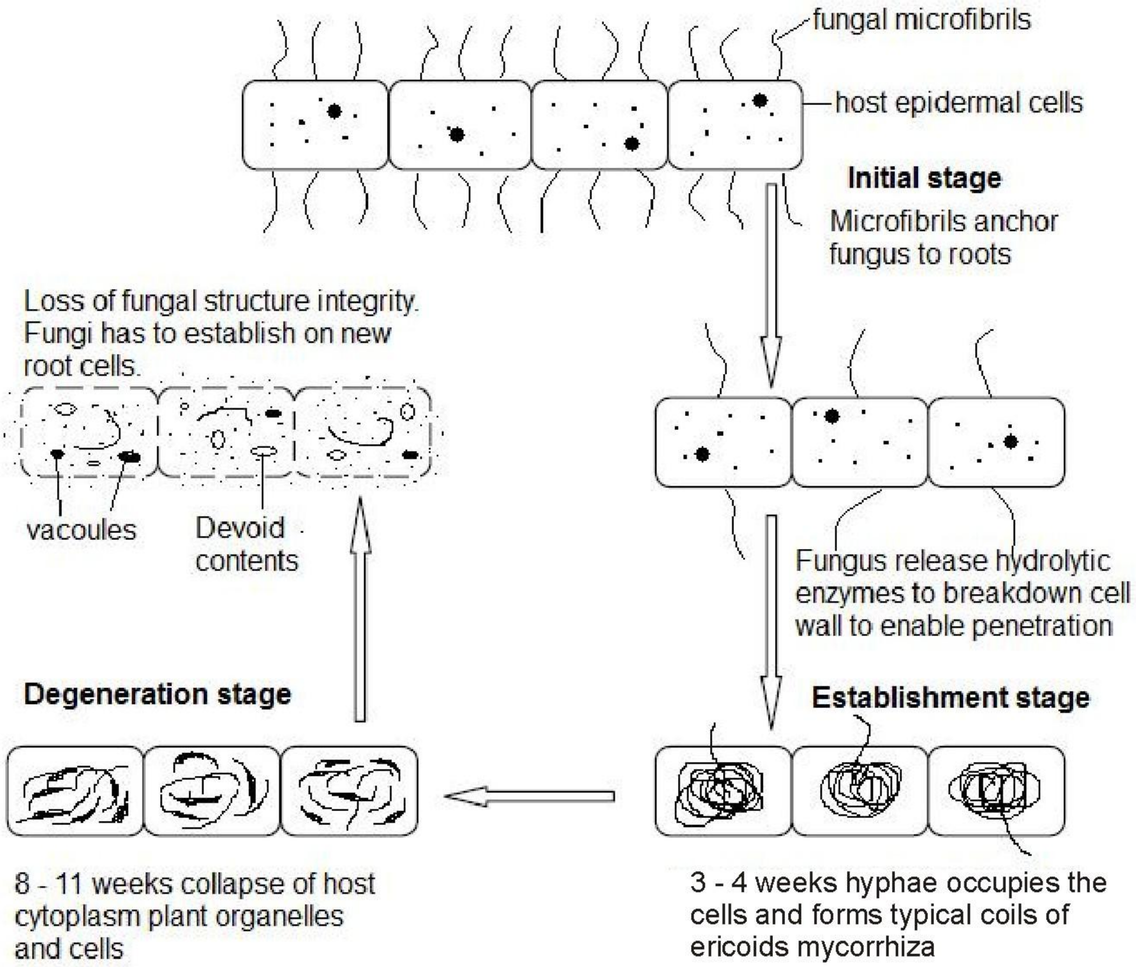

Figure 1.3 Series of events that occur during the formation of the ericoid mycorrhizal association in the Ericaceae hair roots. The stages include initiation, establishment and degeneration and the association lasts up to 11 weeks**.**

Studies have shown that a single ericoid mycorrhiza fungal taxon, usually endophyte groups isolated from a specific field site may differ from those isolated from the same species in another location. This was evident after comparison of ericoid symbionts from *Calluna vulgaris* found in two different locations. The natural heathland sites in south-west England had *Rhyzoscyphus erica* identified as their symbionts while the *Calluna* of the heathland site in northern Italy had *Oidiodendron maius* isolated from their roots (Perotto et al., 1996; Sharples et al., 2001).

1.3.2 Host plants of ericoid mycorrhizal associations

Mycorrhizas are indeed very important for Ericaceae plants and have been studied widely. These plants are found locally in most parts of the world as they grow naturally in areas experiencing unfavorable edaphic and climatic conditions to sustain usual plant growth. Approximately 125 genera and 4500 species are found globally (Luteyn, 2002). The soils are characterized by several edaphic stresses such as low pH; low available nutrients, readily available metals highly are leached. Climatically they experience high and low temperature fluctuations (Cairney and Meharg, 2003). Ericaceae plant species belong to a restricted group of members which include the sub-families Pyrolaceae, Epacridaceae, Ericaceae and Monotropaceae all in the order Ericales. The plants belonging to the Ericaceae family are the most important host taxon for the ericoid symbiosis (Perotto et al., 2001; Straker, 1996).

Ericaceae is a very large family of flowering dicotyledonous shrubs (Magnoliopyta). The most common genera are *Rhododendron, Erica* (Heath), *Calluna* (Heather) and *Vaccinium* (Blueberries; Cranberries) (BassiriRad, 2005; Dod and Bateman, 1979; Smith and Read, 2009,). The *Calluna* were the pioneer ericoid host to be identified when *R. erica* and its anamorph *Scytalidium vaccinii* were recorded. However some families in the Ericales order such as Monotropaceae and Arbutoidaceae do not form ericoid mycorrhizal association but monotropoid and arbutoid mycorrhizas (Dod and Bateman, 1979). In Australia the Epacridaceae family has been established to be a common ericoid fungal host plant as several ericoid endophytes such as *R. erica* have been continuously isolated from plant roots (Chambers et al., 2000). Morphologically the ericoid host plants consist of a woody stem with rolled under edges, a vascular bundle and a layer of cortical epidermal cells. The hair roots are not well defined as in other plant species in their family (Smith and Read, 2009). Most ericoids store their seed underground and may depend on fire to break dormancy to allow germination and ants for seed dispersal. In South Africa the common *Erica* plant species include *E hispidula*, *E. mauritanica*, *E. calcaveophila*, *E. demmissa*, *E. hebdomadalis*, *E. roseoloba* and *E. cerinthoides* among others (Oliver and Oliver, 1998; Straker and Mitchell, 1985). The ericoid host plants contribute between 10-20% of their photosynthate towards the formation, maintenance and function of mycorrhizal structures (Marschner and Dell., 1994).

The use of molecular techniques in the identification of mycorrhizal fungi has enabled researchers to acknowledge the diversity of the ericoid host taxon as well as the diversity of the fungi with respect to groups and number of fungal taxa involved (Grelet et al., 2010; Read, 2000). Vra˚lstad et al. (2000) isolated mycorrhizal endophytes from *Piceirhiza bicolorata* and ectomycorrhiza forming plants which included *Picea abies*, *Pinus sylvestris*, *Betula pubescences*, *Populus tremula*, *Quercus robur* and *Salix phylicifolia*. Some of the fungal genotypes showed up to 95% sequence identity to *R. erica*. It was hypothesized that ericoid and ectomycorrhizal plants share some fungal mycobionts within the *R. erica* aggregate. In another molecular study isolates with a 99% sequence identity to *R. erica* were isolated from the leafy liverwort *Cephaloziella exiliflora* (Chambers et al., 1999). This information broadened the known host range of the ericoid fungi but no mycorrhizal resynthesis trials were conducted to test the ability of these isolates to form ericoid structures (Bergero et al., 2000; Read 2000).

In the Mediterranean region Bergero et al. (2000), isolated genetically identical endophytes from coexisting *Quercus ilex* and *Erica arborea*. The resynthesis experiments illustrated that the fungi from the roots of *Q. ilex* formed ericoid structures when inoculated to *E. arborea*. No true ectomychorhizas were formed by *Q.ilex* after inoculation was done with isolates from both *Q. ilex* and *E. arborea*. These findings further established the hypothesis from prior studies that ericoid fungi associate with ectomycorrhizal and ericoid host plants. Apparently the host range of the ericoid fungi spreads to taxa that coexist with the ericaceous plants in nature. Alternative hosts have been confirmed in the Boreal and Mediterranean regions were Ericales are understorey vegetation of the dominant ectomycorrhizal trees or ecologically growing together (Chambers et al., 1999; Humbleton et al., 1999; KjØller et al., 2010; Selosse et al., 2007; Upson et al., 2007; Villareal- Ruiz et al., 2004; Walker et al., 2008, Zijlstra et al., 2005). In recent studies *R. erica* together with *Lachnum* species were also detected on ectomycorrhizal root tips of two *Kobresia* species in the alpine meadow in China (Gao and Yang, 2010).

1.3.3 Taxonomy of ericoid fungi

Ericoid mycorrhizal fungi are a group of ascomycetous fungi that form symbiotic associations with plants in the order Ericales. Globally, there is great diversity in the taxa of the ericoid mycorrhizal fungi and only a few have been identified (Berch et al., 2002; Chambers and Cairney, 2000; Williams et al., 2004). Fungi are sensitive to physical, chemical and soil biological conditions and ericoid fungi exhibit tolerance to harsh soil and environmental conditions (Classen et al., 1996). Using both molecular and cultural techniques a variety of ericoid fungi have been identified, which include *Rhizoscyphus erica* and its anamorph *Scatalidiun vaccinii*, *Oidiodendron* species and their teleomorphs of the Gymnoascaceae and Myxothricacaeae families. *Acremonium strictum* and *Phialophora finlandica* have also been identified from *Gaultheria shallon* in Canada (Berch et al., 2002; Hambleton et al., 1999). It has been established that several ericoid fungal taxa can interact with a single ericaceous plant root in a particular area (Chambers et al., 2000; Perotto et al., 1996; Williams et al, 2004). Although recently identified in most ericaceous plants, confirmation of basidiomycetes as ericoid symbionts has not yet been established as they are yet to be isolated in pure culture.

The first taxa to be identified as Ericaceae host symbionts were Ascomycetes, *Hymenoscyphus erica* and *Oidiodendron maius*. The mycorrhizal status of *H. erica* was determined in pot culture resynthesis trials on the host plant *Calluna vulgaris*, when isolated in pure culture they produced apothecia (Smith and Read, 2009). Isolates obtained from *Rhododendron* and *Calluna* in France produced small apothecia in culture similar to those produced by the English isolates of *H. erica* (Schulz and Bolye, 2005). This enabled its taxonomic placement in the genus *Hymenoscyphus* (Smith and Read, 2009). Cultural and vegetative characteristics were provisionally used to identify sterile endophytes isolated from ericaceous roots as apothecia are not readily produced in culture. To date apothecia have not been observed under field conditions (Cairney and Chambers, 2000; Hambleton et al., 1999).

H. erica is in the order *Leotiales* and broader class *Leotiomycetes*. It comprises of several species with affinities to the *Leotiomycetes* (Smith and Read, 2009; Vra˚lstad et al., 2000). *H. erica* has recently been reclassified to the genus *Rhizoscyphus erica* in order to accommodate the less well known root associated discomycetes *Hymenoscyphus monotropae* Kernan and Finocchio (Humbleton and Sigler, 2005; Smith and Read, 2009). This ericoid endophyte showed up to 24% divergence in sequence comparison to *H. erica* and *S. vaccinni* (Egger and Sigler, 1993; Williams et al., 2004) and was originally isolated from the roots of *Monotropa uniflora* L.

An anamorph of *R. erica*, *Scytalidium vaccinii* which fragments in culture to produce aseptate arthroconidia was identified as an ericoid endophyte on the basis of phenological and genetic structures (Smith and Read, 2009). In an experiment by Hambleton et al. (1999) isolates from *Ledum groenlandicum* in Western Canada produced apothecium in culture when subcultured on Melin's agar and produced arthroconidia on cornmeal agar. These findings confirmed that *R.erica* and *Scytalidium vaccinii* are indeed different forms of one species. Their anamorph-telemorph state was also confirmed through molecular characterization by Egger and Sigler (1993) in sequence comparisons which showed very low divergence of up to 3.5% range which lies within the acceptable variation in genetically diverse species. The use of arthroconidia as a taxonomic factor has not been fully acknowledged as they are not always formed in culture (Berch et al., 2000; Johansson, 2001).

Ericoid fungi of the Hyphomycete genus *Oidiodendron* (Ascomycota, Onygenales) was also one of the first ericoid symbionts to be identified and one of the most frequently isolated fungus from Ericaceae and Epacridaceae roots (Straker, 1996). *Oidiodendron griseum* Robak was the first species to be identified in this order when it was isolated from *Vaccinium angustifoliun* and *Vaccinium corymbosum*. In pure culture the colonies exhibit white colour owing to the presence of abundant arthroconidia that develop in chains at the apex of conidiophores (Schulz and Bolye, 2005). Other fungal species from the genus *Oidiodendron* which formed ericoid mycorrhiza under axenic conditions include *O. maius* Barron, *O. rhodogenum* Robak, *O. chlamydosporicum* Morral, *O. citrinum* Baron, *O. flavum* Szilvinyi, *O. pericoinioides* Morral and *O. scytaloydes* (Domsch et al., 2007). In axenic ericoid mycorrhiza resynthesis trials conducted by Dalpe (1986) *O. griseum, O. rhodogenum* and *O. cerealis* formed ericoid hyphal coil in the roots of *Vaccinium* species. *Oidiodendron* species *O. truncatum* and *O. tenuissimum* did not form any ericoid structures in these trials and to date no evidence has been published to confirm their ericoid status. Therefore the number of *Oidiodendron* species involved in ericoid association is still uncertain both because of misidentifications of collected species and concept of boundaries within this genus (Perotto et al., 2002; Straker, 1996).

Although not frequently isolated from ericaceous plant roots, the teleomorphs of *Oidiodendron* belonging to the *Myxotrichum* and *Byssoascus* genus both in the ascomycetous family Myxotrichaeceae have been reported to form ericoid mycorrhizas *in vitro* with *Vaccinium angustifolium* (Calduch et al., 2007). There are six telemorph species within Myxotrichaeceae having *Oidiodendron* states (Schulz and Bolye, 2005), these include *Myxotrichum setosum* and *Pseudogymnoascus roseus*. An anamorph of *Geomyces (*Onygenales); *Gymnascella dankaliensis* (Perotto et al., 2002) has also never been isolated from ericaceous plants in nature but ericoid structures have been observed *in vitro* with *V. angustifolium* (Vohník et al., 2007). The *Geomyces* is the closest genus to *Oidiodendron* only differentiated by the unpigmented conidiophores (Domsch et al., 2007). Bergero et al. (2000) isolated *G. pannorum* from roots of *Erica arborea*. In resynthesis experiments by Vohník et al. (2007) they formed structures resembling ericoid coils in the roots of *Vaccinium* cuttings grown on peat based media.

A number of fungi which occur in ERM roots are unculturable hence their status and interpretation is difficult. Basidiomycetes of the genus *Sebacina* most notably *S. vermifera* are prominent (Allen et al., 2003; Berch et al., 2002; Bougoure and Cairney, 2005a; Selosse et al., 2007). Sequencing of the LSU fungal DNA obtained from direct amplification and cloning of *Gautheria shallon* roots revealed that a significant proportion (65%) of the roots contained *Sebacina vermifera* (Berch et al., 2002). These findings highlighted the limitations of the culture based methods of identification at the same time revealing the efficiency of direct amplification and sequencing only in the determination of the fungal population in the roots regardless of their ability to form mycorrhizas. No resynthesis experiments have been done to confirm the mycorrhizal forming ability of basidiomycetes because of their inability to grow on culture media. Culture dependent techniques for identification of mycorrhizal fungi cannot be disregarded as they allow host inoculation experiments to confirm mycorrhizal formation.

Septal pores are a diagnostic feature of the order Auriculariales which encompass the *Sebacina* genus (Smith and Read, 2009). The frequent occurrence of basidiomycete fruiting bodies of

Clavaria species particularly *C. argillaceae* in the soil surrounding roots of ericaceous plants growing in the heathlands, horticultural nursery and in pots is evidence that basidiomycetous fungi may be ericoid symbiont (Norris et al., 1991; Smith and Read, 2009). Basidiomycetes are distinguished morphologically by the presence of dolipore septa in adjacent plant cells. Another common *Clavaria* species is *C. oronoensis* (Norris 1991; Straker 1996). Serological, immune-cytochemical and nutrient transfer techniques have proven that *Clavaria* species can form close associations with ericoid roots, however sequence analysis has failed to identify clavaroid genotypes within ericoid roots this has created controversy regarding their mycorrhizal status (Smith and Read, 2009).

Sterile fungi are commonly isolated from the ericaceous surface sterilized roots native to different parts of the world (Bougoure and Cairney, 2005b; Smith and Read, 2009). Hambleton and Singler (2005) isolated fungi with variable colouration in culture ranging from creamy, white to gray, temporarily named variable white taxon (VWT). Phylogenetic analysis has indicated their affinities to the *R. erica* aggregate. On the basis of morphological and ITS-based characterization they were placed in a new anamorphic genus *Meliniomyces*. Some of the common species include *M. vraolstadine, M. variabilis* and *M. bicolor* both found in the *R. erica* aggregate (Humbleton and Sigler, 2005; Vra°lstad et al., 2002a). *M. variabilis* has however been isolated from non Ericaceae families in the cold–temperate regions of the northern hemisphere such as Fagaceae, Orchidaceae, Pinaceae, Cornaceae and Salicaceae (Humbleton and Sigler, 2005). When inoculated on *Rhododendron* species *M. variabilis* did not colonize their roots but formed structures resembling ericoid coils on *Vaccinium* roots (Vohník et al., 2007). This shows that ericoid endophytes have the potential of being selective of their host symbionts within the Ericales.

A fungus in the genus *Cadophora* previously known as *Phialophora* principally identified as symbionts of ectomycorrhizal and ectendomycorrhizal associations has been isolated from ericaceous roots. Bougoure and Cairney, (2005a) identified this taxon from direct amplification of the root rDNA and not in cultural isolates. This may however imply that they are not readily isolated in culture. *Phialopora finlandia* forms ericoid associations with *Vaccinium* species (Smith and Read, 2009) and ectomycorrhizas on *Pinus strobes* L. (Berch et al., 2002). Williams et al. (2004) identified *Phialocephala fortinii* among the fungi isolated from ericaceous plant roots in sites with low pH and high organic matter from Boreal and Alpine, subalpine and Coastal Heathland forests.

Other hyphomycetes which have been identified as ericoid symbionts include *Acremonium strictum* and *Stephanosporium cerealis* (Thum) these form hyphal structures typical of ericoid mycorrhiza with Ericaceae roots (Janska and Vosatka, 2000). A culturable ascomycetous genera *Capronia* species (Chaetothyriales) has been detected molecularly and their functional status is unknown, Smith and Read (2009) reported them to be mycoparasitic. However Allen et al. (2003) confirmed the mycorrhizal status of *Capronia* species *in vitro* where typical ericoid structures were observed in *Gaultheria shallon* root cells.

1.3.4 Dark Septate Endophytes (DSE)

This refers to a poorly defined group of root associated endophytes (Jumpponen and Trappe, 1998; Vra˚lstad et al., 2002). The taxa in this aggregate has close affinities to the Helotiales ascomycetous fungi (Usiku and Narisawa, 2007) which in culture produce darkly pigmented colonies, lacking distinctive reproductive structures with the exception of *Cadophora finlandia* and *Phialocephala fortinii* which sporulate in culture (Mandyam and Jumpponen, 2005). These species have also been regarded as ericoid fungi until recently when they have been identified from the roots of non ericaceous plants. In a study by Hawley et al. (2008) *Phialocephala fortinii* and *H. erica* were identified through direct root amplification and BLAST, from *Pinus patula* in Sabie, South Africa. This implies that the DSE and ericoid fungi can co-occur in particular host host species. Recently Vohník and Albrechtová, (2011) using root morphology established that DSE and ericoid associations can co-occur in *Rhododendron* species.

The DSE fungi are closely associated with ericoid roots but there is need to establish their functional and ecological roles. DSE have a wide distribution and are also commonly found in cold nutrient deficient habitats thus accounting for their continued identification from the Ericales. In a survey conducted by Newsham et al. (2008) several habitats were investigated which include the South African coastal plains, 25 DSE species were identified. In Polar Regions they are more abundant than mycorrhizal fungi. They have been isolated from a variety of hosts which include ectomycorrhizal, ectendomycorrhiza, endomycorrhizal and non mycorrhizal plants

(Villareal-Ruiz, 2004). Narisawa et al. (2007) isolated *P. fortinii*, *C. finlandica* and *M. variabilis* from herbaceous seedlings of barley, Chinese cabbage, egg plant and melon. Typical DSE fungi root colonization is characterized by hyphal wefts around the root surface and root tips and intracellular colonization within cortical cells (Jumpponen and Trappe, 1998; Usiku and Narisawa, 2004). However several root morphologies have been identified in DSE root associations hence making their placement in any category rather impossible.

In colonized ericaceous plant roots looped branched hypha and occasional hyphal coils have been observed. Lightly pigmented coarse coils which do not align to typical DSE characteristics described by Jumpponen and Trappe (1998) were observed in *in-vitro* resynthesis trials of *V. uliginosum* inoculated with *P. fortinii*. In studies by Zhang et al. (2009) similar structures resembling ericoid mycorrhizas between DSE fungi and *Rhododendron* were observed. In an ericoid synthesis trial conducted by Usiku and Narisawa (2004) *Rhododendron obtusum* var. *kaempferi* was inoculated with *Heteroconium chaetospira* and hyphal coils typical of the ericoid association were reported. These findings are not surprising as it has been established that particular fungal isolate including those of mycorrhizas can form different associations depending on the host plants. Monreal et al. (1999) discovered that *Cadophora finlandia* formed ericoid mycorrhizas with *Gautheria shallon* and they formed ectendomycorrhizas and ectomycorhizas on woody hosts.

The role and ecological functions of DSE in their natural habitats are not well understood (Jumpponen, 1998). DSE root associations do resemble mycorrhizal associations in their host effects which range from mutualism-parasitism, but a consensus has not been reached to whether they should be classified as mycorrhizal. According to Harley (1992) mutualism should be the distinguishing attribute for mycorrhizal associations. Experimentally Koch's postulate has been applied to substantiate effects of DSE on host plants (Newsham et al., 2008). DSE can enhance host growth and nutrient acquisition in a manner typical of mycorrhizal associations. Usiku and Narisawa (2007) inoculated Chinese cabbage with *Heteroconium chaetospira* and the results obtained indicted that the fungi availed nitrogen to the host from amino acids in exchange it attained carbon in the form of sucrose from the host. *P. fortinii* the most commonly identified fungi in the DSE aggregate was inoculated on *Larix deciduas* plants and resulted in a positive growth effect by improving the root system development through the production of indole-3-acetic acid (Romenert et al., 2002).

In another trial *Pinus contorta* plants inoculated with *P. fortinii* in glacier fore front soil supplemented with nitrogen showed enhanced phosphorus and nitrogen uptake and an increased biomass in comparison to the non-inoculated control (Jumpponen et al., 1998). This strongly suggests that DSE has a potential role in mineral nutrient uptake. DSE are also capable of mineralizing organic nitrogen thus enabling host plants to utilize proteins as a source of nitrogen. *Deschampsia antarctica* seedlings showed improved growth in the presence of an organic source of nitrogen and DSE in the growth medium (Newsham et al., 2008). In Ericaceae habitats nitrogen is mainly available in organic forms which cannot be readily assimilated by the plants, thus implying that DSE have great potential to contribute to the growth and establishment of its host plants through the supply of mineral nutrients. DSE fungi which have also been identified include *Leptodontidium orchidicola*, *Phialocephala sphaeroides* and *Heteroconium chaetospira* (Narisawa et al, 2007).

