THE DIVERSITY OF ROOT FUNGI ASSOCIATED WITH *ERICA* SPECIES OCCURRING IN THE ALBANY CENTRE OF ENDEMISM.

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Microbiology

Of

Rhodes University

In the

Department of Biochemistry, Microbiology and Biotechnology

Faculty of Science

By

Christine Bizabani

February, 2015

Abstract

South Africa has the highest species diversity of ericaceous plants belonging to the *Erica* genus. There are over 850 identified species in the Cape Floral Region. The Albany Centre of Endemism (ACOE) is located within this region and is a hotspot of diversity consisting of various plant genera. The success of *Erica* plants is ubiquitously attributed to mycorrhizal relationships they engage in with a diverse group of fungi. This symbiosis is known as the ericoid mycorrhizal (ERM) association. The overall aim of this study was to establish the diversity of root fungi associated with *Erica* plants using morphological, molecular and 454 pyrosequencing techniques.

Six *Erica* species were identified using leaf and flower morphology according to taxonomic keys. The identified plants were *Erica cerinthoides*, *Erica demissa*, *Erica chamissonis*, *Erica glumiflora*, *Erica caffra* and *Erica nemorosa*. Roots from sampled plants were stained and examined microscopically to determine their mycorrhizal status. Ericoid mycorrhizal associations together with dark septate endophyte (DSE) structures and hyphae that did not form any specific structure were observed in all the roots. In addition arbuscular mycorrhizal (AM) structures in the form of vesicles were detected in *E. glumiflora* and *E. cerinthoides*.

In order to identify the culturable fungi associated with the respective hosts, sterilised roots were placed on various culture media for cultivation. Thereafter isolated fungi were morphologically classified into 67 morphotypes. These were mostly sterile and darkly pigmented. Non-sporulating mycelia of variable colouration such as white, cream-yellowish, beige, green and brown were also observed. Further identification was carried out using molecular techniques. DNA was extracted separately from pure cultures and amplified using ITS1 and ITS4 primers in a polymerase chain reaction (PCR). Thereafter sequencing and Basic Local Alignment Search Tool (BLAST) were used to identify the isolates to generic level. The fungi were taxonomically classified into 54 operational taxonomic units and 94% were Ascomycetes and Helotiales was the dominant order. Unclassified Helotiales with affinities to fungi currently identified as *Epacrid* root fungus was common in all hosts. Other isolates that were identified included *Oidiodendron*, *Meliniomyces*, *Phialocephala*, *Cadophora*, *Lachnum*, *Leohumicola*

Cryptosporiopsis, *Chaetomium*, *Acremonium* and *Epicoccum* species. Basidiomycetes were represented by two OTUs belonging to the genus *Mycena*. Four OTUs comprised fungi that had no significant alignments in the reference databases.

Direct root DNA extraction together with 454 pyrosequencing was used to detect the diversity of culturable and unculturable fungi associated with the identified hosts. The ITS2 region was targeted for sequencing. Although Ascomycetes remained the dominant phyla, Basidiomycetes were also detected in all host plants. Glomeromycota was present in *E. caffra* and *E. cerinthoides.* Helotiales was dominant in all *Erica* plants with the exception of *E. cerinthoides* and *E. chamissonis* which were dominated by the order Chaetothyriales. The OTUs identified to genus level included *Epacris pulchella* root fungus, *Oidiodendron* cf. *maius, Acremonium implicatum, Leohumicola*, *Lachnum*, *Capronia* and *Mycena* species. Culture-based techniques and pyrosequencing detected similar fungal composition comprising Ascomycetes, while, pyrosequencing was able to detect Glomeromycetes and Basidiomycetes.

A further objective was to characterise selected ericaceous fungi in terms of various organic and inorganic sources of nitrogen (N), phosphorus (P), and carbon (C) using biomass accumulation in liquid culture. The inorganic N sources used in this study were $(NH_4)_2HPO_4$ and $Ca(NO_3)_2$, and arginine and bovine serum albumin (BSA) were the organic sources. The biomass was variable for the different nutrient sources and inorganic sources were commonly preferred. Isolates *Meliniomyces* and *Leohumicola* species were able to utilise BSA as a sole N source effectively. Phosphorus nutrition was tested in the form of $NaHPO_4.2H_2O$ (orthophosphate), sodium inositol hexaphosphate (phytic acid) and salmon sperm DNA. Most isolates preferred orthophosphate as a substrate. *Meliniomyces* sp. and *Acremonium implicatum* were able to accumulate significant biomass with salmon DNA as a sole source of P nutrition, in comparison to the control that lacked a P source. Phosphomonoesterase activity was detected in controls of unidentified Helotiales fungus, *Leohumicola* sp., and *Cryptosporiopsis erica*.

Carbon utilisation was tested using glucose, cellobiose, carboxymethylcellulose (CMC), pectin and tannic acid as C sources. Variable biomass yields were observed for the different C treatments, with all fungal isolates producing high biomass on glucose and cellobiose. The highest biomass for pectin was recorded in *Meliniomyces* species. Iron assimilation by the selected fungi was assessed using the Chrome Azurol Sulfonate (CAS) assay. Six isolates

produced halos in culture on CAS agar, whereas the liquid CAS assay, as determined by spectrophotometric absorbance readings, showed positive siderophore production in eleven isolates. Fungi that include unidentified Helotiales fungus, *Oidiodendron* and *Meliniomyces* species did not produce halos and had poor growth on CAS agar plates but produced siderophores in liquid CAS assay. From the selected fungi, isolates such as *C. erica* and *Mycena* sp. did not produce siderophores in both these assays.

Anti-pathogenic fungal activity of selected *Erica* root isolates was tested against *Phytophthora cinnamomi* in culture and *in-plantae*. *Leohumicola* sp. was the only isolate that inhibited the growth of *P. cinnamomi* in a dual agar plate assay. *Oidiodendron* and *Leohumicola* species were inoculated on Brightwell blueberry plantlets to assess the effects of *Phytophthora* on root development and plant growth. Although both *Oidiodendron* and *Leohumicola* species formed typical ericoid structures they did not improve the root and shoot biomass in the presence of *P. cinnamomi*. No visible symptoms of disease resulting from *P. cinnamomi* infection were observed on any of the plants including non-ERM plants that were inoculated with the pathogen, indicating that the blueberry variety used in the experiment maybe resistant to *P. cinnamomi.*

Mycorrhizal resynthesis field trials were conducted at Amathole Berries using Misty blueberry variety, which were inoculated with *Leohumicola*, *Oidiodendron* and *Meliniomyces* species respectively. All three fungal isolates formed typical ericoid colonisation in the roots, however, the percentage colonisation was low in all treatments. The highest colonisation percentage of 20.4% was recorded in *Oidiodendron* sp. inoculated plants whereas non-inoculated controls had the least colonisation of 3.7%. Shoot growth and biomass were not significantly different (ANOVA, $p>0.05$) between treatments and the control. Root biomass significantly improved in all inoculated plants. This suggests potential benefits are conferred by inoculation with the three fungal isolates on Misty variety.

Dedication

Dedicated to my parents for who have been a source of inspiration and have shown great faith in me throughout my studies.

Acknowledgements

Firstly, I thank God for granting me the wisdom to complete this research and thesis.

I would like to express my sincere gratitude to my supervisor Professor Joanna Dames, for her guidance, patience and support throughout the course the project.

I am also grateful to the following people and institutions:

Rhodes University and NRF Intergrated Biodiversity Information Programme for funding.

Mr. Tony Dold, Curator Selmar Schönland Hebarium (Grahamstown) for his assistance during the identification of *Erica* plant species. Ryan and Mandilake from Amathole Berries (Stutterheim) for providing the blueberry plants and monitoring the field trials.

Dr Wilbert Kadye, Dr Gwyneth Matcher, Prof. Tony Booth, Dr Ashwani Kumar, Mrs Margot Brooks, Tendai Matsatsa, Prudence (Rhodes University), Dr Leho Terdersoo (Estonia University) and Dr Cecelia Mestre (Argentina) for their technical support and advice during experiments, data analysis and thesis compilation.

Mycorrhizal Research Laboratory team Phumeza, Veronique, Nicolle, Aviwe, Thulisa, Kemi, Mandilive and Roxane for their encouragement and companionship.

My husband, Dennis for his love, patience and believing in my capabilities.

My parents for providing me with the best education possible and unwavering support throughout my studies. You are my source of inspiration.

My siblings Karen, Hope, Tanaka and my daughter Nicole for the relentless support.

Lastly, my friends Lorraine, Rumbidzai, Rose, Gamuchirayi, Beatrice, Perpetual, Nosphiwe and Artwell for babysitting and the moral support.

Table of Contents

	Abstract	**i**
	Dedication	**iv**
	Acknowledgements	**v**
	Table of Contents	**vi**
	List of Figures	**x**
	List of Tables	**xiii**
	List of Abbreviations	**xiv**
1.0	**Literature review**	**1**
1.1	**Taxonomy of *Erica* species**	**1**
1.2	**Geographic distribution of *Erica* plants**	**1**
1.3	**Ecology and reproduction of *Erica* species**	**2**
1.4	**Mycorrhizal and root fungal relations of the *Ericaceae***	**3**
1.4.1	**Ericoid mycorrhizal association**	**4**
1.4.2	**Dark septate endophyte association**	**6**
1.4.3	**Ectendomycorrhizal associations**	**7**
1.4.4	**Cavendishioid mycorrhizal association**	**8**
1.4.5	**Sheathed ericoid mycorrhizas**	**8**
1.4.6	**Arbuscular mycorrhizal association**	**9**
1.5	**Morphological and molecular techniques in ericoid fungal taxonomy**	**10**
1.6	**Ericoid mycorrhizal fungal diversity**	**11**
1.7	**Utilization of various organic and inorganic nutrients**	**15**
1.8	**Antipathogenic activity of ericoid mycorrhizal fungi**	**18**
1.9	**Motivation**	**18**
1.10	**Aims and thesis outline**	**19**
2.0	**Identification of *Erica* species from the Albany Centre of Endemism and their root fungal relations.**	**21**

2.1 Introduction 21

2.2 Methods 22

2.2.1 Root and soil sample collection 22

2.2.2 Root preparation 23

2.2.3 Light microscopy 23

2.2.4 Spore extraction from the rhizosphere of the *Erica* plants 24

2.3 Results 24

2.3.1 *Erica* plants of the Albany Centre of Endemism 24

2.3.2 Soil analysis 25

2.3.3 Mycorrhizal and endophyte status 28

2.4 Discussion 34

3.0 Morphological and molecular detection of culturable fungi associated with the genus *Erica*. 37

3.1 Introduction 37

3.2 Methods 39

3.2.1 Isolation 39

3.2.2 Morphological classification of fungal isolates 39

3.2.3 Molecular characterization of root colonising fungi 40

3.2.4 PCR product sequencing and analysis 40

3.3 Results 41

3.3.1 Molecular analysis 42

3.3.2 Identification of operational taxonomic units of fungal community 42

3.4 Discussion 50

4.0 The diversity of *Erica* root-associated fungi as determined by 454 pyrosequencing. 53

4.1 Introduction 53

4.2 Methods 54

4.2.1	**DNA extraction, PCR and pyrosequencing**	**54**
4.2.2	**Bioinformatics and statistical analysis**	**56**
4.3	**Results**	**57**
4.3.1	**Fungal classification and identification**	**58**
4.0	**Discussion**	**64**
5.0	**Utilisation of organic and inorganic nutrients by selected ericaceous fungal isolates in pure culture.**	**67**
5.1	**Introduction**	**67**
5.2	**Methods**	**68**
5.2.1	**Nutrient assimilation by fungal isolates from organic and inorganic sources**	**68**
5.2.2	**Determination of siderophores**	**70**
5.3	**Results**	**71**
5.3.1	**Siderophore detection**	**75**
5.4	**Discussion**	**77**
6.0	***In-vitro* and *in-plantae* assessment of fungal activity against *Phytophthora cinnamomi* root pathogenic fungi.**	**80**
6.1	**Introduction**	**80**
6.2	**Methods**	**82**
6.2.1	**Qualitative screening**	**82**
6.2.2	**Semi-qualitative analysis**	**82**
6.2.3	***In plantae* experiments**	**83**
6.3	**Results**	**84**
6.4	**Discussion**	**87**
7.0	**The effects of inoculating selected fungi on the mycorrhizal status and growth of *Vaccinium* species.**	**90**
7.1	**Introduction**	**90**
7.2	**Methods**	**91**
7.3	**Results**	**92**

7.4 **Discussion** **96**

8.0 **General Discussion** **98**

8.1 **Conclusion** **105**

References **107**

Appendices **127**

List of figures

Figure 1.1 Geographic distribution of *Erica* species in Europe and Africa. Adopted from McGuire and Kron, 2005. 2

Figure 1.2 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, lasting up to 11 weeks. 5

Figure 2.1 *Erica* plants growing in the Beggers Bush area with the insert emphasising the flower morphology. (a) *Erica chamissonis* (b) *Erica nemorosa* (c) *Erica cerinthoides* (d) *Erica glumiflora*. 26

Figure 2.2 *Erica* plants growing in the Beggers Bush area with the insert emphasising the flower morphology. (a) *Erica Caffra* (b) *Erica demissa* (c) root system of *E. cerinthoides* growing on the soil upper layer with highly branched hair roots. 27

Figure 2.3 The typical ericoid structures observed in the roots of collected *Erica* plants under the light microscope. (a) and (d) hair roots colonised forming ericoid hyphal coils (b) and (c) the ericoid coils within the individual epidermal cells. 29

Figure 2.4 Vesicles observed in *E. glumiflora* and *E. cerinthoides* (a) vesicles with numerous intra-inter-cellular hyphal colonisation in surrounding cells (b) ellipsoidal vesicles supported by aseptate hyphal growth (c) vesicles with round bodies (DSE) growing in adjacent cells. 30

Figure 2.5 Additional structures observed in the roots of the collected *Erica* plants under the light microscope (a) septate hyphae with adjoining round clumped bodies (b) intracellular colonisation by round bodies (c) hyphal hyaline across within epidermal cell with numerous entry points and forming no distinctive structures (d) Pigmented intra-cellular hyphae forming a coil. 31

Figure 2.6 DSE structures observed in the *Erica* roots (a) *Rhizoctonia* DSE type (b) *Phialocephala* Medlar DSE type (c) and (d) intercellular parenchymatous net. 33

Figure 3.1 Distribution of fungal orders isolated and identified from the hair roots of six *Erica* species. Each different colour represents the percentage of isolates detected from a single host plant. Bars represent the overall percentage of the order from all host plants. 43

Figure 4.1 Rarefaction curves for each of the six *Erica* host plants. Showing the number sequences plotted against the number of rarefied operational taxonomic units OTUs a 97% similarity threshold. 58

Figure 4.2 Percentage of OTUs assigned to different fungal phyla. All unidentified OTUs were grouped together under unclassified phyla. Charts representing 59

phyla from (a) *Erica caffra* (b) *Erica cerinthoides* (c) *Erica demissa* (d) *Erica chamissonis* (e) *Erica glumiflora* (f) *Erica nemorosa*

Figure 4.3 Heat map showing the percentage distribution of the Orders detected from six *Erica* host plants. The red colour scale shows the percentage abundance and relative distribution. Colour intensity increases with percentage abundance on a 1 to 4 scale on log transformed percentage data. The hosts were E. caf (*E.caffra*), E. glum (E. *glumiflora*), E. cerin (*E. cerinthoides*), E.nem (*E. nemorosa*) and E.dem (*E. demissa*). 61

Figure 4.4 Nonmetric multidimensional scaling (NMDS) ordination of fungal operational taxonomic units (OTUs) composition as detected from six *Erica* plants at order level. Stress value = 0.08; $R^2 = 0.80$. 62

Figure 5.1 Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in four different N sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were Control (no N), Ammonium (Amm), Nitrate (Nit), Arginine (Arg) and Bovine serum albumin (BSA). 72

Figure 5.2 Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in three different P sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were control (no P), orthophosphate (Orth), phytic acid (PA) and DNA. The Asterisks indicate treatments that tested positive for phosphomonoesterase activity. 73

Figure 5.3 Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in five different C sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were control (no C), glucose (Gluc), cellobiose (Cello), carboxymethylcellulose (CMC) and tannic acid (TA). 74

Figure 5.4 Halos produced by ericaceous fungal isolates on Chrome Azurol Sulfonate (CAS) agar after incubation for 14 days at 27ºC in the dark. Isolates were (a) Ncf91 (b) Caf110 (c) Nem78 (d) Chem038 (e) G828 and (f) Nem15. 76

Figure 5.5 Mean percentage (± standard error) siderophore production by ericaceous fungal isolates in liquid Chrome Azurol Sulfonate (CAS) assay. 76

Figure 6.1 Dual agar plates (a) *Phythopthora cinnamomi* control after 14 days of incubation (b) *Leohumicola* sp. growing against *Phythopthora cinnamomi* and arrow showing the zone of inhibition and (c) *Oidiodendron* sp. showing no growth inhibition against *Phythopthora cinnamomi*. 84

Figure 6.2 Root fungal colonisation of Brightwell blueberry variety. (a) uninoculated control (b) control inoculated with *Phythopthora cinnamomi* (c) and (d) ericoid mycorrhizal coils in roots inoculated with *Leohumicola* sp. (e) and 85

(f) ericoid mycorrhizal coils in roots inoculated with *Oidiodendron* sp. the arrows showing hyphal coils and arrow with Asterisk showing structures like clumped spores.

Figure 6.3 Average shoot height of Brightwell plants after 90 days of growth. Showing the inoculation effects of *Leohumicola* sp., *Oidiodendron* sp. and *Phythopthora cinnamomi* on shoot growth. Different letters above columns indicate significant differences ($p<0.05$, N=8). 86

Figure 7.1 Average shoot height of Misty plants after 56 weeks of growth. Showing the inoculation effects of *Leohumicola* sp., *Oidiodendron* sp. and *Meliniomyces* sp. on shoot growth. No significant differences were recorded. 93

Figure 7.2 Colonisation of Misty plants after inoculation with *Leohumicola* (Chem038), *Oidiodendron* (CafRU082b) and *Meliniomyces* (ECRU075) species after 56 weeks of growth. (a) The inoculated control (b) (c) (d) and (e) ericoid mycorrhizal structures observed from the inoculated plants. 94

Figure 7.3 Percentage root colonization of Misty blueberry plants after 56 weeks of growth following inoculation with Chem038, CafRU082b and ECRU075. (a) Different letters above columns indicate significant differences ($p<0.05$, N=10). 94

Figure 7.4 Shoot and root biomass of Misty plants after 56 weeks of growth following treatment with Chem038, CafRU082b and ECRU075. Shoot biomass showed no significant difference different letters above columns showing root biomass indicate significant differences ($p<0.05$, N=10). 95

List of Tables

Table 2.1 Mineral nutrient analysis of composite soil samples collected from the Beggers Bush area. 28

Table 2.2 Percentage colonisation of hyphal coils typical of ericoid mycorrhizal associations (ERM), dark septate hyphae (DSE), Arbuscules and intermediate hyphae (Inter-hyphae) in epidermal cells of hair roots of six *Erica* species collected from Beggers Bush area. Data shown is the mean percentage colonisation +/- SD (N=12). The proportion of spores present in the rhizosphere of the collected *Erica* plant species per 100 g of soil (N=2). 32

Table 3.1 Identification of isolates from six *Erica* species classified in operational taxonomic units (OTUs). 45

Table 4.1 Analysis of reads and OTUs clustering of sequences obtained for the six *Erica* host plants. 57

Table 4.2 Simpson and Shannon diversity estimators, Evenness and Chao1 richness estimator calculated at 97% sequence identity threshold for the six *Erica* host plants. 58

Table 4.3 Molecular identification of selected OTUs identified to genus and species level from the *Erica* host plants. Identification was based on manual NCBI BLAST search and Ribosomal database project (RDP) classification using UNITE ITS2 reference database. 63

Table 6.1 Treatment application of potential biocontrol fungal isolates Chem038 and CafRU082b against *Phytophthora cinnamomi* infection on Brightwell variety. 83

Table 6.2 Effects of Chem038 and CafRU082b inoculation on the growth of Brightwell blueberry plantlets infected by *Phythopthora cinnamomi*. Average shoot and root biomass +/- SD. 87

List of Abbreviations

^{0}C	degrees celcius
µl	microlitre
ACOE	Albany Centre of Endemism
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
Bp	basepair
DGGE	Denaturating Gradient Gel Electrophoresis
DNA	Deoxyribose Nucleic Acid
DSE	Dark septate endophyte
dNTP	Deoxyribose Nucleotide Phosphate
ERM	ericoid mycorrhizal
g	gram
ITS	Internal Transcribed Spacer
LSU	Large sub unit
Mn	manganese
N	nitrogen
NCBI	National Centre for Biotechnology Information
P	phosphorus
PCR	Polymerase Chain Reaction
SABPA	South African Berry Producers Association
SD	standard deviation
Zn	zinc

1.0 Literature review

1.1 Taxonomy of *Erica* species

Erica species are perennial woody plants that are adapted to harsh edaphic conditions (Schumann and Kirsten, 1992; Oliver, 1967). They belong to family Ericaceae and subfamily Ericoideae. The subfamily consists of twenty-five genera that include *Rhododendron*, *Erica*, *Calluna*, *Empetrum* and *Rhodothamnus* (Schumann and Kirsten, 1992). The genus *Erica* is the second largest genus in the Ericoideae, with approximately 865 identified species globally (McGuire and Kron, 2005). Recently, the Empetraceae and Epacridaceae families were reclassified into Ericaceae. This reclassification is based on a combination of morphological, anatomical and molecular identification together with phylogenetic analysis (Kron et al., 2002; 1999). The relationship between Epacridaceae and Ericaceae plants has been supported by similarities in the phylogeny of mycorrhizal fungi associated with the respective plants (Cairney and Ashford, 2002; Chambers et al., 2000).

1.2 Geographic distribution of *Erica* plants

Ericaceous plants have a broad geographic distribution. *Erica* species are distributed in Africa, Middle East, Madagascar and Europe (McGuire and Kron, 2005). However, the Cape Floral Region (CFR) of South Africa has the greatest species diversity. More than 88% of the identified species are endemic to this area (Fig 1.1) (McGuire and Kron, 2005; Goldbalt and Manning, 2002). The region has 760 species growing between Cape Town and Port Elizabeth, with approximately 235 species being found in the Cape Peninsula (Oliver and Oliver, 2000; Schumann and Kirsten, 1992). In other parts of Africa, 22 species have been identified in eastern Ethiopia, whereas Madagascar and the Mascarene Islands have 50 identified species. In comparison only, 23 *Erica* species have been identified in Europe (McGuire and Kron, 2005).

Species diversity and geographic distribution trends suggest that *Erica* species originated from Africa, specifically the CFR. However, phylogenetic studies by McGuire and Kron (2005) indicate that the European taxa could be the ancestral species. In that study, *Erica arborea* was confirmed to be the only known species found in both Europe and Africa. The ancestral plant showed affinity to the African clade although it was supported in the European clade. Within the

CFR, the genus *Erica* is common in low-temperature mountainous areas that are characterised by acidic soils (Schumann and Kirsten, 1992). This may support the theory that they originated from Europe and the species evolved due to variability in climatic and edaphic conditions, hence, explaining the vast numbers of endemic species in the CFR. Comparatively, Europe has a broader geographic occurrence of the genus that spreads across many countries, whereas in Africa they are confined to the small area of the CFR (Fig 1.1).

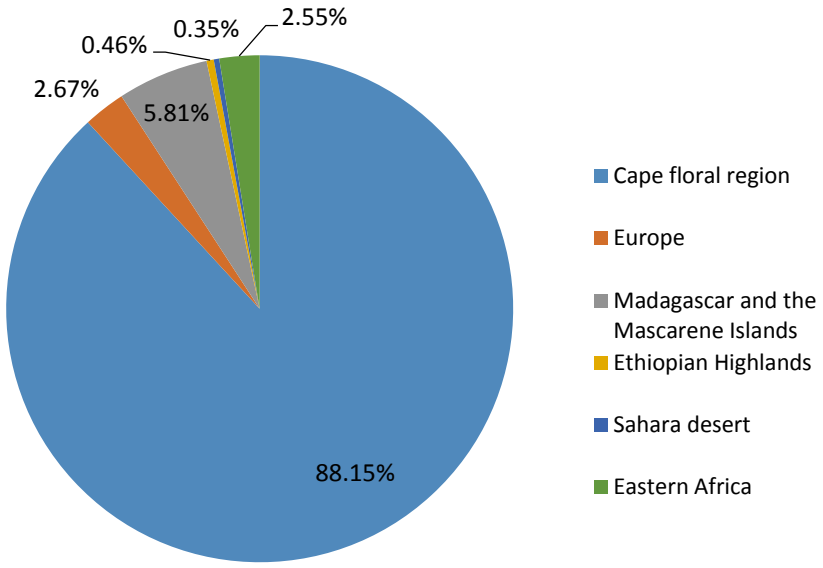

Figure 1.1. Geographic distribution of *Erica* species in Europe and Africa. Adopted from McGuire and Kron, 2005.

1.3 Ecology and reproduction of *Erica* species

Erica species reproduce sexually and asexually (Ojeda et al., 2005; Specht, 1990). The former involves seed germination whereas the latter is achieved through sprouting. In the CFR, 90% of the *Erica* species are non sclerophyllous seeders (Bell and Ojeda 1999; Ojeda, 1998). They produce numerous seeds resulting in high seed banks that are persistent in the soil. This characteristic contributes to their success. Fire, a common feature in their habitats, often results in the death of all plants (Verdaguer and Ojeda, 2005; Verdú, 2000). However, germination from the seed bank re-establishes the population after fire events thus giving the plants a competitive advantage. This mechanism also ensures their survival and success in habitats that experience varying soil temperature conditions over a period of time. Smoke and temperature fluctuations appear to stimulate germination of some seeds (Schumann and Kirsten, 1992).

The conditions that facilitate germination of *Erica* seeds in the wild are generally unknown. Therefore, it is a challenge to enhance conservation of these fynbos species or grow them for horticultural and recreational purposes in South Africa. In an effort to address this problem, Kirstenbosch Botanical Gardens in Cape Town (South Africa) has formulated a commercial product derived from smoke. The seed primer comprises of aqueous smoke extracts and gibberellins that breaks dormancy and stimulates the germination of *Erica* seeds. Examples of *Erica* seeders include *E. hispudula*, *E. pulchella*, *E. coriifolia* and *E. sessiliflora* (Bell and Ojeda, 1999).

Approximately 6% of the *Erica* species in the CFR regenerate from lignotubers (Valbuena et al., 2000; Ojeda, 1998). The lignotubers are buried underground and can re-sprout after fire. Re-sprouting species are generally sclerophyllous, For example they have tough and highly lignified leaves. These include *E. cerinthoides*, *E. chamissonis*, *E. massonii* and *E. articularis* (Verdú, 2000). The remaining 4% of the *Erica* population are mixed species reproducing both sexually and asexually. Examples of mixed species include *E. calycina*, *E. longifolia* and *E. coccinea.*

1.4 Mycorrhizal and root fungal relations of the *Ericaceae*

Ericaceae are obligate mycorrhizal plants that are associated with diverse fungal assemblages. Consequently, they form several distinctive mycorrhizal categories and endophyte relations (Smith and Read, 2008). Formation of these root-fungus relations is influenced by several factors such as fungal taxonomy, host associate and edaphic growth conditions (Setaro et al., 2006a). These associations are distinguished by the morphological structures formed by the fungi within the hair roots. In mycorrhizal associations (MA), these structures serve as interfaces for nutrient and information exchange between the symbionts (Smith and Read, 2008).

Ericoid mycorrhizal (ERM) association is the most common root-fungus relationship in the ericaceous family (Smith and Read, 2008; Midgley et al., 2004). Although they are poorly researched ERM and the dark septate endophyte (DSE) structures have frequently been identified in ericaceous roots (Ucelary, 2002; Jumponeon and Trappe, 1998). Both ERM and DSE associations have a ubiquitous distribution in Ericaceae (Vohník and Albrechtová, 2011). Furthermore, ectendomycorrhizas (EEM), cavendishioid (CVM), sheathed ERM and arbuscular mycorrhizal (AM) associations have also been microscopically detected in ericaceous roots

(Setaro et al., 2013; Chaurasia et al, 2005; Koske, 1990). Root-fungal interactions that result in no clear morphological structures are common in the Ericaceae. These associations can occur either individually or concurrently in the ERM colonised roots (Vohník and Albrechtová, 2011; Massicotte et al., 2005).

1.4.1 Ericoid mycorrhizal association

Ericoid mycorrhizas are the main symbionts of the ericaceous family (Martino et al., 2007; Smith and Read, 2008). They occasionally co-occur with other mycorrhizal associations (Sadowsky et al, 2012; Smith and Read, 2008). Although, ERM fungi can be associated with other non-ericaceous host plants, the ERM association is exclusive to the ericaceous family. Ericaceae roots have narrow lateral roots known as 'hair roots'. These are the sites for ERM fungal colonisation. The ERM fungi penetrate individual cortical cells of the hair roots, forming thin hyphal coils (Smith and Read, 2008; Bergero et al., 2000). There are intercellular hyphal connections between independently colonized cells that enable each hair root to accommodate several fungi (Perotto et al., 1996).

Ultrastructural analysis has been used to establish the ERM life cycle. It comprises three stages; initiation, establishment and degeneration (Duddridge and Read, 1982). ERM fungal inocula come from various sources such as soil and plant debris. The fungi are saprobes and can be free-living in the soil, when a suitable host is absent. It has been suggested that coexisting plants in natural habitats act as a repository for ERM inocula (Chambers et al., 2008). Fungal host recognition is triggered by the secretion of mucilage by the root tip in the form of carbohydrates and organic acids (Peterson et al., 2004).

The ERM fungi have numerous micro-fibrils consisting of polysaccharides cell walls. These attach the fungi to the hair roots forming a loose mycelial network. Thereafter, degrading hydrolytic enzymes such as cellulase and pectinase that break down the epidermal cell walls are secreted. This allows the fungi to penetrate the hair root cells (Fig 1.2). Thereafter, fungal hyphae colonise the epidermal cells to form ericoid coils within individual epidermal cells (Smith and Read, 2008; Perotto et al., 1996). Up to 90% of the colonised epidermal cell can be filled with fungal tissue. The external mycelial growth is limited, extending to about 1 cm from the hair root surface into the rhizosphere.

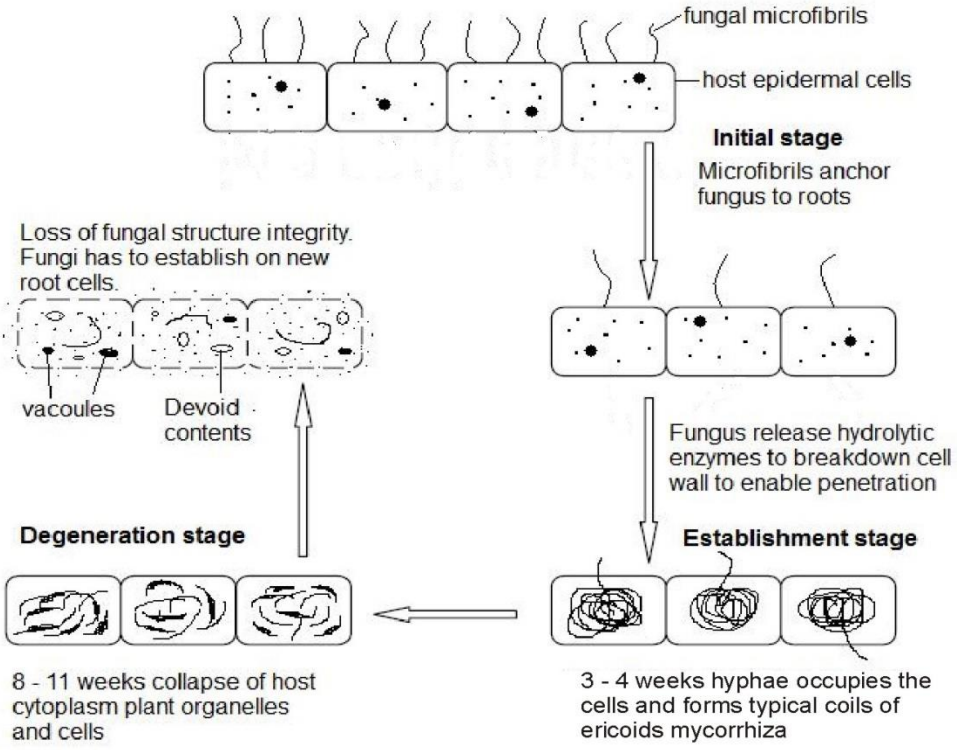

Figure 1.2 Series of events that occur during the formation of the ericoid mycorrhizal association in the Ericaceae hair roots. The stages include initiation, establishment and degeneration, lasting up to 11 weeks**.**

ERM colonisation of ericaceous plants growing in natural soil has been observed after four weeks of inoculation and after three weeks in irradiated soils. The functional life of the ERM is short, lasting less than 11 weeks in natural soils and 8 weeks in irradiated soil. Nutrient transfer occurs within this period, with both partners maintaining structural integrity (Duddridge and Read, 1982). The degeneration stage involves the disintegration of host organelles, cytoplasm and cells. This is followed by the disintegration of fungal structures. The fungi subsequently colonise new hair roots of the host resulting in the recurrence of the ERM cycle. Ericoid fungi are sapro-parasitic partners during a part of the life cycle. They obtain nutrients from dead plant cell material and depend on the hair roots for nutrient requirements for growth (Smith and Read, 2008; Jansa and Vosátka, 2000).

1.4.2 Dark septate endophyte association

Dark septate endophytes were first reported as mycelium of *Radices atrovirens*, in the form of melanised hyphae or micro-sclerotina (Smith and Read, 2008). They are a diverse assemblage of fungi that engage in endophytic relations known as the DSE association. DSE colonise numerous host plant genera. The host associates include ERM, ECM, AM, orchid and non-mycorrhizal plants (Tian et al., 2011; Chambers et al., 2008; Mandyam and Jumpponen, 2005; Rains et al., 2003).

DSE associations have various morphological characteristics that depend on the host and fungi involved (Jummponen and Trappe, 1998). For example, they can be characterised by inter- and intra-cellular melanised thick hyphae. In some cases, the fungal hyphae penetrate cortical cells resulting in micro-sclerotia. In addition, the association can be defined by superficial hyphae forming an intercellular parenchymatous net on the surface or in the rhizodermal cells (Vohník and Albrechtová, 2011; Jummponen and Trappe, 1998). These structures are also referred to as DSE labyrinthine and resemble a Hartig net.

The most common DSE sclerotia morphotypes are *Phialocephala* and *Rhizoctonia* types (Vohník et al., 2005; 2003; Urcelay, 2002). *Phialocephala* consists of intracellular colonisation by melanised, clustered rounded fungal bodies. In comparison, the *Rhizoctonia* morphotype has branched hyphae that produce short branched barrel shaped segments that cluster in cortical cells (Jummponen and Trappe, 1998). Some DSE fungi form ERM structures with Ericaceae hosts (Bergero et al., 2003; 2000; Jummponen and Trappe, 1998; Currah et al., 1993). For example, *Heteroconium chaetospira* a DSE fungus was found to form melanised hyphal coils resembling ERM within the cortical cells of *Rhododendron obtusum* in a resynthesis experiment (Usiku and Narisawa, 2005).

The effects of DSE associations on ericaceous hosts can be either neutral or mutualistic (Schulz et al., 2006; Jummponen, 2001; Jummponen and Trappe, 1998; Johnson et al., 1997). These are influenced by experimental conditions, host and fungal associates involved. There is, however controversy concerning the mycorrhizal status of these associations. This is because no nutrient exchange interfaces have been established for DSE associations that are similar to those of MA. Nevertheless, it has been suggested that mutualism should be the distinguishing attribute for

mycorrhizal associations (Brundrette, 2004; Harley, 1992). Hence, not all DSE associations conform to this classification criterion. In ericaceous hosts, no parasitic tendencies have been reported, whereas neutral effects were observed in *Rhododendron* cv. Belle-Heller inoculated with *Phialocephala fortinii* (Vohník et al., 2003).

Inoculation of *Calluna vulgaris* with *P. fortinii* was found to improve nitrogen uptake, resulting in enhanced seedling biomass (Zijlstra et al., 2005). In non-ericaceous hosts such as Chinese cabbages, bi-directional nutrient exchange has been established (Usiku and Narisawa, 2007). Therefore, it is possible that some DSE associations, particularly those with sclerotia intracellular colonisation, have active interfaces for nutrient and information exchange. These interfaces are yet to be established for the associations that are characterised by the intercellular parenchymatous net. Other benefits conferred by DSE involve the secretion of various extracellular enzymes, such as cellulase, laccases and pectinases by *P. finlandica* and polyphenol oxidase activity in *Leptodontidium orchidicola*. This can be useful in assimilating mineral nutrients from various complex organic substrates. Such evidence suggests that DSE associations may complement the functions of ERM fungi whilst in co-occurrence.

1.4.3 Ectendomycorrhizal associations

Arbutoid and monotropoid mycorrhizas are ectendomycorrhizal (EEM) associations. Morphologically, they are characterised by a hyphal sheath, Hartig net and intracellular hyphal colonisation (Selosse et al., 2007; Setaro et al., 2006a). The intracellular structures are variable between host taxa of the respective associations (Smith and Read, 2008). These associations are classified as a special subtype of ECM rather than ERM because the mycobionts in the symbiosis are mainly basidiomycetes which form ECM (Brundrette, 2004). An example of Ericaceae species involved in Arbutoid mycorrhizas are the sub-family Arbutoideae (*Arbutus unedo* and *Comarostaphylis arbutoides*). Identification of basidiomycete fungi associated with ericaceous plants has been a challenge, especially using microscopy and culture methods of identification. The ultrastructure of basidiomycete hyphal colonisation is not easily distinguishable from ascomycete hyphae in the hair roots using light microscopy (Selosse et al., 2007; Setaro et al., 2006a), therefore they can easily be overlooked. This could explain their less frequent identification in *Erica* roots. The use of the transmission electron microscopy (TEM) for

structural analysis of mycorrhizas has resulted in an improvement in their detection (Selosse et al., 2007; Setaro et al., 2006a). Ultrastructural analysis of *Erica cinerea*, *Calluna vulgaris* and *Gaultheria poeppigii* using TEM showed ERM colonisation. The coils were formed by both ascomycetes and basidiomycetes fungi (Selosse et al., 2007). For comparison ascomycetes are distinguished by septal pores with Woronin bodies whereas basidiomycetes have imperforate parenthesomes and dolipore hyphae forming ERM (Setaro et al., 2006a).

1.4.4 Cavendishioid mycorrhizal association

Cavendishioid mycorrhizal (CVM) association is a mycorrhizal class recently discovered from the hair roots of *Cavendishia nobilis* (Vaccinioideae) (Setaro et al., 2006b). It is characterised by unclamped hyphal sheath, mantles with intercellular fungal tissue that resembles a Hartig net and hyphal colonisation in cortical cells. This morphotype is identical to arbutoid mycorrhizas. However, they are classified as a subcategory of ERM because of their association with ERM basidiomycete fungi. This suggests that the association could be specific for this host taxon. The CVM structures are similar to those previously described in other ericaceous hosts (Massicotte et al., 2005; Rains et al., 2003). However, in those studies the fungi were not identified, therefore no mycorrhizal classification was suggested. The arbutoid and CVM mycorrhiza differ in that arbutoid mycorrhizas have a well-developed Hartig net and hyphal sheath of variable thickness, whereas the CVM has a hyphal sheath, less than 1 µm in diameter. In addition, the CVM Hartig net is looser and not typical of ECM as observed in arbutoid mycorrhizas (Setaro et al, 2006b). The ecological significance of this association is not yet known.

1.4.5 Sheathed ericoid mycorrhizas

A new ERM subcategory known as the sheathed ERM was recently recorded (Vohník et al., 2012). The main features of this association are clamped hyphae that form sheaths surrounding hair roots and intracellular ERM colonisation. The associated fungi are Trechisporales (basidiomycetes). These fungi have lignocellulolytic activity, in comparison with ERM ascomycetes that had no apparent effect on lignin degradation. Therefore, they have a functional role in improving host fitness through the provision of nutrients from highly lignified debris. In contrast, the sheathed ERM has clamped hypha and does not possess a Hartig net that is present in CVM associations (Vohník et al., 2012; Setaro et al., 2006b).

1.4.6 Arbuscular mycorrhizal association

Arbuscular mycorrhizal (AM) associations are ubiquitous endomycorrhiza that associate with approximately 80% of all terrestrial plants (Brundrette, 2009; 2004). Ericaceae are associated with AM fungi mainly from the phylum Glomeromycota (Vega, 2009; Wang and Qiu, 2006). The AM association is distinguished by extensive intracellular hyphae, arbuscules and vesicles within the colonised host cells (Smith and Read, 2008). This AM morphotype is referred as the Arum-type. Another, common AM morphotype is the Paris-type that is characterised by vesicles. It lacks a well-developed system of intracellular hyphae and arbuscles are not always present. Both morphotypes have been reported in the Ericaceae (Obase et. al., 2013; Fukuchi et al., 2011; Abe, 2005; Urcelay, 2002; Zhuang and Chan, 1996). AM can solely colonise ericaceous hosts or co-occur with ERM (Massicotte et al., 2005; Koske, 1990). AM hyphae and vesicles were observed in the hair roots of Epacrids (Davies et al., 2003) whereas a study by Obase et al., (2013) established *Enkianthus campanulatus* to be exclusively associated with AM fungi forming the Paris colonisation.

The primary function of the AM symbiosis is to facilitate phosphorus (P) mineral nutrient assimilation by the host (Smith and Read, 2008). Nutrient and C exchanges between AM fungi and host occur in the arbuscules whereas the vesicles are storage organs (Brundrette, 2004). Some studies have shown that *Glomus faciculatum* and *Glomus mosseae* enhanced the root and shoot growth of *Rhododendron maximum* (Dighton and Coleman, 1992). By comparison, inoculating *Vaccinium corymbosum* (*V. corymbosum*) with AM fungi *G. intraradices* and *G. claroideum* improved the shoot and root biomass but no AM structures were formed. Only hyphal wefts were observed around the host roots (Arriagada et al., 2012). This further supports the suggestion that mycorrhizal structures are particular to the host and fungus involved.

1.5 Morphological and molecular techniques in ericoid fungal taxonomy

The culture-based technique is one of the most common methods for the identification of ERM fungi. This involves isolation of fungi from roots onto culture media and subsequent identification using morphology. Root segments are surface sterilised and cut into small pieces or macerated prior to isolation in order to increase the diversity of fungal isolates. The technique relies on pure culture analysis and has a bias towards the recovery of fast growing, sporulating

ascomycetes (Sun and Guo, 2012; Nafar, 1998). ERM fungi are mostly sterile hence it might not fully represent the diversity of ERM fungi. In addition, some cultures have patches that cannot easily be distinguished as pure or mixed. This can present a challenge for inexperienced researchers. However, the main disadvantage of the technique is that sterile cultures cannot easily be taxonomically identified (Smith and Read, 2008).

Molecular techniques have seen the yield of greater ERM fungal diversity in comparison to culture-base methods. These techniques include Immunocytochemical protocols, direct amplification from the roots and DNA sequencing (Allen et al., 2003; Hutton et al., 1996; Perotto et al., 1996). Immunocytochemical techniques such as Pectin zymograms are not effective in illustrating diversity because they rely on secondary metabolites from ERM fungi. However, fungal DNA can be isolated from surface-sterilised roots and amplified using specific primers in a polymerase chain reaction (PCR). The PCR products are thereafter separated using denaturing gradient gel electrophoresis (DGGE). Different bands are cut and cloned in plasmids. Fingerprinting techniques such as Restriction fragment length polymorphism (RFLP) and PCR-Random amplified polymorphic DNA (RAPD) are used to screen clones for homologous taxa. Representatives from the different groups are then sequenced and taxonomically identified. These techniques were used to establish the presence of several fungal communities in a single root of *Calluna vulgaris* (Perotto et al., 1996) and unculturable fungi that had previously been overlooked (Allen et al., 2003). Hence, this technique improved the knowledge of fungal diversity in ericaceous roots. The RFLP or RAPD techniques compliment the culture method well. However, direct amplification and RFLP analysis is not effective because roots contain numerous fungi, which results in the generation of multiple mixed bands. These cannot be easily separated and, therefore, will not be identified, thus compromising the potential detection of fungal diversity (Monreal et al., 2000).

Direct sequencing techniques have evolved and methods such as Sanger sequencing technology and 454 pyrosequencing have been effectively used in determining root fungal communities (Yamamoto et al., 2014; Tedersoo et al., 2010). Both techniques are reliable in detecting fungal composition, but pyrosequencing has the capacity to emphasise quantitative diversity as well. This was confirmed in ectomycorrhizal fungal studies that showed Sanger and pyrosequencing detect similar fungal community structures (Terdersoo et al., 2010). These techniques enable the

detection of unculturable fungi associated with ericaceous plants such as glomeromycetes and basidiomycetes that have been identified from the host roots, despite their absence from cultures (Obase et al., 2013; Selosse et al., 2007). Allen et al., (2003) identified *R.erica* and *Capronia* sp. in both culture and direct amplification while fungi belonging to the family Sebacinales were only detected by direct amplification.

Nonetheless, pyrosequencing has a higher-throughput, species recovery, better cost and time efficiency in comparison with Sanger technique (Sun and Guo, 2012; Gillevet et al., 2010). The main weakness of the technique in comparison with sequencing from cultures is that it generates short sequences less than 500 basepairs. This can increase taxonomic identification of fungi down to species level. Other methods such as Automated Ribosomal Intergenic Spacer Analysis (ARISA) are also used for fungal community profiling in Ericaceae roots (Gorzelak et al., 2012). This is a fingerprint based method of analysing mixed cultures on the basis of size and variability of the 16S–23S (Internal Transcribed Spacer) ITS sub-regions (García-Martínez et al., 1999). The main disadvantage of this technique is the difficulty in reading fingerprints of uncultured organisms because the analysed regions are small, taxonomic placements are sometimes not definite (Popa et al., 2009). In conclusion, culture-based and molecular analysis allows precise individual taxonomic identification of non-sporulating fungi that cannot be identified based on morphology. On the other hand, direct amplification is more effective in demonstrating fungal diversity.

1.6 Ericoid mycorrhizal fungal diversity

Ericaceae hair roots have been found to harbour numerous fungal taxa that can be pathogenic, endorhizal or mutualistic (Smith and Read, 2008). Of relevance to the current study are taxa that engage in beneficial relations with the ericaceous family. Although ascomycetes have been widely established as ERM fungi, recent research has shown basidiomycete fungi to be beneficial associates of the Ericaceae (Hazard et al., 2014; Selosse et al., 2007, Allen et al., 2003). Culture-based and molecular methods of identification have been used to establish the fungal assemblages associated with Ericaceae roots. *Rhyzoscyphus erica* assemblage, *Meliniomyces*, *Oidiodendron*, *Phialophora*, unnamed Helotiales and fungi in the Sebacinales order are some of the important ericoid fungi (Smith and Read, 2008).

The *R. erica* assemblage (Zhang and Zhuang, 2004) is one of the most common and well-researched ERM fungi. Initially identified as *Pezizella erica* (Read, 1974), the group was renamed *Hymenoscyphus erica* (Read) Korf and Kernan. The nomenclature was recently reassigned to include *Hymenoscyphus monotropae* (Kernan and Finocchio) (Zhang and Zhaung, 2004). This assemblage comprises various fungi (Ascomycota-Helotiales-Helotiaceae), with at least 84% genetic relatedness in the ITS region (Smith and Read, 2008; Vrålstad et. al., 2002). All the fungi in this aggregate have been detected in the ericaceous roots using both culture-based and molecular techniques (Bougoure et al., 2007). However, they were compiled from pure cultures.

Non-sporulating *Meliniomyces* (Bruzone et al., 2014; Kjøller et al., 2010) and teleomorphic in-groups (arthroconidia) *Scytalidium* (Hambleton et al., 1999) and phialidic *(Cadophora)* taxa are part of the *R. erica* assemblage. *Meliniomyces* was previously known as the variable white taxon. They are sterile and their colouration in culture varies from white, cream to grey. This genus has not been reclassified to *Rhyzoscyphus* due to the lack of a teleomorph state (Hambleton and Sigler, 1995). Research has shown that the *R. erica* assemblage are not all ERM fungi (Smith and Read, 2008). The fungi can be non-mycorrhizal, ERM, ECM, EEM, DSE whereas others engage in both ERM and ECM associations (Hambleton et al., 1999).

From a phylogenetic perspective the *R. erica* assemblage consists of 4 main clades (clade 1, clade 2, clade 3 and clade 4) (Smith and Read, 2008; Vrålstad et. al., 2002; 2000; Hambleton and Sigler, 2005). Clade 1 consists of *Meliniomyces* species, including *Meliniomyces variabilis* that are non-mycorrhizal (Hambleton and Sigler, 2005). However, Vohník et al., (2007) showed that *M. variabilis* formed typical ericoid structures in the roots of *Vaccinium* sp., in contrast no colonisation was observed in *Rhododendron ponticum* seedlings. This suggests that the fungus has potential to form ERM with selected host associates (Grelet et al., 2009a; Ohtaka and Narisawa, 2008; Vohník et al., 2007). Studies showing bidirectional nutrient exchange between the associates need to be conducted to determine if the resulting ERM structures are symbiotically functional. Clade 2 has *Meliniomyces vraolstadiae* that engages in ECM or non-mycorrhizal associations (Vrålstad et al., 2002).

Clade 3 is dominated by putative ERM fungi that have been isolated from ericaceous hair roots (Smith and Read, 2008). These include *R. erica* and its anamorph genus *Scytalidium* (Egger and Sigler, 1993; Dalpé, 1989). *Scytalidium* is not commonly detected because it only occasionally produces arthroconidia or apothecia on cereal agar (CER) medium (Read, 1974; Pearson and Read, 1973). To demonstrate their true ERM nature, both *R. erica* and *S. vaccinii* formed typical ericoid structures in *Vaccinium vitis-idaea* host plants but did not interact with ectomycorrhizal hosts (Vrålstad et. al., 2002). *R. erica* has unanimously been confirmed as ERM in numerous experiments (Smith and Read, 2008; Vohník et al., 2005). To date, *R. erica* is the only fungus that can form ERM with ericaceous host regardless of provenance of the isolate (Piercey et al., 2002).

Clade 4 of the *R. erica* aggregate has true ectomycorrhizal isolates such as *Meliniomyces bicolor*. Isolates belonging to the genus *Cadophora (*Lagerb and Melin) (previously *Phialophora*) also belong to this clade. *Cadophora finlandia* is a darkly pigmented slow growing isolate that does not easily sporulate. It engages in both ECM and ERM association with *Piceirhiza bicolorata* and *V. myrtillus* respective hosts (Villarreal-Ruiz et al., 2004; Vrålstad et. al., 2002; Monreal et al., 2000). *M. bicolor* has previously been isolated from both Ericaceae and ectomycorrhizal hosts (Grelet et al., 2010). Grelet et al., (2009) conducted resynthesis experiments with *M. bicolor* and *V. vitis-idaea* that resulted in ERM structures and reciprocal transfer of carbon and nitrogen was observed. This demonstrates a true ERM symbiotic relationship. Ericoid colonisation was also observed in *V. myrtillus* and *V. macrocarpon* following inoculation with *M. bicolar* and positive growth effects were observed in *V. myrtillus* (Villarreal-Ruiz et al., 2004; 2012).

The family Myxotrichaceae (Onygenales) has genera that form ERM associations (Tian et al., 2011; Smith and Read, 2008). Fungal species in the genera include *Myxotrichum setosum, Geomyces pannorum, Gymnascella dnankaliensis* and *Pseudogymnoascus roseus* (Smith and Read, 2008; Vohník et al., 2007; Dalpé, 1986). Within the genus *Oidiodendron, O. maius* (Barron) is the most frequently isolated. It engages in both ERM and ECM associations (Bougoure and Cairney, 2006; Bergero et al., 2000; Perotto et al., 1996). In addition, *Oidiodendron* species that have been confirmed as ERM associates include *O. griseum, O.*

rhodogenum, O. truncatum and O. cerealis, (Bougoure et al., 2005b; Allen et al., 2003; Berch et al., 2002; Monreal, et al., 2000).

DSE are a group of closely related septate ascomycetes that are often darkly pigmented and sterile. Most DSE fungi can easily be identified because they sporulate in culture (Grunig and Sieber, 2008). In the ericaceous family, *Phialocephala fortinii* is the most common DSE (Bruzone et al., 2014; Tian et al., 2011, Vohník et al., 2005; 2003). It engages in ERM, DSE and ECM associations (Walker et al., 2011; Vrålstad et. al., 2002; Zhang et al., 2009). Unidentified fungi that are closely related to *P. fortinii* have been classified into a group, *P. fortinii Acephala applanata* species complex (PAC) (Grünig and Sieber, 2008). The group includes cryptic species in the genus *Phialocephala* such as *P. dimorphosphora, P. europea*, *P. letzii, P. subalpine, P. turiciensis,* and *A. applanata* related species. Other DSE species include *Leptodontidium orchidicola, L. elatius* and *Microdochium* species (Bruzone et al., 2014; Vano et al., 2011, Walker et al., 2011). *Heteroconium chaetospira* is less frequently isolated from ericaceous roots, but it has been confirmed to form ERM with *R. obtusum* host plants (Usuki and Narisawa, 2005).

In addition, other minor associated fungi such as *Cryptosporiopsis erica, Capronia* sp., *Acremonium strictum, Lachnum* and *Lachnuella* species have been detected in ericaceous roots (Walker et al., 2011; Zhang et al., 2009; Allen et al., 2003; Berch et al., 2002; Bergero et al., 2000). ERM status has been confirmed for all of them with the exception of *Lachnum* and *Lachnuella* species. Bizabani, (2012) established *Lachnum* sp. form intracellular colonisation with no specific structures in the roots of *V. corymbosum.* Unidentified ascomycetes with affinities to Helotiales have been increasingly identified from Ericaceae. These fungi are slow growing, darkly pigmented and have a ubiquitous presence in the ericaceous roots. Some of the fungi engage in ERM association but taxonomic placement and resynthesis are still to be conducted to provide more information on these root fungal associates (Smith and Read, 2008).
Sebacinales in the clade B are commonly associated with Ericaceae (Hazard, 2014; Kühdorf et al., 2014; Bougoure et al., 2007; Selosse et al., 2007). *Clavaria argillaceae* collected from fruiting bodies were the first true ERM basidiomycete mycobionts to be identified (Smith and Read, 2008; Englander and Hull, 1980). Most recently, Vohník et al., (2012) proved that *Agrocybe praecox* colonise *V. corymbosum* and positively influence growth although no putative

ericoid structures were observed. Other fungi such as *Laccaria bicolor*, *Lactarius musteus*, *Hyphodiscus hymeniophilus*, *Suillus variegatus, L. musteus* and *Irpex lacteus* have been proved as ERM associates (Villarreal-Ruiz et al., 2012; Walker et al., 2011, Zhang et al., 2009). There are also basidiomycetes such as *Mycena sp.*, *Galerina* sp. and *Tomentellopsis submollis* that are often detected but do not interact with ericaceous hosts (Vohník et al., 2012; Walker et al., 2011). Arbuscular mycorrhizal fungi in the Glomus group A assemblage such as *Glomus indicum* have been identified from the ericaceous roots (Wang and Qiu, 2006; Zhuang and Chan, 1996). AM fungi are detected solely by direct amplification from the roots because they are obligate mycorrhizal fungi, therefore unculturable. In separate studies by Abe (2005) and Obase et al., (2013) Glomeromycota species were identified from six *Enkianthus* species and *Erica campanulatus* respectively, following the observation of vesicles during microscopy.

1.7 Utilization of various organic and inorganic nutrients

The ERM fungi confer numerous benefits to the host. However, its primary function is enhancing nutrient assimilation by the host from various organic substrates in the soil (Smith and Read, 2008). These organic sources include recalcitrant plant debris, phosphomonoesters, phosphodiesters, proteins and sparingly soluble metal complexes (Gibson and Mitchell, 2004, Burke and Cairney, 1997). Unlike other mycorrhizal associations the morphology of ERM is characterised by extraradical mycelia extending less than 2 cm into the soil horizon. They can only access nutrients confined to the organic matrix of the rhizosphere (Smith and Read, 2008). This is facilitated by the fungal secretion of an array of extra-cellular hydrolytic and oxidative enzymes (Perotto et al., 1996).

Soil nitrogen (N) can be in various forms such as inorganic (ammonium and nitrate) and organic (amino acids, peptides and proteins). Ericoid fungi that include *R. erica*, *O. maius* and other unidentified Helotiales have the ability to assimilate both types of N sources (Emmerton et al., 2001; Cairney et al., 2000; Chen et al., 1999). However, most N forms in the soil are organic and are easily bound by recalcitrant compounds such as phenolic acids and tannins that are derived from ericaceous sclerophyll tissues (Bending and Read, 1996). This results in insoluble complex organic compounds that are resistant to microbial decomposition and low in available nitrogen. *R. erica* has been established to utilize such organic N sources through polyphenol-oxidase and

proteinase activities. They depolymerise these phenolic compounds, subsequently conveying the product to the host. During this process, tannin and phenolic acids can be a potential source of C for the fungus (Bending and Read, 1996), hence reducing the C requirement from the host.

Polymeric forms of organic N are also important in ericaceous ecosystems. For example, Chitin contributes 10% of the cell wall component of *R. erica* and other ericoid fungi. It can be a source of N in the form of live or dead mycelia, contributing up to 30% of total soil N (Kerley and Read, 1995; Leake and Read, 1990). *R. erica* and Epacrid mycorrhizal fungi produced chitinolytic activities such endo-acting chitinase that facilitate the utilisation of chitin as a N source by the host (Bougoure and Cairney, 2006). Other fungi that are able to utilize chitin include *O. maius* and *O. griseum*.

Plant debris is comprised of polymers such as lignin, cellulose and hemicellulose that are structural components of the cell wall. These can be a source of organic C (Cairney and Burke, 1998). The ability of ERM fungi to source their own C benefits the hosts by reducing C channelled to the fungi by the host during symbiosis. *R. erica* and other ERM fungi secrete cellulase, cellobiohydrolase and glucohydrolase enzymes. These are beneficial in availing C but are also important for cell wall degradation prior to root colonisation (Smith and Read, 2008). In culture, several unidentified Helotiales (Epacrid and *Woollsia* mycorrhizal fungi) and *R. erica* were able to utilize cellulose, cellobiose and carboxymethylcellulose as a sole C source (Midgley et al., 2006). In the same study, isolates were also able to assimilate pectin, tannic acid and phenylalanine C substrates. This also corroborates suggestions that ERM fungi are facultative mycobionts.

ERM ascomycetes can produce peroxidases and cellulase complexes that can degrade lignin and cellulose (Cairney and Burke, 1998). However, recent research has shown basidiomycetes engaging in sheathed ERM association have better lignocellulolytic activity in comparison to *R. erica* and *O. maius* (Vohnìk et al., 2012). This could explain some of the functional roles of numerous basidiomycetes that have been detected using direct amplification from ericaceous roots. Hemicellulases and pectinases are also some of the enzyme activities of the ERM fungi (Cairney and Burke, 1998). Polygalacturonase activity was detected in various ericoid fungi growing in a medium containing pectin as a sole C source (Perotto et al., 1997).

Phosphorus (P) in the ericaceous soils is mostly derived from plant debris and is in the form phosphomonoesters (inositol phosphate) and phosphodiesters (nucleic acids) (Peterson and Massicotte, 2004). The ERM fungi produce and secrete phosphomonoesterase and phosphodiesterase enzymes that facilitate the breakdown of respective compounds to release P for host utilization. It has been established that in culture *R. erica* exhibits cell-bound and extracellular phosphodiesterase activity. This enabled them to grow in a substrate containing DNA as a sole source of P (Leake and Miles, 1996).

ERM fungi and other ericaceous root-associated fungi produce and secrete siderophores that facilitate the assimilation of Iron (Fe) (Kajula et al., 2010; Bartholdy et al., 2001). Siderophores are a diverse group of compounds that have low molecular weight and high affinity for ferric Fe (Pérez-Miranda et al., 2007). Structurally they are made up of a ligand and a peptide backbone. The ligand is a functional group that ligates Fe^{3+} and is used to differentiate the siderophores. ERM fungal siderophores include hydroxamates, catecholates, carboxylates and mixed ligands (Haselwandter and Winkelmann, 2007).

In the ERM hosts, siderophores of the hydroxamates type are dominant (Haselwandter, 2008). Previous research by Haselwandter et al., (1992) showed that *R. erica* produced three types of siderophores, ferricrocin II (83%), ferrichrome I (11%) and ferrichrome CIII (5%). These siderophore types were also observed for *O. griseum* except for ferrichrome. On the other hand, a *Rhodothamnus chamaecistus* endophyte mainly produced fusigen with small amounts of ferricrocin and ferrichrome. *P fortinii* isolates also produced these hydroxamates and those in the ferrirubin class (Bartholdy et al., 2001). This suggests that the different fungi colonising a single root can produce multiple siderophore classes that help acquire ferric Fe from various sources. The production of different siderophore types can also be a mechanism to prevent exploitation from non- siderophore producing organisms.

1.8 Antipathogenic activity of ericoid mycorrhizal fungi

Root diseases in the production of berries in the Ericaceae such as *V. corymbosum* are becoming increasingly important (Smith, 2000). In other mycorrhizal associations, improvement in host fitness against pathogens has been reported through several mechanisms that include improved nutrient status, competition for colonisation and excretion of antipathogenic fungal exudates (Smith and Read, 2008). Host protection from phytopathogens has recently been established in ericaceous family (Grunewaldt-Stöcker et al., 2013). ERM fungi (*O. maius* and four *Leohumicola* species) were tested against oomycetes *Pythium* and *Phytophthora cinnamomi*. All the ERM fungi colonised *V. corymbosum* and reduced pathogenic infections in the root. External mycelial growth of the pathogen in the substrate was also reduced. The uninoculated controls were vulnerable and easily infected by both pathogens (Grunewaldt-Stöcker et al., 2013). These findings suggest potential biological control of respective diseases caused by these oomycetes.

Dark septate endophytes have also been reported to reduce the incidence of pathogenic infections in their host (Narisawa and Usuki, 2004). *Heteroconium chaetospira* which engages in ERM with *Rhododendron* sp. and *V. corymbosum* (Usuki and Narisawa, 2005, Vano et al., 2011) has been shown to inhibit club root and *Verticillium* yellows in Chinese cabbage (Narisawa et al., 2004; Narisawa et al., 2000). In that study, *Phialocephala fortinii* showed no effect on the pathogen. This suggests that not all DSE or ERM fungi are capable of conferring this benefit to the host. In addition, a recent study has shown that *H. chaetospira* induces resistance to club root in Canola plants through the upregulation of host genes that are involved in several biosynthesis pathways (Lahlali et al., 2014). ERM fungi have the potential to provide host protection against pathogens, therefore more research focused on the antipathogenic activities of the diverse ericaceous fungal associates is needed.

1.9 Motivation

The Cape Floral Kingdom has a significant diversity of ericaceous plants. The genus *Erica* contains approximately 80% of the world's identified species. Their success is attributed to the ERM symbiosis and other endophyte relations they form with soil fungi. These root-fungus relations facilitate host growth by availing nutrients from organic substrates in adverse edaphic growth conditions (Smith and Read, 2008). Most research has been carried out on the fungi

associated with ericaceous plants of the northern hemisphere, consequently, information on the root-associated fungi of genus *Erica* in the Cape Floral Kingdom is limited.

This has prompted research in this area, to provide a better understanding of the genus *Erica* and its associated fungi in South Africa. Characterisation of associated fungi is important because it provides information on their eco-physiological significance in ecosystem. Associations can be manipulated for applications in agricultural biotechnology. For example, inoculation with ericoid mycorrhizal fungi can organically enhance productivity of high-value crops such as *Vaccinium corymbosum* (Scagel et al., 2005, Jansa and Votsák, 2000). Recent studies have shown that ericoid fungi have potential applications in horticulture as biocontrol agents of root-associated diseases (Grunewaldt-Stöcker et al., 2013). This further illustrates potential benefits that can be attributed to their ericaceous fungal isolates, highlighting the important of research to identify any unique qualities that can be utilised for different applications.

1.10 Aims and thesis outline

This thesis seeks to (i) determine the root-fungal structures of *Erica* plants growing in the ACOE (ii) establish the diversity of root-associated fungi, (iii) characterize selected fungal isolates (iv) assess their potential as bio-control agents against *Phytophthora cinnamomi* and (v) determine the mycorrhizal status of selected isolates and evaluate their ability to enhance plant growth of Misty blueberry variety.

These aims were achieved under the following thesis chapters:

Chapter 1. A general introduction and literature review pertaining to the current study.

Chapter 2. The objective was to identify *Erica* species from the Albany Centre of Endemism and their root fungal relations. Identification of *Erica* plants was achieved using taxonomic keys. Root fungal structures were determined by root staining and microscopy.

Chapter 3. The objective was to establish the diversity of culturable fungi associated with the identified *Erica* plants. This was achieved through isolation of fungi from surface sterilised roots onto various culture media. Isolated fungi were identified morphologically using reproductive structures and microscopy. Molecular identification involved deoxyribonucleic acid (DNA)

extraction from pure cultures, polymerase chain reaction (PCR) and sequencing. Basic local alignment search tool (BLAST) analysis was used for taxonomic identification.

Chapter 4. The objective was to establish the diversity of fungi associated with the identified *Erica* plants using tag encoded 454 pyrosequencing techniques. This involved direct DNA extraction from the roots of the respective plants and conducting multiplex PCR, sequencing and identification of sequences through bioinformatics protocols and BLAST analysis.

Chapter 5. The objective was to evaluate the utilisation of organic and inorganic nutrients by selected ericaceous fungal isolates in pure culture. Selected fungal isolates were tested for nitrogen, phosphorus and carbon assimilation on modified Melin Norkrans medium. Iron nutrition was assessed by determining Siderophore activity using liquid and agar-based chrome azure assays.

Chapter 6. The objective was to determine antimicrobial activity of selected fungal isolates against the plant pathogenic fungus *Phytophthora cinnamomi*. This was conducted in culture using the dual agar technique. Biocontrol experiments were conducted on Brightwell blueberry plantlets under controlled conditions in a growth chamber.

Chapter 7. The objective was to conduct mycorrhizal resynthesis using Misty blueberry variety and determine the effects of inoculation on their growth. This was achieved by inoculation of selected fungi on Misty plants under commercial field conditions and monitoring the growth.

Chapter 8. The overall discussion of the current study that harmonizes the research presentation in all chapters.

It is the intention to submit selected chapters alone or in combination to peer-reviewed journals for publication.

2.0 Identification of *Erica* species from the Albany Centre of Endemism and their root fungal relations

2.1 Introduction

The Albany Centre of Endemism (ACOE) is a hot-spot of diversity in the Cape Floral kingdom of South Africa. Within this plant community is the genus *Erica* that comprises approximately 80% of the plants. The dominance of Ericaceae in the ACOE makes it unique and one of the most important floral kingdoms on earth (Schumann and Kirsten, 1992). Previous research has established this genus to favour areas that are highly acidic and rich in recalcitrant organic matter (low in available N and P). They are dependent on the ericoid mycorrhizal association (ERM) and other root fungal associations for their survival (Smith and Read, 2008). These associations enable host plants to acquire essential nutrients from numerous organic sources in the soil.

The identification of *Erica* plants is primarily based on the leaf morphology, which is generally described as ericoid in shape. Using this method to identify the *Erica* genus is challenging due to its subjectivity. The leaves may be similar to those of plants belonging to other families, which may lead to misidentifications; hence, it is crucial to consult experienced botanists or taxonomists for accurate identification. In addition to leaf morphology, flower morphology and reproductive structures are also used to differentiate among species by experienced botanists (Schumann and Kirsten, 1992). Continuous research has shown that Ericaceae plants dominate habitats with highly acidic soils and climates with cold, wet winters and hot dry, summers (Cairney and Meharg, 2003). Similar conditions have been recorded for the Beggers Bush area in Grahamstown, where the mean annual rainfall is between 350-550 mm. The rainfall is unequally distributed over the year, with 60% of the rain being received in summer, whereas 40% is received in winter. However, during drought conditions there is a chance of, not receiving 80% of the mean rainfall anticipated for any particular year. The average maximum temperature range is 29-32ºC while the minimum temperature range is 4-6 ºC (Palmer, 2004). These edaphic and climatic conditions present challenges for sustainable plant growth. The theory is that plants in constained environments have developed beneficial associations with fungi, such as ERM to overcome some of these challenges.

The important *Erica* fungal associates consists of ascomycetes in the *Rhizoscyphus erica* aggregate, dark septate endophytes (DSE), basidiomycetes particularly in the Sebacinales order (Selosse et al., 2007) and arbuscular mycorrhizal fungi belonging to the phylum Glomeromycota (Smith and Read, 2008). Fungi and root relations are variable depending on the fungus involved, the root morphology of the *Erica* species and other ecophysiological conditions. These result in distinctive structures forming in the hair roots such as hyphal coils (ERM), DSE structures, AM, ectendomycorrhizas and ectomycorrhizas (Setaro et al., 2013; Chaurasia et al., 2005). Within mycorrhizal associations, these distinctive structures are interfaces for the exchange of nutrients and information that enables mutually beneficial relations between the symbionts (Smith and Read, 2008). These structures can co-occur in ericaceous plants. The functional role of ERM colonisation has been established (Smith and Read, 2008) whereas the ecophysiological significance of having ERM and DSE or AM association in co-occurrence is not clear. Not all mycorrhizal associations confer the same benefits to the host; hence their co-occurrence could imply specialized functions for the different associations.

The mycorrhizal status of *Erica* plants has been determined successfully using microscopy (Smith and Read, 2008). The traditional staining technique (Smith and Dickson, 1997) is the most widely used because it is inexpensive and reliable. Recent research has seen the introduction of several methods, such as the use of scanning electron microscopy and confocal microscopy (Rath et al., 2014). These methods are all effective, but the main disadvantage of the latest technology is the high cost of equipment. This has resulted in the continued use of the staining technique even though it is laborious and time-consuming.

Considering the diversity of the genus *Erica* in SA and the importance of their mycorrhizal relationships, this study seeks to provide an account of the root fungal interactions of genus *Erica.* The aim of this study was to (i) identify *Erica* plants growing in the Albany Centre of Endemism using phenotypic morphology according to plant taxonomic keys (ii) analyse the mineral nutrient status of the soil and (iii) identify the fungal root structures occurring using microscopy.

2.2 Methods

2.2.1 Root and soil sample collection

Plant root and soil samples were collected in December 2011 (summer) and September 2013 (spring) from the Albany Centre of Endemism. This sample site is located within the Beggers bush area, Wiley farm and along the N2 highway, near Grahamstown (latitude and longitude of 33°19'S and 26° 31'E, respectively). Plants were identified to generic species level using leaf and flower morphology according to taxonomic keys (Schumann and Kirsten, 1992).

From the identified plants, roots were carefully excavated using a hand held spade. A selection of roots was cut off using a scalpel and placed in a bag. Samples were collected in triplicate for each plant species at each collection time. They were brought to the laboratory and processed on the same day. Soil samples of approximately 200 g were collected from around each plant and composite samples were mixed and sent for nutrient analysis. The soil characteristics analysed included organic matter content, exchangeable acidity, soil density and pH. Analysis was conducted at the Döhne Agricultural Development Institute, Analytical Services located in Stutterheim.

2.2.2 Root preparation

Root samples collected from the six identified *Erica* species were washed thoroughly with water order to remove adhering soil particles. Other organic particles and debris were removed under a stereomicroscope (Bergero et al., 2002). Segments with numerous attached hair roots were then collected and cut into 3-4 cm pieces and stored in 50% ethanol until processing. Only attached intact samples of hair roots were used (Massicotte et al., 2005).