1.3.5 Distribution of ericoid mycorrhizal associations in South Africa

There is great diversity in the occurrence of Ericales which are the principal host plants of ericoid fungi (Cairney and Merharg, 2003; Straker, 1996). Ericoid fungi are well established in acidic and nutrient poor environments as diverse as the Heathland of the northern hemisphere, Mediterranean woodlands, tropical forests, the dry plains of Australia, copper sites of Ireland, the UK and the Cape Fynbos of South Africa. The Ericaceae can be found as prominent or understorey vegetation in these habitats. Ericoid mycorrhizas are also found in cloud forests in high altitude (1000 – 3000 m) areas usually characterized by cloud cover such as Central Africa and some parts of Australia (Bougoure and Cairney, 2006).

The genus *Erica* is well represented in southern Africa particularly in the Cape Province were more than 658 species have been identified (Goldblatt and Manning, 2000). In a survey of the mycorrhizal status of the plants growing in the Cape Florist region of South Africa, Allsopp and Stock (1993) revealed that approximately 8% of the 332 fynbos species which were studied showed ericoid mycorrhizas and there were no indigenous ectomycorrhizal species. *Erica* species have the greatest diversity of species in the Cape Peninsula region of South Africa. It comprises of approximately 49% of the endemic vegetation in that area (Helme and Trinder-Smith, 1996). Fig. 1.4 shows the distribution of the range of mycorrhizas found in the Cape lowland vegetations (West Coast Strandveld, West Coast Renosterveld and Sand Plain Lowland Fynbos for mycorrhizas).

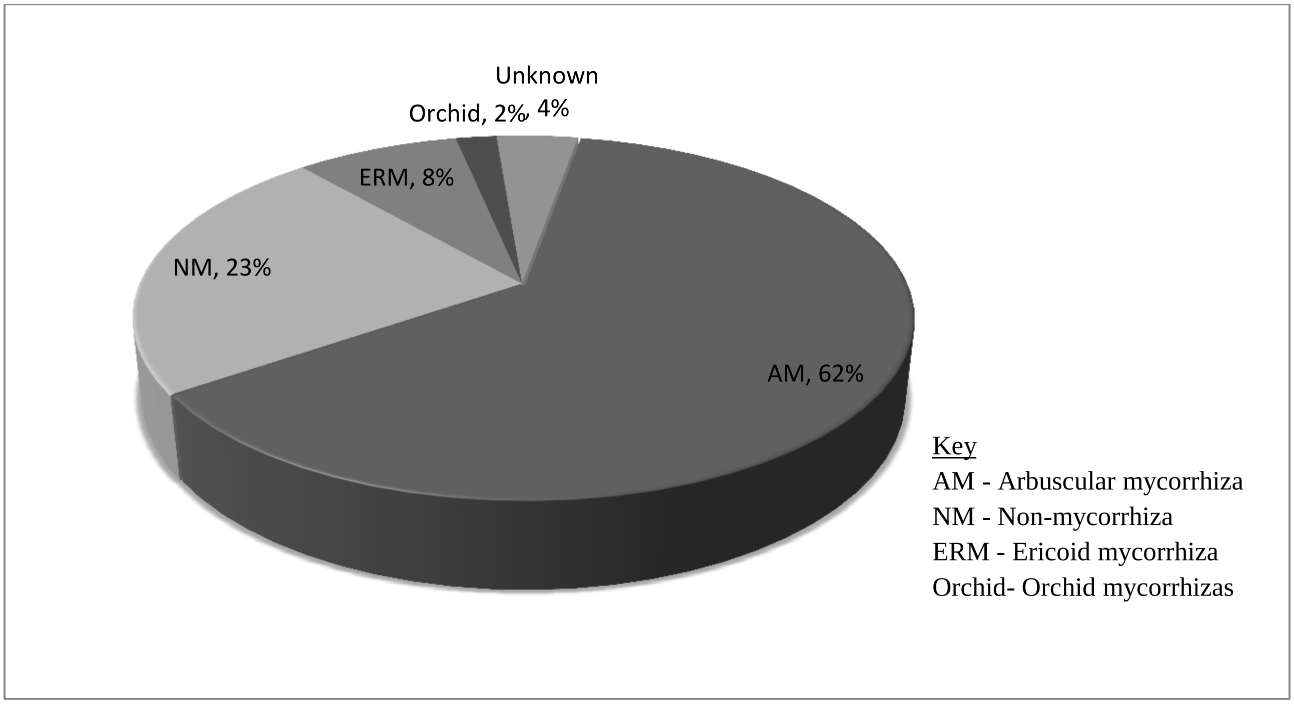

Figure 1.4 Distribution of mycorrhiza typologies compiled from the root assessment of 332 species growing in the Cape lowland vegetation and literature records of mycorrhizas on plants growing in the region. Modified from Allsopp and Stock (1993).

The ericaceous plants are the richest growth form of the fynbos vegetation of South Africa. The fynbos biome experiences climatic conditions resembling a Mediterranean climate of winter rainfall and dry summer regions. The Cape fynbos however specifically shares similar location and climate with the Kwongan of South West Australia (Straker, 1996). The climate of these regions is highly influenced by cold fronts of Antarctica during winters which are also responsible for most of the rainfall. The most notable difference between the two locations is the abundance of the Ericaceae genera in the Cape fynbos while Epacridaceae dominates in the

Kwongan. The vegetation of most South African mountain peaks resemble the fynbos hence explains the unusual mixture of plant types found in these areas.

Identical to the European heaths the fynbos vegetation generally occurs in strongly leached acidic less sandy soils with poor nutrient status, readily available metals, low rates of organic matter decomposition and high temperature fluctuations (Cairney and Meharg, 2003; Oliver and Oliver, 1998; Straker, 1996). The organic matter and total mineral phosphorus and nitrogen content are however lower in the South African soils compared to European soils. These organic forms of nitrogen and phosphorus found in South African soil such as organic phosphates, amino acids, peptides and proteins are high but cannot be readily assimilated by the plants (Leake and Read, 1991).

The fynbos of South Africa also differs from the vegetation of other areas in that it resembles both northern and southern hemisphere areas and has a greater diversity of species. In most communities dominated by Ericaceae, there is often elimination of other plant taxa because the phenolic acids contained in ericaceous leaf litter can be toxic to surrounding plants and fungi, a condition often referred to as ‘toxicity syndrome’. However there are other phenolic compounds such as quinines found in these plants which together with humic acids in the soil bind to decomposer enzymes resultantly slowing down the decomposition of ericaceous leaf litter (Read, 1997). The secretion of proteolytic enzymes by some ericoid fungi enables the plant roots to access organic compounds which are inaccessible to other plants in the vegetation thus conferring a competitive advantage to host plants (Smith and Read, 2009).

1.4 Ericoid mycorrhiza symbiotic benefits

Ericoid fungi have varied functional diversity which is mainly adapted to counter the effects of climatic and edaphic stresses experienced in ericoid habitats. Some of the numerous benefits conferred to the host plants include acquisition of mineral nutrients particularly N and P which are not readily available to the plant roots (Kosola et al., 2007; Smith and Read, 2009; Sokolovski et al., 2002,). The hypha in the ericoid association does not extend far beyond the hair roots unlike other mycorrhizal associations (BassiriRad, 2005), but it produces various extracellular enzymes (Cairney et al., 1998). These break down organic complexes in the soil around the roots into simpler molecules which can be readily assimilated by the roots. This enables the *Erica* plant root to utilize unlikely sources of mineral nutrients such as phosphodiesters and cell wall components such as chitin, lignin and cellulose (Cairney and Burke, 1998). Functional characteristics of ericoid fungi are derived mainly from research on a few strains of *R. erica* hence there is need to broaden research to other ericoid fungi in order to confirm their functional ability (Cairney et al., 2000).

1.4.1 Nitrogen

In ericoid habitats N is limiting, predominantly present in organic forms such as amino acids, peptides and proteins due to moisture stress and poor nutrition retarding organic matter decomposition. Nitrification is low in soils of a pH less than 5.5 which is typical of ericaceous habitats (Midgely et al., 2004). In comparison with other macronutrients N is regarded as a crucial element for the growth and established of the Ericales. Ericoid fungi have been shown to facilitate the access to organic nitrogen to their hosts which are entirely dependent on their mycobiont for the supply of this nutrient (Smith and Read, 2009). *R. erica* produces proteinases which hydrolyses proteins into simpler amino acids which can be absorbed by the plants (Chen et al., 1999). In most soils nitrate is the chief source of N since it has great mobility compared to organic sources of N found in Ericaceae habitats (Kemp et al., 2003).

R. erica makes use of both inorganic and organic sources of nitrogen such as NH_4^+, $NO_3^-$and glutamine (Cairney et al., 2000; Midgely et al., 2004). The utilization of various sources of nitrogen varies between ericoid fungal species and even conspecific fungal isolates (Grelet et al., 2010). Trials conducted by Cairney et al., (2000) showed variation in biomass of different *R. erica* strains, they assimilated inorganic and organic sources of N in the soil at significantly different rates. The isolates having the greatest biomass were abundant in *Calluna vulgaris* of the Devon Great Consols mine compared to those from the Aylesbeare Common site demonstrating that there is natural selection of processes at work.

In a study conducted by Stribley and Read (1976), heathland soils comprising of mycorrhizal inoculated and uninoculated plants were treated with $^{15}NH_4$. The results revealed enhanced plant growth as well as high yields of N content in mycorrhizal plants, however the ^{15}N was more abundant in non-mycorrhizal plants which led to the conclusion that ericoid fungi facilitated the attainment of alternative sources of N such as amino acids, peptides and proteins by the host plant (Yang et al., 2002). Subsequent work by various researchers have confirmed these finding in a series of experiments involving growing mycorrhizal and non-mycorrhizal plants in media amended with various amino acids as a sole N sources (Whittaker and Cairney, 2001,). In most scenarios mycorrhizal plants showed improved growth and higher N content in comparison with the non-mycorrhizal plants. However ericoid endophytes such as *Rhodothamnus chamaecistus* found in alkaline soils and ericoid fungi isolated from the Cape *Erica* plants of South Africa have been deemed least effective in accessing N from organic sources (Mitchell and Gibson, 2006).

Leake and Read (1991) inoculated a broad range of ericoid fungi on *C. vulgaris* seedlings and grew them on medium with protein as the sole source of N. The isolated *R. erica* and *S. vaccinni* significantly enhanced growth of the host plant because they released carboxy-proteinase enzymes which catalysed protein into constituent amino acids. These were subsequently absorbed by the fungi and assimilated by the plants. The enzyme also has an optimum pH range of between 4 – 5 for production and 2 – 3 for enzyme activity. This indicates that the enzyme is well adapted to low pH soil environments were ericaceous plants dominate (Norris et al., 1991).

Polymers such as chitin, cellulose and lignin have been converted into an alternative source of nitrogen for the host plant through enzyme activity and in exchange the fungi acquires carbon from the moribund plant cell wall material in the growth medium (Cairney and Burke, 1998; Scagel and Yang, 2005). This ericoid fungal characteristic enables it to continue to exist as saprotrophic fungi in the absence of the host plant.

1.4.2 Phosphorus

Phosphorus availability in ericaceous habitats are generally low because under acidic conditions the element is firmly bound hence plants are dependent on ericoid fungi for phosphorus nutrition (Gibson and Mitchell, 2004; Mitchell and Gibson, 2006). In soils which have low concentration of phosphorus, the ericoid mycorrhizas enhance the uptake of P by passive diffusion. Polyphosphates are formed within the hyphae which maintain a low internal phosphate concentration (Howarth, 2001).

In ericoid habitats P is usually available in organic forms such as is phosphomonoesters e.g. inositol hexaphosphate and phosphodiesters (Cairney and Burke, 1998; Chen et al., 1999; Midgely et al, 2004). Metal-bound forms of phosphate such as strengite ($FePO_{4.}H_2O$) and variscite ($AlPO_{4.}2H_2O$) commonly found in acidic soils together with inorganic metal-phosphates can be important sources of phosphate under ideal conditions as they are not readily available to the plant (Gibson and Mitchell, 2004). It has been established that ericoid fungi posses the ability to solubilize metal phosphate thereby availing phosphate to the host plant (Straker and Mitchell, 1985). The fungal partner excretes extracellular acid phosphatases which facilitates the degradation of organic complexes into a phosphorus form which can readily be assimilated by the plant. An example is the release of phosphomonoesterase and phosphodiesterase enzymes by *R. erica.* Phosphodiesterase enzymes break down phosphodiesters found in DNA and other nucleic acids of moribund plant tissues thus providing an alternative valuable source of P (Cairney and Burke, 1998).

Ericoid endophytes *H. erica* and *O. maius* are capable of solubilizing metal phosphates such as phosphate hydroxyapatite ($Ca_5(PO_4)_3OH$), Zinc oxide and Zinc phosphate $Zn_3(PO4)_2$) thus making P available to the host plant (Martino et al., 2003; Mitchell and Gibson, 2006). The ability of ericoid fungi to solubilize metal phosphate is determined by several factors which include vulnerability of the compound to chemical weathering, pH of the growth medium as well as the nutrient status of the soil particularly C and N. It has been established that the optimum solubilization of $ZnPO_4$ and $CaPO_4$ occurs under high glucose levels (>600 Mm) in the substrate (Gibson and Mitchell, 2004). There is controversy regarding weather P limitation in the soil is a prerequisite for solubilization by ericoid fungi.

The beneficial effects of mycorrhiza are lost if the concentration of available P in the soil increases as it has the potential to disrupt the electrochemistry of the fungi, in such soils association can be entirely abandoned or lead to growth depression a shift from mutualism to parasitism (Howarth, 2001). Acid phosphatase activity was detected in fungal isolates of *Erica hispudula* in South Africa. These isolates had high phosphatase activity which stimulated enzyme synthesis in low organic phosphate medium (Straker and Mitchell, 1985). These findings suggest that the concentration of phosphorus in the soil has an effect on organic phosphate solubilization.

1.4.4 Mechanisms of metal resistance and tolerance in contaminated soils

Ericoid endophytes have an affinity for habitats which are highly acidic having potentially toxic metals such as Al, Mn and Fe which can also be micronutrients essential for growth in small quantities. Hence their unmistaken success in heavy metal polluted sites (Turnau et al., 2007). Heavy metal contamination in the soil may be naturally occurring but in most cases it is a result of human activities such as agriculture, industry and mining. Although the most important role of ericoid mycorrhizal fungus in nature is promoting the formation of minute hair roots cells detoxification is also regarded as an important function of ericoid mycorrhizal colonization (Norris et al, 1991). It has been established that ericoid fungi alleviate metal toxicity. Studies on metallophytes have shown great emphasis on the mechanisms used by the ericoid endophytes to overcome excess heavy metal toxicity at the same time protecting the host associate. The main mechanisms are exclusion and tolerance which have been seen to work separately or simultaneously (Martino et al., 2000; Vallino et al., 2005).

Metal resistance exhibited by ericoid fungi in their natural soil, probably gives them the ability to resist other metals such as copper, cadmium and zinc which have the same mode of toxicity although they are not commonly found in their natural habitats (Mitchell and Gibson, 2006). Heavy metals have a tendency of binding to the sulphydryl groups of proteins bringing about disturbance to the normal cell activities and structure. This can also facilitate the formation of free radicals and reactive oxygen species which may cause oxidative stress in the plant cells (Vallino et al., 2005). The lethal effects of Al and Fe are exhibited even when the metals are in contact with the extracellular matrix of the plant root cells because toxicity is aggravated by the acidic and reducing conditions of the growth substrate.

Some heavy metals such as Cu, Fe and Zn are essential micronutrients crucial for various chemical and physiological processes that take place in both the plant and fungi partner but in small quantities. For instance Zn ions act as co factors for a number of enzymes and holds together structural components of protein sequences (Martino et al., 2003). Excessive quantities of both essential and nonessential heavy metals results in toxicity generally exhibited as stunted growth, leaf discoloration and death of plants and ericoid fungi have been seen to protect their hosts from these effects.

Ericoid fungi such as *O. maius* and *R. erica* have devised homeostatic mechanisms which allow them to maintain the concentration of essential metals within physiological limits. These include transportation, intracellular detoxification, chelation and extracellular sequestration (mucilage, pH gradient in the rhizosphere). These mechanisms regulate heavy metal uptake, accumulation, transportation and detoxification which results in alleviation of toxicity to host plants (Vallino et al., 2005). The system used by *R. erica* to protect its host in copper contaminated soils was described as an avoidance mechanism in which copper triggers the release of the phosphodiesterase enzyme by the fungi. The phosphotase activity releases HPO_4 in turn reacts with metal (X^{2+}) ions to form an insoluble $MHPO_4$ compound.

$$HPO_4 \; + \; X^{2+} \rightarrow \; XHPO_4$$

This mechanism mobilizes and detoxifies any free metals in the soil. *R. erica* therefore provides P to the host and at the same time protects it from metal toxicity. When P is in excess in the soil ericoid fungi try to counter its toxic effects on the host by accumulating it in the mycelium (Mitchell and Gibson, 2006)

Most ericoid endophytes are capable of alleviating toxicity regardless of the origin of the fungi taxa; however endophytes originating from contaminated environments are more physiologically adapted to withstand excessive metal concentration. Martino et al. (2000) demonstrated *Oidiodendron maius* to be a Zn tolerant endophyte which enabled *Vaccinium myrtillus* to dominate the recolonization of Niepolomice forest trial sites contaminated with Zn, Cd, Al and Fe. The fungal partner *O. maius* was then isolated from the roots of these ericoid species and from *V. myrtillus* growing in unpolluted sites and were cultured in different solid and liquid media in the presence of varying concentrations of Zn ions. It was discovered that in pure culture both isolates were tolerant to Zn ions however isolates from polluted sites could tolerate even higher concentrations of Zn ions. Morphological evidence also suggested production of mucilage material by the isolates as an extracellular sequestration tolerance mechanism (Mitchell and Gibson, 2006).

In prior research mucilage was also noted in the $CdSO_4$ tolerant *O. griseum* endophyte were it was proposed to be a chelating agent. Denny and Ridge (1995) described extracellular glycoprotein slime as the mechanism enabling ericoid isolates from *C. vulgaris* to tolerate high concentrations of metal. Their research findings suggested slime to be the Zn accumulation site as isolates displaying the greatest slime in culture exhibited greater complimentary growth of the host plant under potentially Zn toxic conditions. These findings were confirmed by Jentschke and Godbold (2000) the glycoproteins in the slime attached to Zn in *in-vitro* cultures of fungi hence glycoproteins can reduce the zinc concentration in the growth medium resulting in alleviation of Zn toxicity. The *O. maius* isolate which has been seen to thrive in potentially toxic concentrations of cadmium and zinc was further analyzed by Vallino et al. (2005). The metal tolerant *O. maius* was cultured *in-vitro* under high Zn concentration and the expression of 16 genes was adjusted by being either, up- or down-regulated. Two of the up-regulating genes functioned as an oxidoreductase and a hypothetical protein respectively.

Pigmentation of ericoid fungi which is usually dark has been seen to protect plants from metal toxicity through chelation (Martino et al., 2000). Copper has the ability to bind to the melanin sites in the cell wall of the fungi thus reducing the metal exposure of both mycobiont and host plant (Jentschke and Godbold, 2000). Detoxification of arsenate contaminated soil by *R. erica* was described by Sharples et al. (2001) as a resistant mechanism in which the fungi excludes arsenate at the same time supplying phosphate to the host plant. Arsenate is an analogue of P hence can be transported through the cell membrane through the phosphate co-transporter in both plant and fungi. *R. erica* reduces arsenate to arsenite and accumulate P at the same time as eliminating arsenate. The mechanism of alleviating metal toxicity is dependent on both symbiotic partners.

The ability of ericoid mycorrhizal fungi to tolerate toxic metal concentrations by avoidance mechanism has been due to exclusion of metal from cytoplasm. *R. erica* detoxifies Al and Fe in a similar manner as it sequesters these metals into the extracellular surrounding tissues away from the sensitive parenchyma plant tissues which are susceptible to damage when exposed to high metal concentration. Experiments conducted in a pyrite mine highly contaminated with Al and Fe revealed that these metals were mainly detected in cell walls of epidermal tissues and leaf glandular hairs of *Erica andevalensis* plant species (Turnau et al., 2007). Other fungi can tolerate high intracellular concentrations of toxic metals as a way of protecting the host plant (Martino et al., 2000). Although several mechanisms have been revealed there is still need to continue research on the biochemical reactions involved in these mechanisms.

1.5 Blueberry plants (*Vaccinium corymbosum* L.)

Vacciniun corymbosum plants are members of the ericaceous family which naturally inhabit acidic soils which are low to moderate in mineral nutrient status and have low organic matter content of between 2.5% and 12% (Scagel and Yang, 2005). Blueberries have a shallow root system devoid of hair roots and are not very tolerant to dry soil conditions and require frequent irrigation (Starast, 2008). Hence ericoid mycorrhizal fungi form symbiotic associations with the roots of blueberry plants in order to improve the mineral nutrient acquisition of these plants as well as protect them from environmental/soil adverse conditions such as drought (Jeliazkova and Percival, 2003). The fungi do not only enhance nutrient absorption of soluble inorganic N and P but it also utilizes inorganic and insoluble N and P complexes in the soil. The cultivated blueberries are native to North America and Eastern Asia and vary in size from 10 cm to 4 m in height. The smaller varieties are known as the lowbush while the larger species are referred to as highbush blueberries. The highbush blueberry is amongst the most important commercial berry crops. Sustainable production practices are increasingly becoming important because of their escalating demand in the health food market (Prodorutti et al., 2007).

Under field conditions the ideal growth factors may vary from one region to another but the general conditions are high organic matter and low pH. Acidic growth media is important for the blueberry plants because the symbiotic efficiency of the association is highly dependent on pH. The optimum pH range for the extracellular enzyme activity is between 4.5 and 5.3 (Prodorutti et al., 2007; Scagel and Yang, 2005). Very low pH of less than 3.5 has been seen to reduce the growth of blueberry plants. The growth of *Vaccinium* species is retarded by pH levels above 6 (Darnell and Cruz-Huerta, 2011). In soils with pH above 5 the addition of ammonium sulphate as a source of N can help decrease the soil pH and other cultural practices such as addition of gypsum and acid water irrigation can serve the same purpose (Starast, 2008). There are however some wild varieties such as *V. arboretum* which are tolerant to pH levels as high as 6-6.5 and low organic matter. Depending on the cultivar the plants require full sun to partial shade for optimum productivity however field plants tend to flower more abundantly.

In Oregon trials were conducted on 55 different blueberry fields. It was established that ericoid colonization increases fertilizer plant utilization efficiency and fungal inoculation has resulted in improved growth and significantly high root dry weights (Janska and Votsatka, 2000; Scagel and Yang, 2005). The colonization of blueberry plants by ericoid fungi is variable depending on the variety, age of plant, rate of mineral fertilizer application and the growth medium (pH, soil type) (Scagel and Yang, 2005; Yang et al., 2002). Blueberry plants have very low fertilizer requirements and fertilizer application can enhance growth, development and fruit yield. Depending on the region, cultivar and soil status the optimum N dosage is between 50-100 kg ha^{-1} although other research findings have revealed that dosages in excess of 60 kg ha^{-1} tend to decrease yield. It is also important to note that excessive fertilizer application reduces mycorrhizal colonization and the use of ammonium as a N source promotes mycorrhizal formation (Darnell and Cruz-Huerta, 2011; Starast, 2008). Ericaceous plants form associations with arbuscular mycorrhizas in the absence of ericoid fungi (Brundrett, 2002), however the symbiotic benefits to the Blueberry host are lower in comparison with ericoid benefits (Vega, 2009).

The nutritional and medicinal value of the blueberries has been studied. They contain pterostilbene, anthocyanins, proanthocyanidins, resveratrol, flavonols and tannins which contribute positively to health. Anthocyanins inhibit mechanisms of cancer cell development, inflammation *in-vitro* and have been used in diabetes therapy. Blueberries contain vitamin A, B, C, E, PP and carotenoids which are essential for their anti-oxidant properties. These enable them to shield the body from cellular oxidation reactions. These antioxidant and anticarcinogenic compounds are mainly attributed to the high phenolic compounds present in the fruits. Other hypothesized benefits include prevention of cognitive decline during aging and prevention of the hepatitis C virus (Joseph et al., 2009). Flavanoids have also been used as an alternative for synthetic antioxidants in the cosmetic and pharmaceutical industries (Ghafar et al., 2010).