2.2.3 Light microscopy

Root pieces were further cut using a sterile blade into sections of 1-3 cm. Roots were then placed in McCartney bottles for clearing. In each bottle, 5% (v/w) Potassium hydroxide (KOH) was added prior to heating for 30 minutes in a water bath just below boiling point. To prevent root loss, KOH was discarded through a sieve before rinsing with distilled water. Thereafter roots were bleached in freshly prepared alkaline 10% Hydrogen peroxide (H_2O_2) solution for 30 minutes. Cleared roots were rinsed in water and acidified with 0.1 M Hydrochloric acid (HCl) solution for 3 hours at room temperature. After discarding the acid solution, roots were stained by soaking in lacto-glycerol solution containing 0.05% Trypan Blue at room temperature

overnight. The staining solution was discarded and the roots destained by immersion in lacto-glycerol destain solution (Janska and Vosátka, 2000; Smith and Dickson, 1997). The composition of root clearing and staining solutions is outlined in Appendix A. Root pieces were mounted on microscope slides with the destain solution and covered with cover slips. Thereafter, the roots were observed under a Nikon light microscope at a magnification of 400× and on an Olympus BX50 microscope where images showing the colonisation were captured using an Olympus DP72 camera. Colonisation percentage of the different *Erica* species was determined by microscopy. It was established by examining 50 fields of view of a root sample and recording a positive result if hyphae were present and a negative result for uncolonised cortical cells. The percentage was the determined by dividing the number of colonised cells by the total number of observed cells and multiplying by 100.

2.2.4 Spore extraction from the rhizosphere of the *Erica* plants

The collected soil samples were air dried after which 100 g was measured out for each respective plant and placed in a measuring cylinder. Approximately 500 ml of water was placed in the cylinder and stirred vigorously to suspend the fungal spores. The supernatant was then poured through 425 µm, 250 µm, 125 µm and 45 µm sieves respectively. The soil was then washed off the sieves using water into separate centrifuge tubes. Spore suspension was centrifuged at 1900 g for five min. The supernatant was then carefully discarded. Resuspension of the pellet was done in 60% sucrose solution and centrifuged for five min. A 9 cm gridded filter paper disc (Whatman number 1) was then placed on a Buchner funnel with vacuum suction. The supernatant was decanted onto the filter paper and the spores rinsed with distilled water. The filter paper was transferred to a lid of a clean petri dish and closed. The spores were examined under a Leica S4E stereomicroscope at a magnification of 100× (Smith and Dickson, 1997).

2.3 Results

2.3.1 *Erica* plants of the Albany Centre of Endemism

Using plant morphology, particularly the leaf and flower, six *Erica* plants were successfully identified. The plants were *Erica chamissonis* Klotzsch ex Benth., *Erica cerinthoides* L., *Erica caffra* L., *Erica glumiflora* Klotzsch ex Benth., *Erica demissa* Klotzsch ex Benth. and *Erica*

nemorosa Klotzsch ex Benth. (Fig 2.1 and Fig 2.2). The plants were identified and samples collected in December except for *E. nemorosa* which was sampled in March. They co-occur with other plants mainly grasses and orchids. During sampling the identified plants were flowering, hence leaf morphology was used for primary identification and flower morphology for species verification.

Erica cerinthoides had tough, small, hairy leaves which were needle pointed with low surface area and clustered on the stem. They were branched, forming canopy-like structures with dark pink/reddish flower petals which were globular in shape. The root system was shallow with thick buds. However, hair roots are not numerous and well defined as in the other identified *Erica* plants. They were observed to be flowering both in spring and summer.

Erica chamissonis had numerous soft, velvety, small leaves and a shallow root system less than five cm deep. It had extensive hair roots emerging from the main root which branched forming primary and secondary roots. The roots were parallel to the ground and not vertical as in most plants. The flower morphology observed in spring was characterized by pale pink/deep pink florets. The root morphology for *E. glumiflora* and *E. nemorosa* was the same as described for *E. chamissonis*. Colouration of the other plants was also variable with *E. glumiflora* having brown and white flowers, whereas *E. nemorosa* had purple flowers. *Erica caffra* was up to 3 m tall and growing in a manner that simulates trees, which was not common in the *Erica* plants growing in the Beggers Bush nature area. The root system was shallow and thick with very few hair roots and limited branching in summer while in spring they had numerous hair roots. The plants flowered in summer and had cream to brown florets (Fig 2.2a).

2.3.2 Soil analysis

Erica demissa and *E. cerinthoides* were collected from partially weathered rocks with little soil. Some of the plants had roots extending to partially decomposed grass roots and soil particles. Analysis of the composite soil samples showed that the *Erica* habitat was highly acidic with a pH of 3.63 (Table 2.1). There were no traces of either Mn or Cu in the soil and the concentration of functional elements Ca, K, Mg and was P relatively low.

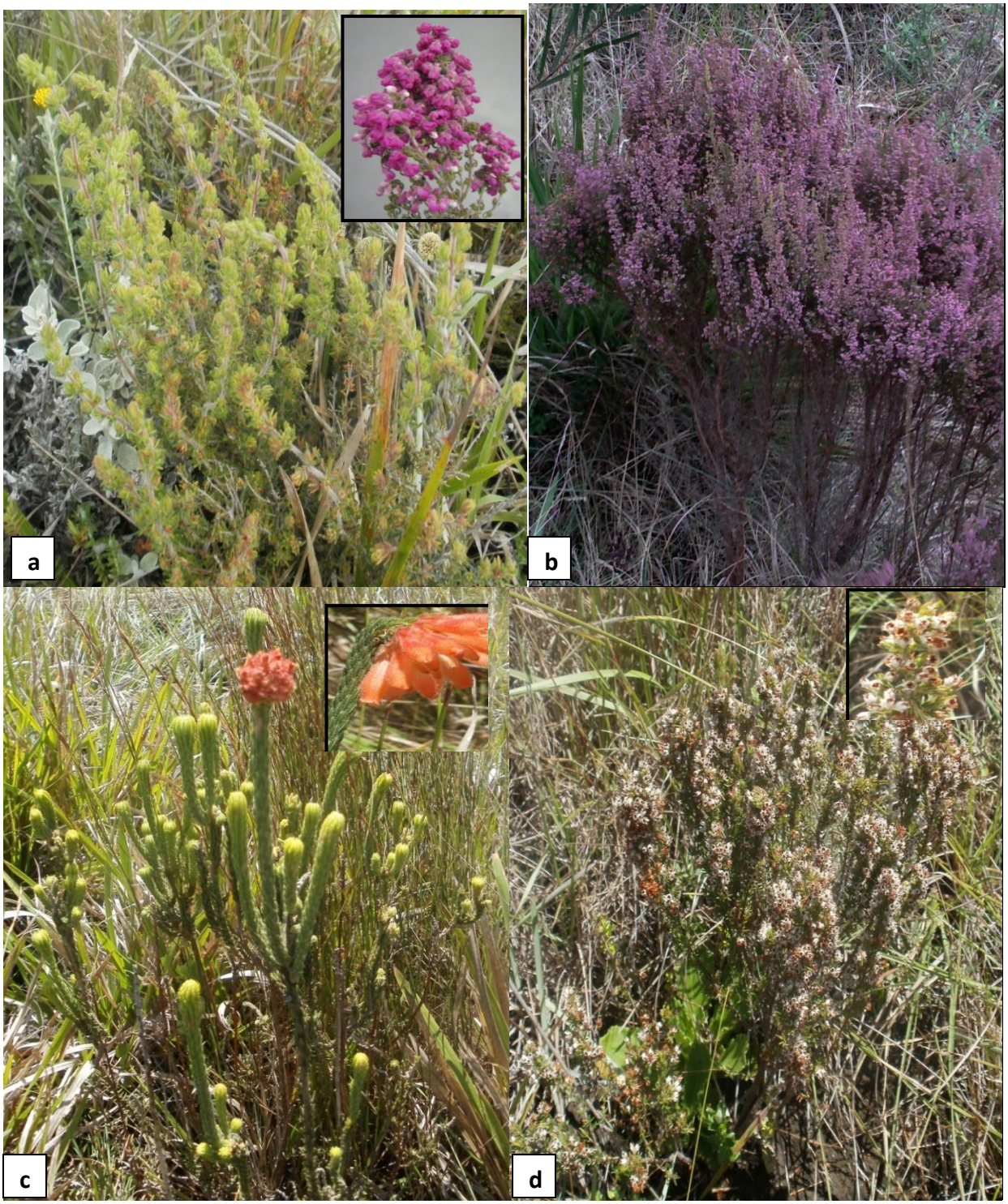

Figure 2.1. *Erica* plants growing in the Beggers Bush nature area with the insert emphasising the flower morphology. (a) *Erica chamissonis* (b) *Erica nemorosa* (c) *Erica cerinthoides* (d) *Erica glumiflora*.

Figure 2.2. *Erica* plants growing in the Beggers Bush area with the insert emphasising the flower morphology. (a) *Erica caffra* (b) *Erica demissa* (c) root system of *E. cerinthoides* growing on the soil upper layer with highly branched hair roots.

Table 2.1. Mineral nutrient analysis of composite soil samples collected from the Beggers Bush area.

Component	**Quantity mg/L**
P	25.0
K	101.0
Ca	626.0
Mg	199.0
Zn	2.4
Mn	0
Cu	0
Soil density g/L	1.0
Exchangeable acidity $cmol/L$	1.4
Total cations $cmol/L$	6.4
Organic carbon %	1.6
pH	3.6

2.3.3 Mycorrhizal and endophyte status

The observed roots showed co-colonisation by a variety of fungal symbionts. These structures include typical ericoid coils, DSE structures, vesicles and hyaline hyphae. All the six species had ericoid structures indicated by hyphal coils observed in the cortical cells of the hair roots (Fig 2.3). The fungal hypha entered the cortical cells forming dense coils. DSE fungal hyphae were observed in cells adjacent to cells forming typical ericoid coils. In older cells, a grainy texture that appeared brown in colour was observed although the coils were still clearly distinguished (Fig 2.3c). The highest percentage colonisation was observed in the *E. caffra* (63%) and *E. chamissonis* (51.6%) species whereas *E. glumiflora* (8%) and *E. cerinthoides* (18.6%) had the least colonisation (Table 2.2).

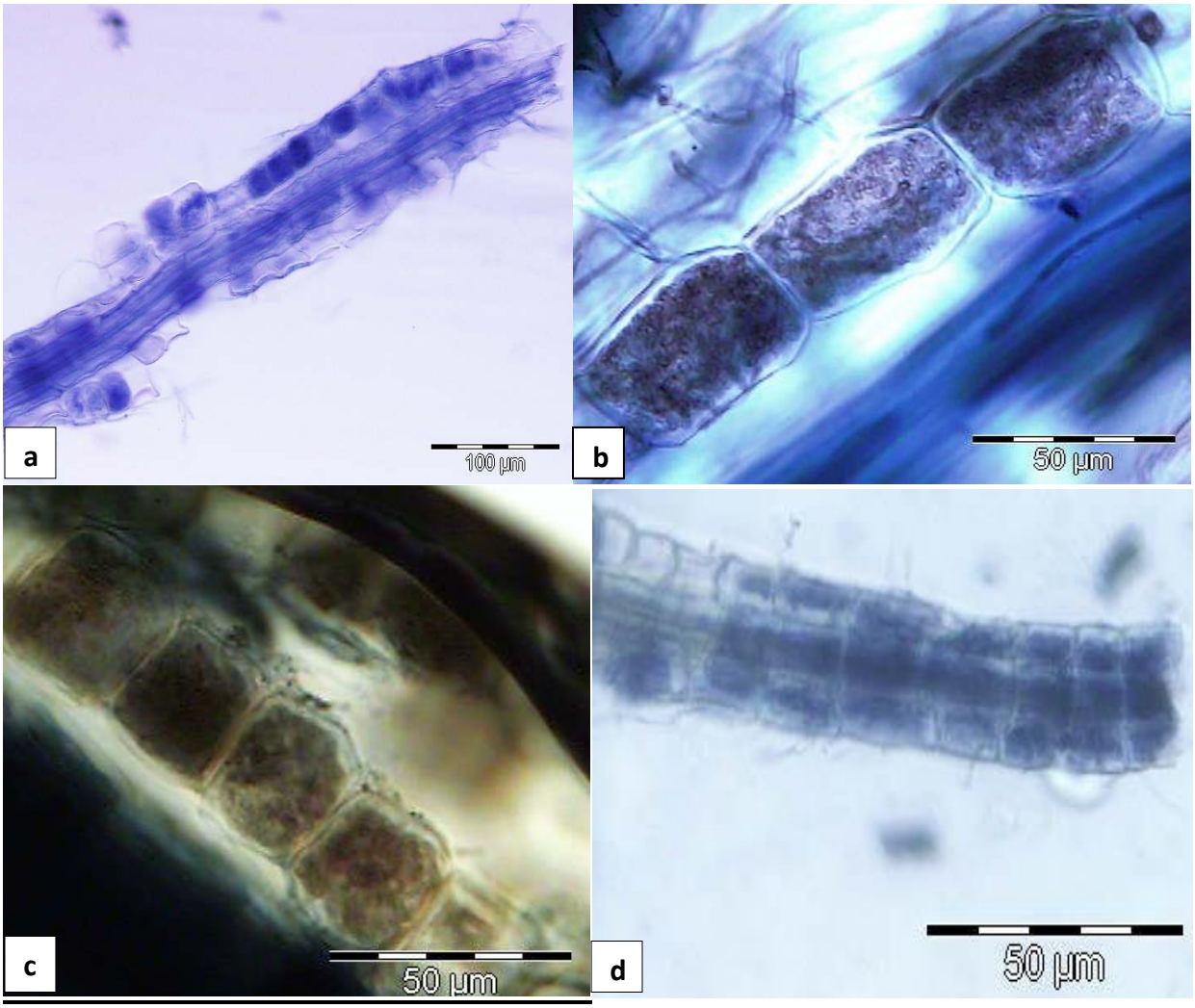

Figure 2.3. The typical ericoid structures observed in the roots of collected *Erica* plants under the light microscope. (a) and (d) hair roots colonised forming ericoid hyphal coils (b) and (c) the ericoid coils within the individual epidermal cells.

Vesicles were detected in the thicker roots of the *E. glumiflora* and *E. cerinthoides* species but not in the roots of all the sampled plants (Fig 2.4). No arbuscules were observed in any of the plants (Table 2.2). The least ERM colonisation was recorded in the plant species where vesicles were present. Colonisation of these roots was characterised by aseptate hyphae with no distinct points of entry running intra- and inter-cellularly. Some of the vesicles were ellipsoidal in shape while others were irregularly shaped and growing from aseptate hyphae. The colonisation percentages of vesicles was significantly higher than other species ($p<0.05$) for *E. glumiflora* and *E. cerinthoides* (Table 2.2). It was not clear if these vesicles were associated with arbuscular mycorrhizal association.

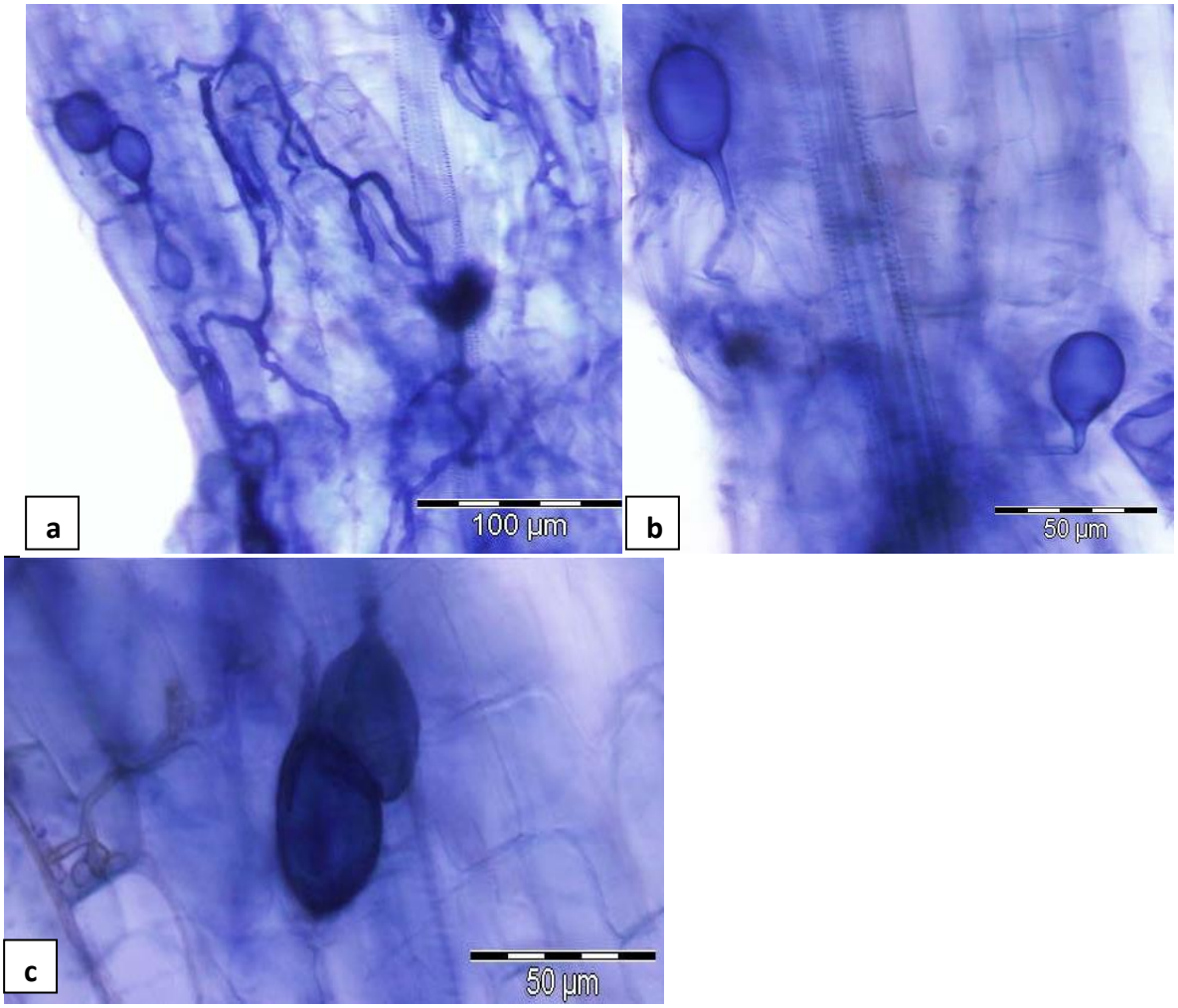

Figure 2.4. Vesicles observed in *E. glumiflora* and *E. cerinthoides* (a) vesicles with numerous intra-inter-cellular hyphal colonisation in surrounding cells (b) ellipsoidal vesicles supported by aseptate hyphal growth (c) vesicles with round bodies (DSE) growing in adjacent cells.

Spore extractions carried out on the soil collected from the rhizosphere of the respective *Erica* plants were successful. The detected spores were variable in size, shape, colour and quantity. *Erica demissa* and *Erica nemorosa* had the highest spore count of 19 and 14 spores per 100 g of soil respectively. Although vesicles were present in *E. cerinthoides* and *E. glumiflora*, they had the lowest spore count (Table 2.2).

Spore-like structures were observed in the *E. glumiflora* roots growing in adjacent epidermal cells colonised by vesicles (Fig 2.4c). More of these stuctures were intracellulary colonising some cells (Fig 2.5a) and, in addition, circular and irregulary shaped round bodies simulating

spores were also present in the *E. demissa roots* (Fig 2.5b). The clamp-like structures were not numerous enough to collect data (Fig 2.5c). Dark septate endophyte fungi were observed forming intracellular micro-sclerotia and other DSE structures (Fig 2.5d). Different types of sclerotia were observed (Fig 2.6).

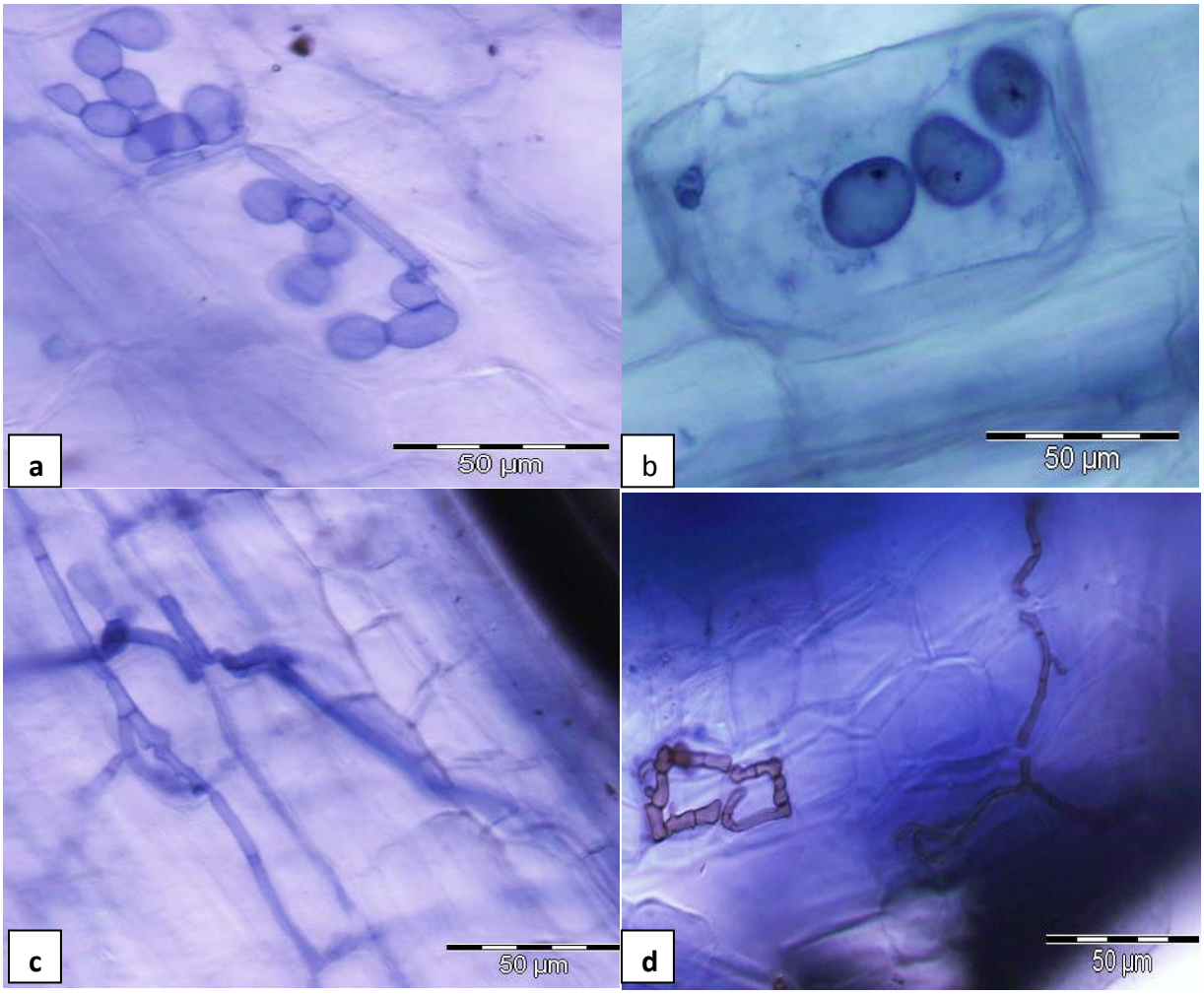

Figure 2.5. More structures observed in the roots of the collected *Erica* plants under the light microscope (a) septate hyphae with adjoining round clumped bodies (b) intracellular colonisation by round bodies. (c) hyphal hyaline across within epidermal cell with numerous entry points and forming no distinctive structures. (d) Pigmented intra-cellular hyphae forming a coil.

Table 2.2. Percentage colonisation of hyphal coils typical of ericoid mycorrhizal associations (ERM), dark septate hyphae (DSE), Vesicles and intermediate hyphae (Inter-hyphae) in epidermal cells of hair roots of six *Erica* species collected from Beggers Bush area. Data shown is the mean percentage colonisation +/- SD (N=12). The proportion of spores present in the rhizosphere of the collected *Erica* plant species per 100g of soil (N=2).

Species	Ericoid mycorrhizal coils (% epidermal cells with coils)	Dark septate hyphae (%colonised cells)	Vesicles (% colonisation)	Intermediate hyphal colonisation	Number of Arbuscular mycorrhizal fungi spores per 100 g of soil
Erica chamissonis	51.9 ± 7.3^{a}	11.7 ± 5.8^{ac}	0^{a}	15.5 ± 5.6^{ad}	3 ± 2.4^{a}
Erica demissa	25 ± 6.7^{b}	16.3 ± 5.4^{ade}	0^{a}	18.3 ± 6.4^{d}	19 ± 8.0^{b}
Erica cerinthoides	18.9 ± 10.6^{b}	25 ± 5.3^{be}	10 ± 5.4^{b}	13.7 ± 6.9^{acd}	8 ± 4.4^{ab}
Erica caffra	62 ± 7.9^{c}	7.4 ± 4.7^{cf}	0^{a}	8.7 ± 5.1^{b}	11 ± 5.1^{ab}
Erica glumiflora	8.7 ± 4.3^{d}	20.5 ± 7.2^{be}	14.4 ± 8.6^{c}	8.9 ± 4.1^{b}	4 ± 2.3^{a}
Erica nemorosa	54 ± 7.9^{a}	7.2 ± 4.1^{f}	0^{a}	12.6 ± 5.0^{abc}	14 ± 7.2^{b}
P-value	F(5,66)=55.32 P<0.000	F(5,66)=19.65 P<0.000	F(5,66)=28.64 P<0.000	F(5,65)=5.35 P<0.004	F(5,20)=2.60 P<0.045

*Means denoted by different letters in each column statistically show significant differences ($p<0.05$) according to Tukeys' LSD.

Hyphae that resembled the *Rhizoctonia* DSE type were observed in the epidermal cells (Fig 2.6a) and numerous round clustered bodies typical of the *Phialocephala* DSE type (Fig 2.6b). Pigmented thick septate hyphae that coiled in a DSE manner as described by Jumpponen and Trappe (1998) were also present. These hyphae were seen randomly in the cells without coiling but as pigmented septate hyphae or loops colonising multiple cells. The cell entry points for some of the DSE were distinct and usually at a single point for each epidermal cell (Fig 2.5c and 2.5d). They were present in all collected species albeit at different intensities.

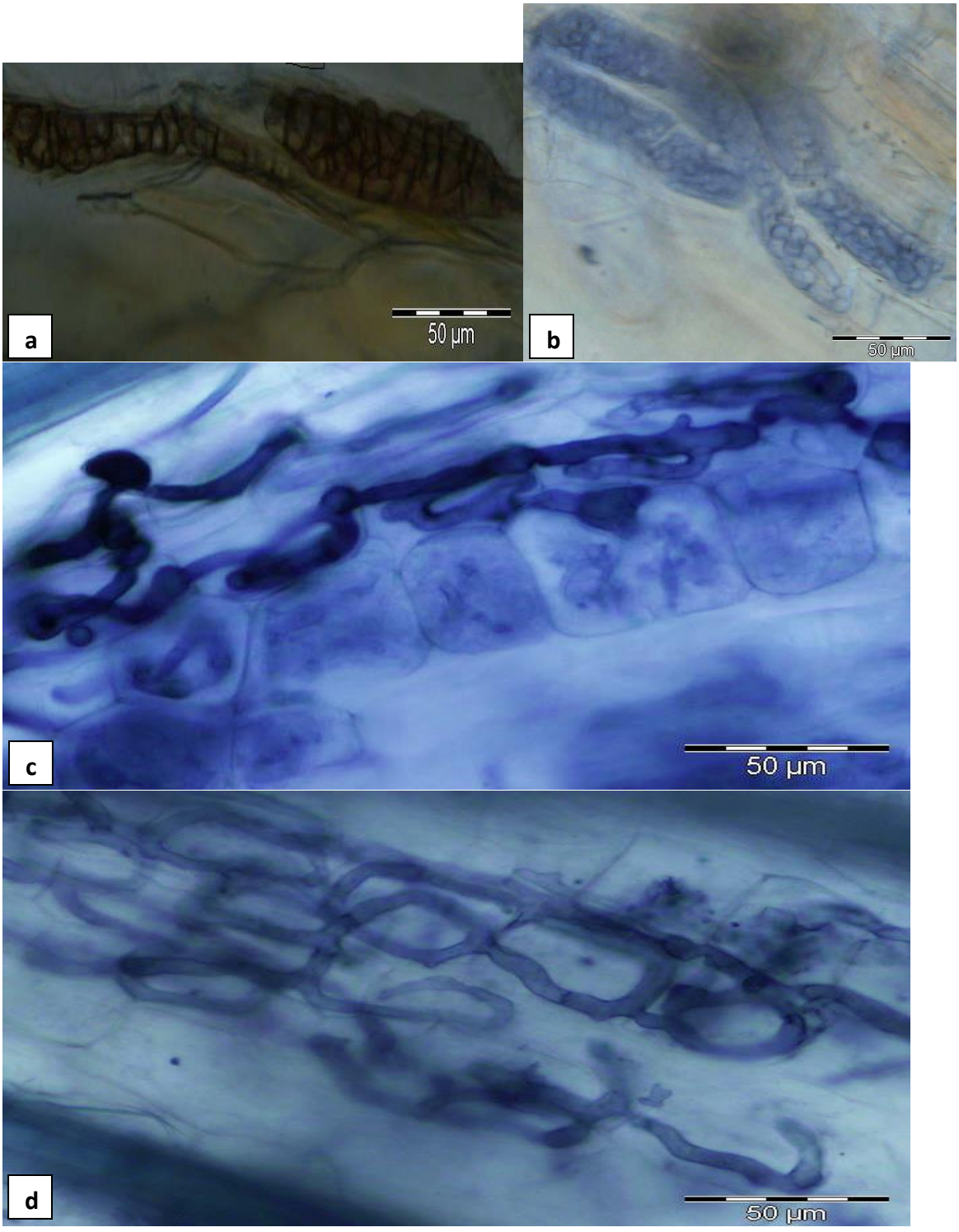

Figure 2.6. DSE structures observed in the *Erica* roots (a) *Rhizoctonia* DSE type (b) *Phialocephala* Medlar DSE type (c) and (d) Intercellular parenchymatous net.

Erica demissa had the highest DSE percentage colonisation of 16% while *E. caffra* had the lowest colonisation 7.4% (Table 2.2). Intercellular parenchymatous net were frequently detected in some of the roots (Fig 2.6c and d). Some of the thick darkly pigmented septate hyphae formed coils typical of the ERM in the epidermal cells. The coils were very similar to ericoid coils, but could be distinguished as ERM coils are much finer. Intracellular hyaline hypha which did not form any distinctive structures in the hair roots, were also observed in all species. They were observed to be inter-and intra-cellular and sometimes formed hyphal wefts surrounding the cells.

2.4 Discussion

The identification of six *Erica* species in the Beggers Bush area, a small area of the ACOE area, illustrates the diversity of the genus *Erica* in this region. These plants include *E. chamissonis*, *E. cerinthoides*, *E. caffra*, *E. glumiflora*, *E. demissa* and *E. nemorosa*. The identified plants have been reported to be endemic to South Africa with the exception of *E. cerinthoides* and *E. caffra* (Foden and Potter, 2005; Schumann and Kirsten, 1992). Amongst the identified species, only *E. glumiflora* is considered vulnerable, and is therefore currently of conservation concern in the Red List of South Africa plants compiled by the South Africa National Biodiversity Institute (Raimondo et al., 2009). *Erica chamissonis* (Grahamstown Heath) is believed to be endemic to this area hence the common name (Schumann and Kirsten, 1992). *Erica cerinthoides* also known as the 'Fire heath' has a more widespread distribution in several parts of South Africa and other Southern African countries, such as Lesotho and Swaziland. Although it occurs in two flower colourations of pink/red and white, in the sampled sites around Grahamstown area, only the pink/red *E. cerinthoides* were identified.

The roots of the *Erica* species were shallow thus confined to the upper soil surface. This is consistent with reports from previous studies where they are generally found in the 0-5 cm zone of the rhizosphere (Smith and Read, 2008). It has been reported that the root system of *Erica* plants is poorly developed and without aerenchyma, therefore, rely mostly on the intracellular spaces to supply oxygen from the aerial plant parts. This also explains their confinement to the upper few centimetres of the soil (Shaw et al., 1990).

The soil analysis results did not differ with those obtained from previous research from various parts of the world (Smith and Read, 2008; Cairney and Meharg, 2003) and locally at the

Mountain Drive area in Grahamstown (Bizabani, 2012). The high soil acidity detected in *Erica* habitats usually results from tannin acid found in the plant debris (Rains et al., 2007). Although Mn deficiency is usually associated with alkaline soils (Bierman and Rosen, 2005), it was absent in the analysed composite soil. It is possible that the mineral is not readily available in the soil but can be acquired through the symbiotic or endophytic associations with soil fungi from alternative sources.

Ericoid mycorrhizal association was detected and co-occurred with DSE association in the roots of all the *Erica* plant species studied. Neither ectomycorrhizas nor ectendomycorrhizas were observed. In *E. cerinthoides* and *E. glumiflora* vesicle structures, with potential to be AM colonisation were also present. These findings correspond to numerous reports from previous studies (Massicotte et al., 2005; Urcelay., 2002, Koske et al., 1990), but, differ from previous reports that portray ERM association as the sole symbiosis of the Ericaceae (Cairney and Meharg, 2003). In research by Urcelay (2002), the distinctive fungal structures were located in different parts of the root system. The ericoid hyphal coils were in the hair roots (diameter 60-120 µm) whereas DSE structures were observed in the 70 µm section of the wider roots and AM association was in the thicker roots (80-120 µm). These mycorrhizal distributions were to some extent similar to the observations made in this study regarding the AM structures found in thicker roots. However, the locality of the DSE and ERM associations was not clearly distinguished as they occasionally occurred in adjacent cells of the hair roots and the former in thicker roots as well.

In a study by Vohník and Albrechtová (2011), six *Rhododendron* species sampled from different parts of Europe engaged simultaneously in ERM and DSE associations. The DSE morphology was characterized by several morphologies such as *Rhizoctonia* type, *Phialocephala* type and the intra or inter cellular parenchymatous net (Vohník and Albrechtová, 2011). In this study, all these morphotypes were observed. This is not unusual as structures formed are determined by the fungus involved as well as root morphology of the host plant. The intracellular morphologies with micro-sclerotia have been proven to be an interface site for nutrient exchange between host and fungus (Usuki and Narisawa, 2007, Mandyan and Jumpponen, 2005). However, it is not clear whether or not DSE loops and the parenchymatous net have the same function. Various

studies have also demonstrated the ability of DSE association to enhance plant growth (Usuki and Narisawa, 2007; Jumpponeon and Trappe, 1998).

The vesicles and hypha observed in this study are possibly AM fungi. This association has previously been established in other genera in the Ericaceae family, including in the genus *Erica* (Fukuchi et al., 2011). Vesicles alone have been observed in *Enkianthus campanulatus* and AM fungi were identified directly from the roots using molecular techniques (Obase et al., 2013). Geographically, the AM association is regarded as the main symbiosis for all ancestral plant species including the Ericaceae (Redecker et al., 2000). However, the association has been substituted by the ERM as the plants evolved (Trappe 1987). This study supports this theory as evidenced by the relatively low account of vesicles observed in the roots of the sampled plants (present in 2 of 6 plants). Although AM spores were present in the rhizosphere of all the sampled ericaceous plants, vesicles that are possibly AM symbiosis were only observed in *E. cerinthoides* and *E. glumiflora* roots. Hyphal growth and spore development are inhibited by acidic soil: this could explain the low vesicle colonisation and lack of vesicles in some cases observed in this study (Postma et al., 2007). The presence of spores in the rhizosphere without corresponding AM structures is not unusual. It is possible they could have dispersed from co-occurring plants that engage in AM association. In previous studies, spore identification has established *Glomus*, *Acaulospora* and *Scutelospora* spores associated with AM in *Erica* species (Urcelay, 2002).

This is the first attempt to describe in detail the mycorrhizal associations of the *Erica* plants of South Africa; hence there is no published data to compare the species used in this study. In conclusion, the Albany Centre of Endemism has a variety of *Erica* species growing in acidic soil conditions that are rich in organic matter. Morphologically, DSE and ERM associations co-occur in the genus *Erica* where ERM is predominant. However, direct amplification and sequencing will help to ascertain the presence of AM association, as potential structures were observed.

3.0 Morphological and molecular detection of culturable fungi associated with the genus *Erica*.

3.1 Introduction

In nature, fungal assemblages associated with the *Ericaceae* family are numerous and diverse, and generally identified using cultural and molecular techniques (Smith and Read, 2008). Culture dependent techniques of identifying root fungal associates have been employed successfully for decades (Smith and Read, 2008) and have played an important role in the discovery of the ericoid mycorrhiza (ERM) fungi. In these earlier studies, *Scytalidium* v*accinii* and *Hymenoscyphus ericae* (Read, 1974) were identified to be ERM fungi in culture. They were described morphologically as darkly pigmented, dematiaceous and non-sporulating slow growing fungi (Pearson and Read, 1973; Smith and Read; 2008). To date, these characteristics play a key role in the primary identification of the ericoid fungi.

Culture-based techniques are used to identify fungi based on the morphological characteristics they exhibit in culture media, especially their reproductive structures. However, most ERM fungi are non-sporulating in culture. This presents a challenge in their identification and taxonomic classification. Efforts to stimulate sporulation using modified artificial media and alternating growth conditions have been ineffective (Guo et al., 2000). The taxonomic classification of sporulating ERM fungi on the basis of reproductive structures can be a challenge for inexperienced non taxonomists. To assess the diversity of these non-sporulating fungi, the fungal cultures are grouped visually into morphotypes based on their cultural characteristics, such as growth rate, colour, texture and exudates (Lacap et al., 2003). Fungi showing similar cultural characters within a morphotype group may comprise distantly related taxa and fungi grouped into different morphotype clusters may belong to the same species. Cultural morphology of sterile isolates, therefore, is not useful in taxonomic classification.

This has prompted the use of molecular techniques to identify and classify sterile fungi (Vohník et al., 2007). Molecular identification involves the analysis of the ribosomal Deoxyribonucleic acid (rDNA) internal transcribed spacer (ITS) region, isolated from pure fungal cultures or

directly from the hair roots (Sharples et al., 2000). In this ITS region, the conserved ITS2 gene is specifically targeted for fungal barcoding and has been used to demonstrate fungal diversity and interrelatedness (Vrålstad et al., 2002).

In comparison to modern fungal identification methods, such as 454 pyrosequencing and direct amplification (Gorzelak et al., 2012; Bruzone et al., 2014), the culture method has the advantage of enabling different root fungus relations to be defined through resynthesis experiments in accordance with Koch's postulates. A mycorrhizal association is described as a healthy structural relationship between fungi and host, resulting in positive growth response of the host. This is subsequently achieved through isolation of the fungus in culture, re-inoculation of host and assessment of growth and lastly re-isolation of the fungal inoculum (Smith and Read, 2008). This method has been used to confirm the mycorrhizal status of *Scytalidium* v*accinii* and *Hymenoscyphus ericae* (Read, 1974) with *Calluna vulgaris* host plants (Allen et al., 2003; Vohník et al, 2003).

Further comparison between culture-based and molecular techniques reveals the weakness of the former for failing to account for unculturable fungi such as Basidiomycetes, which have been identified by direct amplification (Berch et al., 2002; Bougoure and Cairney, 2005a; Selosse et al., 2007). Such fungi also present a problem in their mycorrhizal classification, because they cannot be used for mycorrhizal resynthesis. With respect to the *Ericaceae* family, culturing does not result in the true representation of fungal community in the roots because some slow growing fungi may also be masked by rapid growers during isolation (Smith and Read, 2008). Therefore, complementary use of cultural and molecular techniques to identify the root fungi is more reliable than the use of either technique separately.

The range of fungi that have been established as ERM fungi which associate with the *Erica* host plants include *R. erica* aggregate, *Oidiodendron* sp., *Capronia* sp. and unidentified Helotiales (Vrålstad et al., 2002; Allen et al., 2003; Bougoure et al., 2007). These have been identified through cultural and molecular techniques. In addition, numerous other ascomycetes belonging to the Xylariales, Hypocreales or Sordariales taxa have also been reported (Berch et al., 2002;

Vrålstad et al., 2002), as well as some DSE (Monreal et al., 2000; Gorzelak et al., 2012) and numerous ascomycetes which are not ERM mycobionts (Tian et al., 2011).

The objectives of this study were (i) to isolate in pure culture fungi from the roots of *E. demissa*, *E. cerinthoides*, *E. caffra*, *E. chamissonis*, *E. nemorosa* and *E. glumiflora*, (ii) to carry out morphological analysis of the isolates using microscopy and (iii) to examine species diversity using molecular techniques.

3.2 Methods

3.2.1 Isolation

Root material from sampled plants was initially washed as previously described in chapter 2. Thereafter, surface sterilization was carried out by soaking in 3.3% m/v Sodium Hypochlorite solution for 2 mins and rinsing 3 times in sterile distilled water. In order to dry off excess water, the roots were rinsed in 70% ethanol. The surface-sterilized root segments were cut into 2–3 cm segments and aseptically plated on Petri dishes with agar media. Approximately 10 root segments were placed in each petri dish. In order to improve the diversity of fungi isolated several media were used; (i) modified Melin Norkrans medium (MNN) (Marx, 1969); (ii) Malt extract agar (MEA); Water agar (WA); Modified Fontana media (FTN) (Bonfante-Fasolo and Fontana, 1973) and Maize meal agar (MMA) (Appendix B). To suppress bacterial growth, 50 mg L^{-1} of ampicillin, an antibiotic, was added. Some selected media was amended with 4 mg L^{-1} of Benlate, a fungicide, to eliminate fast growing common root fungi. The procedures were carried out in a laminar flow workstation to ensure aseptic conditions were maintained. The media were incubated in the dark at 27°C and examined at 48 hr intervals. Emerging fungal hyphae were subcultured onto fresh MEA media in order to obtain pure cultures (Kajula et al., 2010). Slow growing cultures were subsequently retained for further analysis.

3.2.2 Morphological classification of fungal isolates

Pure fungal isolates were grown on full strength MEA at 27°C in the dark. The isolated fungi were grouped into morphotypes based on colony pigmentation and texture. Thereafter, the presence of aerial hyphae and other reproductive structures was determined by tape mounts

followed by microscopy. The tape mounts were prepared by placing the sticky side of a Scotch tape on a growing fungal isolate, from the centre towards the margins. Fungal structures were stained using lactoglycerol trypan blue and microscopically examined using a Nikon light microscope at 400× magnification (Smith and Dickson, 1991).

3.2.3 Molecular characterization of root colonizing fungi

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. Integrity of the isolated DNA was evaluated by electrophoresis in a 1% (w/v) agarose gel at 100V 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. The PCR amplification targeted the Internal Transcribed Spacer (ITS) region of the rDNA using the primers ITS1-F and ITS4 (White et al., 1990) (Appendix D). This was carried out in a total reaction volume of 50 µl comprising of Ready mix (KAPA Biosystems Catalogue # kk1006), 5 µl template DNA, 2 µl of the respective primers and 18µl of water. Amplification was conducted in an automated Applied Biosystems 2720 Thermal Cycler. The following cycling conditions were used; initial denaturation at 94ºC for 2 minutes, followed by 25 cycles of denaturation at 94ºC for 30 seconds, annealing temperature of 47ºC for 45 seconds and elongation at 72ºC for 60 seconds. The final elongation period was at 72ºC for 7 minutes. Thereafter, electrophoresis was used as previously described to determine the size of the amplified bands. A 100 bp marker (MBI Fermentas, Vilnius, Lithuania) was used for referencing.

3.2.4 PCR product sequencing and analysis

The polymerase chain reaction (PCR) product was purified using a Promega Wizard SV clean up kit (Catalogue # A9281) according to the manufacturer's instructions. Three times the volume of the membrane binding solution was added to the PCR product in a centrifuge tube to allow binding of the DNA molecules in solution. The mixture was transferred to a column and centrifuged at 13,000 rpm for 1 min, placing the DNA on the membrane filter. Thereafter, the flow through was discarded and 200 µl of membrane wash solution was added to the column in order to wash impurities from the DNA and centrifuged at 13,000 rpm for 1 min. An aliquot of 200 µl membrane wash solution was added again and centrifuged as previously described. To

ensure complete ethanol removal from the filter membrane, the empty column was centrifuged. In a new centrifuge tube, 50 µl of nuclease-free water was added to the filter membrane and incubated on the bench at room temperature for 1 min to allow maximum absorption. Finally, the tubes were centrifuged at 13,000 rpm for 1 min to elute the purified DNA.

The purified PCR product was sent to Inqaba Biotechnology, Pretoria, South Africa for sequencing. The sequencing reaction was carried out using ITS3 primer, sequencing the ITS2 region used for fungal bar-coding (Berch et al., 2002). Nucleotide sequence chromatograms were analysed and edited using Chromas Lite software and compared to sequences in National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov (Altschul et al., 1997) and UNITE http://www.unite.ut.ee (Kõljalg et al., 2005) databases using Basic Local Alignment Search Tool (BLAST) program. Sequences derived from the study and their respective closest matches with homology greater than 95% were pre-aligned in MAFFT http://mafft.cbrc.jp/alignment/software prior to alignment using BIOEDIT Sequence Alignment Editor Version 6 (Hall, 1999). Thereafter, they were clustered into operational taxonomic units (OTUs) with 97% similarity and minimum overlap of 100 bp using CAP3 alignment software (Huang and Madan, 1999).

3.3 Results

Three hundred and eighteen isolates were obtained in pure culture from the hair roots of *E. demissa*, *E. cerinthoides*, *E. chamissonis*, *E. glumiflora*, *E. caffra* and *E. nemorosa*. Media used for isolation were effective with the exception of the following WA, MMA and MNN amended with Benlate which yielded the least isolates. Morphologically, the isolates were initially classified into 67 morphotypes that were predominantly darkly pigmented and non-sporulating. Approximately 40% of these pigmented fungi were isolated from *E. demissa*. Morphotypes comprising darkly pigmented non-sporulating mycelia with black exudate droplets and the yellowish-green mycelia with olive brown soluble pigments were distinctively common to all six *Erica* spp. In addition, morphologies with non-sporulating mycelia of variable colouration such as white, cream-yellowish, beige, green and brown were also observed.

A number of mycelia exhibited radial folds or a cracked appearance and exudates were yellow-brown- purple and black. Common soil fungi such as *Aspergillus, Penicillium* and *Trichoderma* spp. were successfully identified to genus level using their micromorphological characteristics; these are not potential ERM mycobionts and therefore were discarded. The ITS region of the remaining selected isolates was subsequently sequenced.

3.3.1 Molecular analysis

A total of 198 isolates were identified from the six *Erica* host roots. BLAST analysis indicated that the fungal community was dominated by ascomycetes comprising 94% of the total isolates while basidiomycetes and zygomycetes were equally represented in the remaining 6%. The results show variable distribution of taxa from the identified fungal community. In comparison, only the Orders Capnodiales, Onygenales, Pleosporales, Eurotiales and Helotiales were present from all host species. Helotiales were the main culturable fungal associates (Figure 3.1). Among the ascomycetes, 120 isolates were identified to genus level, including 70 isolates that were further classified to species level.

Unidentified root colonising fungi in the Order Helotiales were present in all the sampled plants. The remaining Ascomycete isolates were successfully assigned to their respective families (examples, Herpotrichiellaceae, Davidiellaceae, Dermateaceae and Hyaloscyphaceae) or classes (Leotiomycetes, Eurotiomycetes and Dothideomycetes). Within the Basidiomycetes, only two isolates were recovered from *E. demissa* and *E. glumiflora*. Both fungi belonged to the order Agaricales. On the other hand, zygomycetes isolates were from the Order Mucorales represented by *Umbelopsis* species and unidentified species.

3.3.2 Identification of operational taxonomic units of fungal community

The isolates were classified into fifty-four OTUs showing $\geq$97% similarity with reference sequences. Unidentified Helotiales were the most common comprising five OTUs (OTU3, OTU5, OTU11 and OTU15 and OTU18) (Table 3.1). These had close affinities mainly to *Epacris pulchella* and *Epacris microphylla* root fungus from Australia. OTU15 also aligned with mycorrhizal sp. identified from *Rhododendron fortunei* in China. The genus *Leohumicola* was represented by three OTUs and *Leohumicola minima* was identified to species level (Table 3.1).

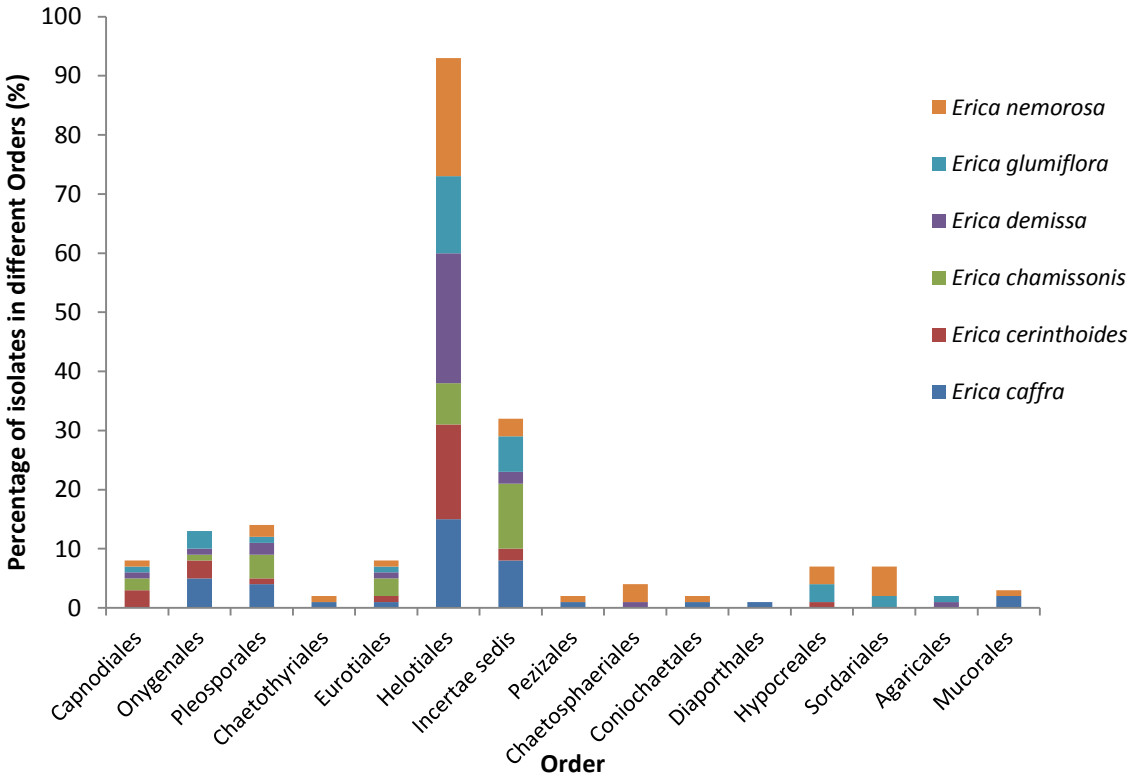

Figure 3.1. Distribution of fungal orders isolated and identified from the hair roots of six *Erica* species. Each different colour represents the percentage of isolates detected from a single host plant. Bars represent the overall percentage of the order from all host plants.

Operational taxonomic units closely related to traditional ERM fungi previously derived from ericaceous roots such as *Oidiodendron maius* and *Meliniomyces* sp. from the *R. erica* aggregate were also identified. Although not present in all host species, OTUs with affinities to DSE genera such as *Phialocephala* sp. (OTU 24 and OTU48), *Cadophora* sp. (OTU28) and *Lachnum* sp. (OTU4 and OTU 49) were detected. Other fungi represented in the OTUs included *Phoma*, *Cryptosporiopsis*, *Chaetomium*, *Acremonium* and *Epicoccum* species. Basidiomycetes were represented by two OTUSs (OTU36 and OTU39) both from the genus *Mycena*. The last four OTUs comprised of fungi that had no significant alignments in the reference databases (Table 3.1).

Isolates were deposited with the Agricultural Research Council (ARC) in South African National Fungal culture collection for preservation and collection numbers assigned. Thereafter, respective sequences were deposited in GenBank in groups and accession numbers were

assigned. The isolates have the following accession numbers in GenBank; KM5880025-KM580062, KM678339-KM678403, KF225580-KF225590, KF270075-KF270088, KF270075-KF270088, KF155899, KF765444-KF765448, KP11468-KP119493 and KC979110-KC979134.

Table 3.1 Identification of isolates from six *Erica* species classified in operational taxonomic units (OTUs).

OTUs	Number of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage/ Similarity (%)	Source of closest match	Host plant species
1	4	KF270075	*Thozetella* sp.	JX244014	87/99	*Populus deltoids (USA)*	*E. nem, E.dem*
2	7	KC979127	*Leohumicola* sp.	JX912155	100/98	*Erica carnea* (Austria)	*E. nem, E. caf, E. cham*
3	24	KP119469	*Epacris pulchella* root associated fungus EP49	AY627805	90/99	*Epacris pulchella* (Australia)	*E. nem, E. caf, E. cham, E. dem, E. cerin, E.glum*
4	4	KM678353	*Lachnum virgineum*	JQ272454	100/99	*Rhododendron* (USA)	*E. dem, E.glum, E. nem*
5	2	KM580061	*Epacris microphylla* root asscociated fungus	AY268204	100/98	*Epacris microphylla* (Australia)	*E. nem*
6	10	KF225580	*Penicillium parviverrucosum*	JX091459	94/100	Fynbos (South Africa)	*E. nem, E.cerin, E. glum, E.dem E.caf, E.cham*
7	6	KC979122	*Lachnum* sp.	JX945580	100/96	*Erica cerinthoides* (South Africa)	*E. nem, E. caf, E. cham,*
8	4	KF765444	*Phomopsis columnaris*	KC145883	100/99	*Vaccinium vitis-idaea* (New Zealand)	*E. nem, E. caf,*
9	4	KM678344	*Cordyceps confragosa*	AB079127	100/100	Japan	*E. glum*
10	8	KM678339	Ascomycota sp.	HQ623459	86/99	*Littorella uniflora* (Norway)	*E. nem, E. glum*

Table 3.1 Continued

OTUs	Number of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage/ Similarity (%)	Source of closest match	Host plant species
11	25	KF270082	Ericoid mycorrhizal fungus	AF072303	90/98	*Woollsia pungens*	*E. caf, E. cham, E.dem, E. cerin, E.glum*
12	8	KM678348	*Oidiodendron cf. maius*	KC180732	100/95	*Gaultheria poeppigii* (Argentina)	*E. nem, E. caf, E. cham, E. dem, E. cerin, E.glum*
13	4	KM678387	Dothideomycetes sp.	JF499840	99/97	*Phaenocoma prolifera* (South Africa)	*E. Nem, E. cham, E. dem, E. cen*
14	2	KC979114	*Penicillium sp.*	KF367503	100/99	Untreated water (Portugal)	*E. cham*
15	8	KF270083	*Epacris microphylla* root associated fungus	AY268189	90/99	*Epacris microphylla* (Australia)	*E. caf, E. dem*
16	5	KM678376	*Westerdykella* sp	HE608773	100/94	*Marine sponge* (Brazil)	*E. caf, E. dem, E.glum*
17	3	KM678371	*Chaetomium cupreum*	JQ676206	99/98	Medicinal plant (China)	*E. dem, E.glum*
18	12	KM678363	*Epacris pulchella* root associated fungus EP15	AY627817	70/99	*Epacris pulchella* (Australia)	*E. caf, E. cerin, E.glum*
19	4	KP119485	*Umbelopsis ramanniana*	HQ608138	99/100	*Trachymyrmex septentrionalis nest* (Brazil)	*E. caf, E.nem*
20	2	KM678393	*Cryptosporiopsis* sp.	GU973506	100/99	Eucalyptus leaves (Australia)	*E. cerin*

Table 3.1 Continued

OTUs	Number of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage/ Similarity (%)	Source of closest match	Host plant species
21	4	KM678385	*Pseudophialophora* sp.	KF689650	94/99	Grass roots of Pine barrens (USA)	*E. cerin, E. cham, E. caf*
22	2	KM678378	*Cladosporium* sp.	KF367544	100/100	Untreated water (Portugal)	*E. cerin*
23	8	KC979123	*Leohumicola* sp.	JX912154	100/98	*Erica carnea*	*E.cham, E.glum*
24	2	KP119488	*Phialocephala* sp.	JN995647	95/98	UK	*E. caf, E. nem*
25	1	KP119480	*Oidiodendron cf maius*	KC180731	98/96	*Gaultheria poeppigii* (Argentina)	*E. caf*
26	1	KP119482	*Coniochaeta gigantospora*	JN684909	100/91	Fresh water (France)	*E. caf*
27	1	KF765447	*Alternaria tenuissima*	KJ083100	100/99	*Bupleurum scorzonerifolium* (China)	*E. caf*
28	1	KM678379	*Cadophora* sp.	JN859262	100/99	*Populus alba* (Hungary)	*E. cerin*
29	1	KM678383	*Epicoccum sp.*	JN113038	86/89	Soil (Norway)	*E. cerin*
30	2	KM678384	*Ilyonectria cyclaminicola*	JN735304	99/100	*Cyclamen* sp. (Netherlands)	*E. cerin*
31	1	KM678386	Fungal endophyte	EU888616	95/99	*Rhododendron fortune* (China)	*E. cerin*
32	1	KM678388	*Meliniomyces* bicolor	HM190124	93/90	*Pinus sylvestris* (Germany)	*E.cerin*
33	1	KC979110	*Lophiostoma* sp.	EU552138	100/94	*Protea cynaroides* (South Africa)	*E. Cham*
34	2	KC979115	*Berkleasmium* sp.	KF850381	100/96	*Pinus tabuliformis* (China)	*E. cham, Nem*

Table 3.1 continued

OTUs	Number of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage/ Similarity (%)	Source of closest match	Host plant species
35	1	KC979120	*Pyrenochaeta sp.*	HM208713	100/90	*Rhododendron* sp.	*E.cham*
36	1	KM678365	*Mycena* sp.	AB846991	99/98	*Enkianthus campanulatus* (Japan)	*E.dem*
37	1	KF225590	*Phoma* sp.	EU551210	99/94	*Phascolarctos cinereus* (Australia)	*E.dem,E.nem*
38	3	KM678356	Ascomyecete sp	AM084476	90/97	Fungal spores (USA)	*E. glum*
39	1	KM678350	*Mycena pura*	KF007948	100/89	fruiting body(USA)	*E.glum*
40	2	KM678352	*Acremonium implicatum*	JQ692168	100/99	Soil (King George Island)	E.glum
41	1	KM678341	*Cryptosporiopsis erica*	AY853167	100/99	Ericaceous host (Canada)	*E.glum*
42	1	KM580040	*Dactylaria appendiculata*	AY265339	100/94	Thailand	*E.nem*
43	1	KM580041	*Pulvinula constellatio*	AF289074	98/83	*Pulvinula constellatio* ectomycorrhizae(Italy)	*E.nem*
44	1	KM580042	*Lecythophora hoffmannii*	KJ957770	100/96	Ginseng leaves(Korea)	*E. nem*
45	1	KM580046	*Beauveria bassiana*	JQ434752	100/100	Insects (India)	*E.nem*
46	1	KM580043	*Phoma sp.*	EU551209	100/94	*Phascolarctos cinereus* (Australia)	*E.nem*
47	1	KM580039	*Phialocephala* sp.	KJ188685	98/94	Grass roots (China)	
48	1	KM580033	*Lachnum* sp.	HQ873703	100/99	*Vaccinium membranaceum* (Canada)	*E.nem*

Table 3.1 continued

OTUs	Number of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage/ Similarity (%)	Source of closest match	Host plant species
49	1	KP119476	*Leohumicola minima*	NR_121307	92/99	USA	*E.caf*
50	1	KM580049	*Phialemonium* sp.	KF367530	100/99	Untreated water(Portugal)	*E. nem*
51	1	KM580059	No significant alignment				
52	1	KM580045	No significant alignment				
53	1	KM580030	No significant alignment				
54	1	KM580030	No significant alignment				

Host plant species abbreviations; *E. nem = Erica nemorosa, E. caf = Erica caffra, E. Cham = Erica chamissonis, E.dem = E. demissa, E. cerin= E. cerinthoides, E. glum = Erica glumiflora.*

3.4 Discussion

Most fungi associated with ericaceous roots are saprobes that can easily be cultivated on artificial media (Smith and Read, 2008; Gorzelak et al., 2012). This is evident in the current study as numerous fungi were successfully isolated in pure culture. However, diversity of fungi that was recovered from each plant varied. This could possibly be due to variability in root morphology or losses incurred during harvesting and processing since the ericaceous hair roots are very delicate. According to Piercey et al., (2002) during surface sterilisation, sterilants even in minute quantities may result in the loss of both external and internal fungi, contributing to reduced recovery rate in culture. Morphological analysis of the isolates showed numerous variable morphotypes comprising sporulating and non-sporulating fungi. These observations are corroborated by reports from previous research on ericaceous hosts (Curlevski et al, 2009; Upson et al., 2007).

The fungal community identified in the current study was diverse and was consistent with findings from previous studies on ericaceous plants from both the northern and southern hemisphere (Bruzone et al., 2014; Walker et al., 2011; Tian et al., 2011, Zhang et al., 2009). There was a noted absence of *R. erica* and related fungi within the *R. erica* aggregate with the exception of *Meliniomyces* sp. that was isolated from *E. cerinthoides*. This isolate had close affinities to *Meliniomyces bicolor* belonging to Clade 4 of the *R.erica* aggregate and is symbiotically associated with both ectomycorrhizal and ERM host plants. In a study conducted by Grelet et al., (2009) resynthesis with fungus *M. bicolor* and *Vaccinium vitis-idaea* host resulted in an ERM, with evidence of reciprocal transfer of carbon and nitrogen. Ericoid colonisation was also observed in *V. myrtillus* and *V. macrocarpon* following inoculation with *M. bicolar* and positive effects observed in *V. myrtillus* (Villarreal-Ruiz et al., 2004; 2012).

Recent studies have noted the absence of *R. erica* aggregate in ericaceous plants (Bruzone et al., 2014, Zhang et al., 2009), thus corroborating our findings. These findings counter the perception that *R. erica* are universal ERM fungi (Smith and Read, 2008, Villarreal-Ruiz et al., 2004; Berch et al., 2002). Although this trend was initially common and associated with host plants in the southern hemisphere, particularly in Australian Epacrids (Chambers et al., 2008; Bougoure and Cairney, 2005b; Berch et al., 2002), recently some parts of the northern hemisphere, such as the

subtropical forests of China also lack the *R. erica* aggregate. Fungal community structure appears to be influenced by the vegetation types (Bougoure et al, 2007), which are intimately linked to climate and soil rather than geography.

It is also possible that *R.erica* and some of the slow growing fungi are masked by faster-growing isolates that result in low or no isolation frequencies. Previously, incorrect identification may have occurred because of reliance on morphological characteristics without supporting molecular identification. Walker et al., (2011) failed to identify *R. erica* in culture which was subsequently identified using PCR-cloning techniques. This highlights the weakness of the culture technique of identification. However, due to the fact that *R. erica* was prominent in other studies where culture technique has been implemented under the same conditions, a conclusion can be reached that it was not present in the *Erica* hosts investigated in the current study. Similar challenges apply to other fungi such as basidiomycetes, which had low isolation frequency in the current study. Culture techniques generally favour the recovery of ascomycetous fungi and, therefore, do not provide a balanced assessment of both basidiomycetes and ascomycetes with respect to ericaceous plants (Allen et al., 2003).

The dominance of Helotiales and *Oidiodendron* species observed in this study is similar to previous reports from numerous ericaceous host plants, for example, *Gaultheria* sp. (Bruzone et al., 2014; Allen et al., 2003), *Vaccinium* sp. (Walker et al., 2011; Ishida and Nordin, 2010), *Rhododendron* sp. (Tian et al., 2011; Zhang et al., 2009), Epacrids (Curlevski et al., 2009) and *Calluna* sp. (Bougoure et al., 2007). Unidentified Helotiales fungi are commonly associated with Epacrids of Australia but have a wide geographical distribution in the Ericaceae (Hazard et al., 2014; Zhang et al., 2009; Chambers et al., 2008; Midgely et al., 2004). The current study was the first to establish these Helotiales species in the roots of genus *Erica* in South Africa. Because of their high isolation frequency in all investigated hosts, it is possible they play an important ecological role in the success of this genus in South Africa. Therefore, additional research is crucial in order to classify them into taxonomic genera and elucidate their ecological significance. The knowledge about their endophytic relations is still unclear although some have been confirmed as ERM (Chambers et al., 2008; Bougoure and Cairney, 2005).

Fungi in the genus *Phialocephala* and *Cadophora* were also detected in ericaceous roots and are associated with DSE relations. Most *Phialocephala* associations have been reported beneficial to the host (Bruzone et al., 2014; Hazard et al., 2014; Zhang et al., 2009; Vohnìk et al., 2005). Also identified were OTUs with *Lachnum* sp. and *Lachnum virgineum*. These are also associated with ectomycorrhizal host plants; however the ERM status was confirmed by Walker et al., (2011) in *V. uliginosum.* In a previous study (Bizabani, 2012) *Lachnum* sp. and *Cadophora* sp. colonised *V. corymbosum* roots, the former without forming any specific mycorrhizas, whereas *Cadophora* sp. produced typical ERM structures and enhanced growth in certain varieties. This suggests that root fungal associations of the ericaceous family are complex and cannot be generalised. *C. erica* has been consistently isolated from various ericaceous hosts and has been confirmed to be ERM (Walker et al., 2011; Zhang et al., 2009; Ziljlstra et al., 2005).

The family Magnaporthaceae is not new to the Ericaceae, *Phialophora botulispora* and *Phialophora lagerbergii* have previously been isolated from *Woollsia pungens* and *Leucopogon parviflorus* (Midgely et al., 2004), however their mycorrhizal status is still unknown. Also present in the roots are species such as *Acremonium implicatum, Alternaria tenuissima, IIyonectria cyclaminicola, Umbelopsis ramanniana* and *Epicoccum* sp. that have been identified as pathogens, saprophytes and Ericaceae root endophytes (Midgely et al., 2004) and their ecological significance in natural occurring plants is not clear. The less common fungal genotypes such as *Phialemonium* sp. could have arisen due to root contact with viable propagules in the natural soil during growth. and importantly certain edaphic and ecological conditions naturally favour.

In conclusion, the roots of *Erica* plants from this study harbour diverse fungal taxa that largely belong to the Helotiales. In culture, they are relatively slow growing and of various coloration, but mostly of dark pigmentation and sterile. *R. erica* is not present in the roots of the sampled plants and the overall fungal community structure in the current study does not differ to those previously reported for some regions of both the northern and southern hemisphere. However, there is need to establish the ecological functions of many of the isolates and to use direct amplification techniques to account for unculturable fungi in the roots.

4.0 The diversity of *Erica* root-associated fungi as determined by 454 pyrosequencing.

4.1 Introduction

The species diversity of fungal taxa associated with ericaceous roots is often underestimated (Smith and Read, 2008). Direct DNA extraction, amplification and sequencing of the internal transcribed spacer (ITS) coupled with fingerprinting techniques have provided better insights on ERM fungal diversity compared to culture-based methods of identification (Allen et al., 2003). However, these techniques still underestimate the level of fungal diversity. To overcome this challenge, Margulies et al. (2005) introduced the 454 pyrosequencing technique. It is an emulsion-based technique of DNA amplification that allows metagenomic and metagenetic barcoding (Margulies et al., 2005). Pyrosequencing has a high throughput determining up to 25 million nucleotide bases in a single 4 hr run with 99% accuracy (Margulies et al., 2005). Direct amplification based techniques are influenced by factors such as DNA extraction protocol, primer used and sequencing techniques (Lindahl et al., 2013).

The ITS region has been universally identified as the barcoding region for fungal DNA (Schoch et al., 2012). It consists of two variable spacers, namely ITS1 and ITS2 and the conserved 5.8S gene. These are flanked by the conserved small subunit (SSU) and large subunit (LSU). The total length of the ITS region is between 450 and 750 bp (Porter and Golding, 2011). Until recently with the introduction of the GS FLX+ system that sequences up to 1000 bases (Hodkinson and Grice, 2015), most genome sequencers such as Roche GS-FLX and Titanium chemistry have the capacity to generate reads less than 450 bp (Margulies et al., 2005). Consequently, the ITS1 and ITS2 with approximately 180 and 170 bp respectively are commonly targeted (De Beek et al., 2014; Blaalid et al., 2013; Davey et al., 2013; Nilsson et al., 2008). Although the 5.8S gene is the only segment that can be aligned for all fungal taxa, it is conserved and has a low taxonomic resolution in classification of fungi (Bruns and Shefferson, 2004). Other ITS subunits such as nuclear SSU and LSU rRNA can be targeted using specific primers (Krüger et al., 2009). For example, the nuclear LSU rRNA gene has been used for arbuscular mycorrhizal fungi barcoding (Terdersoo et al., 2010).

The use of the three sub-regions ITS1, 5.8S and ITS2 and partial flanking subunits has resulted in irregular basic local alignment search tool (BLAST) results (Nilsson et al., 2010; Altschul et al., 1997). The conserved sequence sub-regions (SSU, 5.8S and LSU) easily get BLAST matches in databases with less regard to the variable sub-regions. Therefore, species identification outcome is biased towards the sequence length of the conserved region rather than information provided by the variable sub-regions (Bengtsson-Palme et al., 2013; Bruns and Shefferson, 2004). A BLAST analysis comparison of the different ITS sub-regions showed that the entire sequence did not always (18%) result in the same genus or species compared to individual sub-regions, whereas separate analysis of ITS1 and ITS2 yield the same species (Nilsson et al., 2010). Therefore, analysis of ITS1 or ITS2 separately will provide a better classification to species level (Bazzicalupo et al., 2013; Bengtsson-Palme et al., 2013; Nilsson et al., 2009). The aim of this study was to establish the diversity of fungi associated with *Erica* roots using the pyrosequencing technique.

4.2 Methods

4.2.1 DNA extraction, PCR and pyrosequencing

Surface sterilized root segments from the identified ericaceous plants *Erica caffra*, *Erica cerinthoides*, *Erica demissa*, *Erica chamissonis*, *Erica glumiflora and Erica nemorosa* were initially ground separately using sterile pestle and mortar to improve DNA extraction efficiency. The ZR Plant/Seed MiniPrep™ (Catalog #D6020) was used for DNA extraction following the manufacturer's protocol. Five replicates were extracted from each plant species. Approximately 150 mg of ground root material was placed into a ZR BashingBead™ lysis tube and 750 µl of lysis solution was added. The mixture was vortexed using a standard bench top vortex at maximum speed for 10 mins. Thereafter, the tubes were centrifuged at 10000 rpm for 1 min in a microcentrifuge. From the supernatant, 400 µl was placed into a Zymo-Spin™ IV spin filter that was assembled in a collection tube and centrifuged at 7000 rpm for 1 min. The filtrate was retained and 1200 µl of Plant/Seed DNA binding buffer was added. After thoroughly mixing, 800 µl was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10000 rpm for 1 min. The step was repeated and the flow-through was discarded. A 200 µl aliquot of pre-wash buffer was added to each Zymo-Spin™ IIC column and centrifuged as

previously described. Thereafter, it was washed using a Plant/Seed DNA wash buffer in a new collection tube. It was then transferred to a sterile 1.5 ml microcentrifuge tube and 50 µl of DNA elution buffer added. This was centrifuged at 10000 rpm for 30 seconds to elute the DNA. The eluted DNA was purified to remove PCR inhibitors by transferring into a Zymo-Spin™ IV-HRC spin filter and centrifuging at 8000 rpm for 1 minute. The resulting samples were run on 1% agarose gel to check the integrity of the isolated DNA.