1.6 Motivation

South Africa like most third world countries requires new methods and technologies for improving agricultural productivity with minimal risk to the environment. Studies on ericoid mycorrhizal fungi which are associated with plants that are a significant part of the fynbos biome will contribute positively in the restoration and rehabilitation of the fynbos vegetation of South Africa particularly in the Albany Centre of Floristic Endemism where some *Erica* species are threatened to extinction (Victor and Dold, 2003). Little research has been conducted on ericoid mycorrhiza in South Africa, Straker, (1996) being the last published local contribution. Continuous research is essential in order to improve commercial productivity of high value crops such as Blueberry and as well as growth of the industry. Ericoid mycorrhizal fungi have potential applications in bioremediation, being able to tolerate high metal concentration and ameliorate metal toxicity in some ericaceous plants found in mine dumps and other metal contaminated sites (Martino et al., 2000; Turnau et al, 2007). It is important to research and identify any unique qualities which may be essential for their proposed functional roles. (Perotto et al, 2002). The continued criticism of GMOs has necessitated a shift from such methods of enhancing plant growth to more natural methods which do not alter original end product (such as taste and nutrient status) or interfere with the natural ecosystem.

1.7 Hypothesis and Objectives

1.7.1 Hypothesis

The growth of commercial, blueberry (*Vaccinium corymbosium L.*) species can be enhanced through the inoculation of indigenous ericoid mycorrhizal fungi.

1.7.2 Aims and Objectives

The overall aim of this study was to produce ericoid mycorrhizal fungal inoculum from indigenously colonized ericaceous plant species.

The specific objectives of the study are;

1. To identify selected indigenous ericaceous plant species.
2. To collect root and soil samples.
3. To determine ericoid mycorrhizal association of collected samples.
4. To isolate the ericoid mycorrhizal fungi from root material.
5. To identify fungal isolates using morphological and molecular techniques.
6. To determine optimal growth parameters of the fungal isolates in artificial culture.
7. To determine the tolerance of isolates to Cu^{2+} and Zn^{2+} ions in culture.
8. To formulate a suitable inoculum.
9. To test the infective ability of inoculum on blueberry plants in a green house trial.
10. To determine whether inoculum has enhanced plant growth of the blueberry species.

CHAPTER 2

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2. 1 Collection of plant roots and soil samples

The plant root and soil samples were collected from the Albany Centre of Floristic Endemism (Fig. 2.1) along Mountain Drive, Grahamstown latitude and longitude of 33°19'S and 26° 31'E respectively. Climatically Grahamstown experiences high temperature and seasonal fluctuations daily. The monthly temperature ranges from 10 °C – 21.5 °C as calculated over 12 years while average annual rainfall is 615 mm calculated over 20 years (Merti, 2003).

Plants were identified with the assistance of Mr. Tony Dold curator of the Selmar Schönland Herbarium. From the identified plants the roots were carefully excised using a spade to remove the surrounding soil. A selection of roots were cut off using a scissors and placed in a bag. They were brought to the laboratory and processed on the same day. Approximately 200 g of soil samples was collected from each plant and composite samples were mixed and sent for nutrient analysis. Analysis also included soil nutrient status, organic matter content, exchangeable acicidity, soil density and pH. This was conducted at the Döhne Agricultural Development Institute, Analytical Services, Sutterheim.

2.2 Assessment of ericoid root colonization

A portion of the root samples from both species were washed thoroughly in water in order to remove adhering soil particles. Root pieces were cut using a sterile blade into sections of 1-3 cm. Roots were covered with 5% KOH and heated for 30 min in a water bath just below boiling point. The KOH was discarded through a sieve to prevent loss of roots before rinsing with distilled water. The roots were bleached in freshly prepared alkaline H_2O_2 solution for 30 min. The bleach solution was discarded and the roots were rinsed in water before being acidified for 3 hrs at room temperature in 0.1 M HCl solution. The acid solution was discarded and roots were stained by soaking at room temperature overnight in lactoglycerol solution containing 0.05% Trypan Blue. The staining solution was discarded and the roots destained by immersion in lactoglycerol destain solution (Janska and Vostka, 2000; Smith and Dickson, 1991).

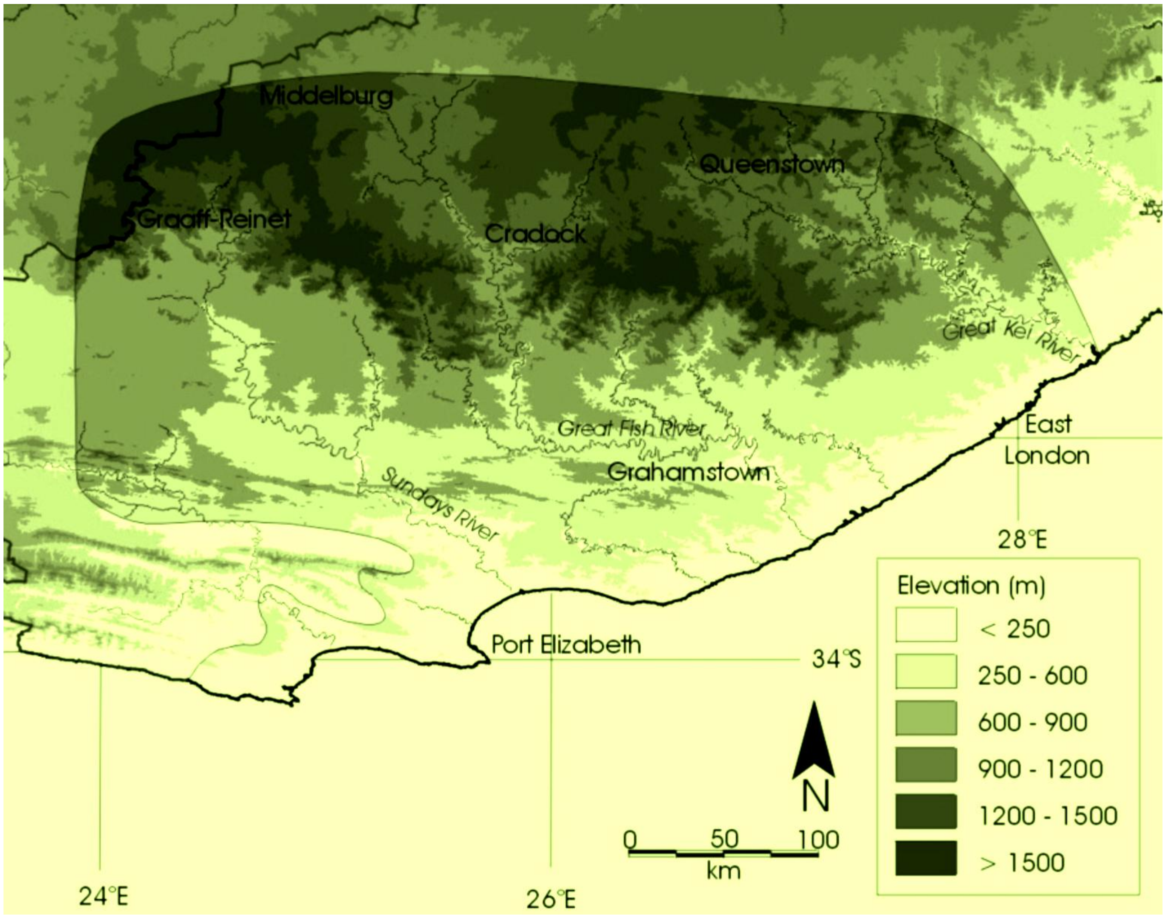

Figure 2.1 The Albany Centre of Floristic Endemism highlighted in the map which includes Grahamstown where samples were collected (Victor and Dold, 2003; Phillipson, 1995).

The composition of root clearing and staining solutions is outlined in Appendix A. The root pieces were mounted onto microscopic slides and covered with cover slips. The roots were observed under a light microscope at a magnification of 400X and on an Olympus BX50 microscope were images showing colonization were captured using an Olympus DP72 camera.

2.3 Isolation and culture of root colonizing fungi

Root samples were washed with tap water and surface sterilized in 70% ethanol for 2 minutes. Samples were cut under sterile conditions into pieces of approximately 1mm and plated on 2% Malt Extract Agar (MEA), modified Fontana media (FTN) (Appendix B) and 1.5 % water agar

(WA) in order to optimize the number of fungal species detected by culturing. The sterile plates sealed with parafilm were incubated at 28 ºC in the dark for about 4 weeks and checked daily for fungal growth. Fungal growth was subcultured onto fresh medium in order to obtain pure cultures (Kajula et al, 2010). Microscope slides were prepared from pure cultures and examined microscopically on an Olympus BX50 light microscope and cultures showing potential ericoid fungal morphotypes were retained for further analysis.

2.4 Molecular characterization of ericoid mycorrhizal fungi

2.4.1 DNA extraction from fungal isolates

Genomic DNA was extracted from pure fungal mycelia using the ZR Fungal/Bacterial DNA Mini-Prep kit (Catalogue #1300-50) according to the manufacturer’s instructions. Approximately 200 mg (fresh weight) of fungal mycelia was scraped from pure cultures using a sterile blade. The mycelium was placed in a Bashing Bead lysis tube prior to adding 750 µl of lysis solution. The tube was vortexed for 5 min on high speed in-order to break the cell membrane and release the intracellular components. The tube was then centrifuged at 10,000 rpm for 1 minute to spin down the solid fungal cell material. The supernatant of volume 400 µl was transferred into a Zymo-Spin IV Spin Filter and centrifuged at 7,000 rpm for 1 min to eliminate organelle debris from the lysate. Thereafter 1200 µl DNA Binding Buffer was added to the filtrate where it binds to the DNA while other molecules remain in the filtrate and 800 µl of the mixture was centrifuged at 10,000 rpm for 1 min in a Zymo–Spin IIC Column. The procedure was repeated with the remaining mixture before adding 200 µl DNA Pre-Wash Buffer to the Zymo- Spin IIC Column to wash away the impurities, prior to centrifuging at 10,000 rpm for 1 minute in a new collection tube. Thereafter 500 µl DNA Wash Buffer were added to the Zymo-Spin IIC Column and was centrifuge at 10,000 rpm for a minute. The empty tube was centrifuged again to remove excess ethanol. The Zymo- Spin Column was transferred into a clean 1.5 ml micro centrifuge tube prior to adding 100 µl of DNA Elution Buffer directly to the column matrix and centrifuging at 10,000 rpm for 30 sec for final DNA elution. The integrity of the isolated DNA was evaluated through electrophoresis in a 0.5µg/ml ethidium bromide stained 1% (w/v) agarose gel (Appendix C) and a 100bp molecular marker (Promega catalogue #G2101) was used to estimate the size of the DNA. Visualization was carried out on a UV fluorescence Uvitec gel doc system.

2.4.2 Polymerase Chain Reaction amplification of fungal genes

Polymerase Chain Reaction (PCR) is an enzymatic reaction which utilizes DNA polymerase to replicate and amplify a target DNA sequence, so as to increase the amount of specific DNA for analysis (Maier et al, 2000). The fungal rDNA region which includes the Internal Transcribed Spacer ITS1, ITS2 regions and 5,8S gene was targeted. The PCR amplifications were performed in a total reaction volume of 50 µl with 5 µl template DNA. In the reaction 25 µl of KapaTaq PCR Ready mix (KAPA Biosystems Catalogue # kk1006) comprising 1.25 µl/1.25 units *Taq* polymerse DNA polymerase, 10 µl PCR buffer, 2 µl dNTPs and 6 µl $MgCl_2$ was added together with 2 µl primers and 18 µl of sterile milliQ water.

The reaction was carried out in an automated Applied Biosystems 2720 Thermal Cycler. This was carried out in 3 steps (Table 2.1); the initial denaturation step involved separation of the double stranded DNA using a high temperature (94 ºC for 1 min). Oligonucleotides primers ITS1F and ITS4 (Table 2.2) (White et al., 1990), were used to define the target region of the single stranded DNA by annealing their complementary sequences on the target region (Fig. 2.2). The primer annealing step which involves the binding of the primers to the target sequence at 47 ºC for 45 s followed. In the final elongation step, the DNA polymerase synthesizes a complementary strand to the original target sequence by adding the dNTPs to the primers that have been bound to the target sequence at 72 ºC for 7 min. A total of 32 cycles were done for the PCR reaction.

Separation of amplified PCR products was done by electrophoresis on a 1% (w/v) agarose gel at 100 V for 75 minutes in 1x Tris-acetate-EDTA (TAE) buffer (Appendix C), stained with 2µl (concentration 0.5 µg/ml) ethidium bromide and visualized under UV fluorescence on a Uvitec gel doc and photographed. In order to determine the size of the amplified bands a 100 bp marker (Promega Catalogue # G2101) was used for referencing (Pitt et al., 2004).

Table 2.1 PCR cycling conditions used in the amplification of the fungal ITS region using the universal fungal primers.

Steps	**Conditions**	**Temperature (ºC)**	**Time (s)**	**Cycles**
Initial 1	Initial denaturation	94	120	1
	Denaturation	94	30	
Step 2	Annealing	47	45	30
	Elongation	72	60	
Final 3	Final elongation	72	420	1

2.4.3 Purification of PCR product

The PCR product was purified using a Wizard SV clean up kit (Promega Catalogue # A9281) according to the manufacturer's instruction. The protocol entailed adding three times the volume of the membrane binding solution to the PCR product in a centrifuge tube in order to adhere to the DNA molecules in the solution. The mixture was transferred to a column and centrifuged at 13,000 rpm for 1min, placing the DNA on the membrane filter. The flow through was discarded and 200 µl of membrane wash solution was added to the column in-order to wash off impurities from the DNA and centrifuged at 13,000 rpm for 1 min. Another 200 µl of the membrane wash solution was added again and centrifuged as previously described. The empty column was centrifuged under the same conditions to ensure all ethanol had been removed from the membrane filter. The column was transferred to a new centrifuge tube and 50 µl of nuclease free water was added, prior to incubation on the bench at room temperature for 1 min to allow the membrane filter to fully absorb the water. Tubes were then centrifuged at 13,000 rpm for a min in order to elute the purified DNA.

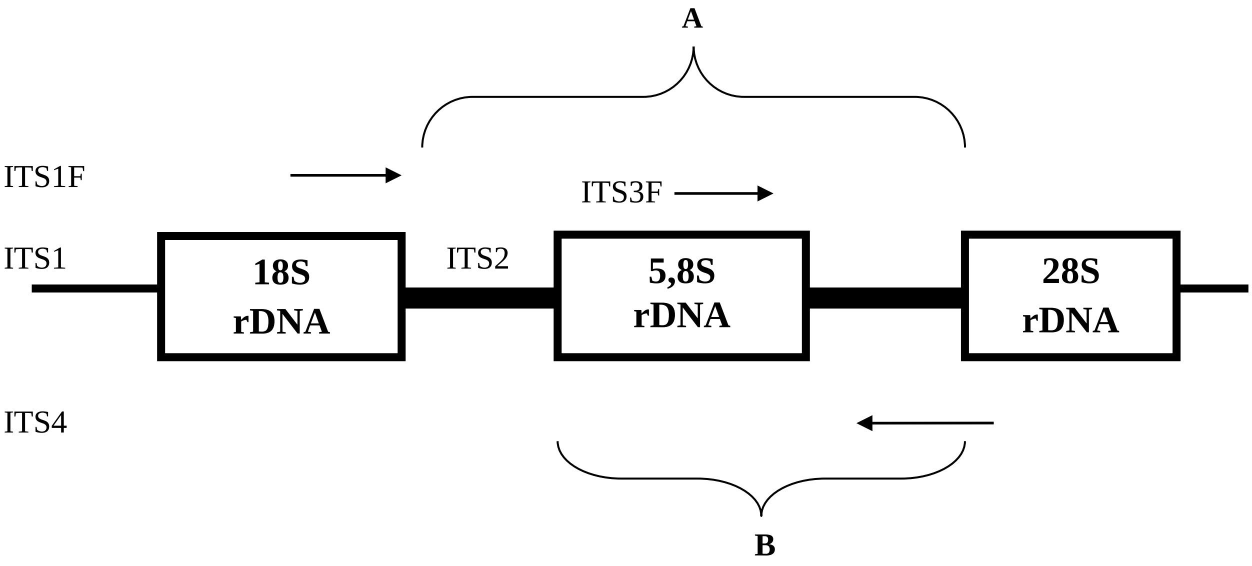

Figure 2.2 Structure of the ITS region of the fungal rDNA. (A). Target region amplified by the ITS1F and ITS4 universal primers during PCR. (B) Highly diverse region sequenced by the ITS3F primer. Adapted from White et al. (1990).

2.4.4 Molecular data analysis

PCR product was sent to Inqaba Biotechnology, Pretoria, South Africa for sequencing. The ITS3F primer (Table 2.2) was used in the sequencing reaction to sequence 5.8s and the ITS2 region of the DNA (Fig. 2.2) (Berch et al., 2002). This primer was used because it targets the ITS2 region which is highly diverse in fungal species and can enable the identification of isolates to species level. Nucleotide sequences were analyzed through the Basic Local Alignment Search Tool (BLAST) searching program on the National Centre for Biotechnology Information (NCBI) site http://www.ncbi.nlm.nih.gov (Altschul et al., 1997) and UNITE http://www.unite.ut.ee site (Kõljalg et al., 2005) for the most similar sequences in sequence databases.

Table 2.2 Primers used in the fungal rDNA amplification and sequencing of the ITS region.

Primer	**Sequence 5’- 3’**	**Tm ºC**	**References**
ITS1F	- CTTGGTCATTTAGAGGAAGTAA-	49.7	White et al. (1990)
ITS3F	-GCATCGATGAAGAACGCACG-	57	White et al. (1990)
ITS4	-TCCTCCGCTTATTGATATGC-	52.1	White et al. (1990)

Tm- Melting temperature which determines the annealing temperature.

2.5 Growth characteristics of fungal isolates

2.5.1 The effects of culture media on fungal growth

Two lines intersecting at right angles were drawn on the base of 9 cm Petri dishes containing 2% MEA, modified Fontana media and WA. Respective culture plugs of 5 mm were centrally placed on the intersection of the lines on each plate (Fig. 2.3). The diameter of the inocula was measured at 48 hr intervals along the lines and the mean value recorded. Five replicate plates were measured on each medium. The plates were incubated at 27 ºC in the dark.

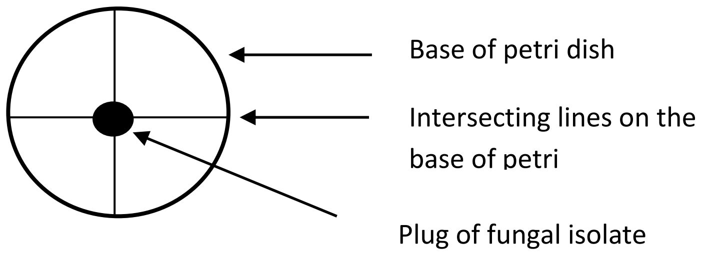

Figure 2.3 Illustration of experimental plate design used for fungal growth measurements.

2.5.2 The effects of temperature on fungal growth

This was conducted on 2% MEA and the plates were prepared as described in section 2.5.1. Five replicate plates were measured for each isolate at each incubation temperature. The plates were incubated at 8 ºC, 27 ºC, 31 ºC and 37 ºC for 3 weeks.

2.5.3 Effects of pH on fungal growth

The plates of acidic pH (pH 3 and 4) were prepared by mixing 40 ml 0.1 M citric acid and 0.1 M $NaHPO_4$ and adjusting to the required pH using 0.1 M HCI. After autoclaving these were then mixed with 40 ml double strength MEA, before pouring plates. For pH 5 and pH 6, MEA was adjusted to the respective pH using NaOH prior to autoclaving and pouring plates. The plates were incubated at 27 ºC for 3 weeks and fungal growth of 5 replicate plates was measured as described in section 2.5.1.

2.5.4 Effects of increasing concentration of Zn ions on fungal isolates

The experiment was carried out on 2% MEA incorporating increasing concentrations of Zn^{2+} ions in the form of $ZnSO_4$. The following Zn concentrations were used 0 (control), 1.5, 2.1, 2.7, 3.4 and 4.2 mM. $ZnSO_4$ was dissolved in sterile water and the pH adjusted to pH 5 prior to autoclaving and then mixing with an equal volume of 2% MEA. The plates were inoculated as described in section 2.5.1. There were 5 replicates for each concentration treatment. Plates were then incubated at 27 ºC and growth of 5 replicate plates was monitored and evaluated weekly over a period of 6 weeks (Martino et al., 2000).

2.5.5 Effects of increasing concentration of Cu ions fungal isolates

The protocol followed was the same as described in section 2.6.4. The following Cu^{2+} ions concentrations were added 0.3, 0.5, 0.7, 1 mM in the form of $CuSO_4$. The plates were inoculated and measured as described in section 2.5.4 (Gibson and Mitchell, 2006; Martino et al., 2000).

2.6 Formulation of inoculum

A liquid formulation was used in preparing inocula. Two agar discs were cut from the edges of selected fungal species cultures using a sterile pasteur pipette. They were placed and grown separately in liquid culture flasks containing 50 ml of 2% Malt Extract Broth media (Appendix B). The cultures were left to grow for 3 weeks in the dark at 27ºC on a rotary shaker. The fungal cultures were then filtered through Whatman No. 1 sterile paper. The collected mycelia was washed with sterile water before being scraped off using a sterile blade and homogenized by blending at high speed using a Waring Blender rinsed in 70% ethanol. The inocula were blended with 200 ml sterile 0.3% water agar for 5 sec in order to make gel inocula (Janska and Vostka, 2000; Yang et al, 1996, 2002).

2.6.1 Mycorrhizal resynthesis on *Erica demissa* seedlings

Seeds collected from the *E. demissa* plants were surface sterilized in 3% sodium hypochloride for 5 min and rinsed thoroughly 5 times in distilled water. They were then soaked overnight in distilled water so as to soften the seed coats and induce germination. Autoclaved cotton wool was placed in 9 cm petri dishes and moistened with distilled water. Under sterile conditions the 10 seeds were then placed on the cotton wool in each petri dish before replacing the lid and sealing with parafilm tape. They were then incubated at 25 ºC with natural daylight conditions. After the seedlings emerged the inocula was prepared as described in section 2.6 above. Using a sterile syringe under sterile conditions 10 ml of respective inoculum was placed across the cotton wool media were the roots were growing and the control were inoculated with 10 ml of distilled water. One petri dish with at least 8 seedlings was used for each treatment. The inoculated seedlings were incubated for 3 months prior to observation for colonization as described in section 2.2. Mycorrhizal resynthesis was also evaluated on Blueberry plantlets in greenhouse trials described in section 2.6.2.

2.6.2 Inoculation on *Vaccinium corymbosum* and mycorrhizal resynthesis

Micropropagated blueberry plantlets were obtained from Amathola Berries Stutterheim, South Africa. Nine plants of each of the five blueberry varieties were obtained which included Bluecrop, Brightwell, Spartan, Chandler and Elliott. The different varieties were inoculated with two isolates which showed potential as ericoid mycorrhizal species. The root system was inoculated by covering the root system with 10 ml of relevant inocula using a sterile syringe. The inoculated plantlets were transplanted into 1 L pots containing pasteurized pine bark compost obtained from Amathola Berries. The pasteurization was conducted in order to eradicate all indigenous fungi that might have been present in the compost. The plantlets were watered with 100 ml of water to ensure settling. Thereafter the pots were placed in a mycorrhizal tunnel with a minimum temperature of 19 ºC and maximum of 27 ºC under natural light conditions. The plants were watered daily with UV sterilized water and a low phosphorus Long Ashton Nutrient solution weekly 10 ml per plant (Appendix D). The experimental design comprised of 3 replicate treatments for each variety of the 2 inoculants and uninoculated controls.

2.6.3 Growth analysis

Weekly shoot height was measured and recorded throughout the trial. After 16 weeks the plants were destructively harvested by cutting the pot on both sides using a blade and carefully removing the pine bark compost from the roots to avoid loss of root material. A sub sample (1 g) of roots were selected from each plant and retained for microscopy. These were stained as described in section 2.2. Plant fresh weight, canopy size and root volume (cm^3), canopy and root density (evaluated subjectively from 1 – 5, 1 = sparse, 5 = dense) were recorded. Canopy size and root volume were estimated using the formula for a cylinder ($\pi r^2 H$), where r = the average radius of canopy or root system, using two measurements per plantlet, H = canopy or root system length. A growth index for canopy and root was developed by multiplying the canopy or root volume by canopy or root density. The plants were then oven dried at 60 ºC for 72 hrs prior to recording the shoot and root dry weights (Yang et al, 1996; 2002). Root weight was corrected for the sub sample removed.