For each host, five replicate DNA eluate samples were mixed in equal volumes into a single composite sample. PCR was performed separately for each *Erica* species using the composite samples. The ITS3 and ITS4 primers (White et al., 1990) were used to amplify and tag the ITS2 region of the rDNA (De Beek et al., 2014; Berry et al., 2011; Davey et al., 2013). Fusion primers were synthesized by Whitehead Scientific (Pvt) Ltd according to Roche prior to emulsion PCR. They contained an emulsion 454 pyrosequencing adaptor, a unique specific 10 bp tag sequence known as multiplex identifiers (MIDs) and either the ITS3 or ITS4 primer (Appendix D). The tags were different from each other by at least 3 bp in both directions. These enabled individual sample recognition during sequence analysis after pyrosequencing.

The PCR reaction contained 25 µl of KAPA HiFi HotStart ReadyMix PCR kit (kk2602) , 2.5 µl of each fusion primer, 5µl of DNA template DNA, 2µg/µl of Bovine Serum Albumin (BSA) (Sigma A-9647) and sterile distilled water in a total 50 µl volume. The BSA was added to optimize PCR by countering the effects of PCR inhibitors such as phenols that may be present in the DNA sample. Amplification was carried out in two cycling conditions for the same reaction. This involved initial heating at 98ºC for 5 mins followed by 5 cycles of denaturation at 98ºC (30 s), annealing at 49.7ºC (45 s) and extension 72ºC (60 s). The second cycling conditions were identical to those in the first with the exception that the cycle was repeated 20 times at an annealing temperature of 52.1ºC. The last step was the final extension cycle at 72ºC for 7 mins. The outcome of the amplification was analysed by gel electrophoresis. Thereafter, amplicons were purified using Wizard® SV Gel and PCR clean-up system (Promega) as previously described in Chapter 3. They were submitted to Rhodes University pyrosequencing facility where they were sequenced on the Genome Sequencer FLX 454 System (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA).

4.2.2 Bioinformatics and statistical analysis

The raw sequences were provided as six separate fasta files created from the original standard flowgram format (SFF) files. Analysis was conducted using Mothur 1.33.3 software (Schloss et al, 2009). Potential sequencing errors were removed from the individual files in a series of steps. Firstly, quality trimming was carried out by removing reads shorter than 150 bp, longer than 600 bp, reads with homopolymers longer than 10 bp or more than 1 ambiguous base were removed from the dataset. These parameters were selected to ensure that sequences representing species with shorter ITS2 would not be filtered and the maximum length was set higher than recommended 450 bp in Mothur to cut off unreliable ends at the same time preserving the ITS2 region. The ITS region of some fungi contains numerous repeats of up to 15 homopolymers, hence justifying the parameters implemented (Lindahl et al., 2013).

Extraction of the ITS2 region was carried out using Fungal ITSx Extractor 1.0.10 software (Bengtsson-Palme et al., 2008). Non target and partial sequences were filtered. Reads were screened for chimeric sequences using the Uchime tool in Mothur and chimeras were removed (Edgar et al., 2011). Sequence comparison was carried out and duplicate sequences were replaced by a single representative sequence. Thereafter, unique sequences were assembled. Final clustering into operational taxonomic units (OTUs) was done with Usearch software with a 97% similarity threshold (Edgar, 2010). The resulting representative OTUs were classified using the ribosomal database project (RDP) (Wang et al., 2007) with UNITE ITS2 as the reference database (Kõljalg et al., 2005) and BLAST against the GenBank database (Altschul et al., 1997). All singletons were disregarded from further analysis as recommended by Terdsoo et al., (2010) on the basis that they may be artifacts.

Rarefaction analysis of OTUs against sequence reads was performed for each host species separately and rarefaction curves constructed. Shannon diversity index, evenness and Chao1 richness were estimated (Chao et al., 2005). These analyses were carried out using Past3 software (http://folk.uio.no/ohammer/past/). The hypothesis that fungal composition is the same among all *Erica* species was tested using two-dimensional nonmetric multidimensional scaling (NMDS) (Kauserud et al., 2012). OTUs abundance data at order level for the six *Erica* host plants was located in ordination space using R software (R Development Core Team, 2014).

4.3 Results

A total of 63 745 reads were recorded from the respective *Erica* host plants. Quality trimming and ITS2 extraction resulted in 30 976 reads after removal of non ITS2 reads, chimeric sequences and non-target lineages consisting mainly of chloroplasts. A total of 7 139 unique reads were subsequently recorded for further analysis. The unique reads from all the host plants ranged from 977 to 1 206 sequences (Table 4.1). Clustering analysis resulted in a maximum of 362 OTUs including 154 singletons in *E. chamissonis*. The least OTUs were recorded in *E. glumiflora* consisting of 161 OTUs that included 73 singletons.

Table 4.1. Analysis of reads and OTUs clustering of sequences obtained for the six *Erica* host plants.

Host plant	***Erica caffra***	***Erica cerinthoides***	***Erica demissa***	***Erica chamissonis***	***Erica glumiflora***	***Erica nemorosa***
Reads (untrimmed)	8 858	6 335	9 300	16 326	7 854	15 072
Fungal ITS2 reads (trimmed)	4691	6151	4171	6381	2081	7501
Unique reads (trimmed)	1 106	1 145	1 206	1 482	977	1 223
OTUs	178	260	219	362	161	227
Singletons (% of OTUs)	69 (38.8%)	112 (43.1%)	90 (41.1%)	154 (42.5%)	73 (45.3%)	124 (54.6%)

The number of OTUs increased with number of sequences, therefore none of the rarefaction curves reached plateau (Fig 4.1). Table 4.2 shows the expected fungal diversity indices. Shannon richness index ranged between (1.83) and (3.32). The highest diversity indices were recorded in *E. caffra, E. cerinthoides* and *E. demissa*. *Erica caffra* and *E. cerinthoides* had the highest eveness of 0.15 whereas *E. nemorosa* had the least value of 0.03. Nonparametric Chao1 diversity estimators showed higher values than OTUs that were observed. These ranged from 256 to 564 OTUs in *E. glumiflora* and *E. chamissonis* respectively (Table 4.2).

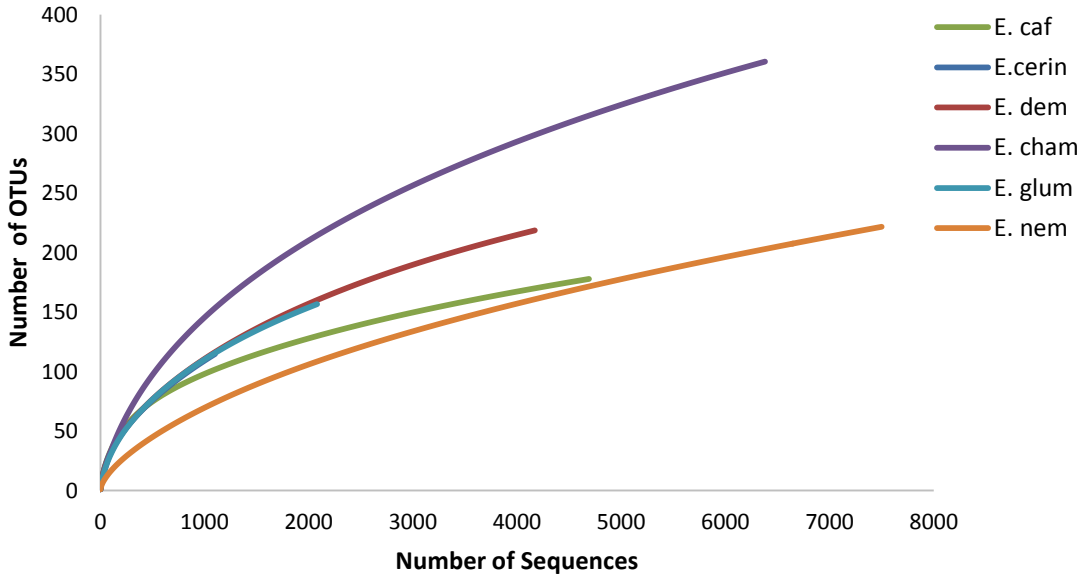

Figure 4.1. Rarefaction curves for each of the six *Erica* host plants. Showing the number sequences plotted against the number of rarefied operational taxonomic units OTUs a 97% similarity threshold.

Table 4.2. Simpson and Shannon diversity estimators, Evenness and Chao1 richness estimator calculated at 97% sequence identity threshold for the six *Erica* host plants.

Host plant	***Erica caffra***	***Erica cerinthoides***	***Erica demissa***	***Erica chamissonis***	***Erica glumiflora***	***Erica nemorosa***
Shannon	3.29	3.31	3.32	2.84	3.02	1.83
Eveness	0.15	0.15	0.13	0.05	0.13	0.03
Chao1	302	448	317	564	256	472

4.3.1 Fungal classification and identification

The dominant taxa (< 2 sequences) fungi were successfully classified into different phyla. Fungal OTUs belonging to Ascomycota and Basidiomycota were present in all the *Erica* hosts (Fig 4.2). Comparatively the distribution of OTUs showed Ascomycota to be a dominant phylum. A

minimum of 30% of the OTUs from *E. glumiflora* and a maximum of 60% identified from *E. chamissonis* were assigned as Ascomycota.

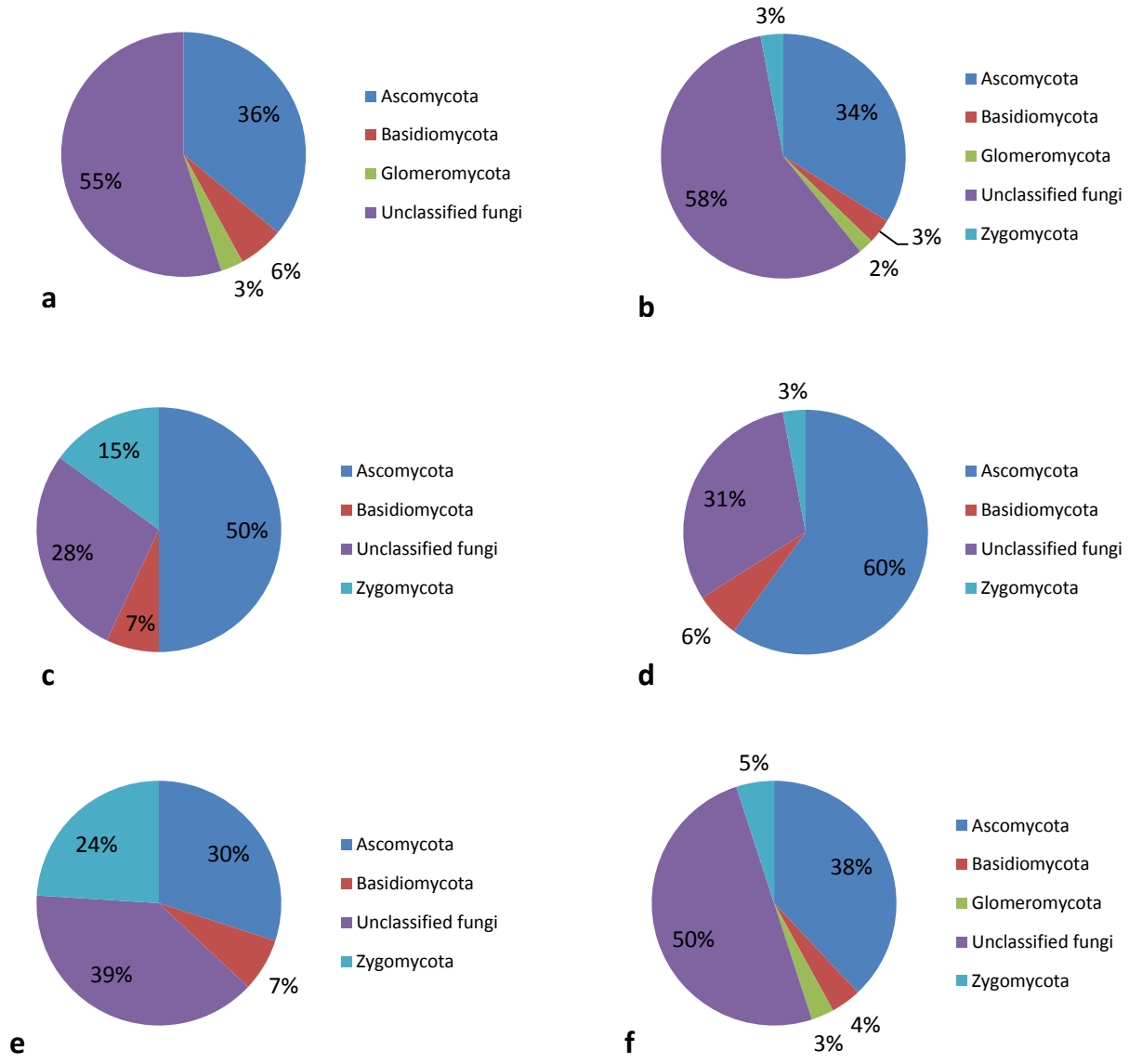

Figure 4.2. Percentage of OTUs assigned to different fungal phyla. All unidentified OTUs were grouped together under unclassified phyla. Charts representing phyla from (a) *Erica caffra* (b) *Erica cerinthoides* (c) *Erica demissa* (d) *Erica chamissonis* (e) *Erica glumiflora* (f) *Erica nemorosa*.

In addition, Basidiomycota was also detected in all the roots with the percentage OTUs ranging between 3% and 7%. Phylum Zygomycota had a similar occurrence except it was absent in *E. caffra*. Although Glomeromycota was present in *E. caffra*, *E. cerinthoides* and *E. nemorosa* it

had low reads. There are fungal species that remain taxonomically unclassified. The highest unclassified OTUs of 58% were recorded in *E. cerinthoides* whereas *E. demissa* had the least unclassified comprising 28% of the OTUs (Fig 4.2). The OTUs belonging to classes Agaricomycetes, Dothideomycetes, Leotiomycetes, Eurotiomycetes were dominant across the six host plants. Other classes such as Glomeromycetes, Tremellomycetes and Sordariomycetes were also detected amongst some of the hosts. Some classes remained unclassified and there was no singular distribution of classes in any of the hosts.

Although most of the orders remained unclassified, Agaricales, Chaetothyriales, Helotiales, Pleosporales and Sebacinales were dominant orders from all *Erica* host plants (Fig 4.3). The highest number of Helotiales species was detected in *E. caffra*, *E. demissa*, *E. glumiflora* and *E. nemorosa* contributing a total of 4.5%, 4.7%, 6.8% and 6.8% OTUs for the respective host plants. The order Chaetothyriales dominated *E. cerinthoides* and *E. chamissonis*. Twenty-eight percent of the orders belonged to Basidiomycetes. However, Agaricales and Sebacinales were not present in all roots. At order level, Glomerales were identified at low percentage OTUs in *E. caffra* (0.5%), *E. cerinthoides* (0.7%) and *E. nemorosa* (4%). Other orders included Mucorales and Mortierellales (Zygomycetes) and Chaetothyriales (Ascomycetes).

A total of 265 OTUs were classified to either genus or species level at 97% similarity and at least 90% bp coverage. The highest number of OTUs identified at genus level was 50 and 39, observed in *E. demissa* and *E. chamissonis* respectively. *Leohumicola* species and unclassified *Epacris* root fungus with affinities to Helotiales were present in all sampled roots. Some of the *Leohumicola* species were identified as *Leohumicola minima* while others had the highest similarities with *L. verrucosa* and *L. incrustata* UNITE hypothesis species (HS). *Oidiodenron maius* had the highest reads of 814 sequences in *E. caffra*.

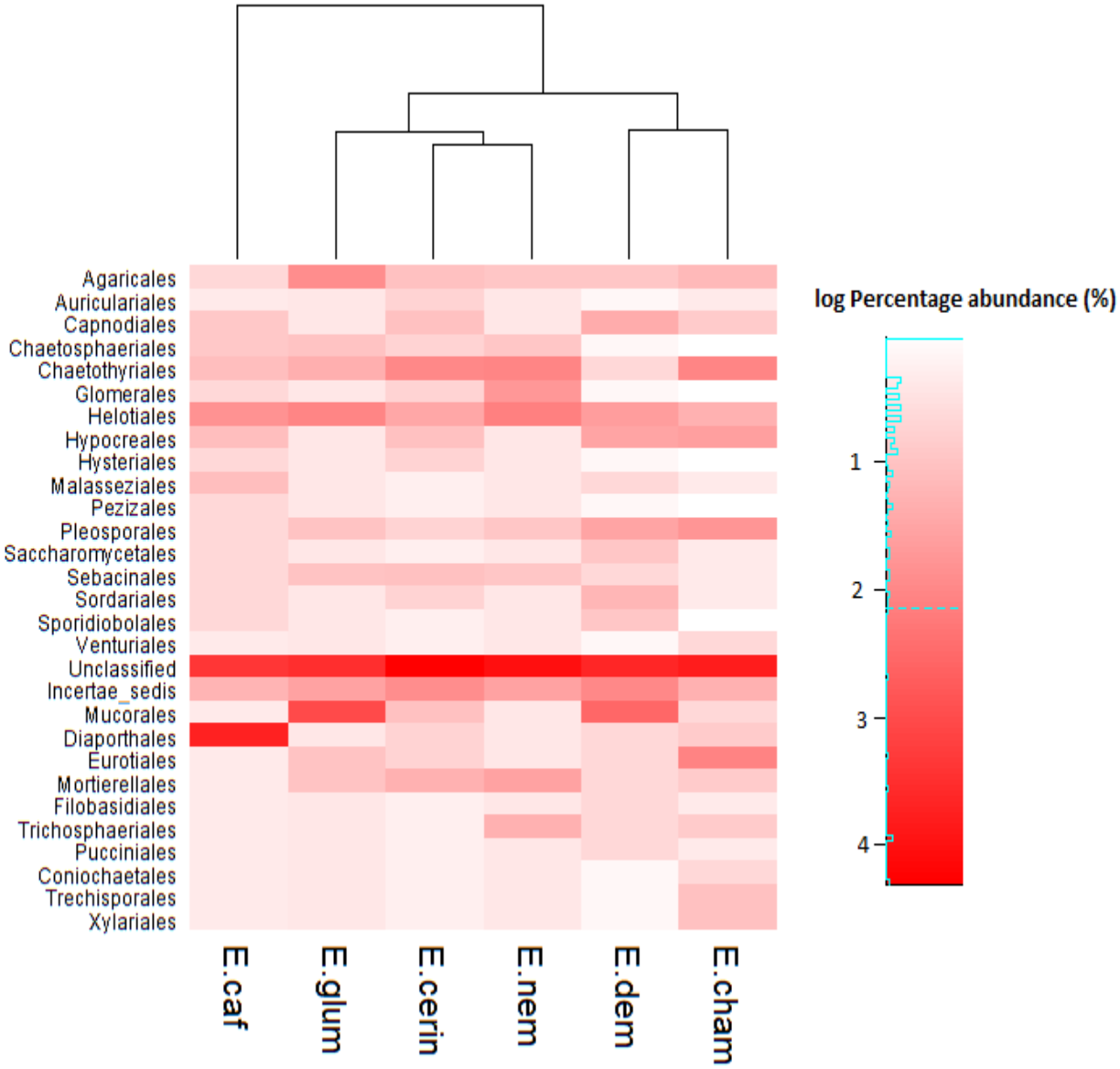

Figure 4.3. Heat map showing the percentage distribution of the Orders detected from six *Erica* host plants. The red colour scale shows the percentage abundance and relative distribution. Colour intensity increases with percentage abundance on a 1 to 4 scale on log transformed percentage data. The hosts were E. caf (*E.caffra*), E. glum (E. *glumiflora*), E. cerin (*E. cerinthoides*), E.nem (*E. nemorosa*) and E.dem (*E. demissa*).

The NMDS analysis further illustrates that at order level there is a difference in OTU composition among the *Erica* species. Considering the distance matrix at order level the OTU composition of *E. chamissonis* and *E. caffra* were distantly located from other Ericas due to the

presence or absence of orders. For example, the presence of Coniochaetales, Trechisporales, Venturiales and Xylariales, and the absence of Pezizales in *E. chamissonis* (Fig 4.3; Fig 4.4).

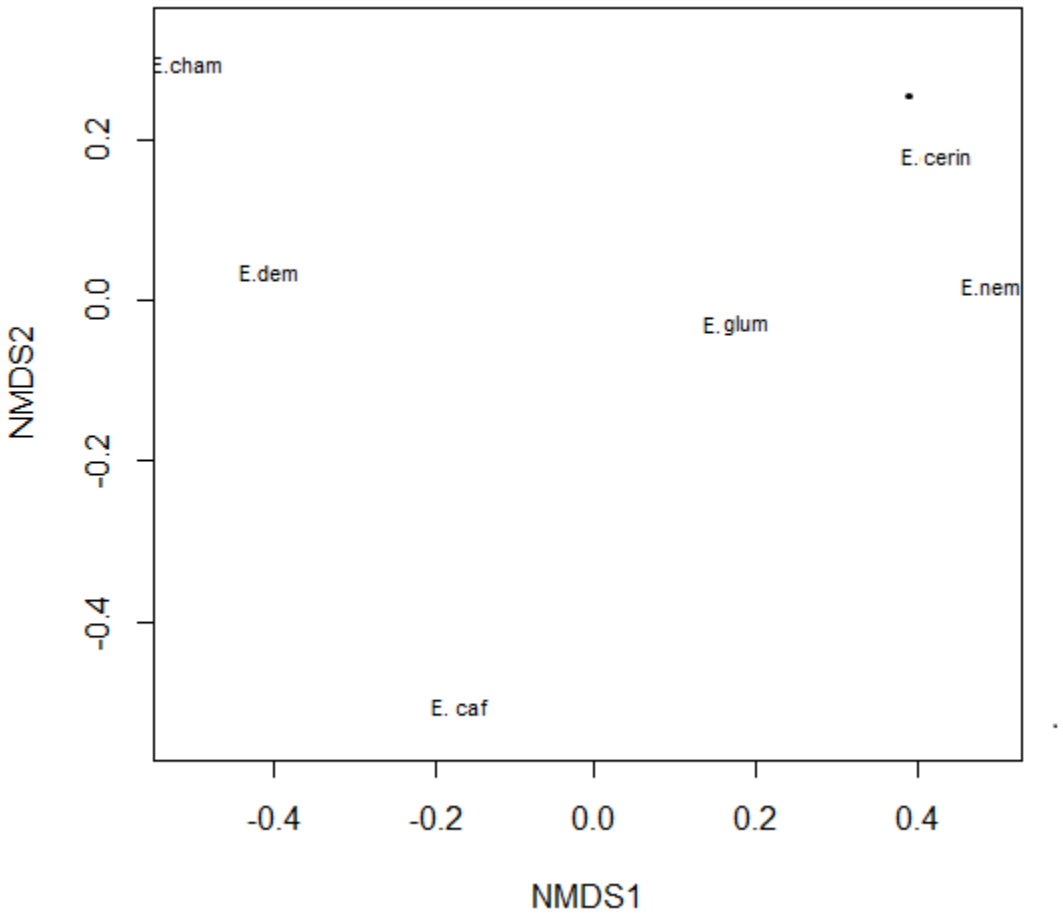

Figure 4.4. Nonmetric multidimensional scaling (NMDS) ordination of fungal operational taxonomic units (OTUs) composition as detected from six *Erica* plants at order level. Stress value= 0.08; R^2 = 0.80.

Other ascomycete included *Acephala* sp. *Cadophora olivo-luteacea*, *Capronia* sp., *Cryptosporiopsis erica*, *Acremonium implanticum* and *Cladosporium* sp (Table 4.3). Within the basidiomycetes, *Mycena* was the only genus identified to genus level in *E. demissa*, *E. glumiflora* and *E. nemorosa*. Zygomycetes *Umbelopsis ramanniana*, *U. angularis and Mortierella elongate* were also detected in most roots. None of the Glomeromycetes were identified to genus level.

Table 4.3. Molecular identification of selected OTUs identified to genus and species level from the *Erica* host plants. Identification was based on manual NCBI BLAST search and Ribosomal database project (RDP) classification using UNITE ITS2 reference database.

OTUs	N	Closest BLAST match	(%) Identity/bp	Accession GenBank/UNITE
Ecaf4	1 013	*Cladosporium* sp.	100/100	KF878321.1/SH196750.06FU
Ecaf 6	814	*Oidiodendron* cf. *maius*	98/98	KC180732.1/SH217743.06FU
Ecaf 9	311	*Acremonium implicatum*	100/100	KJ746997.1/SH191577.06FU
Ecaf 12	267	*Leohumicola* sp.	100/100	JN655658.1/SH209174.06FU
Ecaf 16	183	*Lachnum* sp.	97/100	JN655650.1/SH189775.06FU
Ecaf 17	60	*Lecanicillium* sp.	100/100	KJ862850.1/SH196289.06FU
Ecerin1	5 062	*Umbelopsis ramanniana*	99/100	JN198475.1/SH205578.06FU
Ecerin 2	1 683	*Epacris pulchella* root associated fungus EP7	100/100	AY627809.1/SH209213.06FU
Ecerin 4	310	*Chaetomium cupreum*	100/100	JQ676206.1/SH221746.06FU
Ecerin 7	156	*Cryptosporiopsis ericae*	100/100	HQ889712.1/SH108598.06FU
Ecerin 8	105	*Phomopsis columnaris*	100/100	DQ093770.1/SH194745.06FU
Ecerin 14	63	Ericoid mycorrhizal sp.	97/100	AF072297.1/SH215672.06F
Edem2	303	*Devriesia fraseriae*	99/100	HQ599602.1/SH189975.06FU
Edem 5	182	*Mycena hudsoniana*	97/95	EU846300.1/SH208809.06FU
Edem 8	55	*Cadophora* cf. *olivo-luteacea*	100/100	KC180676.1/SH103239.06FU
Echam 2	198	*Capronia* sp.	98/97	AF284128.1/SH226829.06FU
Enem 4	129	*Mycena leptocephala*	97/95	HQ604773.1 /SH237319.06FU

Selected OTUs were from Ecaf (*E.caffra*), Ecerin (*E. cerinthoides*), Enem (*E. nemorosa*) and Edem (*E. demissa*). (N) is the number of sequences in each OTU. (bp) is base pair coverage. Annotation of identities are according to manual NCBI BLAST and the respective UNITE accession number used for classification is shown.

4.0 Discussion

Most identification of Ericaceae root-associated fungi has been achieved through culture-based techniques followed by molecular analysis. There are limited reports derived from 454 pyrosequencing techniques, therefore the data was primarily compared to reports from direct DNA amplification from ericaceous roots and sequencing using Sanger based techniques. The Shannon diversity indices were high with an average of (2.96), however, Chao1 estimates showed that none of the plants fungal species diversity was sampled to completion. This implies that continuous sampling could result in the identification of additional fungal species.

Most of the ITS2 sequence reads obtained in the current study had an average of 250 bp that is sufficient to allow taxonomic identification of the OTUs to genus and species levels (Nilsson et al., 2009). A large proportion of the OTUs remained unclassified after classification using both non-redundant GenBank and curated UNITE databases. This has also been reported in previous studies (Toju et al., 2012; Buèe at al., 2009). In a study on fungal diversity of Ericaceae in the tundra vegetation, 41% of the sequences obtained could only be assigned to kingdom taxonomic level (Walker et al., 2011). Hence, there is urgent need for taxonomists to identify the unclassified fungi to enable detailed and conclusive information on the diversity of fungi. In the current study, plant DNA was also amplified. This is not surprising because the DNA was extracted from the roots and ITS primers can amplify a broad range of organisms that include plants (Obase et al., 2013; Gardes and Bruns, 1993; White et al., 1990).

Ascomycota and Basidiomycota belonging to the subkingdom Dikarya were detected in all samples. The taxonomic resolution on the classification of OTUs was low and most OTUs could not be classified to genus level. Similar phyla distribution has been reported from molecular identification of fungi in Ericaceae (Selosse e al., 2007; Bougoure and Cairney, 2005; Allen et al., 2003). Ascomycetes were also established to be the dominant phylum (Walker et al., 2011; Allen et al., 2003). Most notably in Basidiomycetes and Glomeromycetes, OTUs were mostly identified to order level. This was also observed in *Epacris pulchella* after direct amplification and sequencing of the ITS1 region (Bougoure and Cairney, 2005b). This shortcoming has been addressed by the use of specific primers (De Beeck et al., 2014; Selosse et al., 2007). For example, Glomeromycetes have been identified to genus level in *Enkianthus campanulatus* after

amplification with AM specific primers NS31 and AM1 that amplify the SSU (Obase et al., 2013). While the use of specific primers may have resulted in a higher rate of detection in the half of *Erica* plants in this study in which detection did not occur, their usage may have the disadvantage of eliminating general fungal taxa (Lindahl et al., 2013).

Helotiales was pedominant in all *Erica* plants with the exception of *E. cerinthoides* and *E. chamissonis* in which Chaetothyriales had the highest percentage OTUs. This differs from previous findings that have established Helotiales as the dominant order associated with most ericaceous roots (Bruzone et al., 2014; Tian et al., 2011; Walker et al., 2011; Curlevski et al., 2009). Chaetothyriales is a common order in ericaceous roots and contains genera such as *Cladophialophora* and *Capronia* that can be ericoid mycorrhizal or root endophytes (Allen et al., 2003). The fungal OTUs classified to genus level in this study showed similar identities to those obtained in several studies using both culture-based and molecular techniques (Bougoure and Cairney, 2005b). However, in the current study there is a notable absence of *Rhyzoscphus erica* (*R. erica*) which is typically present in ericaceous roots (Smith and Read, 2008; Villarreal-Ruiz et al., 2004; Berch et al., 2002). It has not been detected in several recent molecular based studies (Bruzone et al., 2014; Zhang et al., 2009), which could imply they are not as ubiquitously present in ericaceous roots as previously envisioned. Most previous reports were based on morphology, therefore, there is a possibility of misidentifications that had a bias towards *R. erica*.

Although fungi such as *Leohumicola* sp., *Epacris pulchella* root associated fungus EP7, *Cryptosporiopsis ericae* and *Acremonium implicatum* were shared across all host plants, fungal composition of OTUs at order level was variable. At phylum level variability was illustrated by the absence of Glomeromycetes and Zygomycetes in some of the *Erica* plants. There is no consensus pertaining to the distribution of fungal composition between co-occurring ericaceous plants within a single ecosystem (Bougoure et al., 2007). In a study conducted in the Heath temperate ecosystem, it was established that the fungal community between coexisting *Vaccinium myrtillus* and *Calluna vulgaris* were distinct. Contrasting findings were reported from a similar study conducted in the tundra ecosystem in which there was no divergence in the fungal composition (Walker et al., 2011; Kjøller et al., 2010). Ericaceae plants *Empetrum Hermaphroditium*, *Andromeda polifolia*, *Vaccinium ulinginosum* and *Vaccinium vitis-idaea* had the same fungal community (Kjøller et al., 2010). This illustrates the importance of conducting

research in different ecosystems separately, in order to establish accurate conclusions on the distribution of ERM fungal communities.

In conclusion, Ascomycetes and Basidiomycetes are the main phyla associated with *Erica* roots growing in the Albany Centre of Endemism. They are dominated by Helotiales, Chaetothyriales and Sebacinales and *R. erica* is not part of the fungal community. Although the *Erica* plants shared some fungal taxa, overall fungal composition was variable among the different host plants.

5.0 Utilisation of organic and inorganic nutrients by selected ericaceous fungal isolates in pure culture.

5.1 Introduction

Ericaceous plants grow in habitats that are rich in recalcitrant organic matter but deficient in mineral nutrients that are essential for plant growth, such as nitrogen (N), phosphorus (P) and Iron (Fe) (Cairney and Meharg, 2003; Cairney and Burke, 1998; Shaw et al., 1990). In ericoid mycorrhizal symbiosis, the fungi acquire photosynthetic C from the host and in return avail mineral nutrients required by the host (Grelet et al., 2009; Smith and Read, 2008, Straker, 1996). Fungi facilitate nutrient acquisition from organic substrates through an array of extracellular enzyme activities that exploit organic compounds (Piercey et al., 2002; Cairney and Burke, 1998). It has been established that ericaceous roots are associated with numerous fungal taxa and potentially beneficial fungi can be selected in pure culture.

The culture-based techniques are employed on the basis that fungi can utilise nutrients they assimilate for individual growth. Culture media typically used are either modified agar or liquid media (Nurfadilah et al., 2013, Vohník et al., 2012). The use of agar media in nutrient assimilation assays is limited because bacteriological agar, the solidifying agent, contains numerous nutrient sources such as peptone and glucose that can compromise the intended analysis. Nonetheless, they can be used effectively for qualitative and semi-quantitative assays to determine production of microbial secretions such as siderophores, cellulase and ligninase enzymes. In such assays, colour change and formation of halos in the media are used as positive indicators (Neilands, 1995). Vohník et al. (2012) used modified Melin-Norkrans media (MMN) that was altered with cellulose azure dye to detect both ligninolytic and cellulolytic activities in fungi. Clearing of azure dye illustrated the lignocellulolytic capabilities of the fungi tested.

Liquid assays are mostly used for nutrient assimilation studies (Cairney and Burke, 1998). They can be manipulated to suit the specific requirements for a particular study. In addition, they enable quantitative analysis and identification of respective enzymes together with organic compounds such as primary and secondary metabolites, secreted by fungi. Medium such as modified Melin Norkrans medium (MMN) has been used in N, P and C utilisation studies by altering the media with specific amounts and nutrient sources (Nurfadilah et al., 2013; Grelet et

al., 2005; Midgely et al., 2004). For example, ammonium, Bovine serum albumin (BSA) and glutamine can be added separately to MMN media as sole sources of nitrogen (Cairney et al., 2000). This also applies to inorganic and organic P and C sources. Utilising the respective nutrients can be evaluated through fungal biomass production. Further analysis to determine extracellular and cell-bound enzyme activities can be conducted from culture filtrates (Tibbett et al., 2002; Burke and Cairney, 1997; Leake and Read, 1990).

Siderophore assays are carried out on iron deprived media. The Chrome Azurol Sulfonate (CAS) assay, developed by Schwyn and Neiland (1987), is commonly used. It uses CAS and hexadecyltrimethylammonium bromide (HDTMA) as indicators. A colour change resulting from removal of Fe from the CAS complex signifies the presence of siderophores. However, this method is not universal to all organisms because HDTMA that is used to achieve the blue colour can be toxic to fungi. Modified CAS assays have been developed for fungal siderophore analysis (Haselwandter and Winkelmann, 2007; Pérez-Miranda et al., 2007, Machuca and Milagres, 2003). Siderophores can also be identified in liquid assays using spectrophotometric techniques. These tests involve analysis of culture supernatants using the Arnow and Atkins tests that identify catechols and hydroxamates siderophore types respectively (Arnow, 1937; Atkin et al., 1970).

The aims of this study were to (i) assess the ability of selected ericaceous fungi to utilise various inorganic and organic sources of N, P, and C using biomass accumulation in liquid culture, (ii) detect phosphomonoesterase and phosphodiesterase activities in P substrate, and (iii) determine siderophore activity in selected isolates.

5.2 Methods

5.2.1 Nutrient assimilation by fungal isolates from organic and inorganic sources

Selected fungal isolates used in this study were previously isolated and identified in Chapter 3. The fungal isolates used were *Meliniomyces* sp. (ECRU075), unidentified Helotiales fungus (EdRU083), *Acremonium implicatum* (Nem83), *Leohumicola minima* (Chem038), *Cryptosporiopsis erica* (Glum686) and *Oidiodendron* sp. (CafRU082b).