Canopy size = $\pi r^2 H$

Where, r = average radius of canopy (cm) ***Equation 1***
H = canopy length (cm)

Root size = $\pi r^2 H$
Where, r = average radius of root (cm)
H = root length (cm) ***Equation 2***
$\pi = 3.1428$ **(**for all calculations)

Canopy growth index (cm^3) = Canopy size x Canopy density ***Equation 3***
Where, Canopy size = $\pi r^2 H$
Canopy density = Determined subjectively

The same formula was used to determine the root growth index.

Root growth index (cm^3) = Root volume x Root density ***Equation 4***

Where, Root size = $\pi r^2 H$
Root density = Determined subjectively
(Yang et al., 1996)

2.7. Colonization

Colonization percentage of the different highbush Blueberry varieties was determined by microscopy. It was established by examining 50 fields of view of a root sample and recording a positive result were hyphae was present and a negative result for uncolonized cortical cells. The percentage was the determined by dividing the percentage of colonized results by 50

$$\textit{Percentage Colonization} = \frac{\textit{Colonized cells}}{50} \times 100 \qquad \textit{Equation 5}$$

2.8. Statistical analysis

All data collected after harvest of the blueberry plants was evaluated using one way ANOVA. A repeated measure ANOVA was used to determine the effects of different treatments (pH, temperature, media, and metal ions) on the growth of the isolates. The effects of the isolates on the shoot height of the different blueberry cultivars was analyzed by a 3 factor factorial ANOVA. The statistical evaluation was carried out using the STATISTICA package and the Tukey's Test was used to determine the least significant differences.

CHAPTER 3

RESULTS

3.0 RESULTS

3.1 Identification of Ericaceous plants

The identification of the Ericales is highly dependent on their flowering morphology which is predominantly exhibited in summer. Two ericaceous species were identified *Erica cerinthoides* L. and *Erica demissa* Klotzsch ex Benth. Commonly known as the Fire heath or Red hairy heath, *E. cerinthoides* is native to South Africa. They have hairy stems with brightly coloured petals and sharp small leaves (Fig.3.1a). They require long photoperiods, good drainage and acidic soils for their establishment. *E. demissa* is variable and complex with three forms of the same species having been recorded. The species identified in Grahamstown was up to 1m in length with highly branched and small sharp leaves (Fig.3.1b). These species grow in edaphic conditions similar to those of *E. cerinthoides*.

Figure 3.1 (a) *E. cerinthoides* and (b) *E. demissa* identified from the Mountain Drive in Grahamstown.

Five plants of *E. cerinthoides* were sampled but only one shrub of *E. demissa* was positively identified due to the absence of flowers at the time of sample collection and similarity to other non ericaceous plants in the area. Three samples were taken from one shrub for analysis.

3.2 *Soil nutrient analysis*

Soil analysis (Table 3.1) revealed that *E. cerinthoides* and *E. demissa* are growing in acidic soil with pH 5 and 4 respectively. Mineral nutrient requirements vary from one species to another and (Table 3.1) shows the mineral nutrient status of the soil in Ericaceae habitats. The macronutrients are relatively high and are adequate to promote plant growth and development. Micronutrients were however not detectable in the soil from both species.

Table 3.1 Soil analysis results for soil collected from *Erica cerinthoides* and *Erica demissa* plants growing in the Mountain Drive area of Grahamstown. Values are an average of 3 composite samples.

Mineral nutrients gL^{-1}	**Origin of soil sample**	
	E. cerinthoides	***E. demissa***
P	14	68
K	228	94
Ca	1163	1378
Mg	204	251
Zn	0	0.2
Mn	0	0
Cu	0	0
Exchangeable acidity cmolL^{-1}	0.2	1
Soil density gmL^{-1}	1.09	1.0
Organic matter %	3.4	5.94
pH (KCI)	5	4

3.3 *Assessment of root colonization*

Microscopy revealed that the roots of both *E. cerinthoides* and *E. demissa* had some hyphal association within the epidermal cells of their roots with some of them forming coils typical of ericoid mycorrhizas (Fig.3.2). This confirmed the ericoid mycorrhizal status of the identified Ericaceae plants in Grahamstown. Percentage root colonisation was not determined because there was not enough root material. However other structures were observed in the roots similar to those previously described for DSE colonization (Fig. 3.3).

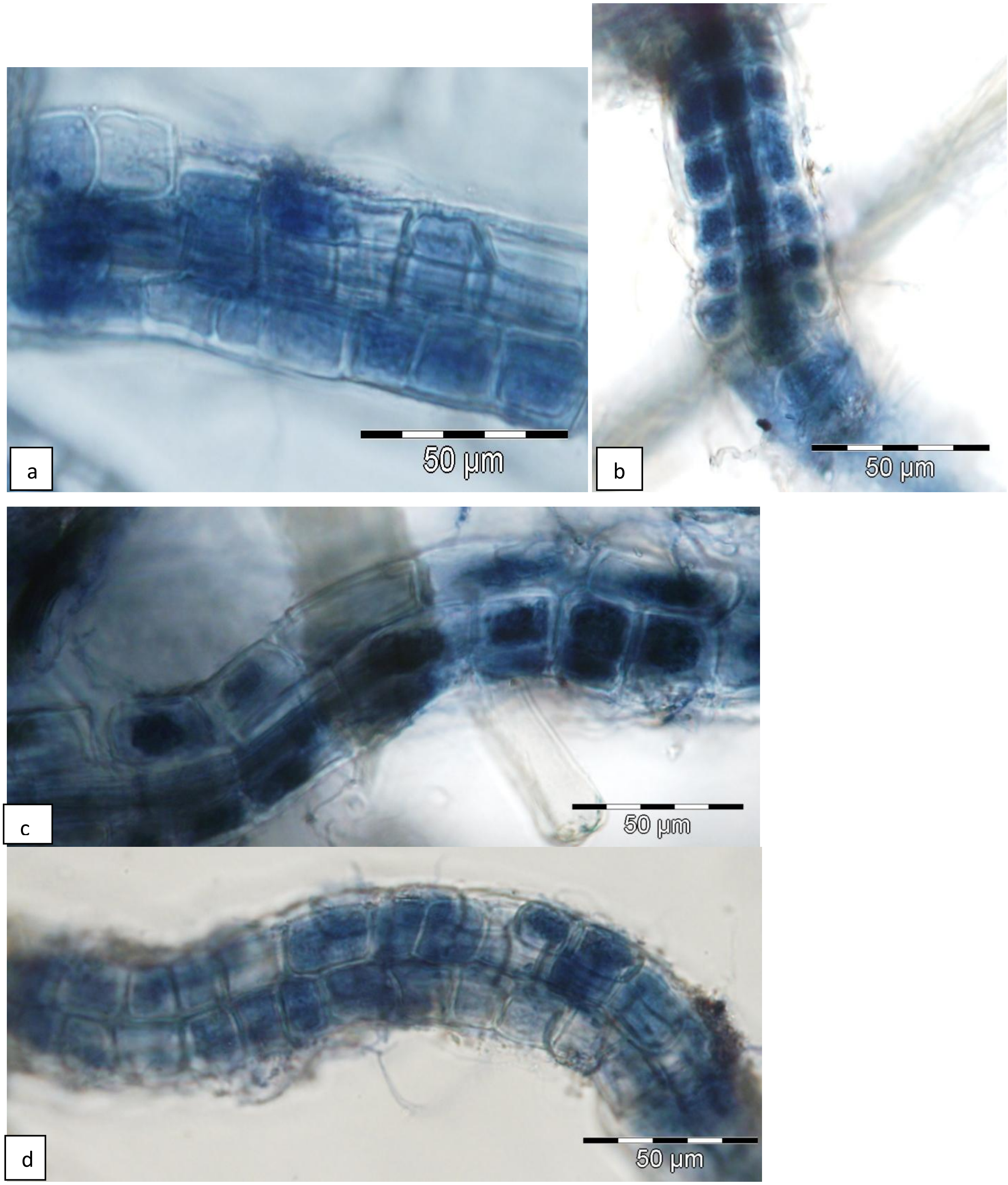

Figure 3.2 Intracellular hyphal colonization with coils typical of ericoid association. *Erica cerinthoides* (a) and (b) and *Erica demissa* (c) and (d) as observed under a light microscope.

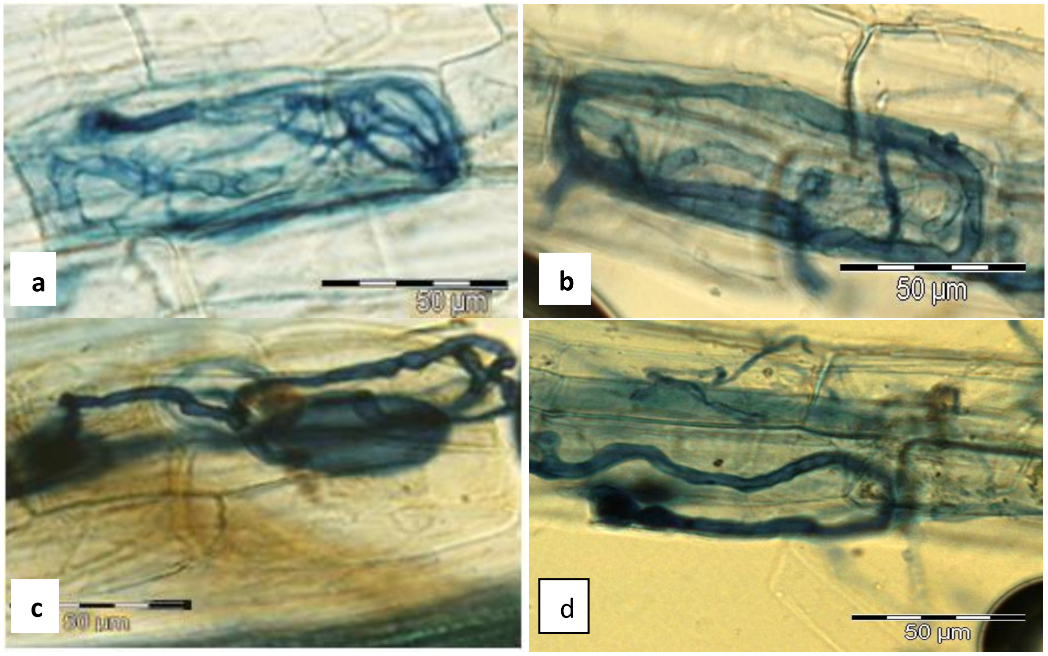

Figure 3.3 Intracellular hyphal colonization with DSE association structures. *Erica demissa* (a) and (b) and *Erica cerinthoides* (c) and (d) roots as observed under a light microscope.

3.4 Isolation and morphological identification

Approximately 25 isolates were obtained from both *E. cerinthoides* and *E. demissa*. Using morphological characteristics the fast growing and heavily sporulated isolates were discarded. These included common soil fungi such as *Aspergillus*, *Penicillium* and *Trichoderma*. One of the isolates from *E. cerinthoides* (ECHh) and one from *E. demissa* (Ed6d) cultured on MEA were both relatively slow growing and exhibited different morphological characteristics and were designed as potential ericoid mycorrhizal fungi. ECHh was olive brown in colour and the fungal hyphae were septate and sterile (Fig. 3.4a and b). The Ed6d was darkly pigmented, septate and sporulated in culture (Fig. 3.4c and d).

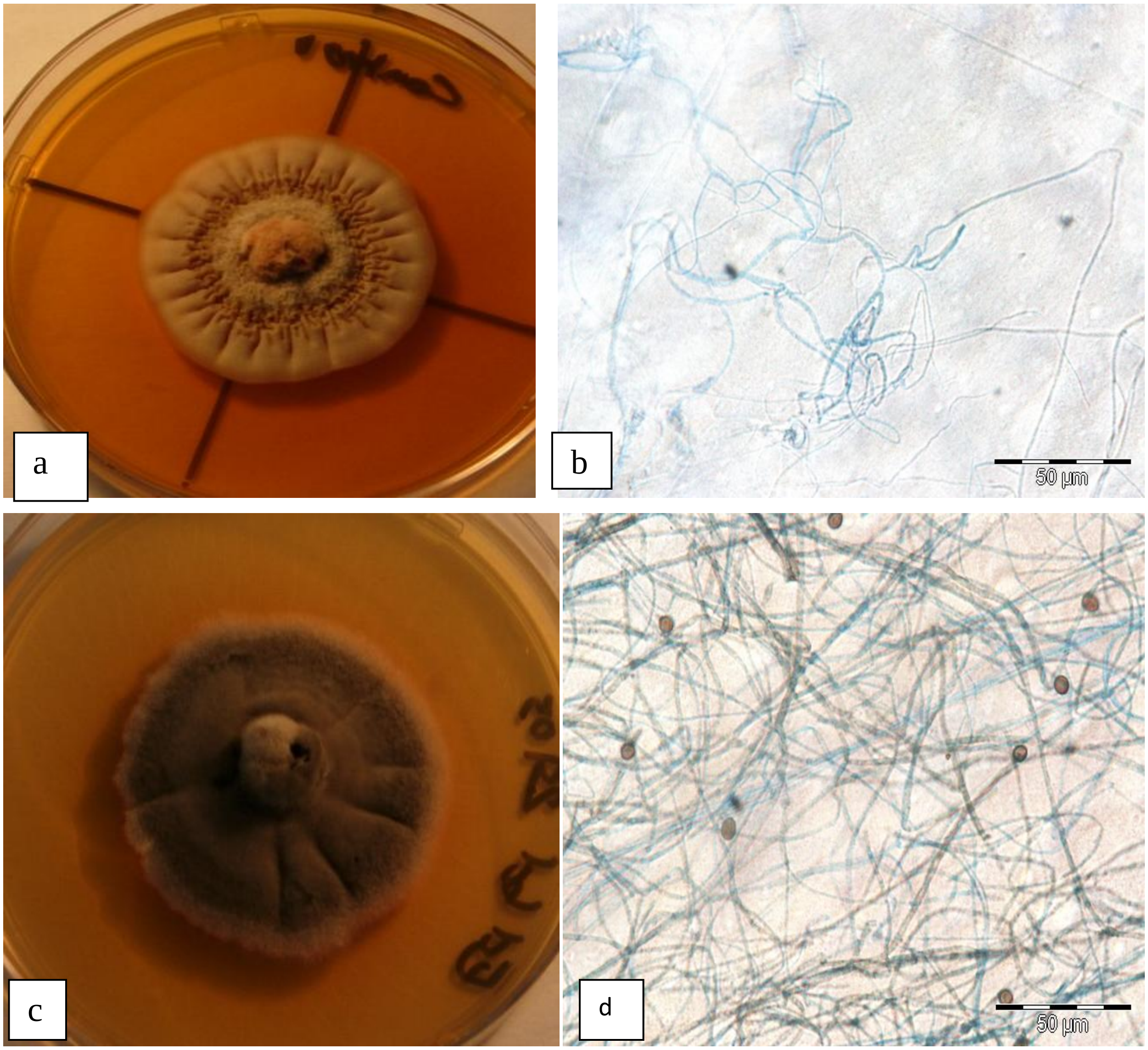

Figure 3.4 The cultural morphology of the isolate from ECHh (a) and the sterile septate mycelium as observed under the microscope (b) and Ed6d isolate cultural morphology (c) after 3 on MEA culture media and the septate mycelium with brown oval spores (d).

3.5 *Molecular identification*

The ribosomal DNA regions of ITS1 and ITS2 were amplified in a PCR. For the ECHh isolate approximately 571 nucleotide bases were amplified while approximately 604 bp were obtained for *E. demissa* (Fig. 3.5). The diverse ITS2 region was successfully sequenced and the sequences obtained from ECHh and Ed6d (Appendix E2) were compared through BLAST on NCBI and

UNITE databases for matching sequences. The matching sequence for the two isolates obtained through BLAST on NCBI database revealed the ECHh isolate was 98% identical to *Lachnum* species Retz. While the E. *demissa* isolate had 99% similarity to the *Cadophora* species. On the UNITE database ECHh had 91% identity to *Lachnum brevipilosum* while Ed6d was 97% identical to *Cadophora luteo – olivaceae* (Table 3.2) (Appendix E1).

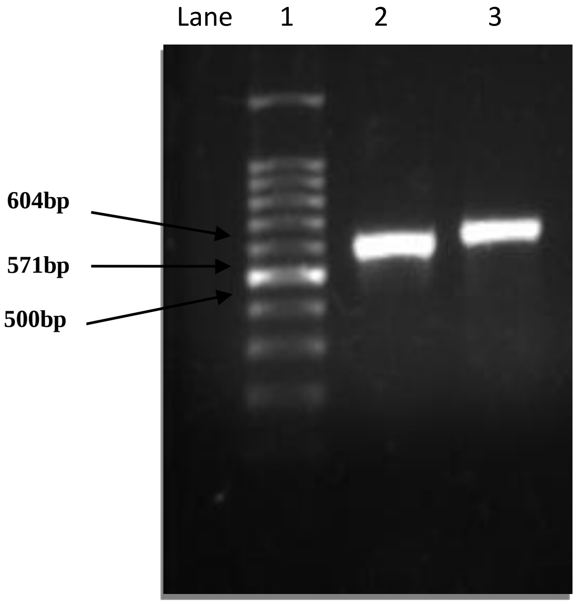

Figure 3.5 PCR products amplified from pure fungal cultures using ITS1F and ITS4 primers. Lane 1; is the 100bp ladder marker; Lane 2: isolate ECHh with 571bp and Lane 3: isolate from Ed6d with 604bp. As visualized on 1% agarose gel stained with ethidium bromide.

Table 3.2 Taxonomic affinities of ericoid endophytes isolated from the roots of *Erica cerinthoides* (ECHh) and *Erica demissa* (Ed6d) obtained from Mountain Drive in Grahamstown according to BLASTn searches of ITS sequences in the GenBank/EMBL and UNITE databases.

Isolate	**NCBI/UNITE Accession number**	**Most significant alignment**	**% Similarity identity**	**E-value**
NCBI				
ECHh	UDB 003974	*Lachnum* species	98%	0.000
Ed6d	GU 212390	*Cadophora* species	99%	0.000
UNITE				
ECHh	UDB003048	*Lachnum brevipilosum*	91%	0.000
Ed6d	DQ404348.1	*Cadophora luteo – olivaceae*	97%	0.000

3.6 Resynthesis trials

Resynthesis experiments were conducted on micropropagated blueberry plantlets and *E. demissa* seedlings germinated from seeds. These studies are important in the determination of the mycorrhizal status of the isolated fungi. In the hair roots of *E. demissa* seedlings inoculated with *Cadophora* species hyphal colonization and spores were observed (Fig.3.6b) after resynthesized under laboratory conditions. However in *E. demissa* seedlings no colonization was observed within the roots or surrounding the epidermal cells after inoculation with *Lachnum* species (Fig.3.6a). In the non-inoculated plants no hyphal colonization was observed. In blueberry plants root staining revealed inter and intracellular hyphal colonization on roots of species inoculated with *Lachnum* species with no distinctive ericoid structures were formed. In the Elliott and Brightwell varieties plants inoculated with *Cadophora* species showed typical ericoid structures in their roots (Fig.3.12 and 3.13) although no distinctive structures were observed on the other varieties.

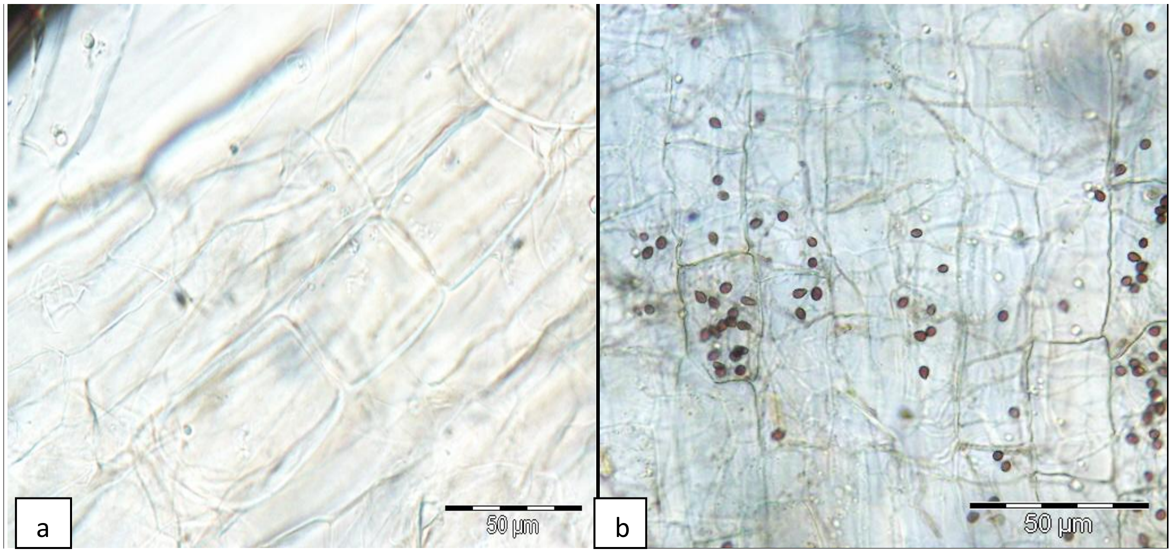

Figure 3.6 The colonization of the roots of *E. demissa* after inoculation with *Cadophora* and *Lachnum* species. (a) Uncolonized roots of *E. demissa* seedlings inoculated with *Lachnum* species. (b) Inter-intra cellular colonization and fungal spores observed in *E. demissa* seedlings after 8 weeks of inoculation with *Cadophora* species.

3.7. Characterisation of fungal isolates

3.7.1 Growth analysis on different media

The growth of microorganisms in culture is influenced by several factors which include growth medium, pH and temperature. MEA, modified FTN and WA were the culture media used to determine the medium which supported optimum growth of *Lachnum* and *Cadophora* species. Both species grew poorly in WA developing only an unpigmented light mycelia growth. Although growth of *Lachnum* species was recorded on both MEA and FTN media, optimum growth was on MEA while *Cadophora* species grew better on the FTN media. The exponential growth rate was better for *Lachnum* species grown in MEA medium (1.3 mmd^{-1}) while in *Cadophora* species the best growth rate of 1.88 mmd^{-1} was recorded in FTN (Table 3.3). MEA was subsequently used to maintain both cultures.

Table 3.3 Mean growth and exponential growth rate of *Lachnum* and *Cadophora* species on MEA and FTN incubated at 27°C in the dark and measured at 48 hr intervals for 3 weeks. *Lachnum* species ($F_{(2,39)} = 66.196$; $p < 0.0001$) and *Cadophora* species ($F_{(2.39)} = 532.860$; $p < 0.0001$) ($N = 5$).

Culture	Mean growth (mm)		Exponential growth rate (mmd^{-1})	
Medium	*Lachnum* species	*Cadophora* species	*Lachnum* species	*Cadophora* species
2% MEA	12.923^a	22.127^a	1.300^a	1.280^a
FTN	11.083^b	25.807^b	1.249^b	1.883^b

Means denoted by the different letters in each column are significantly different.

3.7.2 Growth analysis at different temperature

There were significant differences ($p < 0.0001$) in the growth of *Lachnum* and *Cadophora* species at different temperatures. The optimum growth temperature was for both species was 27ºC and the highest exponential growth rates were recorded for both species. The growth of *Lachnum* species decreased as temperatures exceeded the optimum temperature while *Cadophora* species were more sensitive to low tempearure (8ºC) as the lowest growth was recorded of 3.93 mm after 3 weeks of incubation (Table 3.4). The *Cadophora* species had significant growth under all given temperatures. No growth was however observed at 37ºC for both species. The morphology of *Lachnum* and *Cadophora* species on 2% MEA after 3 weeks of incubation is shown in (Fig. 3.4a and c).