To assess the ability of the fungal isolates to assimilate N, P and C from different inorganic and organic sources, modified MMN was used as a basal medium. The MMN medium originally contained (L^{-1}) 5.0 g glucose, 0.30 g KH_2PO_4, 0.25g $(NH_4)_2HPO_4$, 0.14 g $MgSO_4.7H_2O$, 50 mg $CaCl_2$, 25 mg NaCl, 3 mg $ZnSO_4$, 12.5 mg ferric EDTA and 0.13 mg thiamine. It was adjusted to a pH between 4 - 4.5 prior to autoclaving. The medium was altered accordingly for the different nutrient sources. The inorganic N sources used in this study were $(NH_4)_2HPO_4$ and $Ca(NO_3)_2$ and arginine (basic amino acid) (CalBiochem cat#181003) and BSA (complex protein) (Sigma cat #A-9647) were the organic sources. Ammonium $(NH_4)_2HPO_4$ was omitted from the basal media and each N source was added separately as treatments. The initial N concentration for all the treatments was 7.6 mM (Chambers et al., 2000). Inorganic N sources were sterilized together with the basal medium by autoclaving. Organic sources were filter-sterilized into autoclaved medium through 0.8/0.2 µm Millipore filters because they are unstable to heat sterilization. The control treatment contained no N (Nurfadilah et al., 2013; Chen et al., 1999). Two plugs of fungi were cored from the margins of actively growing mycelia using a sterile corer. These were inoculated into 9 cm petri dishes containing 25 ml liquid basal media amended with the respective treatment. Each treatment was replicated four times with each of the selected cultures and incubated at 27ºC for 21 days in the dark (Whittaker and Cairney, 2001). Mycelia were harvested by filtration on a pre-dried and weighed sterile Whatman number 1 filter paper and dried in an oven at 60ºC for 48 h prior to weighing the dry mass (Grelet et al., 2009).

To determine P utilisation, the basal medium was initially adjusted by omitting P sources $(NH_4)_2HPO_4$ and KH_2PO_4. A double proportion consisting of 0.606 g L^{-1} NH_4NO_3 and 0.164 g L^{-1} KCl was added to compensate for the omitted N and K. The following P treatments were used; $NaHPO_4.2H_2O$ (orthophosphate), sodium inositol hexaphosphate (phytic acid) (Chemindustry cat. 14306-25-3) and salmon sperm DNA (Sigma-Aldrich #D1626). Each treatment had a starting P concentration of 5.0 mM. The inorganic P sources were sterilized by autoclaving whereas phytic acid was dissolved in 10 ml of basal medium and pH was adjusted to between 4 - 4.5 and filter sterilized into the basal medium. For DNA treatment, glucose was reduced to 4.02 g L^{-1} and NH_4NO_3 omitted from the basal medium based on the concentration of C and N already present in salmon DNA. The DNA was sterilised by soaking in 3 ml of 70% ethanol for 48 hr prior to adding into the autoclaved basal medium. The controls had no P source

added to the medium. The fungi were inoculated, incubated and harvested as previously described for nitrogen (Nurfadilah et al., 2013; Leake and Miles, 1996).

Further analysis was carried out to determine phosphomonoesterase and phosphodiesterase extracellular activity of the fungal isolates. This was done by measuring the release of *p*-nitrophenyl ion (*p*NP) from *p*-nitrophenylphosphate (*p*NPP) for phosphomonoesterase and bis(*p*-nitrophenyl) phosphate (bis-*p*NPP) for phosphodiesterase. Each treatment contained 0.2 µl of culture filtrate, 1 ml of buffer and 1 ml (0.4 mg) of the substrate in a test tube. The buffer was made with 0.2 M sodium acetate/acetic acid and adjusted to pH 4.5. Substrates used were either Phosphatase substrate (Sigma-Aldrich #P4744) or bis-*p*NPP substrate (Sigma-Aldrich #123943). The test tubes were incubated at 37ºC on a shaker for 2 hours. To stop the reaction, 2 ml of 0.5M NaOH was added to 0.5 ml of the assay solution. Absorbance was measured at 410 nm using a power wave Spectrophotometer (Bio-Tek instruments). Four replicate samples were used for each treatment. Decrease in absorbance and colour change of the assay from clear to yellow showed the presence of the *p*NPP and *p*NP ions (Leake and Miles, 1996).

Carbon utilisation was tested using glucose (monosaccharide), cellobiose (disaccharide), carboxymethylcellulose (CMC), pectin (polysaccharides) and tannic acid (phenolic carbon). Glucose was omitted from all basal media but was added as a treatment. These C sources were filter sterilised and added to liquid basal media to a final concentration of 2 g L^{-1}. A carbon free control treatment was included. Respective inoculations, incubation and biomass collection were carried out as previously described. All the biomass data collected was analysed separately for each fungal isolate using one-way analysis of variance (ANOVA). The Shapiro-Wilks and Bartlett tests were used to check for normality and homogeneity of variance respectively. Significant differences between mean biomass were determined by Turkey's post hoc test at $p < 0.05$. Means for each treatment and isolate were used to plot histograms illustrating utilisation (Nurfadilah et al., 2013). All analysis was conducted using R software (R Development Core Team, 2014).

5.2.2 Determination of siderophores

All glassware was prepared by washing in 1M HCl and rinsing in milliQ water at pH 7 to remove traces of Fe. Initial screening tests were done using an overlay (O)-CAS assay (Pérez-Miranda et

al., 2007). Selected isolates were inoculated on both MMN and Fe free MMN media and incubated at 27ºC in the dark. Fe free medium was used to stimulate siderophore production through iron deprivation. After three weeks, the O-CAS agar medium was prepared as described by Schwyn and Neilands, (1987) (Apendix E). It was then poured over the agar plates containing cultivated fungi to be tested for siderophore production. Thereafter, plates were observed for colour change over a 1 hour period. A colour change from blue to purple indicated the presence of catechol, whereas blue to orange indicated hydroxamates. A total of 50 fungal isolates were tested with two replicates on each medium for each isolate.

The isolates that tested positively for siderophores were further inoculated onto CAS medium to confirm the colour changes. Liquid CAS culture assay was used for spectrophotometric quantitative analysis (Tortora et al., 2011). Two plugs of fungus were cored from the margins of actively growing mycelia using a sterile corer. These were inoculated into 50 ml flasks containing 25 ml of Fe free MNN liquid medium. Each treatment was replicated four times and incubated at 27^0C for 21 days on a rotary shaker. Thereafter, respective fungal mycelia were filtered off and the supernatant retained. For the assay, 100 µl of supernatant was added to 100 µl of CAS 2X solution and 2 µl of sulfosalicylic *a*cid (0,2 mM) and incubated for 20 minutes at room temperature. The absorbance was read at 630 nm. To calibrate a blank MMN medium was used and the reference media contained 100 µl MMN medium, 100 µl CAS2X solution and 2µl sulfosalicylic acid. Percentage siderophore was determined by the following equation $\% = [(Ar(R) - Ar(S) / Ar(R)] \times 100$ where Ar is absorbance at 630 nm, *Ar(R)* is absorbance of the reference sample and *Ar(S)* is absorbance of supernatant (Tortora et al., 2011).

5.3 Results

Fungal biomass differed on the various N sources for each of the isolates (Fig 5.1). Treatments with inorganic sources $Ca(NO_3)_2$ (Nit) and $(NH_4)_2HPO_4$ (Amm) accumulated the highest biomass in ECRU075, EdRU083, Nem83 and Chem038. The biomass yield of CafRU082b was highest in arginine treatment. Although inorganic sources were commonly favourable, notably high yields were observed with BSA for ECRU075 and Chem038 isolates, which had biomass of 68.75 mg and 75.25 mg, respectively. In P treatments, all isolates had observable biomass (Fig 5.2).

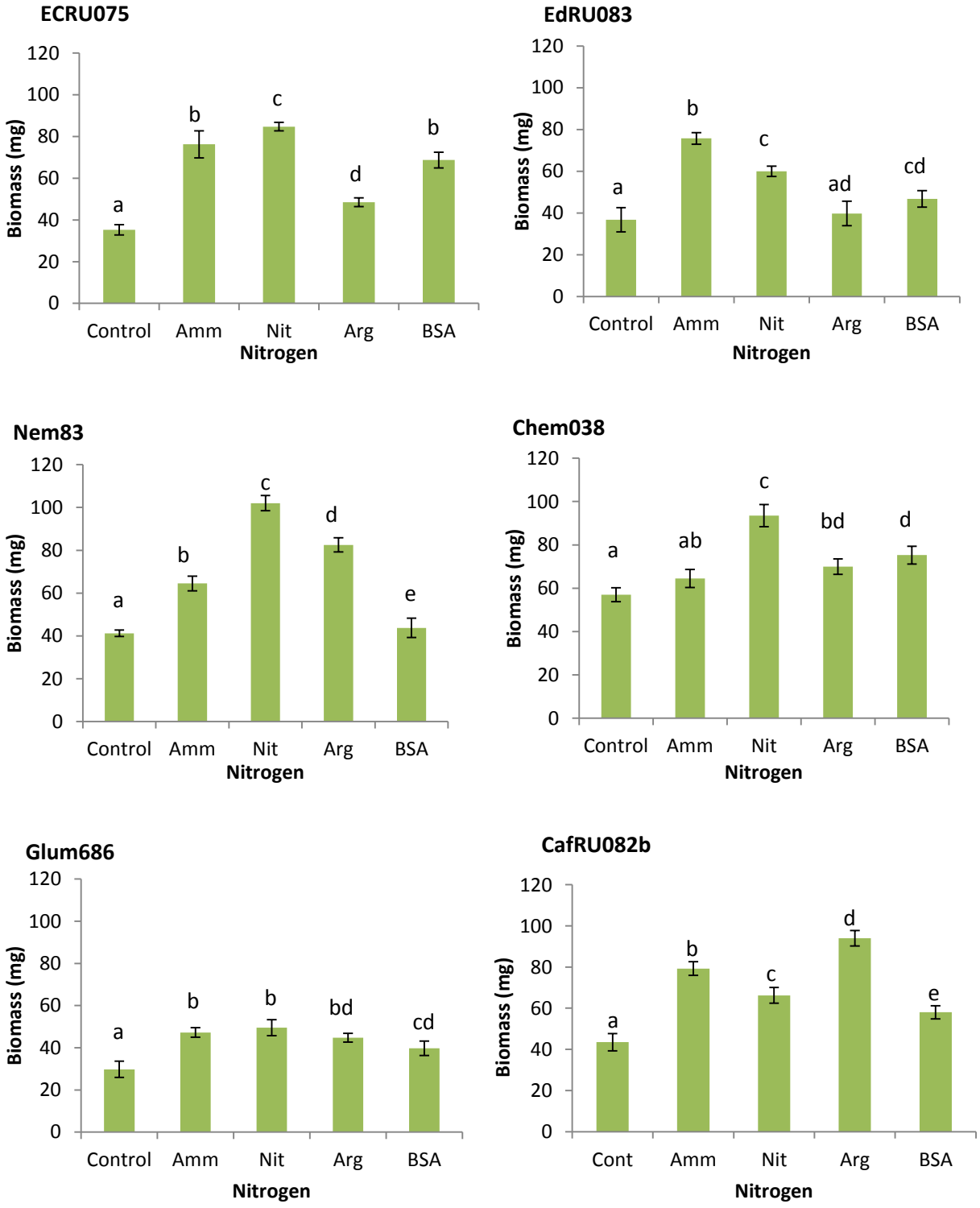

Figure 5.1. Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in four different N sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were Control (no N), Ammonium (Amm), Nitrate (Nit), Arginine (Arg) and Bovine serum albumin (BSA).

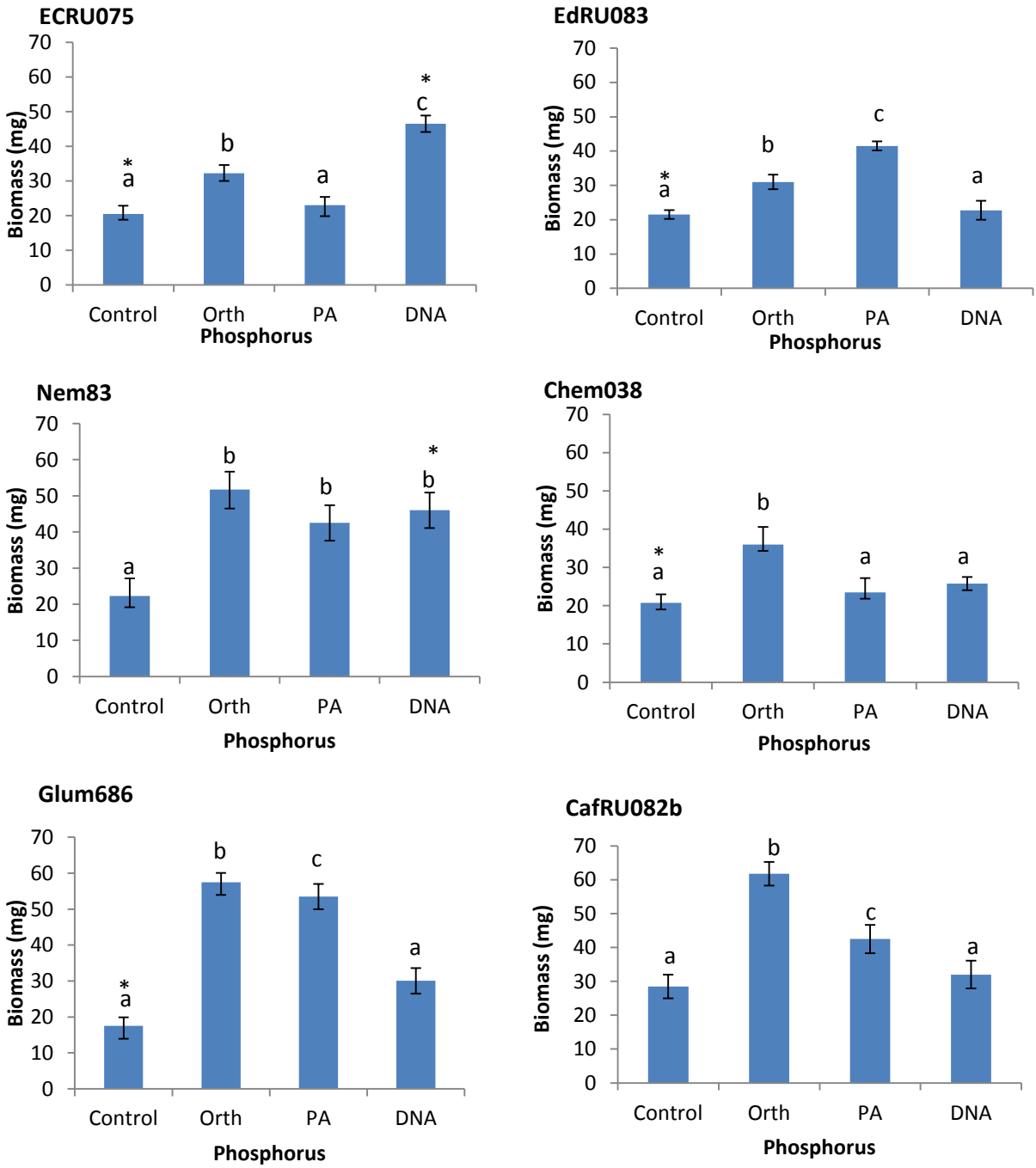

Figure 5.2. Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in three different P sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were control (no P), orthophosphate (Orth), phytic acid (PA) and DNA. The Asterisks indicate treatments that tested positive for phosphomonoesterase activity.

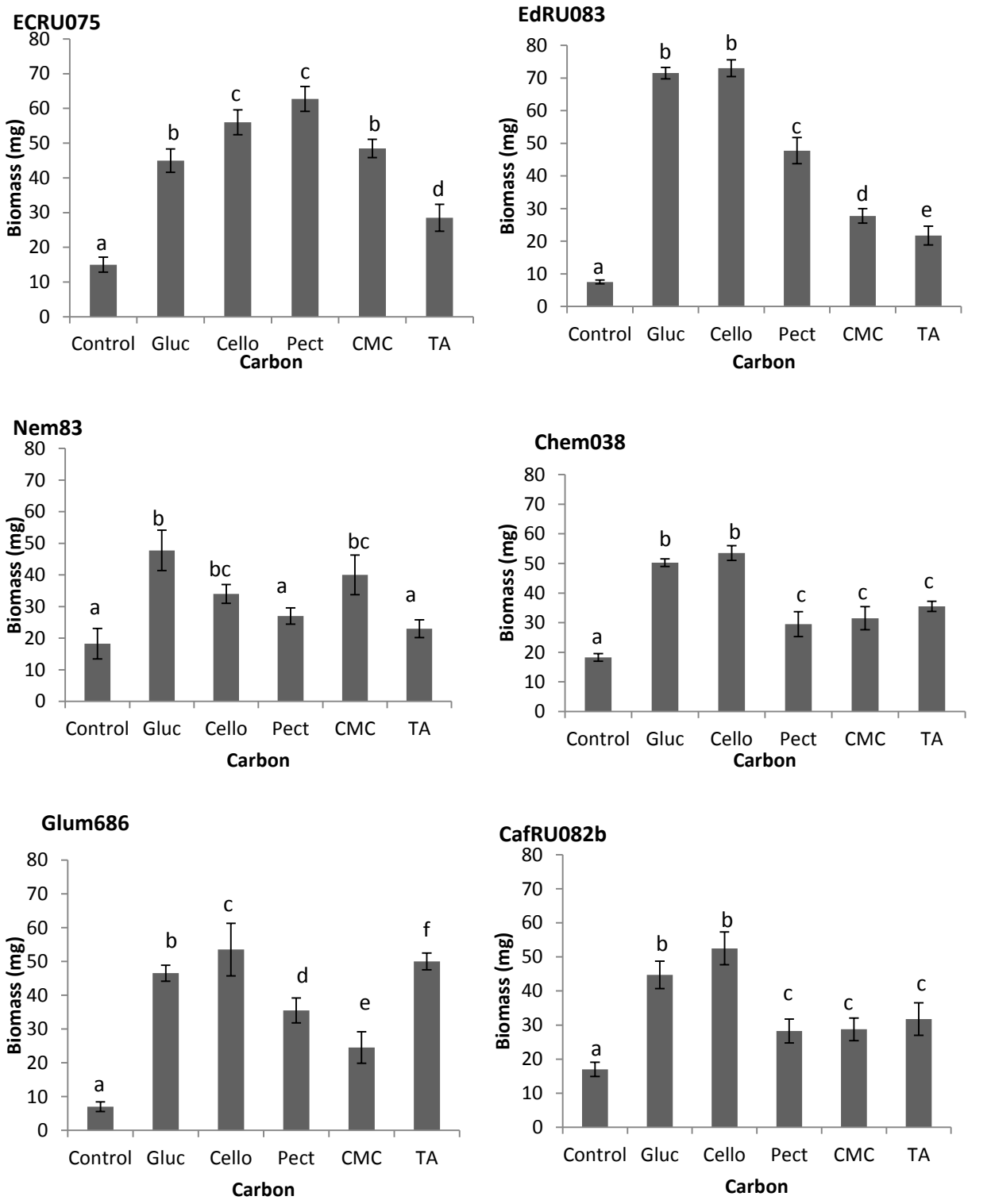

Figure 5.3. Mean (± standard error) biomass production of six ericaceous fungal isolates grown for 21 days in five different C sources. Letters above columns indicate significant differences ($p<0.05$, N=4). Treatments were control (no C), glucose (Gluc), cellobiose (Cello), carboxymethylcellulose (CMC) and tannic acid (TA).

Isolates Nem83, Chem038, Glum686 and CafRU082b had the highest biomass on $NaHPO_4.2H_2O$. There were no significant biomass yields (ANOVA, $p>0.05$) for most isolates growing in DNA substrate, except for ECRU075 and Nem83. For Nem83, assimilation of orthophosphate, phytic acid and DNA did not differ. Phytic acid was utilised preferentially in isolates EdRU083, Nem83 and Glum686. Phosphomonoesterase activity was detected in isolates EdRU083, Chem038, and Glum868 that lacked a P source (control) (Fig 5.2). Isolates ECRU075 and Nem83 that grew in substrates containing DNA tested positively for phosphomonoesterase activity. No phosphodiesterase activity was detected in any of the isolates. A colour change from clear to yellow was initially observed in the respective phosphomonoesterase assays after stopping the reaction. The results were further confirmed by a decrease in absorbance. Variable biomass yields were also observed for the different C treatments. All fungal isolates produced high biomass on glucose and cellobiose. Pectin accumulated the highest biomass in ECRU075 of 62.75 mg. In contrast, Nem83 showed no significant differences in biomass yields (Tukey HSD, $p=0.12$; $p=0.69$ respectively) in pectin and tannic acid (Fig 5.3)

5.3.1 Siderophore detection

A total of fourteen isolates tested positively for siderophore activity on the O-CAS assay. Further analysis on CAS agar assays confirmed development of visible halos in six isolates. These halos had various colourations ranging from pale yellow to orange in colour. Additional isolates from nutrient assimilation that produced siderophores were included as unidentified Helotiales (Nem84 and Nem78), *Phialemonium* sp. (Ncf54), *Chaetomium cupreum* (Ncf91), *Penicillium* sp. (CafRU028), Leotiomycetes fungus (CafRU110), Dothidiomycetes fungus (Nem15) and Leotiomycetes fungus (Glum828). Isolate Caf110 had a ring like halo surrounding the fungus at least 3 cm from the margin (Fig 5.4). Fungal growth on the CAS was minimal and some fungi did not show any growth. In the liquid CAS assay, eleven of the 14 isolates produced siderophores as determined by spectrophotometric absorbance readings. Isolates Nem84, CafRU082b, ECRU075, Nem54 and CafRU028 did not produce halos and had poor growth on CAS agar plates but produced siderophores in liquid CAS assay (Fig 5.5). Percentage siderophore production ranged from 11 to 61% among the isolates. The remaining three isolates Glum686, Ed002 and EdRU018 did not produce siderophores in both these assays.

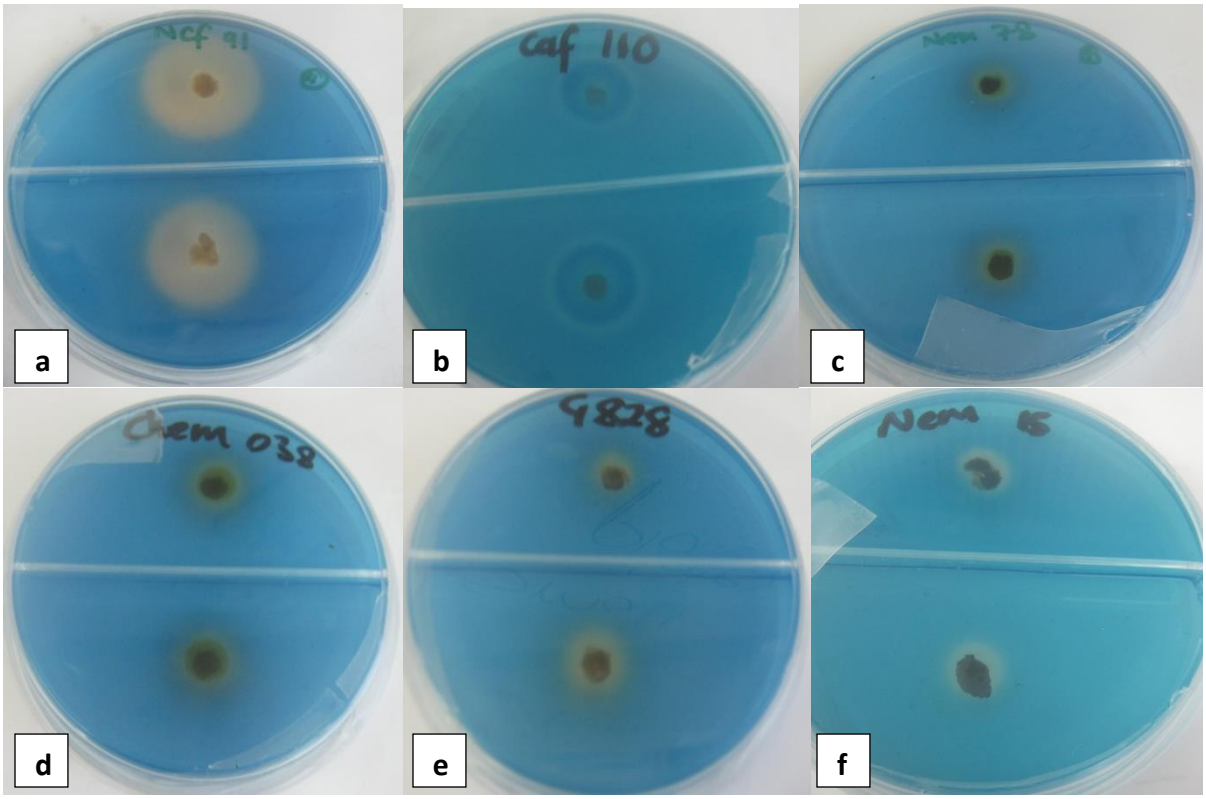

Figure 5.4. Halos produced by ericaceous fungal isolates on Chrome Azurol Sulfonate (CAS) agar after incubation for 14 days at 27ºC in the dark. Isolates were (a) Ncf91 (b) CafRU110 (c) Nem78 (d) Chem038 (e) Glum828 and (f) Nem15.

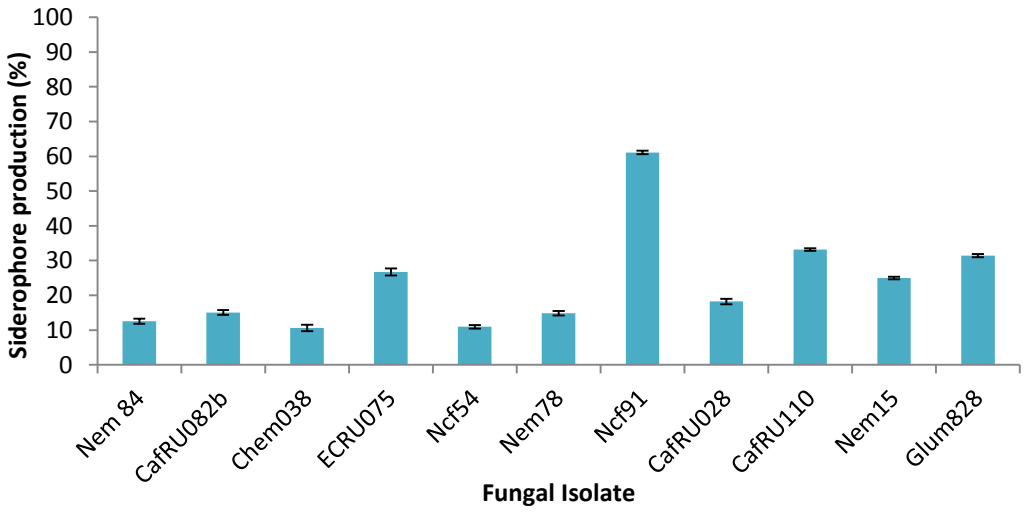

Figure 5.5. Mean percentage (± standard error) siderophore production by ericaceous fungal isolates in liquid Chrome Azurol Sulfonate (CAS) assay.

5.4 Discussion

The isolates selected for nutrient assimilation were able to utilise all inorganic and organic sources of N confirming findings from previous research (Grelet et al., 2005; Cairney et al., 2000; Whittaker and Cairney, 2001). These studies concluded that only the efficiency of utilisation varies between different ericoid fungal species. The current study showed that inorganic N (ammonium and nitrate) was readily assimilated, in contrast to organic sources. Under natural soil conditions, organic matter is mineralised to ammonium that can subsequently be oxidised to nitrate that can be utilised directly by the host or fungus (Read and Perez-Moreno, 2003). However, this is not common in ericaceous habitats where there are low rates of mineralisation (Cairney and Meharg, 2003). This results in the use of alternative organic N substrates that would have accumulated in the soil from plant litter and debris. The utilisation of BSA implies that the isolates secreted protease activity (Leake and Read, 1990). Protease enzymes hydrolyse BSA into amino acids or peptides that can be absorbed directly by the respective fungi. This characteristic is an important defining feature of ericoid fungi because assimilation of N from protein is regarded as the primary function of the ericoid mycorrhizal association.

Phosphate utilisation was variable between isolates. Orthophosphate was readily assimilated by all the fungi. Previous researchers have reported similar observations in ericoid fungi (Smith and Read, 2008; Cairney and Burke, 1998). This is because P is directly absorbed from the substrate and utilised in the form of orthophosphate. Organic forms of P have to be hydrolysed by phosphatase activity into orthophosphate prior to assimilation (Straker and Mitchell, 1986). Biomass yield in Phytic acid substrate suggests the presence of phytase activity. DNA was utilised by ECRU075 and Nem83 suggesting phosphatase activity. Although the use of DNA as a P source requires the production of phosphomonoesterase, phosphodiesterase and other nucleotidase enzymes (Midgely et al., 2006), only phosphomonoesterase was detected. This suggests that the enzymes were able to adequately hydrolyse DNA to provide P to the fungi. Phosphomonoesterases have been reported to hydrolyse diesters and various other monoester substrates (Tibbett, 2002). The remaining isolates failed to utilise DNA, confirming their inability to produce the required enzymes. Although control treatments that lacked P source in ECRU075, EdRU083, Chem038 and Glum686 isolates tested positive for phosphomonoesterase,

biomass yield remained significantly low. This suggests that the enzyme is released by the respective isolates through a negative feedback mechanism whereby lack of P would trigger the release of the enzyme in order to make P available from potential organic sources in the growth substrate (Marschner, 1994; Cairney and Burke, 1998).

The fungal isolates were able to assimilate all C sources with the exception of Nem83 that failed to utilise tannic acid and pectin. This utilisation trend has previously been reported in literature (Midgely et al., 2006) and indicates the ability of fungi to produce several enzymes, such as pectinase and tannase (Perotto et al., 1995; Cairney and Burke, 1998). They break down polysaccharides and other complex forms of C into simple monosaccharides that can easily be utilised by the fungi. Perroto et al., (1997) detected polygalacturonase (PG) enzymes in several ericoid fungi after growth in Czapek liquid medium containing pectin as a sole C source. However, three isolates did not produce PG but had high biomass yields. This suggests the presence of other pectin enzymes and shows the importance of individual enzyme assay tests for the different fungal species. Enzymes involved in C utilisation are also essential for root penetration prior to colonisation (Smith and Read, 2008). Biomass was recorded in all the control treatments lacking the test nutrients because of the starting concentration present in the core with the inocula.

Poor fungal growth and failure to produce siderophore halos observed in the CAS agar plates could have resulted from HDTMA toxicity and has been previously reported (Milagres et al., 1999). The growth inhibition by CAS agar technique was confirmed by the presence of siderophores in isolates Nem84, CafRU082b, ECRU075, Nem54 and CafRU028 in the O-CAS and CAS liquid assays but not in CAS agar. The colour change observed in the halos from blue to yellowish to orange indicates the presence of hydroxamates siderophore types. This result corroborates previous findings that identified hydroxamates as the main siderophore of the ericoid fungi (Haselwandter, 2008; Bartholdy et al., 2001). Among the fungi that produced siderophores Ncf54, CafRU028 and Nem15 have been established to be non-mycorrhizal (Smith and Read, 2008).

The current study demonstrated that the selected fungi were able to utilise a range of inorganic and organic N, P and C sources for growth in pure culture. In addition, of isolates that were used

for nutrient analysis ECRU075, Chem038 and CafRU082b also produced siderophores. These are characteristics of potentially beneficial fungi. There is need to conduct further studies that establish the specific enzymes and siderophores responsible for the utilisation of the various nutrient sources.

6.0 *In-vitro* and *in-plantae* assessment of ericoid mycorrhizal fungal activity on plant growth and antagonism against *Phytophthora cinnamomi* root pathogen.

6.1 Introduction

Plants in the Ericaceae family have numerous uses ranging from ornamental to fruit crops. Amongst the fruit crops, international production of blueberries has increased due to high demand (Prodorutti et al., 2007). The common varieties include wild lowbush (*Vaccinium angustifolium*), northern highbush (*Vaccinium corymbosium*), southern highbush (*Vaccinium* sp.) and rabbiteye bush (*Vaccinium ashei*) (Strik and Yarborough, 2005). In order to optimize the commercial production of the blueberries, use of organic modified media has been widely adopted (Williamson and Miller, 2009). This has resulted in the persistence of root-rot diseases (Vohník et al., 2012). Prevalent pathogens include *Pythium*, *Phytophthora* and *Alternaria* species. In comparison to other fruit crops, blueberries are not easily affected by diseases. However, most blueberry varieties are susceptible to *Phytophthora* root rot (Smith, 2000).

Phytophthora root rot is important in blueberry production because it results in severe plant loss. *Phytophthora cinnamomi* (*P. cinnamomi*) is the causal agent of this disease and *Vaccinium corymbosium* is highly susceptible. *Phytophthora cinnamomi* is an oomycete that depends on free water for survival and reproduction. Hence, it thrives in poorly drained or waterlogged soils. Once established it is difficult to eradicate because it produces overwintering structures known as chlamydospores which are adapted to variable environmental conditions. Pathogen infection and spread from one plant to another is stimulated once excess water conditions are present. Symptoms of *P. cinnamomi* infection are characterised by formation of lesions and change in colouration of the hair roots from brown to black. This discolouration may also spread to larger roots and crowns. The damage to the roots results in limited water and nutrient supply to the plant. Consequently, the infected plants shows above ground symptoms that include yellow to pale reddish leaves, marginal leaf scorch, death of terminal buds and leaves, stunted growth and in severe cases death (Bryla and Lindermann, 2008; Prodorutti et al., 2007).

Phytophthora cinnamomi is not host specific and infects a wide range of hosts that include plants in the ericaceous family (Krebs and Wilson, 2002). However, there are varieties that are resistant to *P. cinnamomi*. These include Toro, Star, South moon and Gulfcoast. Susceptible varieties

include Bilox, Misty, Jubilee, Elliot and Duke (Smith, 2012; Larach et al., 2009). Screening different blueberry varieties for resistance to root rot diseases is important. It can provide a germplasm pool for breeding varieties that are resistant to *P. cinnamomi* and other pathogens of economic importance. Although there are fungicides such as metalaxyl that have been administered to control *Phytophthora* root rot, culturing practises have been established to be more effective (Smith, 2012; Krebs and Wilson, 2002). Culturing practises include using well-drained medium such as composted hardwood and pine bark, preventing over irrigation or fertilisation and use of mulches (Prodorutti et al., 2007).

Mulches improve the drainage properties of the medium that helps with the control of the pathogen. An additional mechanism involving free-living microbes in the mulch has been established to suppress *P. cinnamomi*. Structurally *P. cinnamomi* has cellulose microfibrils that are easily degraded by cellulase enzymes produced by fungi and other microbes present (Richter et al., 2011; Downer et al., 2001). A study by Downer et al. (2001) demonstrated that the addition of cellulase to *P. cinnamomi* inhibited zoospore and chlamydospore development and concentrations as high as 25 units per ml destroyed the mycelia. Previous research showed *Epicoccum purpurascens* isolated from flax plants inhibited the growth of *P. cinnamomi* in culture and further analysis identified cellulase and β, 1-3 glucanase activities as the antifungal compounds after the fungus utilised *Phytophthora* sp. as a sole C source (Brown et al., 1987).

Some ericoid fungi secrete enzymes such as cellulase, proteolytic and polyphenol oxidases that could contribute to biocontrol (Lin et al., 2011; Schulz et al., 2002), therefore they have potential biocontrol capabilities against *P. cinnamomi*. In addition, some ericoid fungal strains belonging to the *Oidiodendron* and *Leohumicola* genera have been established to reduce root rot infection and enhance plant growth of *Rhododendron* plants (Grunewaldt-Stöcker et al., 2013).

The aims of this study were to (i) determine anti-pathogenic fungal activity of selected ericoid isolates against selected *P. cinnamomi* in culture and (ii) determine the effects of selected fungi on *Phytophthora* root rot development and plant growth.

6.2 Methods

6.2.1 Qualitative screening

Anti-pathogenic fungal activity was tested in a dual agar plate experiment on Malt extract agar (MEA). Formation of an inhibition zone was used for initial screening. Twenty-five selected fungal isolates previously identified in Chapter 3 were tested against fungal pathogen *Phytophthora cinnamomi* that was previously isolated from infected Avocado plants and sourced through the Agriculture Research Council, National Collection of Fungi (PPRI number. 15460). In preparation for the experiment 9 mm petri dishes were divided into two equal segments marked by lines on the underside. Thereafter autoclaved MEA was poured. Selected ericoid fungal isolates (5 mm) were cored from actively growing margins using a sterile corer then inoculated centrally on one-half of the plate. The plates were incubated at 27 ºC in the dark for 14 days before inoculating *P. cinnamomi* because the isolates were relatively slow growing in culture in comparison with the pathogen. After inoculating *P. cinnamomi*, the plates were further incubated and observed every 48 hrs to examine for the zone of inhibition and the experiment lasted for 7 days. There were two replicates of each experiment (Grunewaldt-Stöcker et al., 2013).

6.2.2 Semi-qualitative analysis

The isolates that antagonized the growth of *P. cinnamomi* in culture were used for semi-quantitative fungal growth inhibition analysis. Isolate CafRU082b (*Oidiodendron* sp.) did not show inhibition potential in the screening assay but was selected for further analysis on the basis that literature reports it as a typical ERM fungus, therefore its ability to colonize the host roots might enable it to improve host fitness against *P. cinnamomi* (Grunewaldt-Stöcker et al., 2013; Smith and Read, 2008). Isolates were grown in 2% ME broth (50 ml) on a rotary shaker for 21 days at 27ºC. The plates were prepared by drawing intersecting lines on the base and MEA medium was cored halfway along the lines using a sterile pipette. Thereafter *P. cinnamomi* was inoculated centrally on the plate. Absorbance of broth extract was measured at 600 nm using a Power Wave Spectrophotometer (Bio-Tek instruments). Thereafter, an aliquot of 100 µl with absorbance of OD_{600}=0.78 for *Leohumicola* sp. and OD_{600}=0.65 of *Oidiodendron* sp. were placed in respective cored holes surrounding *P. cinnamomi*. Four replicates for each treatment were

incubated as above. Colony diameter of the *P. cinnamomi* was measured and recorded daily until no change in growth was measured (6 days). Percentage growth inhibition was determined using the following equation: $\% = [\theta CP - \theta(P+T)/\theta CP] \times 100$ where θCP is the diameter of pathogen control and $\theta(P+T)$ is the diameter of pathogen with fungal treatment (Rahman et al., 2014).

6.2.3 *In plantae* experiments

Leohumicola and *Oidiodendron* species were inoculated into separate 2% ME broth cultures and incubated as previously described. Thereafter mycelium was harvested using sterile Whatman no. 1 filter paper and washed using sterile water. Inocula were prepared by homogenizing harvested fungal mycelia in 0.03% w/v agar solution. Micro-propagated blueberry plants of the Brightwell variety obtained from Microprop Plant Nursery Company were used. The plants were transplanted onto pasteurised substrate consisting of equal parts of compost and sand. For each fungus, 10 ml of mycelial suspension was inoculated on the respective roots using a sterile syringe. Treatment application was applied as described in Table 6.1. Thereafter plants were grown for 60 days under controlled greenhouse conditions with a minimum temperature of 19ºC and maximum of 27ºC under natural light conditions. The plants were watered daily with sterilized water and a 10 ml aliquot of low phosphorus Long Ashton Nutrient solution was applied per plant weekly (Appendix F) (Hewitt, 1966).

Table 6.1. Treatment application of potential biocontrol fungal isolates Chem038 and CafRU082b against *Phytophthora cinnamomi* infection on Brightwell variety.

Treatment	Fungal Isolate	Pathogen *P. cinnamomi* (Presence +)
1	Control	
2	Control	+
3	*Leohumicola* sp. (Chem038)	
4	*Leohumicola* sp. (Chem038)	+
5	*Oidiodendron* sp. (CafRU082b)	
6	*Oidiodendron* sp. (CafRU082b)	+

Phytophthora cinnamomi inoculum was prepared as previously described for the isolates. Thereafter 10 ml of mycelial suspension (OD_{600}=1.27) was injected on the respective plants for the different treatments (Table 6.1). There were eight replicates for each treatment. The initial shoot height was recorded prior to placement in a controlled environment growth chamber at 75% humidity, 27ºC and 12 hours day/night conditions. Plants were flood watered daily with sterile distilled water (Silva et al., 2000). They were grown for another 90 days. Final data was recorded on shoot growth (cm). Fungal colonisation was determined by staining technique as previously described in Chapter 2. Shoots and roots were then oven dried at 65 ºC for 36 hours prior to recording respective biomass weights (Yang et al, 1996; 2002). The data was analysed using R software (R Development Core Team, 2014). The data did not assume normality using Shapiro Wilk test ($P<0.05$) therefore Kruskal–Wallis one-way analysis of variance was used. Tukey multiple test comparisons were used to separate the means at 95% confidence level.

6.3 Results

In the dual agar assay, all tested fungi showed no effects on the growth of *P. cinnamomi* with the exception of isolate *Leohumicola* sp. An olive brown soluble pigment was produced in the media. These soluble pigments resulted in of 45.1% growth inhibition of the pathogen. Although *Oidiodenderon* sp. produced a soluble brown pigmentation in broth no inhibition was established (Fig 6.1).

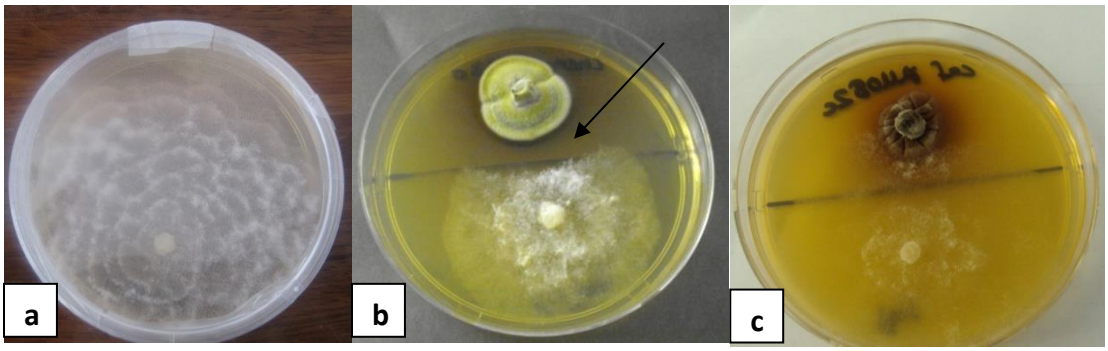

Figure 6.1. Dual agar plates (a) *Phythopthora cinnamomi* control after 14 days of incubation (b) *Leohumicola* sp. growing against *Phythopthora cinnamomi* and arrow showing the zone of inhibition and (c) *Oidiodendron* sp. showing no growth inhibition against *Phythopthora cinnamomi*.

Both *Leohumicola* and *Oidiodendron* species formed ericoid structures in the roots of Brightwell plants (Fig 6.2). Ericoid mycorrhizal (ERM) colonisation was variable within the same hair root.

Some segments were intensely colonised whereas others had no colonisation. There were some hair root cells that had clustered spore-like structures that could not be identified as ERM structures with certainty.

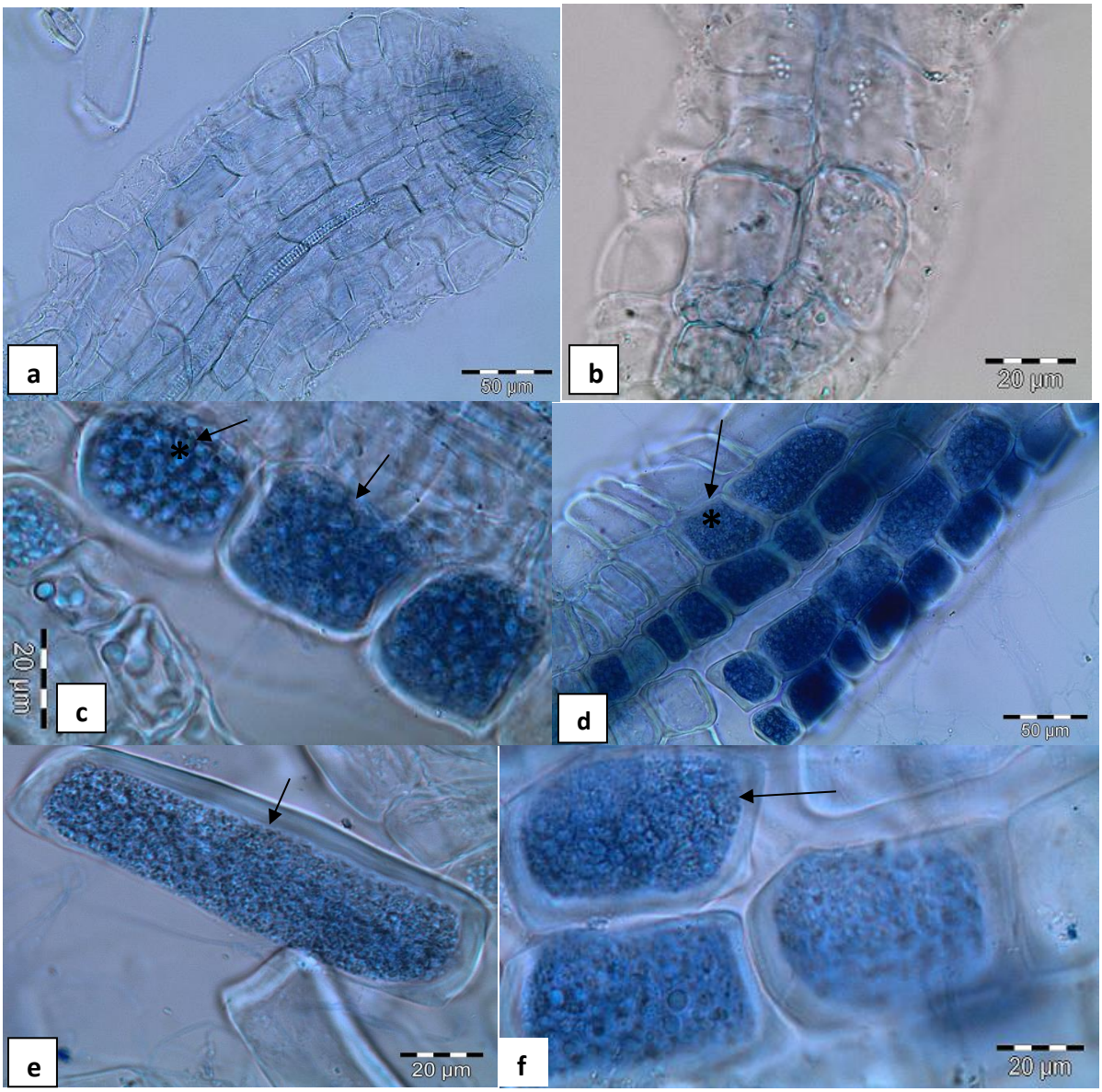

Figure 6.2. Root fungal colonisation of Brightwell blueberry variety. (a) uninoculated control (b) control inoculated with *Phythopthora cinnamomi* (c) and (d) ericoid mycorrhizal coils in roots inoculated with *Leohumicola* sp. (e) and (f) ericoid mycorrhizal coils in roots inoculated with *Oidiodendron* sp. the arrows showing hyphal coils and arrow with Asterisk showing structures like clumped spores.

The control plants had no colonisation, but random intra and extracellular hyphal colonisation was observed. In the plants inoculated with *P. cinnamomi* the pathogen was not clearly stained

by trypan blue; therefore it was not easily distinguishable. Therefore, respective fungal treatments with *P. cinnamomi* were not clear enough to detect infection levels.

After 90 days of infection with *P. cinnamomi*, no visible symptoms of *Phythopthora* root rot were observed on the shoots or roots of any plants. Absence of secondary symptoms was confirmed after no significant differences in shoot growth were recorded in comparison with *P. cinnamomi* infected control. However, there were significant differences (p=0.02) in average shoot height growth between treatment responses. Plants inoculated with *Leohumicola* sp. had the highest growth of 1.03 cm. The average shoot growth in the remaining treatments did not differ significantly (p>0.05) from both infected and uninfected controls (Fig 6.3).

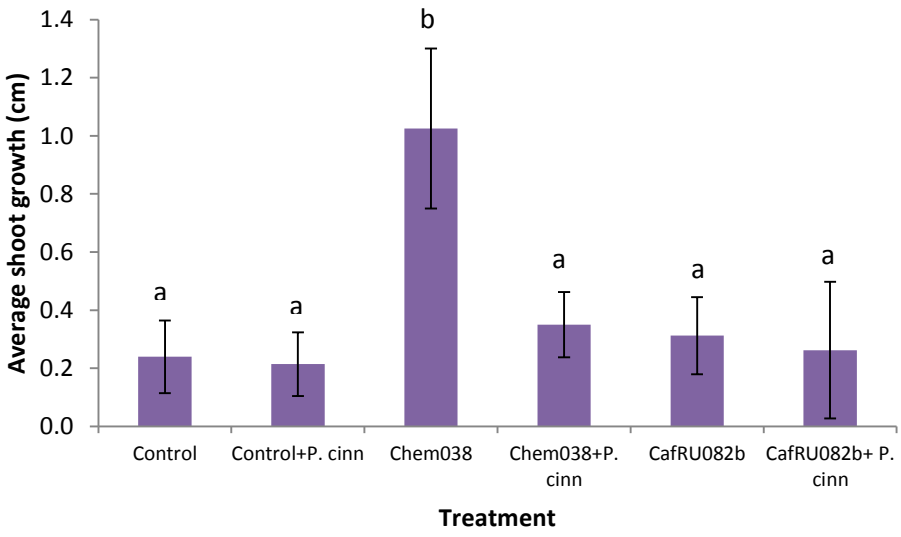

Figure 6.3. Average shoot height of Brightwell plants after 90 days of growth. Showing the inoculation effects of *Leohumicola* sp., *Oidiodendron* sp. and *Phythopthora cinnamomi* on shoot growth. Different letters above columns indicate significant differences ($p < 0.05$, N=8).

The shoot biomass was not significantly different (p=0.79) across all treatments (Table 6.2). This shows that *P. cinnamomi* had no effect on the shoot development of the Brightwell variety. Similar results were obtained for the root biomass (p=0.65).

Table 6.2. Effects of Chem038 and CafRU082b inoculation on the growth of Brightwell blueberry plantlets infected by *Phythopthora cinnamomi*. Average shoot and root biomass +/- SD.

Treatment	Shoot biomass (g)	Root biomass (g)	Ericoid colonisation (+/-)
Control	0.23 ± 0.12^{a}	0.36 ± 0.15^{a}	-
Control+*P. cinnamomi*	0.27 ± 0.06^{a}	0.35 ± 0.008^{a}	-
Chem038	0.34 ± 0.1^{a}	0.36 ± 0.12^{a}	+
Chem038+*P. cinnamomi*	0.27 ± 0.13^{a}	0.42 ± 0.19^{a}	+
CafRU082b	0.32 ± 0.16^{a}	0.40 ± 0.12^{a}	+
CafRU082b+*P. cinnamomi*	0.23 ± 0.08^{a}	0.33 ± 0.16^{a}	+

Means followed by the same letters in the same column are not significantly different according to Tukey's test ($p<0.05$)

6.4 Discussion

Ericoid fungi have been used solely for the purpose of enhancing the growth of ericaceous plants. Most studies have demonstrated nutrient uptake as the mechanism of growth promotion (Jansa and Vostka, 2000). In comparison with other mycorrhizas, little research has been conducted on other potential benefits regarding root diseases (Steinkellner et al., 2012; Whipps, 2004). In this study *Leohumicola* sp. showed antimicrobial activity against *P. cinnamomi* in culture. The olive-brown secretions could be polyphenol oxidases (Lin et al., 2011), suggesting cell wall degradation as the mechanism used by *Leohumicola* sp. in suppressing the growth of *P. cinnamomi* in culture. This corroborates findings by Grunewaldt-Stöcker et al., (2013) in which *Leohumicola verrucosa* and two other *Leohumicola* species inhibited the growth of phytopathogens *P. cinnamomi* and *Pythium* species. This illustrates the potential biocontrol properties of *Leohumicola* sp. against *P. cinnamomi*.

Analysis of *Oidiodendron* sp. against *P. cinnamomi* in the dual agar and broth extracts assays did not exhibit antifungal characteristics. A positive outcome is dependent on both the pathogen and *Oidiodendron* sp. involved. Hosoe et al. (1990) tested three *Oidiodendron* sp. (*O. flavum*, *O. griseum* and *O. truncatum*) against fungi *Aspergillus fumigatus* and *A. niger* and bacteria *Staphylococcus aureus* using broth extracts. Inhibition zones were observed for *O. truncatum* only. In contrast, all *Oidiodendron* sp. showed antimicrobial activity against *S. aureus*. Ericoid

mycorrhizal fungi have potential to inhibit undesirable microbial growth by stimulating or inducing plant defence mechanism that suppresses pathogen infection (Grunewaldt-Stöcker et al., 2013; Smith and Read, 2009). Despite the lack of evidence of anti-pathogenic fungal activity in culture, *Oidiodendron* sp. improved plant growth and reduced pathogen infection in *Rhododendron* plants (Grunewaldt-Stöcker et al., 2013).

This study established that both isolates form ERM association with Brightwell. In addition *Leohumicola* sp. significantly improved the shoot height of this variety. *Oidiodendron* sp. is a typical ERM fungus and has been widely associated with the functional role of improving growth and productivity in blueberry production (Scagel and Yang, 2005; Scagel, 2005; Yang et al., 2002). In this study *Oidiodendron* sp. did not significantly enhance the growth of Brightwell. This has previously been reported and it was suggested that mycorrhizal benefits may not be apparent if nutrients are adequate to support plant growth (Vega et al, 2009; Starret, 2002). Commercial products have been formulated using selected *Oidiodendron* sp. (United States Patent, No. 6,146,880). *Leohumicola* species have been mostly identified from burnt ecosystems, commercial blueberry cultivation fields and ericaceous roots in the fynbos of South Africa (Nguyen and Seifert, 2008). Their ability to form ERM structures in ericaceous roots has been established, but the functional roles are still vague. Recently, Grunewaldt-Stöcker et al., (2013) demonstrated their ability to promote growth and inhibit *P. cinnamomi* root infection in *Rhododendron*. This study has demonstrated an increase in shoot height, but field studies are essential in establishing the growth promoting ability of both *Leohumicola* and *Oidiodendron* species from the current study.

Effects of *Leohumicola* and *Oidiodendron* species with respect to antagonising *P. cinnamomi* infection could not be established. Traces of *P. cinnamomi* were observed in infected roots, but no detrimental effects were evident in shoot height or shoot and root biomass of the both inoculated and uninoculated controls. It is possible that the variety is resistant to *P. cinnamomi* infection. There are some blueberry varieties that possess multiple genes that enable partial or complete resistance to *P. cinnamomi* (Larach et al., 2009; Krebs and Wilson, 2002). In addition, Brightwell is a rabbiteye variety and other varieties belonging to this bush type such as Tifblue and Primier have established resistance to *Phythopthora* root rot (Larach et al., 2009; Smith, 2000).

In conclusion, *Leohumicola* sp. has antimicrobial activity against *P. cinnamomi* in culture. The soluble olive-brown secretions produced by the isolate contain compounds that inhibit the growth of this pathogen. Both *Leohumicola* and *Oidiodendron sp.* form ERM with Brightwell host plants. *Phythopthora cinnamomi* inoculation did not induce *Phythopthora* root rot in Brightwell variety plants or affect their growth. However, there is need to conduct more studies under a controlled environment that simulate field conditions, using zoospores isolated from diseased blueberry plants before concluding that Brightwell is resistant to *P. cinnamomi*.

7.0 The effects of inoculating selected fungi on the mycorrhizal status and growth of *Vaccinium* species in large scale field conditions.

7.1 Introduction

Commercial blueberry cultivation is a fast growing division of the horticulture industry in South Africa. According to Ryan Davies, the chairperson of South African Berry Producers' Association (SABPA), growers export three-quarters of the total produce. They find a ready market in Europe and the United Kingdom during the (September to mid-February) summer season (Personal communication), taking advantage of the market gap because the northern hemisphere is off season during winter. There is also potential for expanding to other market regions globally due to escalating demand (Meyer and Prinsloo, 2003).

Blueberries have variable uses which range from direct consumption as a fruit to pharmaceutical and cosmetic applications (Ghafar et al., 2010, Riihinen et al., 2008). They are rich in phenolic compounds such as anthocyanin pigments which give them the deep blue colour, chlorogenic acid, procyanidins and flavonoids phytochemicals that enable them to provide therapeutic health benefits (Smith et al., 2000). The benefits include antioxidant and anti-carcinogenic properties. In addition, they prevent cognitive decline during aging and diseases caused by the hepatitis C virus (Joseph et al., 2009; Koca and Karadeniz, 2009). Such benefits have resulted in increased blueberry consumption. However, the benefits can be reduced in processed blueberry products because they may require enzymes and extensive procedures that alter some of the phytochemical properties (Rossi et al., 2003).

In nature ericoid mycorrhizal (ERM) fungi engage in symbiotic relationships with blueberry varieties. This enables the host root to access organic nutrients, a transfer facilitated through extracellular fungal enzyme activities secreted by the respective fungi (Cairney and Meharg, 2003). Previous studies have confirmed the ability of several ERM fungi such as *Oidiodendron* sp., *Rhyzoscyphus erica* and *Phialocephala fortinii* to enhance growth of blueberry varieties *in-vitro* and under field conditions (Yang et al., 2002). Consequently, some countries have formulated ericoid fungal inocula to complement their cultural practices in order to enhance growth and productivity of the blueberry commercially (Vega et al., 2009). Responses to fungal

inoculation vary from neutral to positive (Yang et al., 1998) and are influenced by several factors such as variety-fungus specificity, cultural practices and possibly ecosystems (Vega et al., 2009; Scagel et al., 2005b).

Although there are no published reports that contradict the efficacy of ericoid mycorrhizal products if applied in South Africa, it has been suggested that they may not render optimum effectiveness in non-native regions owing to the sensitivity of the association and contrasting outcomes in different ecosystems of the same region (Vega et al., 2009). Therefore, there is a need to develop ERM fungal inocula using native fungi that have evolved and adapted to a specific ecosystem.

The aims of this study were to (i) determine the mycorrhizal status of selected native fungal isolates and (ii) establish effects of inoculation on the growth of Misty southern highbush variety grown in South Africa.

7.2 Methods

Leohumicola (Chem038), *Oidiodendron* (CafRU082b) and *Meliniomyces* (ECRU075) species identified in Chapter 3 were selected for field trials. Respective inocula were prepared in 500 ml of 2% Malt extract broth. They were incubated for six weeks with three replicate flasks for each fungus and incubated at 27ºC on a shaker. These were then mixed into a single composite inoculum for each fungal treatment. From each inoculum, 1 ml was subsampled for quantification using the spectrophotometer. They were then formulated in 0.03% gel as previously described in Chapter 6. The Optical Density (OD) was recorded at a wavelength of 600 (OD_{600}). The following ODs were recorded 1.30 *Leohumicola*, 1.57 *Oidiodendron* and 0.45 *Meliniomyces* species.

A field trial was conducted at Amathole Berries located in the Eastern Cape Province (latitude 32.57 S; longitude 27.60 E). Four-year-old Misty plants that had already established in pine bark substrate of pH 4.5-5 in 20 litre pots. Prior to inoculation plants in two rows were selected alternating the treatments in a zig zag pattern. Different coloured wool was secured around the base of the stem for each treatment and control plants were marked, but left uninoculated. There were thirty replicates for each treatment. An inoculum aliquot of 10 ml was applied to each plant

in the respective treatments using a sterile syringe at least 5 cm into the root zone close to the stem. Control plants received the same aliquot comprising 0.03% v/w agar only.

The plants were grown under a black shade cloth with a light transmission ranging between 61-63% and a Shade factor of 36%. Drip irrigation and fertilisation were applied per commercial practice. Each plant was watered with 10 litres of water daily and at least 15 litres when temperatures were above 30ºC. Fertilizers were applied to give a total mineral nutrient supply of Nitrogen 4.35 g, Phosphorus 1.34 g, Potassium 4.30 g, Calcium 1.94 g, Magnesium 0.50 g, Sulphur 5.01 g, Iron 0.06 g, Manganese 0.03g, Zinc 0.04 g and Boron 0.04 g per plant for the growth period. Other important culturing practices conducted include prunning and manual weed control (Ryan Davies, Amathole Berries, Personal communication).

Shoot height was measured at 11, 19, 28 and 56 weeks after inoculation. After 56 weeks 10 plants were randomly selected and destructively harvested for each treatment. The pot was slit open on both sides using a blade and the pine bark compost carefully removed from the roots to avoid loss of root material. A subsample (1 g) of roots was selected from each plant and retained for microscopy. These were stained as described in Chapter 2. The shoots and roots were separately oven dried at 60ºC for 72 hours prior to recording the shoot and root dry weights (Yang et al, 2002; 1996). Root weight was corrected for the sub sample removed. The collected data was analysed using one-way ANOVA on R software (R Development Core Team, 2014). Tukey multiple test comparisons were used to separate the means at 95% confidence level.

7.3 Results

Shoot height fluctuated in some of the plants due to pruning and other culturing practices that were carried out during the growing season. Repeated measures were analysed using ANOVA and there were no significant differences between control and inoculated plants. Plants that were not severely pruned were selected to determine average shoot growth after 56 weeks of inoculation. At least 20 plants in each treatment were suitable for analysis using this criterion. There was no significant difference in the average shoot growth among inoculated plants and the control (ANOVA, $p=0.548$) (Fig 7.1).

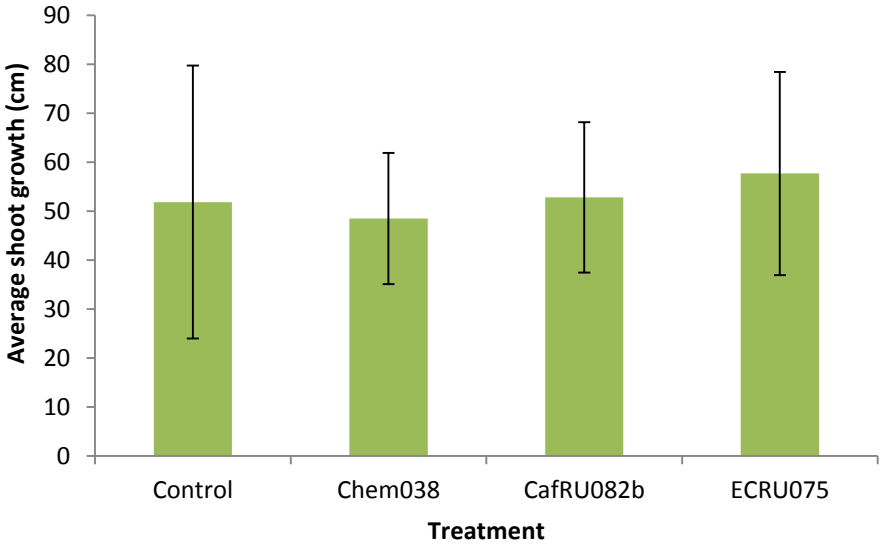

Figure 7.1. Average shoot height of Misty plants after 56 weeks of growth. Showing the inoculation effects of *Leohumicola* sp., *Oidiodendron* sp. and *Meliniomyces* sp. on shoot growth. No significant differences were recorded.

Although the control roots remained mostly uncolonised they had traces of ERM coils and inter- and intracellar hyphae that did not form any particular structure. Ericoid structures were observed in all treatments (Fig 7.2) together with other hyphal structures reported in the control. The ERM colonization was significantly lower (Tukey HSD, $p<0.005$) in the control in comparison with inoculated plants (Fig 7.3). Plants inoculated with *Oidiodendron* sp. had the highest colonization of 20% recorded, which was not significantly different to 17.5% from *Leohumicola* inoculated roots (Tukey HSD, $p=0.49$).

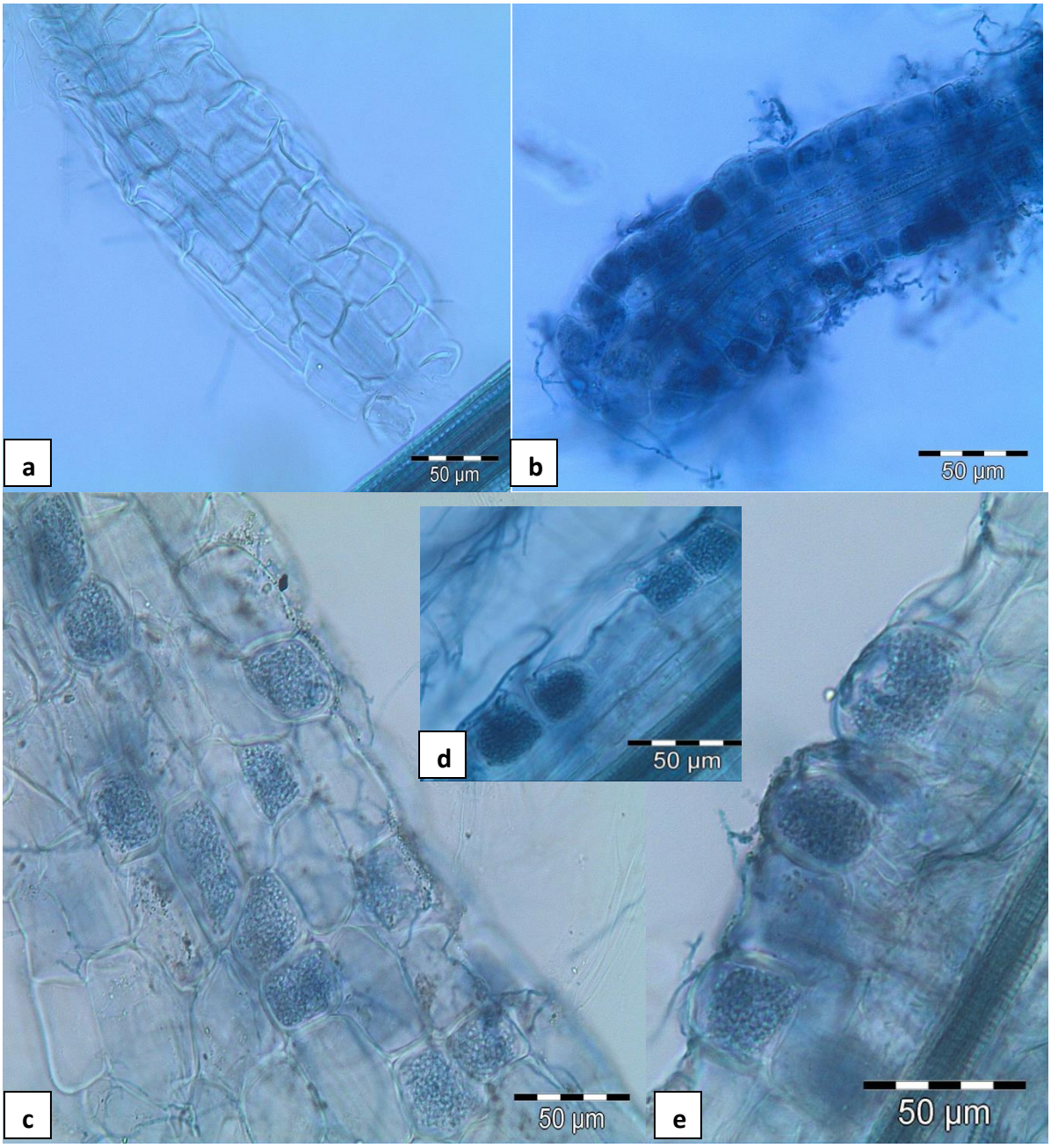

Figure 7.2. Colonisation of Misty plants after inoculation with *Leohumicola* (Chem038), *Oidiodendron* (CafRU082b) and *Meliniomyces* (ECRU075) species after 56 weeks of growth. (a) The un-inoculated control (b) (c) (d) and (e) ericoid mycorrhizal structures observed from the inoculated plants.

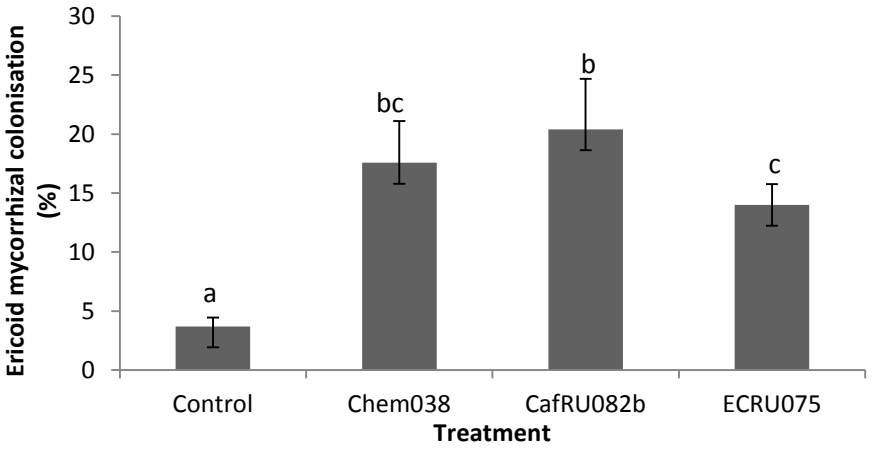

Figure 7.3. Percentage root colonization of Misty blueberry plants after 56 weeks of growth following inoculation with Chem038, CafRU082b and ECRU075. (a) Different letters above columns indicate significant differences ($p<0.05$, N=10).

Shoot biomass was not significantly different (ANOVA, p=0.34) between the inoculated plants and the control (Fig 7.4). However, the root biomass showed significantly increased (ANOVA, p=0.03) biomass in all inoculated Misty plants. Further analysis using Tukey HSD showed that the root biomass among treated plants was not significantly different ($p>0.005$).

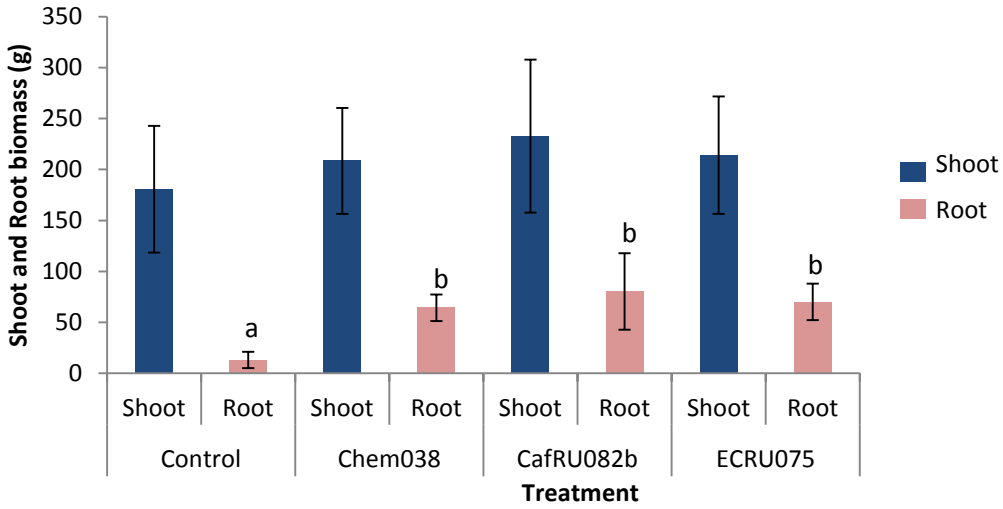

Figure 7.4. Shoot and root biomass of Misty plants after 56 weeks of growth following treatment with Chem038, CafRU082b and ECRU075. Shoot biomass showed no significant difference. Different letters above columns showing root biomass indicate significant differences ($p<0.05$, N=10).

7.4 Discussion

Culturing practices such as pruning are common in blueberry production. Pruning removes excessive vegetative growth and maintains the shrub at suitable height for harvest. This promotes the quality and yield of blueberry fruits (Waters et al., 2008). In this study out growing shoots were constantly pruned as per commercial practice. Consequently, the recorded average shoot growth was not significantly different between the untreated controls and inoculated plants. Therefore, the study could not assess the effects of inoculation with regard to shoot growth.