3.7.3 Growth analysis at different pH

The growth of both *Lachnum* and *Cadophora* species increased with an increase in pH. Optimum growth was recorded at pH 5 for both species. There was however a decrease in mycelia growth when pH of both species exceeded 5. There were significant differences in fungal growth at the different pH values (Table 3.5) and growth decreased with as pH became highly for both species as shown by the mean growth was recorded.

Table 3.4 Mean growth and exponential growth rate of *Lachnum* and *Cadophora* species on MEA incubated at different temperatures and measured at 48 hr intervals fro 3 weeks. *Lachnum* ($F_{(4,118)} = 2.55.12$; $p < 0.0001$) and *Cadophora* species ($F_{(4.118)} = 597.27$; $p < 0.0001$) ($N = 5$).

Temperature	Mean growth (mm)		Exponential growth rate (mmd^{-1})	
(°C)	*Lachnum* species	*Cadophora* species	*Lachnum* species	*Cadophora* species
8	3.92^{a}	6.15^{a}	0.401^{a}	0.7^{a}
27	12.94^{b}	20.15^{b}	1.75^{b}	2.1^{b}
31	2.22^{c}	13.64^{c}	0.400^{a}	1.05^{c}

Means denoted by the different letters in each column are significantly different.

Table 3.5 Mean growth and exponential growth rate of *Lachnum* and *Cadophora* species cultured on 2% MEA of different pH and measured at 48 hr intervals for 3 weeks. *Lachnum* species ($F_{(6,158)} = 78.99$; $p < 0.0001$) and *Cadophora* ($F_{(6,158)} = 834.44$; $p < 0.0001$) ($N = 5$).

pH	Mean Growth/mm		Exponential growth rate (mmd^{-1})	
	Lachnum species	*Cadophora* species	*Lachnum* species	*Cadophora* species
3	8.41^{a}	3.71^{a}	0.6^{a}	0.3^{a}
4	8.70^{b}	14.197^{b}	0.61^{a}	1.187^{b}
5	10.44^{c}	24.99^{c}	0.7^{b}	2.5^{c}
6	9.516^{d}	24.22^{d}	1.12^{c}	2.542^{c}

Means denoted by the same letter on each column are not significantly different

3.7.4 Growth analysis of isolates at different metal ion concentrations.

Ericoid fungi are known for their role in the amelioration of metal toxicity. The potential of *Lachnum* and *Cadophora* species to withstand elevated concentrations of metal in their growth medium was conducted using Zn^{2+} and Cu^{2+} ions. The growth of *Cadophora* significantly decreased with an increase in the concentration of Zn^{2+} ions. At concentration 3.4 and 4.2 mM mycelia was lightly pigmented (brownish in colour) with slimy substances on the surface (Fig 3.7 and 3.9). *Lachnum* species were more tolerant to Zn^{2+} ions as their mycelia growth actually improved at 1.5 and 2.1 mM concentrations. Significantly lower mycelial growth ($p < 0.000121$) was recorded in Zn^{2+} concentrations above 2.7 mM for both species (Fig 3.8 and 3.9).

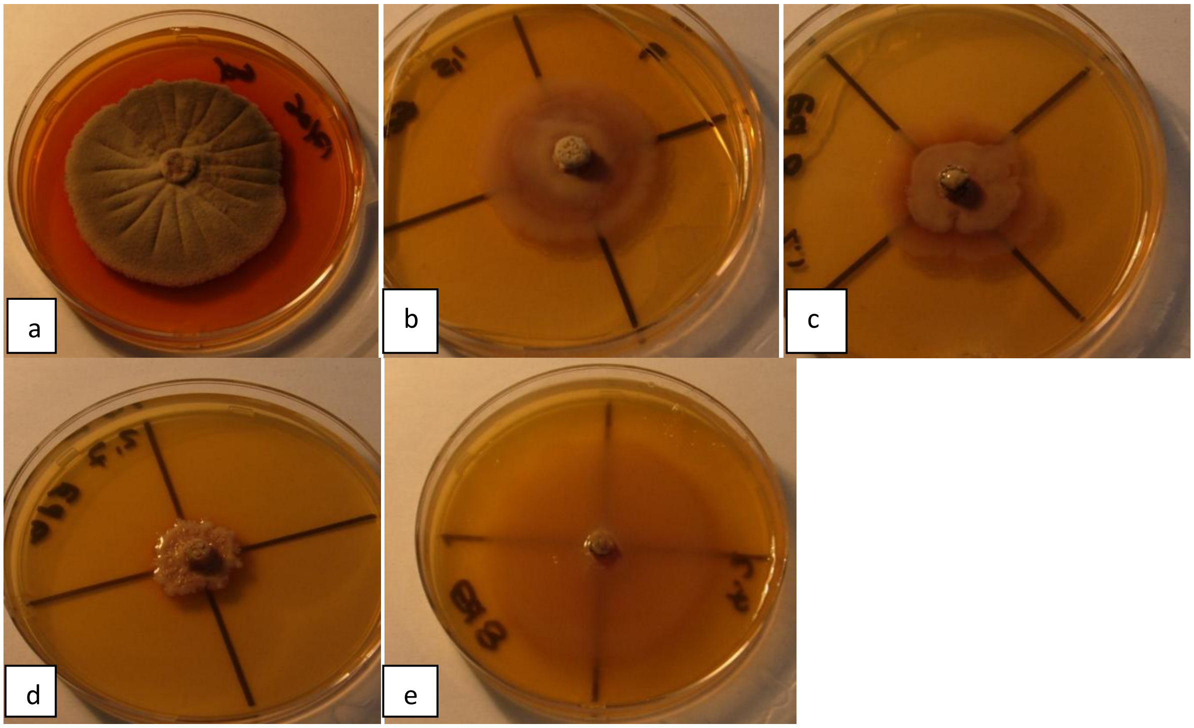

Figure 3.7 The growth of *Cadophora* species incubated in increasing Zn^{2+} ions after 6 weeks. (a) Control (b) 1.5 mM (c) 2.1 mM (d) 2.7 mM and (e) 3.4 mM.

The fungal growth of *Cadophora* species was not significantly reduced with an increase in ion concentration in the medium. *Lachnum* species had relatively high fungal growth on all the Cu^{2+} ion concentration tested. There was no difference in the growth of fungi in the metal free media and the 1 mM concentration (Fig.3.10).

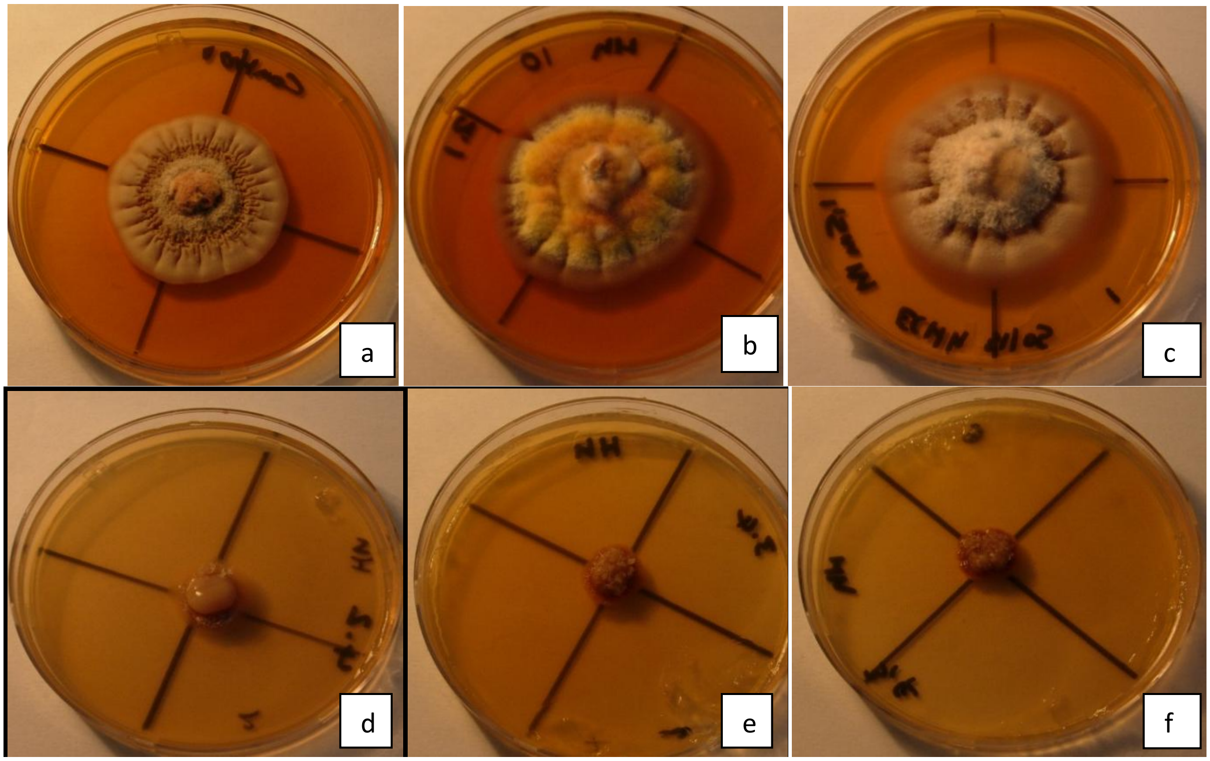

Figure 3.8 The growth of *Lachnum* species incubated in increasing Zn^{2+} ions after 6 weeks. (a) Control (b) 1.5 mM (c) 2.1 mM (d) 2.7 mM (e) 3.4 mM and (f) 4.2 mM.

3.8 Growth analysis of different varieties of Blueberry inoculated with *Lachnum* and *Cadophora* fungal isolates.

3.8.1 Mycorrhizal colonization

The root colonization assessed at 15 weeks was low in all Blueberry plantlets and significant different ($p < 0.0001$) were recorded between varieties. The *Cadophora* species had greater root colonization in all species except in Chandler variety were colonization was 12.5%. Within varieties the roots of Elliott and Brightwell plantlets inoculated with *Cadophora* species had high colonization (35% and 31% respectively) (Fig.3.11). In some uninoculated controls a few traces of hyphae were observed which were however not significant enough to make a proportion of the percentage colonization. However no re-isolation was done from the inoculated Blueberry plants to confirm Koch's postulate.

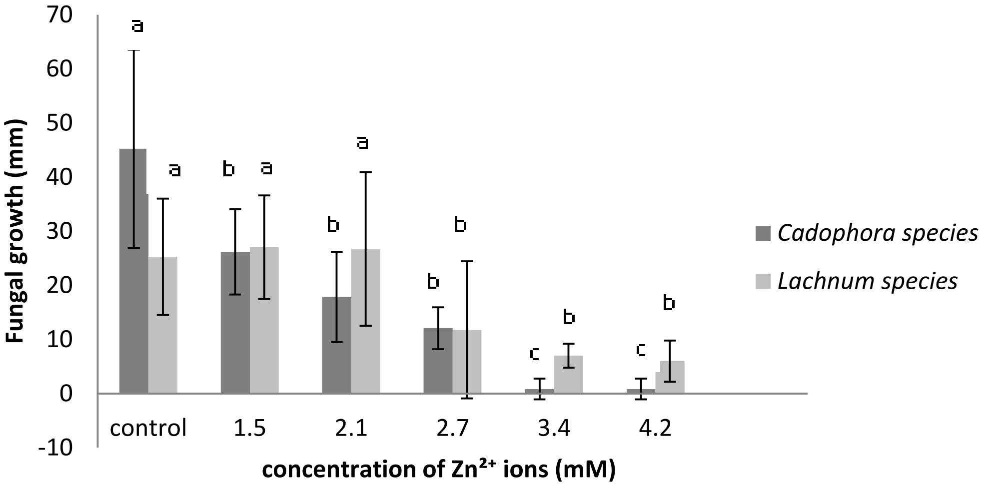

Figure 3.9 The effect of increasing Zn^{2+} concentration on the growth of *Cadophora* and *Lachnum* species. Column represents Mean ± SD (N = 5). Mycelial growth of *Cadophora* species ($F_{(10,202)}$ = 30.048; $p < 0.0001$) and *Lachnum* species ($F_{(10, 202)}$ = $p < 0.0001$) measured at weekly intervals for a period of 6 weeks. The same letter in each treatment indicates that means in each treatment are not significantly different.

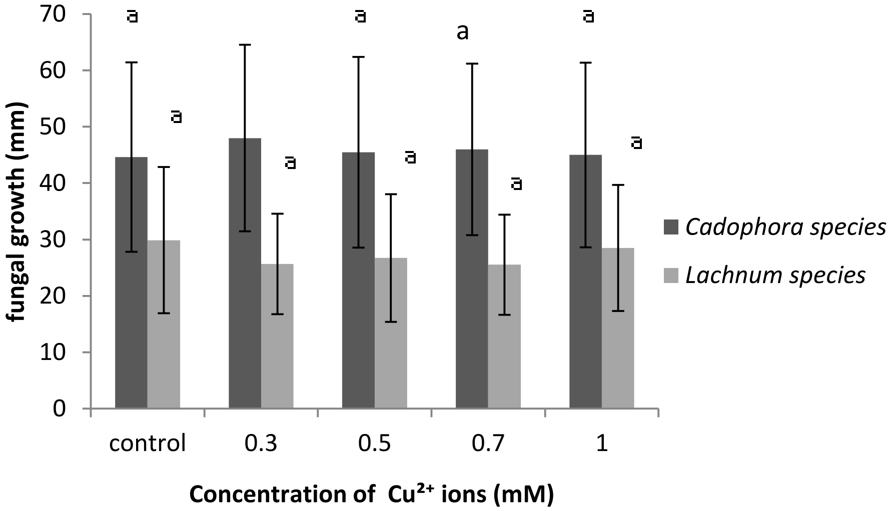

Figure 3.10 The effect of increasing Cu^{2+} concentration on the growth of *Lachnum* and *Cadophora* species. The column represents Mean ± SD (N = 5) mycelial growth of *Cadophora* species ($F_{(8,168)}$ = 0.44913; $p < 0.88982$) and *Lachnum* species ($F_{(8, 168)}$ = 3.8239; $p < 0.37000$) measured at weekly intervals for a period of 6 weeks. The same letter in each treatment indicates that means in each treatment are not significantly different.

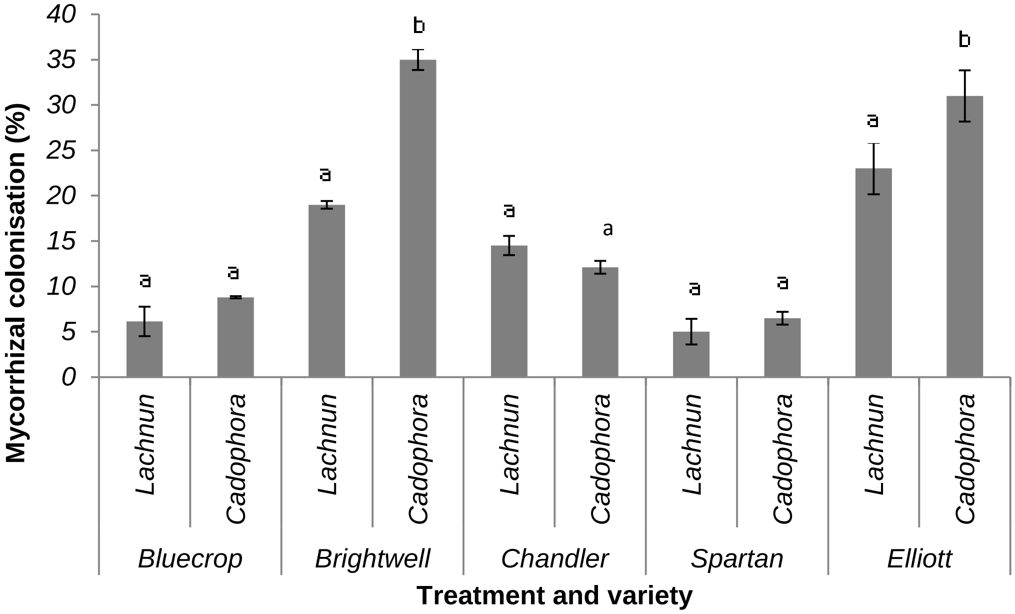

Figure 3.11 Percentage mycorrhizal of *Vaccinium corymbosum* varieties after 14 weeks of inoculation by *Lachnum* and *Cadophora* species. Columns represent Mean ± SD (N = 3) ($F_{(14,269)} = 1.615$; $p < 0.0001$). The uninoculated controls showed no significant colonization. Treatment within varieties denoted by the same letter show no significant differences in percentage colonization.

The structures formed upon the interaction of the roots of *V. corymbosum* varieties and *Cadophora* and *Lachnum* species were variable. The hypha infected the cortical cells intracellulary and there was inter-cell hyphal connections were observed (Fig.3.12). However the *Cadophora* inoculated plants had hyphal loops surrounding the root in a manner that simulates a net which was not found in *Lachnum* inoculated plants. The hyphal colonization pattern observed in Elliott/*Cadophora* inoculated plantlets showed ericoid potential (Fig.3.12c and d) as hyphae could be observed penetrating the cells and forming condensed structures. This was also observed in the Brightwell/*Cadophora* inoculated plantlets (Fig.3.13).

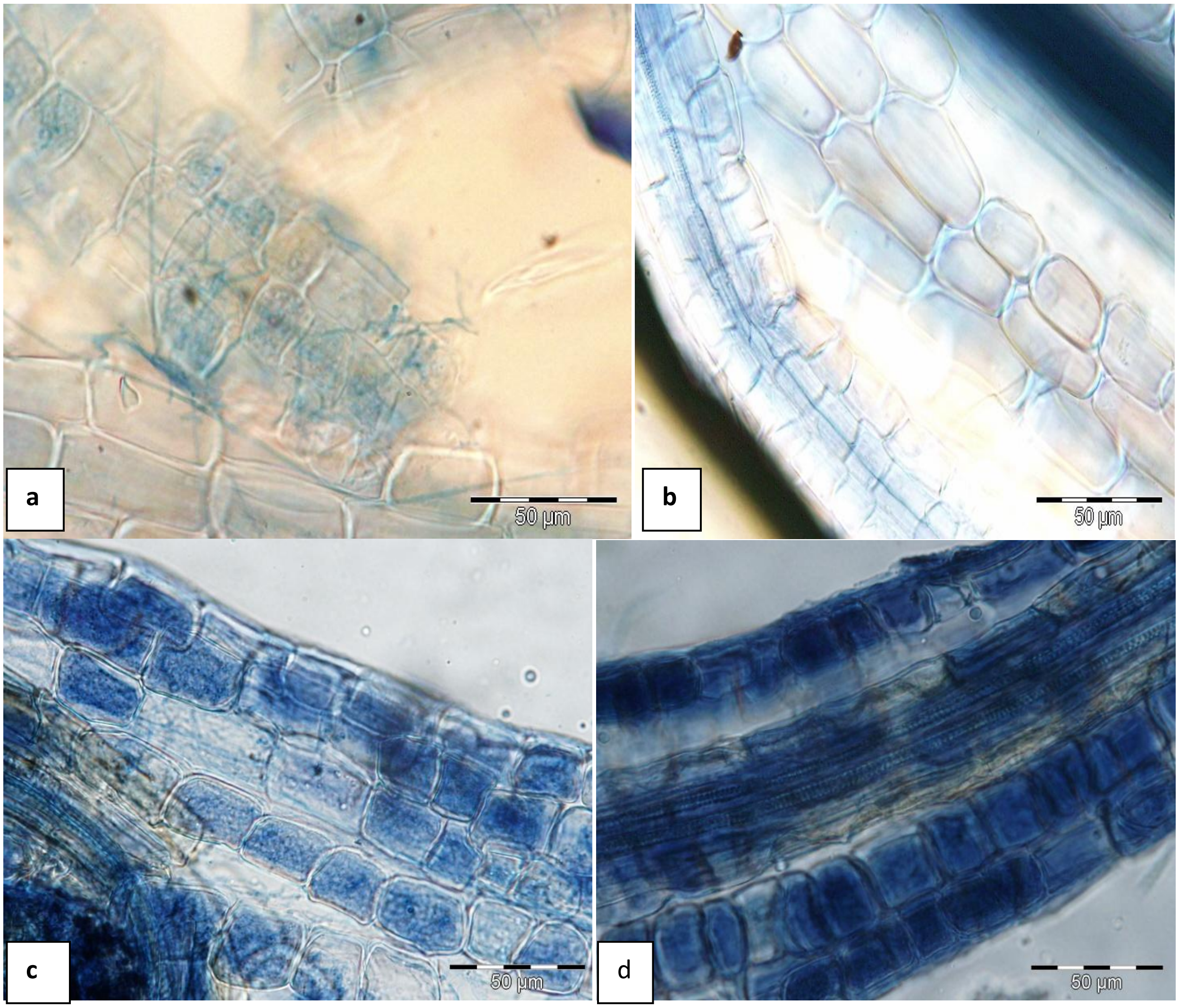

Figure 3.12 Structures formed in *Vaccinium corymbosum* Elliott variety plants after 14 weeks of inoculation. Root colonization by (a) *Lachnum* species (b) uninoculated control (c) *Cadophora* species (d) the root tip of the Elliott plantlet with coiled ericoid structures.

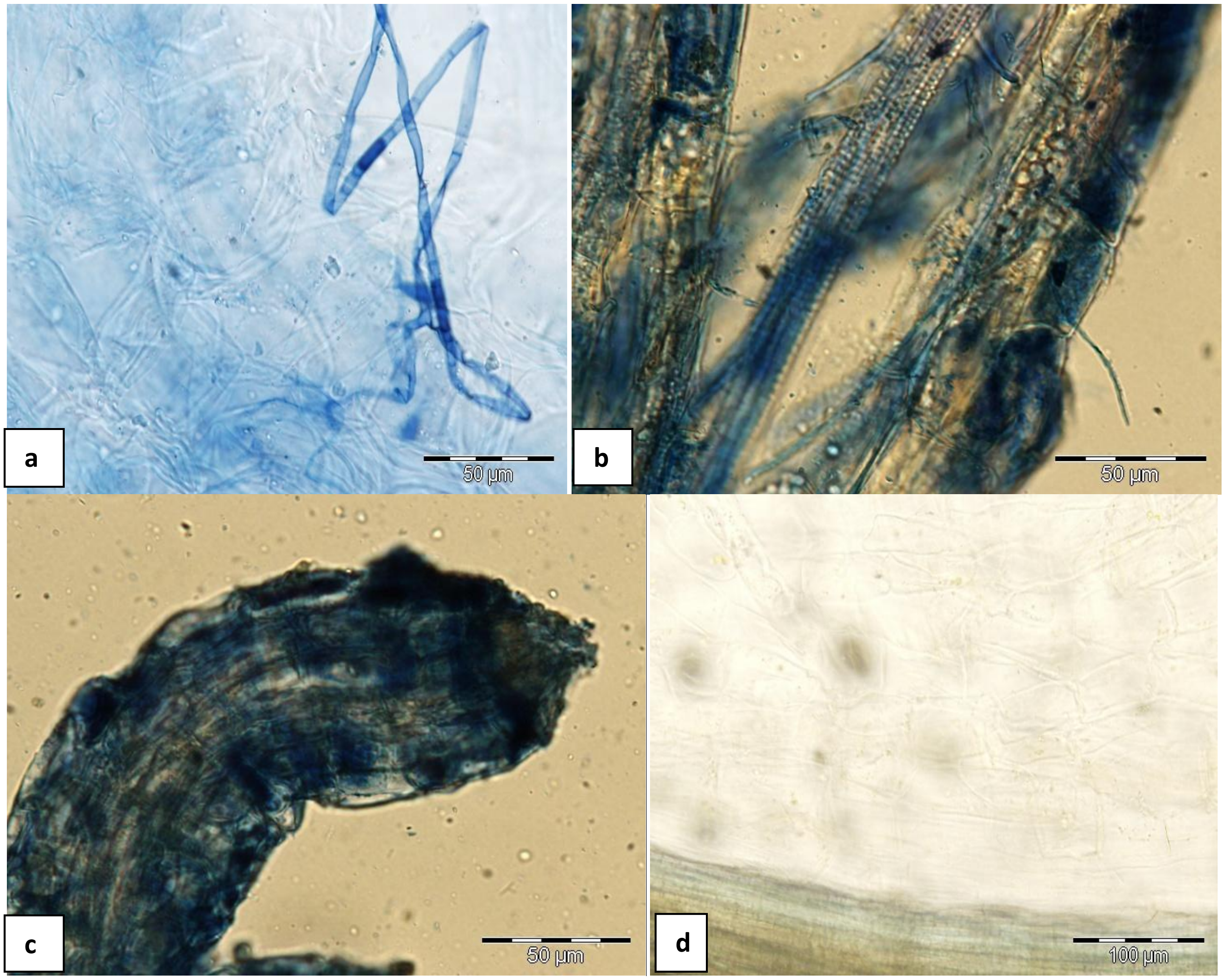

Figure 3.13 Structures formed in *Vaccinium corymbosum* Brightwell variety after 14 weeks of inoculation (a) *Lachnum* inoculated root (b) *Cadophora* inoculated (c) the root tip of a *Cadophora* inoculated plantlets and (d) Uninoculated control.

3.8.2 Shoot height

Analysis of the shoot height was carried out using a 3 factor (fungi, variety and time) factorial ANOVA. This indicated significant differences ($p < 0.001$) in shoot height of the different blueberry varieties because of single treatments and some of the interactions between treatments (Table 3.6). The average shoot growth was compared at weekly intervals over a period of 14 weeks. Although colonization was observed fungal treatment did not improved the shoot height of all plantlets of the different varieties.

The Brightwell plants inoculated with *Cadophora* species showed the greatest response to inoculation with mean shoot growth of 14.25 cm recorded and *Lachnum* species inoculated plants with 7.77 cm. A significant increase in shoot height of the *Lachnum* inoculated plantlets was recorded after 3 weeks of inoculation (Fig. 3.14). The untreated plantlets had the least mean shoot height (3.16 cm). In the Elliott variety greatest mean shoot height (4.68 cm) was also recorded in *Cadophora* species inoculated plantlets, while those treated with *Lachnum* species had a mean shoot height of (2.86 cm) and the least height recorded in the control (1.7 cm) (Fig 3.15). The Spartan variety was significantly fast growing while Elliott had low vegetative growth (Fig. 3.16).The mean shoot height of the inoculated Brightwell and Elliott (Fig.3.17 and 18) plantlets was significantly ($p < 0.05$) higher than the corresponding uninoculated plants.

Table 3.6 Analysis of variance for the mean weekly shoot height of the 5 *Vacinium corymbosum* varieties inoculated with *Lachnum* and *Cadophora* species as compared to uninoculated controls.

Effect	df	Ms	F	p
Week	13	208.31	448.18	0.000*
Variety	4	738.11	588.04	0.000*
Fungi	2	42.92	92.34	0.052
Week x Variety	52	5.78	12.44	0.000*
Week x fungi	26	2.43	5.23	0.000*
Variety x fungi	8	314.35	676.32	0.000*
Week x variety x fungi	104	4.81	10.35	0.000*
Error	211	0.46		

*significantly different

Statistical analysis showed that there were no significant differences ($p < 0.735$) in shoot height between Chandler plantlets inoculated with *Lachnum* and uninoculated controls plants. After 12 weeks a notable increase in shoot growth was recorded in the *Lachnum* species inoculated plants. Inoculation of Spartan and Bluecrop blueberry plantlets with *Lachnum* and *Cadophora* species did not significantly enhance the shoot height development (Fig.3.14 and 3.15).

Table 3.7 The growth rate (cmd^{-1}) of the 5 *Vacinium corymbosum* varieties inoculated with *Lachnum* and *Cadophora* species as compared to uninoculated controls after 7 weeks of inoculation. Mean ± SD (N = 3) ($F_{(104,211)} = 10.347$; $p < 0.0001$).

Treatment	**Blueberry variety growth rate (cmd^{-1})**				
	Bluecop	Brightwell	Chandler	Spartan	Elliott
Control	0.803^{a}	0.342^{a}	2.075^{a}	1.183^{a}	0.3^{a}
***Cadophora* species**	0.508^{b}	2.425^{b}	2.95^{b}	1.333^{b}	0.775^{b}
***Lachnum* species**	0.317^{c}	1.55^{c}	1.487^{c}	1.506^{c}	0.492^{c}

Means denoted by the same letter on each column are not significantly different.

In the first 7 weeks post inoculation growth rate in the Bluecrop variety was not improved for the plants inoculated with both *Lachnum* and *Cadophora* species. In this case the uninoculated controls had a better growth rate of 0.803 cmd^{-1}. In comparison with the controls inoculation improved the growth rate in the Brightwell, Chandler and Elliott varieties. *Cadophora* species inoculated plants recorded the highest growth rate followed by the *Lachnum* species inoculated plants. In the Spartan variety significantly higher growth rate was recorded in the *Lachnum* inoculated plants.

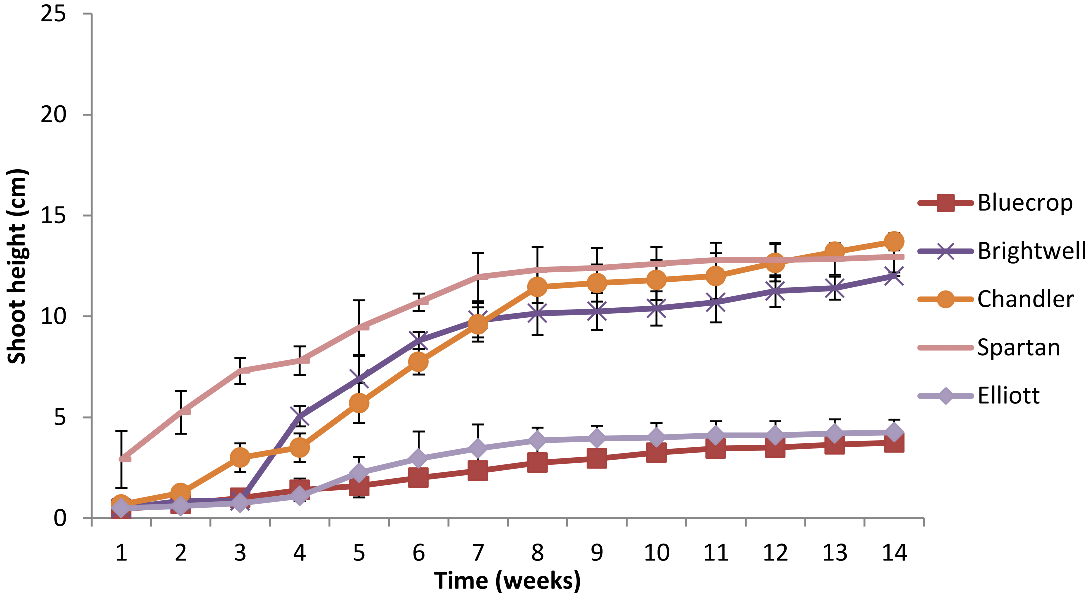

Figure 3.14 Interactive effects of *Lachnum* inoculation, variety and time on the shoot growth of the *Vaccinium corymbosum* varieties measured weekly over a period of 14 weeks. The graphs represent Mean ± SD (N = 3) ($F_{(104,211)} = 10.347$; $p < 0.0001$).

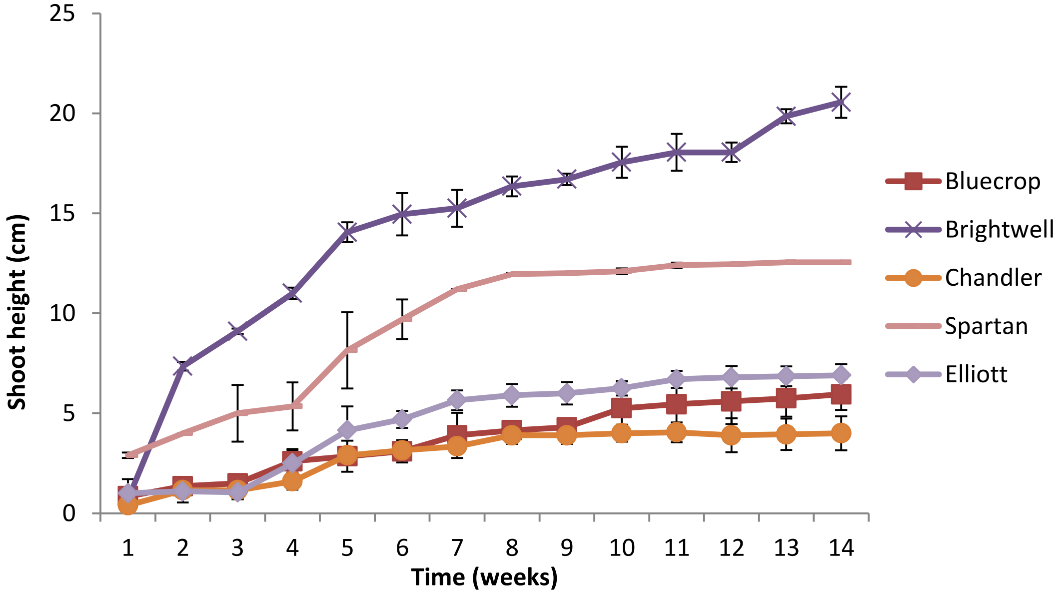

Figure 3.15 Interactive effects of *Cadophora* inoculation, variety and time on the shoot growth of the *Vaccinium corymbosum* varieties measured weekly over a period of 14 weeks. The graphs represent Mean ± SD (N = 3) ($F_{(104,211)} = 10.347$; $p < 0.0001$).

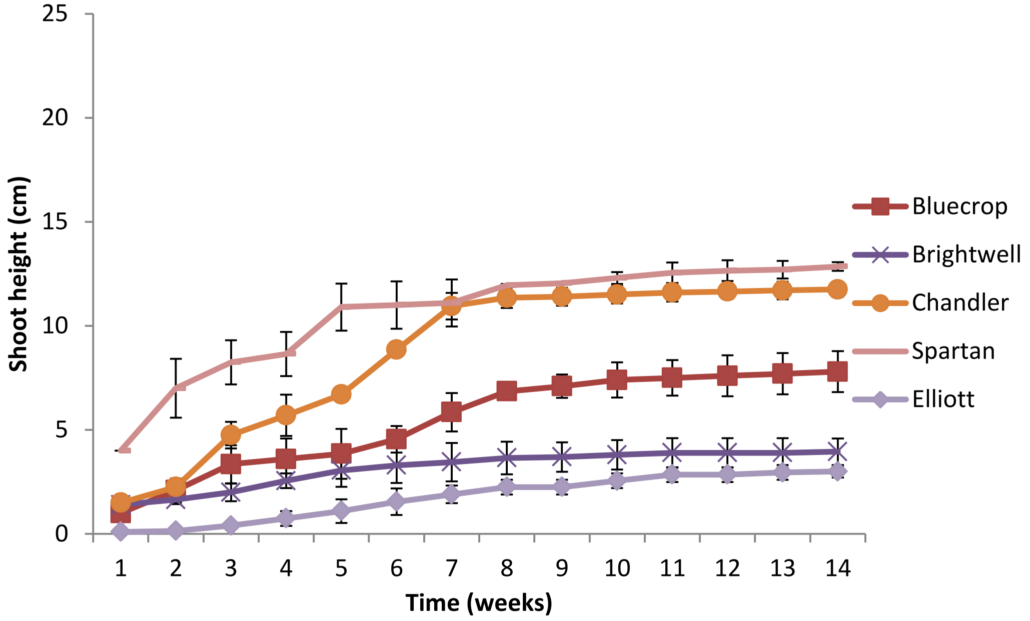

Figure 3.16 Interactive effects of uninoculated plants, variety and time on the shoot growth of the *Vaccinium corymbosum* varieties measured on weekly over 14 weeks. The graphs represent Mean ± SD (N = 3) (F $_{(104,211)}$ = 10.347; $p < 0.0001$).

3.8.3 Post harvest growth analysis

A significant increase in shoot dry weight was recorded for Chandler variety inoculated with *Lachnum* species (1.8g). There were no significant improvements in the dry shoot weight of the other inoculated plants of the remaining varieties.

Significant differences in root dry weight were recorded in *Cadophora* inoculated plants. The root dry weight of Elliott variety plantlets was improved as a result of inoculated with *Lachnum* species. However inoculation with *Cadophora* species ($p > 0.899$) did not differ from the uninoculated controls. No significant differences in root dry weight were recorded for the other treatments (Fig.3.19).

The data in for the growth indices was transformed because of the high variability within the replicates (3) used in the pot experiments. The variation ($p < 0.0001$) observed in canopy growth index and root growth index was mainly between varieties. Within treatment varieties, inoculated Bluecrop plants had a high canopy growth index particulary the *Cadophora* inoculated plants which were significantly different from the control. Brightwell variety inoculated with *Lachnum* species showed significantly improved canopy and root growth index. In the Chandler variety inoculation did not enhance either canopy or root growth index. *Cadophora* inoculated Spartan plants had higher canopy growth index while inoculation had no effect on the canopy or root growth index on all the other varieties (Fig.3.20).

Figure 3.17 Elliott Blueberry variety (A) uninoculated control, (B) inoculated with *Lachnum* species and (C) inoculated with *Cadophora* species after 14 weeks of inoculation.

Figure 3.18 Brightwell Blueberry variety (A) uninoculated control, (B) inoculated with *Cadophora* species and (C) inoculated with *Lachnum* species after 14 weeks of inoculation.

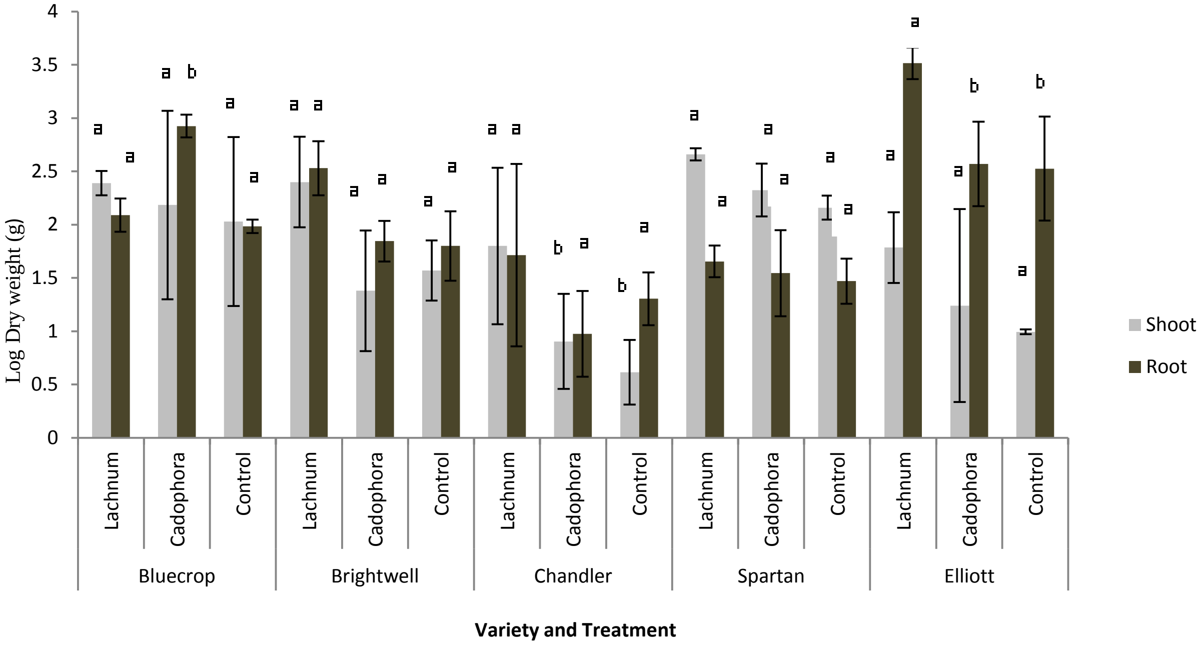

Figure 3.19 Shoot and root dry weights of inoculated *Vaccinium corymbosum* varieties in comparison with uninoculated control measured after 14 weeks of inoculation. Column represents Log. Mean ± SD (N = 3) .Shoot dry weight ($F_{(14;\,15)} = 3.029$; $p < 0.2055$) and root dry weight ($F_{(14,15)} = 7.317$; $p < 0.0002$). Log transformed means denoted by the same letter shows that means are not significantly different for each treatment within varieties.

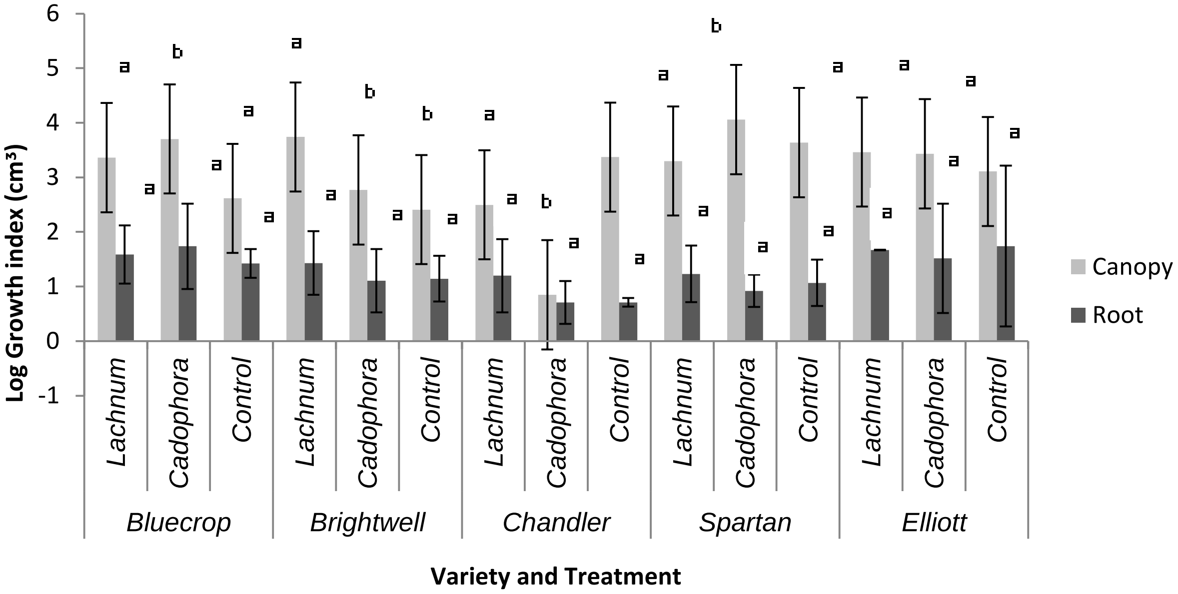

Figure 3.20 Canopy and root growth index of inoculated *Vaccinium corymbosum* varieties in comparison with uninoculated control measured after 14 weeks of inoculation. Column represents Log. Mean ± SD (N = 3). Canopy growth index ($F_{(14,\ 15)} = 17.383$; $p < 0.00019$) and root growth ($F_{(14,\ 15)} = 17.383$; $p < 0.0001$). Log transformed means denoted by the same letter shows that means are not significantly different for each treatment within varieties.

CHAPTER 4

DISCUSSION

4. Discussion

4.1 Identification of *Erica* plant species

Two ericaceous plants were identified *E. cerinthoides* and *E. demissa* and they commonly occur in low altitude areas. *E. cerinthoides* is native to South Africa, Lesotho and Swaziland. They have been identified growing in summer rainfall regions, in soils with a pH range of 5.5 – 6.7 and are susceptible to frost. *E. cerinthoides* is the most abundant ericaceous species in southern Africa (Schumann et al., 1992). The size, shape and colour of the leaves vary depending on the habitats they are found. *E. demissa* has been previously identified in South Africa from Cedarberg in the North West and the Klein Karoo, Van Stadens area near Port Elizabeth (Oliver and Oliver, 1998) and also in Grahamstown (Victor and Dold, 2003). *E. cerinthoides* are widely distributed and does not form part of the threatened species (Foden and Potter, 2005). There are two common forms of *E. demissa* differentiated mainly by their leaf morphologies; one has broad leaves which are recurved while the other has narrower sub-erect leaves. In Grahamstown the *E. demissa* differs from the above described morphologies mainly because of their unique habitat. The leaves are ternate with slender branches and have pale colored flowers (Bolus et al., 2009).

4.2 Soil nutrient analysis

The Ericaceae plants were growing in acidic soil with low micronutrient status (Table 1). Most ericaceous species in nature are well adapted to low pH soil of range 4 – 5.5 (Starast, 2008). These edaphic conditions are characteristic for Ericaceae habitats (Cairney and Meharg, 2003; Oliver and Oliver, 1998, Smith and Read, 2009; Straker, 1996). The unfavourable soil conditions could have arisen due to very low rates of evapotranspiration which do not promote decomposition of plant matter (Cairney and Burke, 1998). These edaphic factors together with the harsh climatic conditions often leads to the development of tough foliages consisting of a tissue rich in lignin and cellulose but deficient in N and P (Setaro et al., 2006; Straker 1996). Hence the ericoid mycorrhizal system is critical in the mobilization of nutrients from alternative sources in the soil.

The soil found in Grahamstown is predominantly weakly developed acidic lithosols which are low in mineral nutrients and is characterized by dark grey top soil (Schumann et al., 1992). This agrees with the analysis results and physical observations made on the collected samples. The soil characteristics of the locations where the plants were collected differed; *E. cerinthoides* was growing on a rocky outcrop with partially weathered rocks with very little vegetation while *E. demissa* was near the valley of the mountain where there was no rock material in the soil and significant ground cover vegetation. There were also large trees growing in close proximity to the *E. demissa* species which are predominantly arbuscular mycorrhizal (Skinner, 2001; Hawley and Dames, 2004). These distinct characteristics from the sampling sites could explain the differences observed in mineral nutrient status of the composite sample (Table 3.1).

Soil organic matter is comprised mainly of dead roots; decompose leaf litter and various plant residues contributed by plants inhabiting a particular site. The percentage organic matter of *E. demissa* soil samples (5.94%) was twice that observed in *E. cerinthoides* (3.4%) because of the abundance of partially weathered rocks in the soil which do not contribute to the soil organic matter content. However in soil samples analyzed for both *E. demissa* and *E. cerinthoides* the soil organic matter content was less than 10% which aligns with previous research findings for typical Ericaceae habitats (Meharg and Cairney, 2003). The amount of organic matter content varied from 3 – 5% in both soils. The organic matter found in *E. cerinthoides* 3.4 % has a potential of supplying 4.5% Nitrogen to the plant (Hofman, 1988). The soil density for both sampling sites was 1.0 gL^{-1} and 1.09 gL^{-1} recorded for *E. demissa* and *E. cerinthoides* respectively. These soils may not be ideal to support plant growth and root development. This could explain the poor vegetation observed in the site where *E. cerinthoides* was collected; hence imply that *E. cerinthoides* are dependent on the ericoid fungi for their survival in this area. The pH of the soil samples indicated that they were both acidic which is expected of the Ericaceae habitats.

4.3 Mycorrhizal status of *E. cerinthoides* and *E. demissa*

Ericoid mycorrhizas were confirmed for *E. cerinthoides* and *E. demissa* (Fig. 3.2) and DSE association was also observed in the roots (Fig. 3.3). Vohník and Albrechtová, (2011) established that DSE and ericoid associations can occur at the same time in plants and in such scenarios the predominant association is determined by the environmental conditions of the sampling site. In *Rhododendron* species investigated the ericoid associations were mainly found in Southern and central Europe while DSE were predominant in the northern Finland and in the Carpathians subalpine sites (Vohník and Albrechtová, 2011). From the roots sampled in this study the ericoid association was more dominant during microscopy. The abundance of the root hair and the intensity of the hyphal colonization within the infected cells are determined by factors such as season of collection (Kemp et al., 2003) and the fungal life cycle. Hutton et al. (1994) recorded that in *Epacridaceae* species of South West Australia, root hair abundance was greatest in winter and spring and no hair roots during the summer season. The latter is likely to be relevant to areas that experience Mediterranean climate were the hair roots die back as the soil dries out during the dry summers (Smith and Read, 2009). South Africa experiences similar climate and this could explain the findings as the roots observed were collected at the end of summer.

4.4 Isolation and identification of endophytes

The growth morphology of the pure isolates in culture was variable and the rate of growth was consistent with prior research findings on ericoid fungi which reported them as slow growing in culture (Smith and Read, 2009). *Cadophora* species have previously been isolated from ericoid, ectomycorrhiza and ectendomycorrhiza hosts (Bougoure, 2005; Tedersoo et al., 2009). Their ecological roles range from plant pathogens to root associates and colonizers of wood. The nature of the association is determined by various factors such as host plant, edaphic and climatic conditions (Blanchette et al., 2010). In the Antarctica *Cadophora* species comprise 35% of the total isolates identified as potential wood decay agents. *C. luteo – olivaceae* (strain 18) and *C. malorum* were the only *Cadophora* species isolated from soil (Blanchett et al., 2010). This distribution illustrates the diversity of the ecological roles of *Cadophora* species in a particular ecosystem. The Antarctica experiences extreme weather and poor soil conditions hence the abundance of *Cadophora* species implies that they well adapted to stressful conditions.

Cadophora is a generic name recently used to refer to *Phialophora* species with resemblance to the Dermateaceae in the Helotiales and these species exhibit various morphologies but the hypha is usually pigmented (Gams, 2000). This morphological trait was also observed (Fig.3.4) in this study. *Cadophora* species were identified from *Vaccinium myrtillus* in a spruce forest and *Vaccinium vitis-idae* in a *Pinus* forest. Ishida and Nordin, (2010) conducted fungal habitat preference trials which established that *Cadophora* species preferred Pine forests habitats. This agrees with the findings of this current research as the site of collection of samples was previously colonized by exotic Pine trees which have been excavated for the past 20 years in attempts to clear alien vegetation. Both *Cadophora* and *Lachnum* species are shared by ericaceous and non ericaceous plants hence these endophytes could have been shared by the Pine trees which are involved in ectomycorrhizal associations and the Ericaceae plants as they co-existed. No studies from this time on mycorrhizal fungi in the area could be found. The fungal colonies of some *Cadophora* species have previously been described as olivaceous grey with dark brown to black colouration in older cultures. The conidiophores are differentiated although sometimes it is not very distinct. The conidiogenous cells vary in shape and colour (light brown or olivaceous) and occur laterally or terminally on the apex of the conidiophores. This description indicates that the spores observed in culture of Ed6d could belong to the *Cadophora* genus (Williams and Spooner, 1991).

Although the associations are not clearly understood *Cadophora finlandia* has been recorded to form ericoid associations with *Vaccinium* species (Smith and Read, 2009) and ectomycorrhizas on *Pinus strobes* L. (Berch et al., 2002). Direct DNA extraction and amplification from the roots could have been used to determine the fungal taxa within the ericaceous plants. This would facilitate the identification of unculturable fungi which are potential ericoid endophytes (Allen et al., 2003; Bougoure et al., 2007). The *Cadophora* species like most DSE are well adapted for growth at pH 4.5 and do not favor high pH of the range 6.5 - 7 (Postma et al., 2007). Although the ericoid fungi are generally adapted to low pH the *Cadophora* species could be more tolerant to the pH 4 giving it a colonizing advantage as seen in this study (Table 3.5).

Lachnum species are saprotrophic fungi belonging to the Hyaloscyphaceae and are common root associates which are distributed widely especially in the tropical regions. They exhibit different morphological characteristics (Cantrell and Haines, 1997) and pigment under selected culture media (Ye et al., 2011). In a study conducted Ye et al., (2007) it was established that *Lachnum* species have an optimum growth at 25°C and pH 5.5. This was similar to the findings of this current research (Table 3.3 and 3.4). *Lachnum* species are not host specific and have been identified from different plant species which are known to form different mycorrhizal associations which include the ericoid mycorrhizas. However they have not been confirmed as mycobionts of the ericoid association (Walker et al., 2008). The pH of the culture media is also pivotal in the growth of fungi as some fungi are well adapted to acidic or alkaline or prefer neutral conditions for optimum growth. Both *Lachnum* and *Cadophora* species had optimum growth on acidic pH 5 which is not surprising as it is within the pH range of the sites of identification.

4.5 Resynthesis

In mycorrhizal resynthesis experiments carried out on blueberry plantlets and *E. demissa* seedlings. Inter and intracellular hyphal colonization was observed together with potential typical ericoid structures in *Cadophora* inoculated blueberry plantlets (Fig. 3.13). Read, 1983 as cited in Jeliazkova and Percival, (2003) described the association of ericoid fungi and *Vaccinium* as characterized by a loose hyphal network surrounding the root surface and hyphal penetration of the cortical cells. These findings were consistent with the DSE fungi root colonization described by Jumpponen and Trappe, (1998); Usiku and Narisawa (2007), were hyphal wefts around the root surface and intracellular colonization within cortical cells was recorded.

In colonized ericaceous plant roots, looped branched hypha and occasional hyphal coils with lightly pigmented coarse coils were observed by Walker et al. (2011) in *in-vitro* resynthesis trials of *V. uliginosum* inoculated with *P. fortinii*. Similar structures resembling ericoid mycorrhizas were observed in *Rhododendron* plants inoculated with DSE fungi (Zhang et al, 2009). These findings are not consistent with the typical DSE characteristics described by Jumpponen and Trappe (1998). This is not unusual as it has been established that particular fungal isolates have the ability to form different associations depending on the host plants and environmental factors.

Monreal et al. (1999) discovered that *Cadophora finlandia* formed ericoid mycorrhizas with *Gautheria shallon* and engaged in ectendomycorrhiza and ectomychorhizas on woody hosts. Tedersoo et al. (2009) suggested that identification of DSE as ericoid mycorrhiza or ectomycorrhiza mycobionts is a case of mistaken identity which is incurred because of the use of universal fungal specific primers during molecular identification. This is true for some cases were ericoid coils have not been recorded in resynthesis experiments for these associations however in light with the other positive results recorded, it can be hypothesized that the outcome of the association is determined by the host and mycobiont in the association.

Lachnum species have largely been isolated from ectomycorrhiza host plants (Bergemann and Garbelotto, 2006; Gao and Yang, 2010) although in areas of co-occurrence with ericoid plants they also have been identified in the Ericaceae roots (Walker et al., 2008). Resynthesis trials conducted on *V. uliginosum* with fungal isolates identified from Ericaceae plants indicated that *L. pygamaeum* and *L. virgineum* formed typical ericoid mycorrhiza coils. This shows that *Lachnum* species are ericoid mycorrhizal endophytes. In resynthesis trials the hyphal within the ericaceous roots (Fig 3.12a and 3.13a) did not form typical coils in both *E. demissa* and *V. corybosum*. This can also be explained as that coils observed during staining (Fig. 3.2) could have been formed by unculturable fungi such the basidiomycetes (Berch, 2002). In a study conducted to identify ericoid root endophytes, Walker at al. (2011) established that culture based methods of identification revealed a DSE complex *Phialocephala-Acephala* as the principal mycobiont of 3 co-occurring ericaceous plants while in direct amplification *Rhizoscyphus erica* complex was mainly identified.

These findings highlight the short comings of culture based methods of identification as rapidly growing fungi trend to conceal the presence of the relatively slow growing fungi in the roots. If this is the case it would imply that the Ericaceae roots in Grahamstown contain different fungal taxa within a single root which include the identified fungi. These coexisting endophytes may vary in their functions and may all be important for host survival and diversity in soil environmental conditions that are not ideal for plant growth (Midgely et al., 2004). The lack of ericoid structures in resynthesis trial could have resulted from the growth conditions which did not support ericoid mycorrhizal formation such as excess mineral nutrients and high pH (Darnell and Cruz-Huerta, 2011).

R. erica fungi are the main ericoid endophytes identified from the heathlands of the northern hemisphere (Smith and Read, 2009), these endophytes were however not identified in this study as well as in prior studies by Straker and Mitchell, (1985) where comparison of South African and European *R. erica* isolate by anti-body labeling showed that they were different. This supports the findings by Ishida and Nordin, (2010) which suggested that the environmental factors influence the fungal communities found at a particular site. The environmental factors experienced in the northern hemisphere differ from those of the fynbos of South Africa (Straker, 1996). Resynthesis experiments were carried out on pine bark which is the ideal growth medium used for the production of Blueberries in Stutterheim (Amatola berries); however this could not have been the best medium for the fungus and could account for the variable results that were obtained. The use of soil from the localities of original collection could have been ideal for the fungus and yield different results.

4.6 Growth characterization of identified isolates

4.6.1 Growth on different culture media

Culture media is a growth medium designed to provide microbes with essential nutrients to support microbial growth. They vary in their composition and do not support fungal growth in the same way because different fungi have different nutritional requirements essential for growth (Maier et al., 2000). Poor growth was observed in WA for both fungal species because it does not contain supplements essential for their growth. WA is an extract from algae which promotes slow growth of fungi hence has been used successfully for isolation of fungi from plant material. However during this study isolation was regarded as unsuccessful on this media. This indicates that *Lachnum* and *Cadophora* species require mineral nutrient supplements for their growth which were present in MEA and FTN media. *Cadophora* species sporulated and were darkly pigmented in 2% MEA while FTN it was lightly pigmented and non sporulating although growth was faster. This implied that 2% MEA is rich in mineral nutrients which induce spore formation while FTN promotes hyphal vegetative growth of the *Cadophora* species. The growth rate of *Lachnum* species on FTN was less in comparison with growth on MEA because the pH of the FTN was adjusted to pH 7 which is neutral and might not be conducive for growth of these species as they were identified from acidic soil conditions. An oversight at the time of experimentation although growth was recorded indicating their ability to grow on a wider range of pH.

4.6.3 Growth on different temperature

Temperature is influential in the metabolic activities of the cells of organisms hence there is variation in the optimum temperatures for growth of different organisms. Both *Cadophora* and *Lachnum* (Table 3.4) had an optimum growth temperature at 27°C which are classified as mesophilic. However they can be described as cold tolerant mesophiles because of their ability to grow in low temperatures (8°C). Mesophilic temperature range between 10 - 35°C and optimum temperatures vary between 15 - 30°C. The growth rate of the *Lachnum* and *Cadophora* species decreased after 27°C and no growth was observed at 37°C although it has been reported in literature that they can tolerate up to 40°C. In a study conducted by Duncan et al. (2006) it was established that two different strains of *Cadophora malorum* had optimum growth at 15°C and 25°C respectively. This shows that the optimum growth temperatures may differ between species. The *Cadophora* species were also able to grow at temperatures as low as 4°C thus showing their tolerance to low temperatures.

4.6.3 Growth of isolates on increasing Zn and Cu ions

In South Africa Cu and Zn naturally occur in areas located in close proximity to Cu ore outcrops where Cu is mined and Zn mines respectively. In these areas the soils are unsuitable for any agricultural activities e.g. Cu suppresses the uptake of Mn by crops as a mechanism of toxicity while Zn disturbs the uptake and utilization of other mineral nutrient such as Cu and Fe (Copper, 2005). The analysis of fungal growth *in-vitro* enables the determination of fungal responses to elevated concentrations of metals under controlled conditions. *H. erica* is one of the ericoid fungi that have been broadly acknowledged for its metal insensitivity thus its functional role in alleviation of metal toxicity to its hosts in polluted sites. In studies conducted by Gibson and Mitchell, (2006), *H. erica* showed tolerance to Cu and Zn with the concentration of metal resulting in 50% reduction mycelia dry mass (EC_{50}) in the range 0.3 - 0.9 mM and 1.2 - 5.5 mM for Cu and Zn respectively. Lacourt et al. (2000) discovered that *O. maius* was sensitive to Zn concentrations above 4 mM as the ericoid isolates showed reduced growth as metal ion increased. These findings are similar to those obtained in this trial for Zn^{2+} tolerance for both *Lachnum* and *Cadophora* species. Growth was reduced by the ion concentration of between 1.5 - 4.2 mM. However the concentration of Cu^{2+} ions between 0.3 and 1mM did not have a significant effect on the mycelial growth of both species. This indicates that both *Lachnum* and *Cadophora* species have potential to alleviate Cu^{2+} toxicity to its ericoid hosts. A decrease in mycelial growth in culture media with elevated metal ion concentration growth is consistent with a decrease in growth of the mycorrhizal host plants (Bradley et al., 1982). The findings in this trial on Cu^{2+} sensitivity trials did not agree with results obtained by Bradley et al., (1982) where 3 ericoid endophytes tested grew vigorously up-to 0.7 mM with little growth at concentrations exceeding 0.7 mM and no growth at 1mM.

Several mechanisms of tolerance have been established for different fungal isolates and particular metals. Zinc tolerance mechanism has been seen to be achieved by the secretion of mucilage and extracellular pigments which were also identified later as low molecular weight polypeptides (Martino et al., 2000; 2002). The *Cadophora* species produced slimy substances as the concentration of Zn^{2+} increased. No further tests were carried out on these secretions to confirm their identity and functional roles. Although growth was reduced with an increase in Zn^{2+} concentration the morphology of the mycelia was thin and lightly pigmented which could be a mechanism of tolerating toxicity. No secretions were however noted for the *Lachnum* species and growth was completely inhibited by concentrations above 4.5 mM.

In trials conducted by Gibson and Mitchell, (2005) it was established that pH has an effect on metal sensitivity. Hence the adjustment of the pH to 5.5 similar to the natural soil habitat would give a more precise insight on determining the metal tolerance of *Lachnum* and *Cadophora* species. The natural soil growth media for the isolates did not contain any toxic metals, *Cadophora* species were isolated from roots growing in soil containing 0.2 mgL^{-1} of Zn, which is low and might serve as a micronutrient to the plant or fungi while *Lachnum* species had no traces of Zn. This may contribute to sensitivity observed in *Lachnum* species as they may lack the down regulated genes which were seen to enable some ericoid fungi to resist Zn toxicity (Martino et al., 2002). It was however established that ericoid endophytes have the ability to alleviate metal toxicity regardless of the metal status of their soil of origin. In this current trial the results also indicate the same conclusion but may vary from one species to another as both

Lachnum and *Cadophora* species were insensitive to elevated concentration of Cu^{2+} when the sites did not contain Cu conversely *Cadophora* species was insensitive to Zn^{2+} and *Lachnum* species was sensitive.

4.7 Colonization and growth analysis

Colonization of Blueberry roots are characterized by intense hyphal infection of the cortical cells. Approximately 80% of the cells comprise hyphal material. The colonization of roots is influenced by several factors which include host, type of fungal endophyte, environmental conditions and most importantly the edaphic conditions and cultural practices. These edaphic factors include pH of the growth medium, N application rates, source of organic media and organic matter (Goulart et al., 1996; Yang et al., 2002). Percentage colonization was low and varied between isolates and across the varieties that were investigated in this study (Fig 3.10). Similar results were observed by (Scagel and Yang, 2005) and they were within the colonization range (0.5 - 44%) observed by Scagel and Yang, (2005) in a field analysis of blueberry plants. The fungi colonizes a small area of the root hence it is important to analyze a large number of root pieces.

In this trial the Elliott variety had 23% and 31% colonization for *Lachnum* and *Cadophora* species inoculated plants respectively. These observations were better than the percentage colonization observed for Elliott Blueberry variety in greenhouse trials after the first season of growth where percentage colonization ranged from 10.4 - 20.9% (Yang et al., 1996; 1998). They were however similar to the colonization (25.3%) of this variety observed under field conditions (Yang et al., 2002). These findings contradict the conclusion by Yang et al., (2002) that field plants have greater colonization in comparison with greenhouse plants. This however might be true for the region were the trials were conducted, proving that colonization of a particular host plant is indeed influenced by several factors such as environmental conditions, growth medium and cultural practices.

The hair roots of the Ericaceae are minute and delicate (Read, 1997; Smith and Read, 2009) and have also been described as devoid of hairs (Starast, 2008). Minimal colonization observed might have resulted from the loss of true hair roots during handling of plantlets at harvesting. This would result in the analysis of old hair roots for colonization during microscopy. According to the life cycle of the ericoid association the old hair roots contain traces of fungi with no typical ericoid structures as the fungi would have re-established in the new hair root near the meristem. Researchers agree that the work on the ericoid associations is limited due to difficulty experienced in handling them owing to their minute size (Smith and Read, 2009). The set back of hair root loss in future could however be prevented by harvesting the roots on a 50 µm sieve and retaining the fine hair roots from the sieve for analysis.

The percentage colonization can be also influenced by the mineral nutrient status of the soil. In both field and controlled conditions low N levels in the growth medium increase the ericoid fungal colonization (Goulart et al., 1996). The Pine bark is regarded as a very good growth medium for Blueberries (Greef, 2005) as it comprises highly of lignin which is not easily decomposed and N levels are less than 20 mM. The natural pH ranges between 4.0 and 5.0 (Krewer and Ruter, 2009) which is optimal for Blueberry plant growth (Scagel and Yang, 2005; Prodorutti et al., 2007). Although pine bark is ideal for Blueberry production the mineral nutrients have to be supplemented because it has a poor anion exchange capacity. The Long Ashton nutrient solution used in the trial to supplement mineral nutrients and N was in the form of NH_4SO_4 which effectively lowers soil pH as well. The solution was applied weekly so as to avoid excessive nutrient supply and no mineral nutrient analysis was conducted on the pine bark media. However the effects of N concentration on the ericoid fungal colonization are also influenced by the host. Trials conducted by Ishida and Nordin, (2010) showed an increase in N had no effect on ericoid fungal response in *V. myrtillus* and *V. vitis-idaea*. *Rhododendron* species showed declined colonization (Moore-Parkhurst and Englander, 1982) while *V. macrocarpon* had no colonization observed after being grown in media with elevated concentration of N (Stribley and Read, 1976).

In cases where N enrichment results in reduced colonization, the N enhances vegetative growth of the plant consequently C supply to the fungi is reduced which is critical for the growth, development and completion of the fungal life cycle. For ericaceous plants (*Calluna vulgaris*) the addition of N does not improve the rate of growth or photosynthesis, hence the host C transfer to the fungi in the roots remains consistent (Johansson, 2001). The colonization intensity of *C. vulgaris* was rather varied within seasons.

The Blueberry plants were propagated from tissue culture and grown in pasteurized pine bark in this trial so as to eliminate contaminants. The introduction of inocula through agents such as rain and wind were also eliminated by growing the plants in a mycorrhizal tunnel under controlled conditions. Although these precautions were taken traces of hypha were however observed in some untreated controls. This could be infection from fungi as that are widely distributed and found in numerous habitats such as the air, wood pulp and paper mill (Yang et al., 1998). Blueberry plants can however be naturally colonized by ericoid mycorrhizal fungi in nurseries or in the field (Scagel and Yang, 2005). This means that pasteurization is an effective method of maintaining the non mycorrhizal status of the tissue cultured Blueberry plantlets. Similar findings were obtained by Yang et al. (1998) after fumigating the growth medium with methyl-bromide; the untreated controls had minimal infection by soil fungi in the first growing season as they randomly colonized the cells without forming any distinctive mycorrhizal structures.

Although nutrient availability influences colonization and growth responses of Blueberry plants to ericoid fungi, the host plant cultivar and fungal compatibility and availability of the inoculum play a critical role in ericoid mycorrhizal colonization (Scagel, 2005). The presence of inocula is critical for the growth responses which are analyzed. In this trial during the formulation of the inoculum no measures were put in place to ensure the presence and to measure the concentration of the inoculum prior to inoculation as dry weight (mg) (Yang et al., 1998) to ensure adequate and consistent application to reduce variability owing to this factor. Although inocula was thoroughly homogenized and mixed variation in the concentration could still occur.

The Blueberry (*Vaccinni Corymbosum* L.) is native to North America and several cultivars have been developed for commercial purposes. These cultivars include Elliott, Brightwell, Chandler, Spartan and Bluecrop. They are well adapted to varying growth conditions and exhibit different vegetative growth patterns. The different varieties also produce fruits of different characteristics and in different seasons which is important in the commercial production of the berries. Ericoid fungi have potential to enhance plant growth under favorable conditions. The effects of ericoid fungal treatment on the growth of blueberry varieties have been inconsistent and higher colonization percentage does not imply a corresponding induced higher growth. Rogers, 2001 established that the greatest colonization had a corresponding elevated fruit yield. The *H. erica* fungi have been seen to improve the growth of Blueberries in field conditions and has shown little effect on trials conducted *in-vitro* or under greenhouse conditions. This suggests the use of field conditions may be important during research especially when the plants are being grown commercially in the field.

In 3 varieties which are fairly new to South Africa Chandler, Bluecrop and Spartan (Greef, 2003) no improved shoot growth was recorded on plantlets inoculated with either *Lachnum* or *Cadophora* species. Similar results were observed when a DSE (*Heteroconium chaetospira*) was inoculated on Blueberry plants; they colonized the cortical cells in a manner resembling an ericoid association but no growth was enhanced and the percentage colonization (10.1%) was relatively low (Vano et al., 2009). Inoculation of *H. chaetospira* on *Rhododendron obstusum var. kaempferi* had a colonization of between 13 - 20% and typical ericoid structures present but no effect on growth of the plants. These research findings as well as the current research confirm the ability of DSE endophytes to form ericoid structures with Ericaceae species (Usiku and Narisawa et al., 2004). In the Bluecrop variety investigated by Vega et al. (2009) inoculation with *H. erica* did not enhance shoot growth. This shows even typical ericoid fungi can have no effect on a particular ericaceous variety.

The Elliott and Brightwell highbush Blueberry varieties had enhanced shoot height (Fig 3.13) after inoculation with both *Cadophora* and *Lachnum* species. In a study conducted by Scagel, 2005 the colonization of the Blueberry plants was between 15 - 30% colonization was greater in organic fertilized plants. Plant shoot growth was enhanced but root: shoot ratio decreased. The inoculation of Elliott variety with *O. maius* improved the dry plant weight and the canopy volumes (Yang et al., 2002). This was also observed in the shoot and root dry weight of the *Lachnum* inoculated plantlets in this study. The length of the trial may have an effect on the outcome of the experiment e.g. growing the plants for 1 or 2 growing seasons.

CHAPTER 5

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

5.1 Ericaceae plants and their habitats

The identification of indigenous Ericaceous plants was carried out successfully. *E. demissa* and *E. cerinthoides* were identified although several species have been reported in literature to be present in the Mountain Drive area. There are more than 14 *Erica* species in the Albany Centre of Endemism which flower from November to April. The samples in this study were collected in March so diversity of endophytes would be important for all species. Continuous sampling in different seasons e.g. flowering season would have optimized the chances of identifying more species thus acknowledging the diversity of these species in the area of study. This is because their flowering morphology is the main key feature used for identification and the *Erica* species flower at different seasons’ e.g. *E. cerinthoides* flower in summer (December to late April) while no flowers were noted in March and December during sampling of *E. demissa*.

Soil samples collected from the Ericaceae habitats were analyzed for their nutrient status and other physical and chemical properties. The N content of the soil is difficult to analyze because it is usually variable owing to several factors such as soil temperature and moisture, organic matter content and other soil properties. Hence it is usually determined from the nitrate and protein in the soil and was not carried out in this study. The results from the analysis also confirmed that the Ericaceae species occur in acidic soil conditions low in organic matter content and available nutrients (Cairney and Meharg, 2003). These soil conditions coupled with the harsh climatic conditions experienced in the area are suitable for the growth of particular plant species and reports from various Ericaceae habitats universally have confirmed this. This has lead to the conclusion that the *Erica* plants grow and establish in these conditions because of their mycorrhizal relations with the ericoid fungi (Smith and Read, 2009). The plant roots from both *E. demissa* and *E. cerinthoides* were shown microscopically to have hyphal coils within their cortical cells which are typical for ericoid associations as well as other fungal structures which are possible DSE. Hence our findings support literature as it is evident that the association enables the plants to acquire nutrients from organic matter particularly nitrogen essential for growth and establishment.

5.2 Isolation and identification

The isolation of endophytes from the roots was conducted using the traditional culture method on various media. This was effective in isolating a diversity of isolates attained for both *E. demissa* and *E. cerinthoides* as isolates were sensitive to media during the initial isolation stages. In this research it has been established that ericaceous fungi are not always sterile and may exhibit different morphologies in culture depending on the composition of the culture media i.e. the *Cadophora* species sporulated in FTN media and remained sterile in 2% MEA. This has shown that precautions should be taken when screening isolates as their morphology may be complex and not easily distinguishable for non taxonomist (Domsch et al, 2007). Morphological identification was mainly used for screening common soil fungi such as *Aspergillus*, *Penicillum* and *Trichoderma* which were discarded.

Molecular techniques were used with the intention of identifying potential ericoid fungi to species level. However we not able to verify the identity of the isolates to species level as the sequence identities was higher for genus only in comparison with the species identity. It is important to note that the UNITE database which had the closest matches to species level is dedicated to mycorrhizal fungi and is more specific in comparison to the NCBI database which has a diversity of environmental microbes. Hence UNITE is an important reference for future work on mycorrhizas and there is a need to continuously update the database so as to have a greater collection of mycorrhizal species for research purposes. The identification of *Lachnum* and *Cadophora* species as ericoid endophytes has shown the fungi involved in the ericoid association are not highly specific as it can form other associations with different host plants (Jumpponen, 1998; Walker et al., 2008). *Erica* roots can harbor multiple fungal taxa in their roots all with different functional roles (Cairney and Meharg, 2003) hence there is still a possibility that there are some potential ericoid fungi which were not identified because they are unculturable such as Basidiomycetes or masked in culture by other fungi which are fast growing. For future work it is also important to do direct root DNA amplification so as to get the fungal taxa of both culturable and unculturable fungi. This could be done by Denaturating Gradient Gel Electrophoresis (DGGE) or 454 pyrosequencing both methods which involves analyzing fungal DNA extracted directly from the roots in order to determine the microbial genetic diversity in the root system. During pyrosequencing ITS copies are generated from a small initial amount of

DNA and either the ITS1 or ITS2 are targeted for sequencing making it possible to identify environmental samples of fungi to species level (Nilson et al., 2009). In DGGE the DNA directly extracted from the roots is amplified and denatured with a denaturation agent resulting in small genomic fragments of DNA bands being identified and individually sequenced prior to comparison to sequences in the database (Ferns et al., 1996). Nilson et al. (2009) described pyrosequencing as the most important technique in the future identification of fungi and other groups of organisms in environmental samples.

5.3 Growth characterization

Cadophora and *Lachnum* species were both established to be cold tolerant mesophiles (Duncan et al., 2006) as they have optimum growth at 27°C and tolerate temperatures as low as 8°C. Both species had optimum growth on acidic media of pH 5 which is consistent with the soil pH of their original soil habitat. The isolates are not selective on the culture media and require mineral nutrient supplements as shown by their retarded growth on WA in comparison with nutrient rich FTN and 2% MEA. These factors are important for the development of batch cultures for inocula production because there is need to optimize production especially for commercial purposes. The ericoid fungi have diverse functional properties in nature hence it is importance to examine their other characteristics such as protease, cellulase, chitin and pectinolytic activities. These studies may contribute positively in different industries, such as Agriculture where chitin enzymes have a potential use in plant disease management (Dubey, 2011). Ericoid fungi with pectinolytic activity can also be used in the industrial production of pectinases which can be utilized in the wine making industry (Jayani et al., 2005).

5.4 Metal sensitivity

Cadophora species has the potential to alleviate Zn^{2+} toxicity as shown by their growth in concentrations of up to 4.5 mM. These species showed reduced mycelial growth as metal concentration increased but secreted pigmented substances which were spread on the media in a circular manner simulating the normal mycelial growth. Further tests should to be conducted to identify the secreted substances and to establish the mechanism of tolerance for Zn^{2+} toxicity. Greenhouse trials with plants inoculated with *Cadophora* species must be conducted in media with elevated metal concentration so as to avoid speculation on the role of *Cadophora* species in metal toxicity alleviation. *Lachnum* species were sensitive to increasing concentration of Zn^{2+} metal hence do not have any potential uses in bioremediation.

Both *Lachnum* and *Cadophora* species showed tolerance to elevated Cu^{2+} concentrations of between 0.3 - 0.1 mM. Literature has reported 0.9 mM to inhibit the mycelial growth of *H. erica* (Gibson and Mitchell, 2006). This study has established that the investigated isolates have potential to alleviated Cu^{2+} toxicity in the Ericaceae plants. In light with these findings the *Lachnum* and *Cadophora* species have potential to alleviate other metal toxic effects of metals such as Al, Fe, Cd and Mn. There is need to conduct further research on the potential uses of these isolates in bioremediation with respect to metal toxicity alleviation. This research will be useful in South Africa as it has a vast amount of land occupied by mining properties and is one of the most environmentally impacted countries in the Sub-saharan region (Behnassi et al., 2011). The mining activities have resulted in heavy metal polluted soils contaminated with Ca, Mg, Fe, Mn, Cd, SO_4 and Zn. Research can also be broadened to all the metals of importance so as to rehabilitate soil in the mine dumps and other soils which are naturally rich in toxic heavy metals and excessive trace elements. The restoration of the soil will enable it to be used for agricultural or recreational purposes.

5.5 Effects of inoculation on Blueberry plant growth

Inoculation significantly enhanced the shoot height of Elliott and Brightwell blueberry plantlets. There was no improvement in the shoot and root dry weight of the same plants. The post harvest growth analysis had variable results which were not consistent with the shoot growth, i.e. the Bluecrop variety plants inoculated with *Cadophora* species had no improved shoot height but had improved root dry weight as well as shoot and root growth index. This means that *Cadophora* species have the potential to improve growth of the Bluecrop variety when all attributes are considered. In light with the findings there is need to conduct more research with the same isolates under field commercial conditions with more replicate plantlets to reduced standard errors. It is also possible that effects of the fungal inoculum (*Lachnum* and *Cadophora* species) did not have adequate time to have significant effects on the plants as duration of the study was limited. Hence in future better results could be attained with the same species if research was conducted for 2 - 3 growing seasons (Yang et al., 2002). Apart from monitoring growth other attributes such as nutrients e.g. C or N content in the leaves, photosynthetic rate and berry productivity can also be evaluated in order to determine the effects of inoculation on the blueberry plants. Three methods of formulating inocula which are what have successfully been implemented in inoculation experiments. The gel inocula however is ideal for experiments run for a limited period as inoculum is directly applied to the roots and the gel sticks to the hair roots thus enhancing colonization (Smith and Read, 2009).

Most research on *V. corymbosum* has been conducted in the northern hemisphere countries which experience difference climatic conditions and have different soils growing conditions in comparison to South Africa. The variations in the results could be attributed to these factors. Very little research has been conducted on Blueberries in South Africa (Greef, 2003) and there is little published work. The colonization and fungal inoculation effects differ with fungal species and host involved, *Lachnum* and *Cadophora* species have not been tested on blueberry plants before. This study has further affirmed that ericoid fungi have potential of enhancing productivity of *V. corymbosium* especially for commercial purposes, hence there is need to intensify research in this field in South Africa.

5.6 The Blueberry industry in South Africa

Blueberry is one of the smallest and successful fruit divisions in the horticulture (Agriculture) industry of South Africa. There are between 40 - 50 growers in the country and over the years production of blueberries has been consistently high and of great quality with approximately 1000 tonnes exported in 2011 (Hartigh, 2011). The global demand for the berries has increased because of the numerous health benefits they confer. However the production in the UK and Europe has decreased due to poor weather conditions, high production cost and the strict pesticide control legislation (Prodorutti et al., 2007). The land for agriculture has also become limited because of industrialization. This has increased the demand for the local produce with most exports being to the UK and Europe. In the northern hemisphere the supply of blueberry in winter is entirely depended on imports from the southern hemisphere because of the difference in seasons. In the southern hemisphere Argentina is South Africa's main competitor and the latter has potential of topping the list as they have been producing consistently good berries (Hartigh, 2011). However with research support the South Africa blueberry industry has potential of being the largest producer in the southern hemisphere. Research on ericoid endophytes like *Lachnum* and *Cadophora* species will enable the SABPA to have the resources they require to enhance plant productivity while maintaining or improving the quality of the berries.

5.7 Conclusion

The overall aim and objectives of the current study were fulfilled. Ericaceous plants, *E. cerinthoides* and *E. demissa* were successfully selected and identified from Grahamstown. The analysis of the soil from the *Erica* sample sites characterized them as acidic and low in mineral nutrients. Microscopy confirmed the ericoid mycorrhizal status of *E. cerinthoides* and *E. demissa* and the presence of DSE association within the roots of the same plant species. Potential ericoid fungi were isolated from the sampled roots in culture and different morphological characteristics were exhibited which were used for initial identification and screening of common soil fungi. Relatively slow growing isolates which did not sporulate heavily in culture were identified molecularly and *Lachnum* and *Cadophora* species were selected as potential ericoid fungi hence were retained for further experimentation. The optimum growth conditions for both fungi were acidic media containing mineral nutrients and incubated at 27°C. *Cadophora* species were insensitive to elevated concentrations of Cu and Zn ions, while *Lachnum* species were only tolerant to a high concentration of Cu ions. A gel inocula was formulated from both *Lachnum* and *Cadophora* species and tested on blueberry plantlets in a greenhouse. Inoculation had variable effects on the attributes evaluated. The shoot height of both *Lachnum* and *Cadophora* inoculated Elliott and Brightwell varieties was enhanced while the root growth index and biomass of *Cadophora* species inoculated Bluecrop plants was improved.

In conclusion, there is need to conduct continuous research on the ericoid fungi in South Africa as they have important potential functions in the commercial berry production and further research can result in potential uses in bioremediation.

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Appendices

APPENIDIX A

Roots clearing and staining solutions (Smith and Dickson, 1997)

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 HCI (MW36.46)

22.79 ml HCI

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

B2. 2% Malt extract agar

Constituent components	**GramL^{-1}**
Malt extract	**30**
Soy peptone	**5.0**
Agar	**15**

B3. Modified Fontana media (Fontana, 1963)

Constituent component	GramL^{-1}
D- Glucose	6.5
Peptone	1.65
Potassium di-hydrogen orthophosphate	0.33
1% Mg Sulphate heptahydrate	150 µl
1% Ferric chloride	150 µl
1% Zinc sulphate	150 µl
1% Manganese sulphate monohydrate	150 µl
1% Calcium chloride	150 µl
Bacteriological agar	15
Distilled water	950 ml
After autoclaving add:	
BSA	0.33
Chloramphenical	0.01
Ampicilin	0.01

APPENDIX C

C1. 1% agarose gel

1g 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:

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

To make make 1X TBE Buffer working solution

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

Storage is at room temperature.

APPENDIX D

LONG ASHTON'S PLANT NUTRIENT SOLUTION (Hewitt, 1966)

Stock A

Chemical	**Quantity in 1L distilled water**
$MgSO_{4.}7H_2O$	36.9 g
$MnSO_4$ or $MgSO_{4.}H_2O$	0.223 g
$CuSO_{4.}5H_2O$	0.24 g
$ZnSO_{4.}7H_2O$	0.029 g
H_3BO_3	0.19 g
$(NH_4)_6Mo_7O_{24.}4H_2O$	0.0035 g
$CoSO_{4.}7H_2O$	0.0028 g
NaCl	0.585 g

Stock B

Chemical	**Quantity in 1L distilled water**
FeEDTA	3.0 g

Stock C

Chemical	**Quantity in 1L distilled water**
$CaCl_2$	50 g

Stock D

Chemical	**Quantity in 1L distilled water**
K_2SO_4	21.7 g

Stock E

Chemical	**Quantity in 1L distilled water**
$(NH_4)_2SO_4$	105.0 g

Stock F

Chemical	**Quantity in 1L distilled water**
$NaH_2PO_4.2H_2O$	2.5 g

Stock solutions were stores at 4ºC.

Preparation of 1L of Nutrient Solution

The stock solutions were removed from the fridge and were brought to room temperature. Thereafter 200 ml of distilled water was placed in a 1L measuring cylinder. From each stock solution 10 ml L^{-1} was added. Distilled water was used to make upto 1L.

APPENDIX E1

Erica cerinthoides isolate (ECHh) alignment with GenBank isolate *Lachnum species*.

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CLUSTAL W (1.83) multiple sequence alignment


ECHh  34    AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGAGTTCATGCCC--TTCGGGGT  91
            |||||||||||||||| |||||||| ||||||||||||||||||||||||  |||   ||
L.spp.5     AAGGTTTCCGTAGGTG-ACCTGCGG-AGGATCATTACAGAGTTCATGCCCTTTTC-ACGT 61
            **************** ******** ************************  ***   **

ECHh  92    AGGTCTCCCACCCTTGTGTATCATTATAGAATGTTGCTTT--GGCGGGCCGC--GCCTCG  147
            || |||||||    |||||||||||||||||||||| |||  ||||||||||  |    |
L.spp. 62   AGATCTCCCAAAAATGTGTATCATTATAGAATGTTG-TTTCCGGCGGGCCGCGGGGAAAG 120
            ** *******    ********************** ***  **********  *    *

ECHh  148   -TGCGCCTAGATCCGCGTCTAGCGTGCCCGCCAGAGGACCCCTAAACTCTGAATGTTAGT  206
               ||||| ||| ||||||  | ||||||||||||| |||||||||||||||||||||||
L.spp. 121  ACACGCCTGGATTCGCGTCCGGGGTGCCCGCCAGAGAACCCCTAAACTCTGAATGTTAGT  180
               ***** *** ******  * ************* ***********************

ECHh  207   GTTGTCTGAGTACTATTAAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT  266
            || |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
L.spp. 181  GTCGTCTGAGTACTATTAAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT  240
            ** *********************************************************

ECHh  267   CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC  326
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
L.spp. 241  CCATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC  300
            * **********************************************************

ECHh  327   GAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTC  386
            |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
L.spp. 301  GAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTC  360
            ************************** *********************************

ECHh  387   ATTTATACCAATCTAGCCTGGCTAGGTGTTGGGCTTCGCCGTCTGGCGGGCCTTAAAAAT  446
            ||||||||||||||||||||||||||||||||| |||||||||||||||||||||||| |
L.spp. 361  ATTTATACCAATCTAGCCTGGCTAGGTGTTGGGGTTCGCCGTCTGGCGGGCCTTAAAACT  420
            ********************************* **************************

ECHh  447   TAGTGGCGGTGCTCTTGGGCTCTACGCGTAGTAATTTTCTCGCTATAGGGTCCTGGGAGA  506
             |||||||| |||||| |||||||||||||||||||||||||||||| ||||||||||||
L.spp. 421  -AGTGGCGGGGCTCTTAGGCTCTACGCGTAGTAATTTTCTCGCTATAAGGTCCTGGGAGA  479
             ******** ****** ****************************** ************

ECHh  507   GGCTAGCCAACAACCCCAAATTTTTCTAGGTTGACCTCGGTTCAGGTAGGGATACCCGCT  566
             ||| |||| |||||||||||||||||||| ||||||||| |||||||||||||||||||
L.spp.  480 TGCTGGCCAGCAACCCCAAATTTTTCTAGGGTGACCTCGGATCAGGTAGGGATACCCGCT  539
             *** **** ******************** ********* *******************

ECHh  567   GAACTTAAGCATATCAATAA-GCGG-AGGAA  595
            |||||||||||||||||||| |||| |||||
L.spp.540   GAACTTAAGCATATCAATAAAGCGGGAGGAA  570
            ******************** **** *****
```

Erica cerinthoides **isolate (ECHh) alignment with UNITE isolate** ***Lachnum brevipilosum*****.**

```
CLUSTAL W (1.83) multiple sequence alignment


echh_B10_004.ab1      -----------------------------ACGTAAGGTTTCCGTAGGTGA-CCTGCGGA- 29
L.brevipilosum        TCTTGGTCATTTAGAGGAAGTAAAAGTYGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA 60
                                                       ***************** ********  

echh_B10_004.ab1      GGATCATTACAGAGTTCATGCCCTTTTCACGTAGATCTCCCAAAAATGTGTATCATTATA 89
L.brevipilosum        GGATCATTACAGAGTTCATGCCCTTCG-GGGTAGGTCTCCCACCCTTGTGTATCATTATA 119
                      *************************     **** *******    **************

echh_B10_004.ab1       GAATGTTGTTTCCGGCGGGCCGCGGGGAAAGACACGCCTGGATTCGCGTCCGGGGTGCCC 149
L.brevipilosum        GAATGTTGCTTT-GGCGGGCCGCGCCT---CGTGCGCCTAGATCCGCGTCTAGCGTGCCC 175
                      ******** **  ***********          ***** *** ******  * ******

echh_B10_004.ab1      GCCAGAGAACCCCTAAACTCTGAATGTTAGTGTCGTCTGAGTACTATTAAATAGTTAAAA 209
L.brevipilosum        GCCAGAGGACCCCTAAACTCTGAATGTTAGTGTTGTCTGAGTACTATTAAATAGTTAAAA 235
                      ******* ************************* **************************

echh_B10_004.ab1      CTTTCAACAACGGATCTCTTGGTTCTGGCATCCATGAAGAACGCAGCGAAATGCGATAAG 269
L.brevipilosum        CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG 295
                      ******************************** ***************************

echh_B10_004.ab1      TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTG 329
L.brevipilosum        TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTG 355
                      ********************************************************* **

echh_B10_004.ab1      GTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTATACCAATCTAGCCTGGCTAGGTGT 389
L.brevipilosum        GTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTATACCAATCTAGCCTGGCTAGGTGT 415
                      ************************************************************

echh_B10_004.ab1      TGGGGTTCGCCGTCTGGCGGGCCTTAAAA-CTAGTGGCGGGGCTCTTAGGCTCTACGCGT 448
L.brevipilosum        TGGGCTTCGCCGTCTGGCGGGCCTTAAAAATTAGTGGCGGTGCTCTTGGGCTCTACGCGT 475
                      **** ************************  ********* ****** ************

echh_B10_004.ab1      AGTAATTTTCTCGCTATAAGGTCCTGGGAGATGCTGGCCAGCAACCCCAAATTTTTCTAG 508
L.brevipilosum        AGTAATTTTCTCGCTATAGGGTCCTGGGAGAGGCTAGCCAACAACCCCAAATTTTTCTAG 535
                      ****************** ************ *** **** *******************

echh_B10_004.ab1      GGTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAAGCGGGAGG 568
L.brevipilosum        GTTGACCTCGGTTCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA-GCGGAGGA 594
                      * ********* *************************************** ****  * 

echh_B10_004.ab1      AAA------- 571
L.brevipilosum        ACACTCCTTA 604
```

Alignment for isolate from *Erica demmissa* (ed6d) with Genbank isolate *Cadophora species.*

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CLUSTAL 2.0.12 multiple sequence alignment


ed60_B11_003.ab1      ANGTNAGGTTTCGTAGGTGACCTGCGGAGGATCATTACTAGAGCAAAGGANAGANTCNNN 60
Cadophora spp.        ------------GGAAGTAAAAGTCGTAACAAGGTCTCCGTAG----GTGAACCTGCGGA 44
                                  * * ** *    ** *  *   *  *   **    *   *    *

ed60_B11_003.ab1      TACTTCGGGGNANAANNCGGCGGGCCTCTCGGAGAAGNTTNCCCCCTGAACTCCAGAAAA 120
Cadophora spp.        AGGATCATTACAGAGAATATCGCGCCTCACGGGTGACTCTCC------AACCCTA----- 93
                          **     * *      ** ***** ***   *   * *      *** * *

ed60_B11_003.ab1      NAATAAATTACCTTTGTTGCTTTGGCGGGCC-GCCTCGCGCCAGCGGCTTCGGNTGTTGA 179
Cadophora spp.        TGTTATATAACCTTTGTTGCTTTGGCGAGCCCGCCTCG-GCCGCTGGCTTCGGCTAGCGA 152
                         ** ** ****************** *** ****** ***   ******** *   **

ed60_B11_003.ab1      GTGCCCGCCAGANGACCACAA-CTCTTGTTTTTAGTGATGTCTGAGTACTATATAATAGT 238
Cadophora spp.        GTGCTCGCCAGAGGACCCCAAACTCTGAATATTAGTGTCGTCTGAGTACTATATAATAGT 212
                      **** ******* **** *** ****   * ******  *********************

ed60_B11_003.ab1      TAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG 298
Cadophora spp.        TAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG 272
                      ************************************************************

ed60_B11_003.ab1      ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 358
Cadophora spp.        ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 332
                      ************************************************************

ed60_B11_003.ab1      CTCTGGTATTCCGGGGGGNATGCCTGTTCGAGCGTCATTATAACCACTCAAGCTCTCGCT 418
Cadophora spp.        CTGTGGTATTCCGCAGGGCATGCCTGTTCGAGCGTCAGTACAAC-ACTCAAGCTCT-GCT 390
                      ** **********  *** ****************** ** *** *********** ***

ed60_B11_003.ab1      TGGTATTGGGGTTCGCGGTT-TCGCGGCCCCTAAAATCAGNGGGGGTGCCTGTCGGCTCT 477
Cadophora spp.        TGGTATTGGGCCCTGCCGTCGCCGGCAGCCTTAAAATTAGTGGCGGCGCAT-CTGGCTCT 449
                      **********    ** **   **    ** ****** ** ** ** ** *   ******

ed60_B11_003.ab1      ACGCGTAGTAATACTCCTCGCGATTGAGTCCGGNAGGTCTACTTGCCAGCAACCCCCAAT 537
Cadophora spp.        AAGCGTAGTAATACTCCTCGCTACAGAGTCCGTTGGA--TGCTTGCCAGCAACCCCCAAC 507
                      * ******************* *  *******   *   * ******************

ed60_B11_003.ab1      TTTTACAGGGTGACCTCGGATCAGGNAGGGATACCCGCTGAACTTAAGCATATCAATAAG 597
Cadophora spp.        TTTCTAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAG 567
                      ***   *** *************** *** ******************************

ed60_B11_003.ab1      CGGAGGAA 605
Cadopho               CGGAGGA- 574
                      *******
```

Alignment for isolate from *Erica demmissa* (ed6d) with UNITE isolate *Cadophora luteo-olivacea.*

```
CLUSTAL W (1.83) multiple sequence alignment


Ed6d  3       TGTGCATTGCAG-ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTA  61
              |||| ||||||| |||||||||||||||||||||||||||||||||||||||||||||||
C.luteo-oliv   TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTA 420
              **** ******* ***********************************************

Ed6d  62     TTCCGGGGGGCATGCCTGTTCGAGCGTCATTATAACCACTCAAGCTCTCGCTTGGTATTG  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.luteo-oliv TTCCGGGGGGCATGCCTGTTCGAGCGTCATTATAACCACTCAAGCTCTCGCTTGGTATTG   480
             ************************************************************

Ed6d  122    GGGTTCGCGGTTTCGCGGCCCCTAAAATCAGTGGCGGTGCCTGTCGGCTCTACGCGTAGT  181
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
C.luteo-oliv GGGTTCGCGGTTTCGCGGCTCCTAAAATCAGTGGCGGTGCCTGTCGGCTCTACGCGTAGT   540
             ******************* ****************************************

Ed6d  182    AATACTCCTCGCGAT-TGAGTCCGGTAGGTCTACTTGCCAGCAACCCCCAATTTTTACAG  240
             ||||||||||||| | || |||||||||||||||||||||||||||||||||||||||||
C.luteo-oliv AATACTCCTCGCG-TCTGGGTCCGGTAGGTCTACTTGCCAGCAACCCCCAATTTTTACAG   599
             ************* **********************************************

Ed6d  241    GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA  299
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.luteo-oliv GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA   658
             ***********************************************************
```

E2 Fungal nucleotide sequences

```
>ECHh_ITS3F_D03_12.ab1
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCMTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGT
CATTTATACC
AATCTAGCCTGGCTAGGTGTTGGGCTTCGCCGTCTGGCGGGCCTTAAAACTAGTGGCGGTGCTCTTAGGC
TCTACGCGTA
GTAATTTTCTCGCTATAGGGTCCTGGGAGATGCTGGCCAGCAACCCCAAATTTTTCTAGGTTGACCTCGG
ATCAGGTAGG
GATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA


>ED6d_ITS3F_E03_15.ab1
GWTGTGCATTGCAGATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGG
GCATGCCTGT
TCGAGCGTCATTATAACCACTCAAGCTCTCGCTTGGTATTGGGGTTCGCGGTTTCGCGGCCCCTAAAATC
AGTGGCGGTG
CCTGTCGGCTCTACGCGTAGTAATACTCCTCGCGATTGAGTCCGGTAGGTCTACTTGCCAGCAACCCCCA
ATTTTTACAG
GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA
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