The traces of ERM colonisation observed in control plants could have arisen from fungal propagules that are naturally occurring in the bark compost used as a growth substrate. This observation corroborates previous studies that have reported the presence of ERM structures in un-inoculated blueberry varieties growing in either nurseries or field conditions (Scagel and Yang, 2005). Scagel et al., (2005b) recorded less than 5% colonization in several non- inoculated blueberry varieties including Misty. Such observations also suggest that the growth substrate which is not pasteurized may have low ERM inoculum potential. Inoculation with *Leohumicola*, *Oidiodendron* and *Meliniomyces* species resulted in ericoid mycorrhizal structures confirming the symbiotic association. The ERM status of similar *Erica* isolates has previously been established with various Ericaceae hosts (Grunewaldt-Stöcker et al., 2013; Villarreal-Ruiz et al., 2012; Vega et al., 2009). *Oidiodendron* sp. has widely been inoculated in blueberry plants, and in addition to ERM structures it enhanced growth (Yang et al., 2002).

Colonisation was low in inoculated Misty plants, ranging between 14 and 20% in roots inoculated with *Meliniomyces* and *Oidiodendron* species respectively. Low ERM colonisation is common in blueberry varieties that are grown commercially (Scagel and Yang, 2005; Yang et al., 1998). Several factors influence the colonisation frequency, these include the high turnover rate of the ERM life cycle, which lasts up to 11 weeks before the ERM structure disintegrates and fungus establishes in new hair roots near the meristem (Smith and Read, 2008). Therefore, colonisation observed is influenced by the stage of the ERM cycle. Percentage colonisation may also be influenced by the mineral nutrient status of the growth substrate. Studies conducted in the field and controlled conditions have shown that low N levels in the growth medium increase the ericoid fungal colonisation (Goulart et al., 1996). In contrast, the current study had all mineral

nutrients adequately supplied to the plants through fertilisation. It is also important to note that blueberry hair roots are very delicate (Read, 1996). Therefore, they are difficult to handle during processing; resulting in loss of hair roots, some of which are possibly colonised. Researchers agree that work on ERM is limited due to the difficulty experienced in handling the hair roots (Smith and Read, 2008; Scagel et al., 2005b).

Lack of significant differences in shoot biomass amongst all treatments corresponds with the outcome of shoot growth analysis. Therefore, similar factors could have led to this result. Improved root biomass recorded in the study suggests that the respective ERM fungi promoted biomass allocation to the roots. This could imply the plants had greater root surface area for nutrient uptake. Scagel et al., (2005b) reported low root colonization and increased root biomass in the first growing season of Misty after inoculation. However, shoot biomass and colonisation significantly improved in the second growing season. This shows the need for continued assessment over a longer period of time. This assessment was not done in this study due to restraints in removing plants from the commercial production facility. In addition to plant growth, biomass accumulation and colonisation, crop yield should also be assessed in order to determine the beneficial effects of inoculating the respective fungi.

In conclusion *Leohumicola*, *Oidiodendron* and *Meliniomyces* species engage in ERM association with Misty blueberry variety. Inoculation improved the root biomass of Misty, showing that inoculation with the respective fungi was potentially beneficial. Therefore, there is a need to continue assessing the effects of inoculation over two or three year period, measuring yield and quality of fruit. To improve the quality of data for the effects of ERM on plant growth, evaluation over longer periods of time in a dedicated trial block would be valuable.

8.0 General discussion

Erica plants have a wide geographic distribution. The greatest species diversity has been identified in the Cape Floral Region of South Africa (Schumann and Kirsten, 1992). In the current study Chapter 2, the identification of six *Erica* species; *E. caffra*, *E. cerinthoides*, *E. demissa*, *E. chamissonis*, *E. glumiflora* and *E. nemorosa* in a small area of the Beggers Bush illustrates the potential species richness of the Albany Centre of Endemism (ACOE). This supports its status as a hotspot of diversity in the Cape Floral kingdom (Schumann and Kirsten, 1992). Flower morphology is key to species identification with plants being classified only once they started flowering, which was generally in December except for *E. nemorosa* that flowered in March.

The soil was highly acidic (pH 3.6) and the roots were confined to the soil top layer that is rich in organic matter. This corroborates the growing conditions that have been established for ericaceous plants over the years (Smith and Read, 2008; Cairney and Meharg, 2003; Straker, 1996). The shallow root system suggests that they rely on the organic matter in their root zone for growth and establishment as reported in the literature (Smith and Read, 2008; Cairney and Burke, 1998). Recalcitrant organic matter does not contain nutrients that can readily be utilised by Ericaceae plants for growth, yet this plant family is very successful under such harsh edaphic conditions. Previous studies have established the important role played by the ericoid mycorrhizal (ERM) symbiosis in the survival of the Ericaceae (Smith and Read, 2008; Cairney and Meharg, 2003; Read, 1996). Analysis of root-fungal structures associated with the identified *Erica* plants showed ERM, dark septate endophyte (DSE) and hyaline, aseptate hyphae and vesicles. Hyaline hyphae and vesicles are potentially arbuscular mycorrhizal (AM). Ericoid mycorrhizal and DSE associations have established roles in enhancing nutrient assimilation from organic sources through the secretion of various enzymes (Usiku and Narisawa, 2007; Piercey et al., 2002; Cairney and Burke, 1998).

Although co-occurrence of these associations has previously been reported in the family Ericaceae (Massicotte et al., 2005; Urcelay, (2002), this is the first study to report ERM, DSE and AM associations in the genus *Erica*. The ecological significance of the simultaneous

occurrence of the associations in the hair root is yet to be established. It is possible that they broaden the range of nutrients that can be utilised by the host, depending on the fungal partner involved, or improve host fitness (Smith and Read, 2008). For example DSE comprising either looped hyphae or a parenchymatous net do not possess a defined interface for nutrient exchange (Vohník and Albrechtová, 2011), but may improve nutrient assimilation through secretion of enzymes and facilitate nutrient exchange through other alternative mechanisms, such as transfer across the hyphal wall, as previously described for the ectomycorrhizal association involving *Cenococcum geophilum* fungus (Paris et al., 1993). They can also be multifunctional, thus conferring other numerous benefits to the host in addition to nutrient acquisition (Mandyam and Jumpponen, 2005; Narisawa et al., 2004; 2002).

Although arbuscular mycorrhizas are not commonly associated with Ericaceae plants, several studies have reported their presence in this family (Obase et al., 2013; Brundrett, 2009). Inoculation of field blueberry plants with arbuscular fungi resulted in colonisation percentage of 80% but with lower symbiotic benefits compared to ERM fungal inoculated plants (Vega et al., 2009). Their ecological roles in association with Ericaceae have not been clearly established. Considering the concept of a symbiosis the AM fungus is likely to benefit from the *Erica* host by acquiring carbon it requires for the completion of its life cycle. However, the mechanism by which the host would benefit is not clear. In a typical AM association with non-ericaceous hosts, the fungi would increase the root surface area for absorption, thus enhancing nutrient uptake, particularly phosphorus (P) (Smith and Read, 2008). Ericaceous hair roots are confined to the top soil layer rich in organic matter. Therefore, AM associations may increase the hair root surface area in this soil profile area and enhance P nutrition from organic matrices (Feng et al., 2003). It can be suggested that AM fungi work together with ERM fungi. As they are co-occurring, it would seem likely that the AM increases the absorption surface area of the hair roots and specialises in P uptake, whereas ERM facilitates nutrient uptake of other nutrients through secretion of enzymes. There is need to conduct studies that determine the significance of AM when associated with ericaceous hosts.

To identify fungi responsible for the formation of the respective structures in chapter 3 culture-based techniques were initially used. Numerous fungal taxa showing variable pigmentation

ranging from white to black were isolated. Although a few isolates sporulated in culture, most of them were slow growing and sterile. These morphological traits have been reported continuously from more recent studies on ericaceous associated fungi (Curlevski et al., 2009; Upson et al., 2007). This has improved the information on morphological keys of the fungi that were initially described as slow growing, darkly pigmented and sterile mycelia (Read, 1974). Although keys are being modified to include other characteristics such as sporulation and pigmentation in culture, the ericoid fungi seem to be consistently slow growing. Therefore, it is a reliable trait that can be used in screening potential ericoid fungi during isolation in culture.

Ascomycetes belonging to the order Helotiales dominated the culturable fungi isolated and identified from all the six *Erica* hosts. This corroborates studies that have established Ascomycetes as the dominant phylum that forms ERM relationships (Smith and Read, 2008). There were operational taxonomic units (OTUs) that were shared in all *Erica* hosts. Most dominant were unclassified fungi with affinities to Helotiales that have previously been isolated from Australian Epacrids. These isolates have been temporarily annotated with names such as *Epacris pulchella* root fungus, *Epacris microphylla* root fungus and ericoid mycorrhizal fungus (Chambers et al., 2008; Midgely et al., 2004). Resynthesis studies have confirmed the ERM status of these fungi (Bougoure and Cairney, 2005). These fungi seem to be more dominant in the southern hemisphere, for example, Australia and in the current study. Could they be major ERM fungi of these regions? It is possible they have evolved with the respective hosts in their specific ecosystems, and are therefore well adapted to confer mycorrhizal benefits under these conditions. This theory is supported by the current study and those from other by the absence of *Rhyzoscyphus erica* (*R. erica*) in the current study and other regions (Bruzone et al., 2014; Zhang et al., 2009). *Rhyzoscyphus erica* has previously been regarded as the main ERM fungus of most regions of the world particularly in the northern hemisphere (Smith and Read, 2008).

Other fungi such as *Meliniomyces* sp., *Leohumicola* sp., *Oidiodendron maius* and *Cryptosporiopsis erica* were also identified. It has been established that they form ERM associations (Walker et al., 2011; Bougoure et al., 2007; Allen et al., 2003). Molecular identification of fungi such as *Phialocephala* sp., *Cadophora* sp. and *Lachnum* sp. that have been previously associated with DSE fungi could possibly explain the DSE structures observed in this

study (Hazard, 2014; Vohník et al., 2005). According to the literature, none of the culture isolated fungi were potentially AM or synonymous with the vesicles. Arbuscular mycorrhizal fungi are obligate mycobionts, are therefore unculturable (Obase, 2013). Some of the remaining fungal isolates could be root fungi that formed no distinctive structures. However, root fungal structures formed are dependent on fungus, host and growth conditions (Smith and Read, 2008), therefore it is important to conduct mycorrhizal synthesis to draw accurate conclusions on the mycorrhizal status of the identified fungal isolates. Basidiomycetes were poorly represented by two OTUs both belonging to the genus *Mycena*. This is a common result because the culture technique favours the recovery of Ascomycetes (Selosse et al., 2007; Allen et al., 2003).

Further analysis using direct root DNA extraction and 454 pyrosequencing in Chapter 4 was performed in an effort to account for fungi that are either unculturable or not easily culturable. The technique allowed for additional species identification of the ERM fungi such as *Capronia* and *Cladophialophora* species and DSE fungi *Acephala* species. Glomerales were also identified in some of the roots accounting for the vesicles observed in Chapter 2 as AM associates. Numerous basidiomycetous fungi were identified through pyrosequencing. However, no distinctive basidiomycete fungal structures were observed using root staining and light microscopy. The use transmission electron microscopy (TEM) could have improved the identification of basidiomycetes in the roots (Selosse et al., 2007), by increasing visibility of hyphal clamps.

Both culture-based and pyrosequencing techniques in Chapters 3 and 4 respectively detected a diversity of fungi from the different host roots in which Ascomycetes were the dominant phylum. This is uniformally consistent with previous research (Walker et al., 2011; Smith and Read, 2008; Allen et al, 2003). More Basidiomycetes were identified using pyrosequencing with a total of 27% abundance in comparison with 3% recorded in culture. This implies that Basidiomycetes are not easily cultured. Similar conclusions have been reached in other studies (Allen et al., 2003). Glomeromycetes were also detected in *E. caffra*, *E. cerinthoides* and *E. nemorosa* from direct amplification. The identification of these phyla using 454 pyrosequencing

techniques clearly illustrates its effectiveness in detecting the fungal communities, including unculturable fungi or taxa that are easily overlooked by the culture-based techniques.

According to the culture-based identification Helotiales were dominant in all host roots whereas pyrosequencing showed Chaetothyriales as the most abundant taxa in *E. cerinthoides* and *E. chamissonis*. This could suggest that Helotiales are favoured by culture isolation technique in the respective hosts. Some fungi from both techniques remained unidentified. These included fungi with affinities to Helotiales previously identified from *Epacrid* roots. This emphasises the urgent need for studies aimed at taxonomic classification using different barcoding genes simultaneously to enable accurate and conclusive fungal diversity analysis.

Fungi taxonomically identified to species level using both culture-based technique and pyrosequencing belonging to the phylum Ascomycota were relatively similar. However, there was a notable detection of *Capronia* sp. from pyrosequencing which were absent in culture. The same was observed by Allen et al., (2004) in a study that compared diversity of fungi in Sasal roots using Sanger direct amplification and sequencing and culture-based identification. On the other hand, *Phialophora* sp. was identified in culture but not pyrosequencing in this study. This could have resulted from sample preparation such as DNA extraction and amplification that selected against this DSE fungus (Lindahl et al., 2013). Such observations highlight the weakness of pyrosequencing and illustrate the importance of using both techniques which complement each other in fully exploring the root fungal communities in ericaceous roots. *Rhyzoscyphus erica* was not detected in any roots using either technique. This confirms their absence in *Erica* species in the Albany Centre of Endemism and further illustrates that its occurrence is not as widely universal as suggested (Smith and Read, 2008; Villa-Ruiz et al., 2004).

Pyrosequencing has helped to reveal greater fungal diversity, highlighting particularly Glomeromycetes and additional Basidiomycetes which were not identified in culture. However the taxonomic classification to genus and species level was poor. This could have been improved by the use of specific primers for the different phyla (Obase et al., 2013; Selosse et al., 2007). In addition, it shows that the use of this technique in diversity studies must include the consideration of using multiple primers for improved identification. In the current study, the

primers used were suitable for the study as it was aimed at identifying ERM fungi that have extensively been described as ascomycetes.

It has been established that fungi associated with ericaceous plants such as ERM fungi and DSE facilitate nutrient assimilation from various organic substrates (Smith and Read, 2009). However, there are numerous other root fungi associated with *Erica* plants whose ecological roles have not been established. The current study found in Chapter 2 that the Erica roots were confined to the organic layer of the soil. Therefore Chapter 5 aimed to determine the effectiveness of selected *Erica* fungal isolates in utilising both organic and inorganic nutrient sources. The tested isolates were able to utilise various inorganic and organic substrates for biomass accumulation. The inorganic substrates were readily assimilated in comparison to organic substrates. This is expected as inorganic compounds can directly be utilised in their form whereas the latter has to be degraded to simple molecules prior to assimilation (Cairney and Burke, 1998). *Meliniomyces*, *Leohumicola* and *Oididendron* species were able to accumulate significantly higher biomass in Bovine Serum Albumin (BSA) compared to the respective controls. This could be due to the secretion of protease enzymes by the isolates (Leake and Read, 1990). This characteristic is important in determining potential ERM fungi because facilitating assimilation of nitrogen from complex organic substrates is the principal function of the ERM mycobionts (Smith and Read, 2008).

Erica isolates *Meliniomyces* sp. and *Acremonium implicatum* effectively utilised salmon sperm deoxyribonucleic acid (DNA) as a sole source of phosphorus. Phosphomonoesterase activity was detected from both isolates. This suggests that these isolates produced phosphomonoesterase enzymes that facilitated the breakdown and utilisation of DNA as a P source instead of the phosphodiesterases (Leake and Miles, 1996). However, the control treatments of all isolates, with the exception of *Acremonium implicatum* and *Oidiodendron* sp. that lacked a P source tested positively for phosphomonoesterase activity. This could imply that a negative feedback mechanism triggers the secretion of phosphomonoesterase enzymes in the absence of available P (Cairney and Burke, 1998). The remaining isolates were not able to utilise DNA for the production of biomass. All selected *Erica* isolates were able to utilise organic carbon (C). This could be important during an ERM association. It ensures saprophytic growth because the fungal

partner is able to secure C from other sources in the soil. This could reduce its C requirements from the host resulting in more symbiotic benefits channelled to the host.

Production of siderophores by fungal partners can facilitate the utilisation of Iron (Fe) by ericaceous hosts. Fe deficiency is not usually a common problem in acidic growth habitats that have a pH below 6.5 (Marschner and Dell, 1994). However in acidic soils Fe can be bound in metal complexes such as Strengite ($FePO_4.H_2O$) rendering it unavailable to the plant roots (Gibson and Mitchell, 2004). In the current study, siderophores were detected in several isolates that include *Meliniomyces, Leohumicola* and *Oidiidendron* species. This shows the potential of fungi to improve Fe nutrition of the host.

Chapter 5 showed the potential ecological roles of the different fungi in facilitating the availability of nutrients to their respective hosts. Such techniques have been successfully used in determining the roles of the ERM fungi (Chambers et al., 2000). Considering the effectiveness of selected *Erica* isolates to accumulate biomass utilising various organic nutrient sources, as well as reports from literature, *Meliniomyces*, *Leohumicola* and *Oidiidendron* species, were selected for further mycorrhizal synthesis analysis.

Further analysis of potential functions of the various root fungi was conducted in chapter 6. Their biocontrol capabilities were tested against *Phytophthora cinnamomi* a causal agent of root rot disease in blueberry plants. In this study *Leohumicola* sp. inhibited the growth of *Phytophthora cinnamomi* in culture. The Brightwell blueberry plants, including the uninoculated control plants, did not exhibit any symptoms of infection 90 days after pathogen inoculation. This could imply that the Brightwell variety is resistant to this pathogen. Some blueberry varieties are resistant to *P. cinnamomi* particularly those belonging to the rabbiteye bush type which includes Brightwell (Larach et al., 2009; Smith, 2000). However, further experimentation is required under field conditions using *P. cinnamomi* that has been isolated from blueberry plants in order to establish beyond doubt that Brightwell is a resistant variety.

Both *Leohumicola* and *Oidiodendron* species formed typical ERM colonisation with Misty variety host plants. Mycorrhizal synthesis and the effects of inoculation on Misty variety were assessed in Chapter 7. Inoculation of *Leohumicola, Oidiodendron* and *Meliniomyces* species resulted in ERM colonisation and enhanced root biomass. This further confirmed the ERM status

of *Oidiodendron* sp. corroborating findings from previous studies on the mycorrhizal association with *Vaccinium* sp. (Scagel, 2005). Although *Leohumicola* sp. has been reported to form ERM association with other Ericaceae plants (Grunewaldt-Stöcker et al., 2013), to my knowledge this is the first study that reports on the mycorrhizal relationship with *Vaccinium* sp.. In the current study, *Leohumicola* sp. formed ERM structures in two resynthesis experiments with two different blueberry varieties, Brightwell and Misty. In another separate experiment in which mycorrhizal synthesis was conducted in culture using aseptically germinated *E. caffra* seedlings, *Leohumicola* sp. formed ERM structures (data not shown). In addition *Leohumicola* sp. enhanced root biomass in Misty. The identification of root fungi in earlier Chapters 3 and 4 using both culture-based and pyrosequencing techniques detected *Leohumicola* sp. in all the respective hosts. This strongly suggests that *Leohumicola* sp. is an ericoid mycorrhizal fungus and is a major mycobiont of the ERM association in the ACOE.

8.1 Conclusion

To my knowledge, this is the first attempt to establish the diversity of ericoid mycorrhizal fungi associated with ericaceous plants of South Africa. The study has established that *Erica* plants of the Albany Centre of Endemism have ERM, DSE and AM associations. The roots harbour a diversity of fungi that are largely ascomycetes which include common ERM and DSE fungi. However, *R. erica* was not detected using both culture-based and pyrosequencing techniques. Comparatively, pyrosequencing detects greater fungal diversity in *Erica* roots than culture-based techniques. The diversity of Ascomycetes identified using cultural techniques yields are similar to those detected in pyrosequencing. The latter further illustrates the quantitative abundance of the various taxa. This shows that the concurrent use of both techniques in determining root fungal diversity gives a better outcome than either technique used alone.

Most of the characterised *Erica* fungal isolates showed potential to facilitate organic nutrient supply to the host. *Leohumicola* sp. is potentially an important ericoid fungus in the ACOE. This has been demonstrated throughout the study by its presence in all six *Erica* plants, ability to assimilate BSA as a sole N source, produce siderophore and antimicrobial activity against the phytopathogen *Phytophthora cinnamomi* in culture. In addition *Leohumicola* sp. engaged in ERM associations with *Vaccinium* sp., both Brightwell and Misty blueberry varieties. These are

important characteristics that are typical of ERM fungi. *Oidiodendron* and *Meliniomyces* isolates were also able to utilise BSA and produce siderophores and *Meliniomyces* sp. accumulated biomass with DNA as a P source. In addition both fungi formed ERM association with *Vaccinium* plants. This shows that there can be more than a single ERM fungus in the hair roots of an *Erica* host. This highlights the need to conduct further studies to determine the ecological roles of the numerous fungi associated with the Ericaceae.

The South African Fungal Diversity Network developed a fungal research strategy for South Africa in 2013. The strategy highlighted the need to investigate biodiversity of soil fungi including those forming mycorrhizal relationships (Prof. Dames, Personal communication). This study has significantly contributed to the understanding of fungal biodiversity in the ACOE of South Africa. Isolated fungi from this study have been submitted to the ARC, PPRI National Collection of Fungi. A fungal species list compiled using the Darwin core standard will be submitted to the South African Biodiversity Institute.

References

Abe, J. P. (2005) An arbuscular mycorrhizal genus in the Ericaceae. Inoculum 56:6

Allen, T. R., Millar, T., Berch, S. M., & Berbee, M. L. (2003). Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytologist, 160(1), 255-272.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research, 25(17), 3389-3402.

Arnow, L. E. (1937). Colorimetric determination of the components of 3, 4-dihydroxyphenylalaninetyrosine mixtures. Journal of Biological Chemistry, 118(2), 531-537.

Arriagada, C., Manquel, D., Cornejo, P., Soto, J., Sampedro, I. & Ocampo, J. (2012). Effects of the co-inoculation with saprobe and mycorrhizal fungi on *Vaccinium corymbosum* growth and some soil enzymatic activities. Journal of soil science and plant nutrition, 12(2), 283-294.

Atkin, C., Neilands, J. & Phaff, H. (1970). Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*, Journal of Bacteriology, 103(3), 722-733.

Bartholdy, B. A., Berreck, M., & Haselwandter, K. (2001). Hydroxamate siderophore synthesis by *Phialocephala fortinii*, a typical dark septate fungal root endophyte. BioMetals, 14(1), 33-42.

Bazzicalupo, A. L., Bálint, M., & Schmitt, I. (2013). Comparison of ITS1 and ITS2 rDNA in 454 sequencing of hyperdiverse fungal communities. Fungal Ecology, 6(1), 102-109.

Bell, T. L., & Ojeda, F. (1999). Underground starch storage in *Erica* species of the Cape Floristic Region–differences between seeders and resprouters. New Phytologist, 144(1), 143-152.

Bending, G. & Read, D. (1996). Effects of the soluble polyphenol tannic acid on the activities of ericoid and ectomycorrhizal fungi. Soil Biology and Biochemistry, 28(12), 1595-1602.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., ... & Nilsson, R. H. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods in Ecology and Evolution, 4(10), 914-919.

Berch, S. M., Allen, T. R., & Berbee, M. L. (2002). Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. In Diversity and Integration in Mycorrhizas (pp. 55-66). Springer Netherlands.

Bergero, R., Girlanda, M., Bello, F., Luppi, A., & Perotto, S. (2003). Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. Mycorrhiza, 13(2), 69-75.

Bergero, R., Perotto, S., Girlanda, M., Vidano, G. & Luppi, A. (2000). Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). Molecular ecology, 9(10), 1639-1649.

Berry, D., Mahfoudh, K. B., Wagner, M. & Loy, A. (2011). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Applied & Environmental Microbiology, 77(21), 7846-7849.

Bierman, P. M., & Rosen, C. J. (2005). Nutrient cycling and maintaining soil fertility in fruit and vegetable crop systems. University of Minesota.

Bizabani C. (2012). Ericoid mycorrhizal fungi and potential for inoculation of commercial berry species (*Vaccinium corymbosium* L.) (Masters Dissertation), Rhodes University.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., & Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. Molecular ecology resources, 13(2), 218-224.

Bonfante Fasolo P. & Fontana A. 1973. Sulla nutrizione del mecelia di Tuber melanosporum Vitt in coltura. Atti della. Academia delle Scienze di Torino 107: 713–741.

Bougoure, D. S. & Cairney, J. W. G. (2006). Chitinolytic activities of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae). Mycological Research, 110(3), 328-334.

Bougoure, D. S., & Cairney, J. W. (2005a). Assemblages of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae) as determined by culturing and direct DNA extraction from roots. Environmental Microbiology, 7(6), 819-827.

Bougoure, D. S., & Cairney, J. W. (2005b). Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. Environmental Microbiology, 7(11), 1743-1754.

Bougoure, D. S., Parkin, P. I., Cairney, J. W., Alexander, I. J., & Anderson, I. C. (2007). Diversity of fungi in hair roots of Ericaceae varies along a vegetation gradient. Molecular Ecology, 16(21), 4624-4636.

Brown, A. E., Finlay, R. & Ward, J. S. (1987). Antifungal compounds produced by *Epicoccum purpurascens* against soil-borne plant pathogenic fungi. Soil Biology and Biochemistry, 19(6), 657-664.

Brundrett, M. C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant and Soil, 320(1-2), 37-77.

Brundrett, M. (2004). Diversity and classification of mycorrhizal associations. Biological Reviews, 79(3), 473-495.

Bruns, T. D. & Shefferson, R. P. (2004). Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. Canadian Journal of Botany, 82(8), 1122-1132.

Bruzone, M. C., Fontenla, S. B., & Vohník, M. (2014). Is the prominent ericoid mycorrhizal fungus *Rhizoscyphus ericae* absent in the Southern Hemisphere's Ericaceae? A case study on the diversity of root mycobionts in *Gaultheria* spp. from northwest Patagonia, Argentina. Mycorrhiza, 1-16.

Bryla, D. R., Linderman, R. G. & Yang, W. Q. (2008). Incidence of *Phytophthora* and *Pythium* infection and the relation to cultural conditions in commercial blueberry fields. HortScience, 43(1), 260-263.

Buee, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S. & Martin, F. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist, 184 (2), 449-456.

Burke, R. & Cairney, J. (1997). Purification and characterization of a beta-1, 4-endoxylanase from the ericoid mycorrhizal fungus *Hymenoscyphus ericae*. New Phytologist, 135(2), 345-352.

Cairney, J. W. & Ashford, A. E. (2002). Biology of mycorrhizal associations of Epacrids (Ericaceae). New Phytologist, 154(2), 305-326.

Cairney, J. & Burke, R. (1998). Extracellular enzyme activities of the ericoid mycorrhizal endophyte *Hymenoscyphus ericae* (Read) Korf & Kernan: their likely roles in decomposition of dead plant tissue in soil. Plant and Soil, 205(2), 181-192

Cairney, J. W. G., & Meharg, A. A. (2003). Ericoid mycorrhiza: a partnership that exploits harsh edaphic conditions. European Journal of Soil Science, 54(4), 735-740.

Cairney, J. W. G., Sawyer, N. A., Sharples, J. M., & Meharg, A. A. (2000). Intraspecific variation in nitrogen source utilisation by isolates of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan. Soil Biology and Biochemistry, 32(8), 1319-1322.

Chambers, S. M., Curlevski, N. J. & Cairney, J. W. (2008). Ericoid mycorrhizal fungi are common root inhabitants of non-Ericaceae plants in a south-eastern Australian sclerophyll forest. FEMS microbiology ecology, 65(2), 263-270.

Chambers, S. M., Liu, G., & Cairney, J. W. G. (2000). ITS rDNA sequence comparison of ericoid mycorrhizal endophytes from *Woollsia pungens*. Mycological Research, 104(02), 168-174.

Chao, A., Chazdon, R. L., Colwell, R. K., & Shen, T. J. (2005). A new statistical approach for assessing similarity of species composition with incidence and abundance data. Ecology Letters, 8(2), 148-159.

Chaurasia, B., Pandey, A. & Palni, L.M.S. (2005). Distribution, colonization and diversity of arbuscular mycorrhizal fungi associated with central Himalayan Rhododendrons. Forest Ecology and Management, 207(3), 315-324.

Chen, A., Chambers, S. M., & Cairney, J. W. G. (1999). Utilisation of organic nitrogen and phosphorus sources by mycorrhizal endophytes of *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae). Mycorrhiza, 8(4), 181-187.

Curlevski, N. J., Chambers, S. M., Anderson, I. C., & Cairney, J. W. (2009). Identical genotypes of an ericoid mycorrhiza-forming fungus occur in roots of *Epacris pulchella* (Ericaceae) and *Leptospermum polygalifolium* (Myrtaceae) in an Australian sclerophyll forest. FEMS microbiology ecology, 67(3), 411-420.

Currah, R. S., Tsuneda, A., & Murakami, S. (1993). Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum*. Canadian Journal of Botany, 71(12), 1639-1644.

Dalpé, Y. (1989). Ericoid mycorrhizal fungi in the Myxotrichaceae and Gymnoascaceae. New Phytologist, 113(4), 523-527.

Dalpé, Y. (1986). Axenic synthesis of ericoid mycorrhiza in *Vaccinium angustifolium* Ait. by *Oidiodendron* species. New phytologist, 103(2), 391-396.

Davey, M. L., Heegaard, E., Halvorsen, R., Kauserud, H. & Ohlson, M. (2013). Amplicon-pyrosequencing-based detection of compositional shifts in bryophyte-associated fungal communities along an elevation gradient. Molecular ecology, 22(2) 368-383.

Davies, P. W., Mclean, C., & Bell, T. (2003). Root survey and isolation of fungi from alpine Epacrids (Ericaceae).

De Beeck, M. O., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J. & Colpaert, J. V. 2014. Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. PloS one, 9(6), 97629.

Dighton, J. & Coleman, D.C. (1992). Phosphorus relations of roots and mycorrhizas of *Rhododendron maximum* L. in the southern Appalachians, North Carolina. Mycorrhiza, 1(4) 175-184.

Downer, A., Menge, J. & Pond, E. (2001). Effects of cellulytic enzymes on *Phytophthora cinnamomi*, Phytopathology, 91(9), 839-846.

Duddridge, J., & Read, D. J. (1982). An ultrastructural analysis of the development of mycorrhizas in *Rhododendron ponticum*. Canadian Journal of Botany, 60(11), 2345-2356.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. Bioinformatics, 27(16), 2194-2200.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26(19), 2460-2461.

Egger, K. N., & Sigler, L. (1993). Relatedness of the ericoid endophytes *Scytalidium vaccinii* and *Hymenoscyphus ericae* inferred from analysis of ribosomal DNA. Mycologia, 219-230.

Englander, L., & Hull, R. J. (1980). Reciprocal transfer of nutrients between ericaceous plants and a *Clavaria* sp. New phytologist, 84(4), 661-667.

Emmerton, K. S., Callaghan, T. V., Jones, H. E., Leake, J. R., Michelsen, A., & Read, D. J. (2001). Assimilation and isotopic fractionation of nitrogen by mycorrhizal fungi. New Phytologist, 151(2), 503-511.

Feng, G., Song, Y. C., Li, X. L., & Christie, P. (2003). Contribution of arbuscular mycorrhizal fungi to utilization of organic sources of phosphorus by red clover in a calcareous soil. Applied Soil Ecology, 22(2), 139-148.

Foden W, Potter L. (2005). Cyperus latifolius Poir. National Assessment: Red List of South African Plants version 2011.1. South African National Biodiversity Institute, Pretoria.

Fukuchi, S., Obase, K., Tamai, Y., Yajima, T. & Miyamoto, T. (2011). Vegetation and colonization status of mycorrhizal and endophytic fungi in plant species on acidic barren at crater basin of volcano Esan in Hokkaido, Japan. Eurasian Journal of Forest Research, 14, (1), 1-11.

García-Martínez, J., Acinas, S.G., Antón, A.I. & Rodríguez-Valera, F. (1999). Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. Journal of microbiological methods, 36(1–2), 55-64.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular ecology, 2(2), 113-118.

Ghafar, M. F. A., Prasad, K. N., Weng, K. K. and Ismail, A., 2010. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. African Journal of Biotechnology, 9(3): 326-330.

Gibson, B. R., & Mitchell, D. T. (2004). Nutritional influences on the solubilization of metal phosphate by ericoid mycorrhizal fungi. Mycological research, 108(8), 947-954.

Gillevet, P., Sikaroodi, M., Keshavarzian, A. & Mutlu, E. (2010). Quantitative Assessment of the Human Gut Microbiome Using Multitag Pyrosequencing. Chemistry and Biodiversity, 7(5), 1065-1075.

Goldblatt, P., & Manning, J. C. (2002). Plant diversity of the Cape region of Southern Africa. Annals of the Missouri Botanical Garden, 281-302.

Gorzelak, M. A., Holland, T. C., Xing, X., & Hart, M. M. (2012). Molecular approaches for AM fungal community ecology: a primer. Journal of microbiological methods, 90(2), 108-114.

Goulart, B. L., Demchak, K., & Yang, W. Q. (1996). Organic matter and nitrogen level effects on mycorrhizal infection in 'Bluecrop' highbush blueberry plants. Journal of Small Fruit and Viticulture, 3(4), 151-164.

Grelet, G., Meharg, A. & Alexander, I. (2005). Carbon availability affects nitrogen source utilisation by *Hymenoscyphus ericae*. Mycological Research, (109), 469-477.

Grelet, G., Johnson, D., Paterson, E., Anderson, I.C. & Alexander, I.J. (2009). Reciprocal Carbon and Nitrogen Transfer between an Ericaceous Dwarf Shrub and Fungi Isolated from *Piceirhiza bicolorata* Ectomycorrhizas. New Phytologist, 182(2) 359-366.

Grelet, G. A., Johnson, D., Vrålstad, T., Alexander, I. J., & Anderson, I. C. (2010). New insights into the mycorrhizal *Rhizoscyphus ericae* aggregate: spatial structure and co-colonization of ectomycorrhizal and ericoid roots. New Phytologist, 188(1), 210-222.

Grelet, G., Meharg, A. A., Duff, E. I., Anderson, I. C. & Alexander, I. J. (2009). Small Genetic Differences between Ericoid Mycorrhizal Fungi Affect Nitrogen Uptake by *Vaccinium*. New Phytologist, 181(3), 708-718.

Grunewaldt-Stöcker, G., von den Berg, C., Knopp, J., & von Alten, H. (2013). Interactions of ericoid mycorrhizal fungi and root pathogens in *Rhododendron*: In vitro tests with plantlets in sterile liquid culture. Plant Root, 7(0), 33-48.

Grünig, C.R., Queloz, V., Sieber, T.N. & Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the *Phialocephala fortinii* sl-*Acephala applanata* species complex in tree roots: classification, population biology and ecology. Botany, 86(12), 1355-1369.

Guo, L. D., Hyde, K. D., & Liew, E. C. Y. (2000). Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New phytologist, 147(3), 617-630.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series 41, 95-98.

Harley, J. L. (1992). Mycorrhizae, 7th edn. (Encyclopedia of Science and Technology 11).

Hambleton, S., & Sigler, L. (2005). *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (*Hymenoscyphus ericae*), Leotiomycetes. Studies in Mycology, 53, 1-27.

Hambleton, S., Huhtinen, S., & Currah, R. S. (1999). *Hymenoscyphus ericae*: a new record from western Canada. Mycological Research, 103(11), 1391-1397.

Haselwandter, K. (2008). Structure and function of siderophores produced by mycorrhizal fungi. Mineralogical Magazine, 72(1) 61-64.

Haselwandter, K., Dobernigg, B., Beck, W., Jung, G., Cansier, A. & Winkelmann, G. (1992). Isolation and identification of hydroxamate siderophores of ericoid mycorrhizal fungi. BioMetals, 5(1), 51-56.

Haselwandter, K., & Winkelmann, G. (2007). Siderophores of symbiotic fungi. In Microbial siderophores (pp. 91-103). Springer Berlin Heidelberg.

Hazard, C., Gosling, P., Mitchell, D. T., Doohan, F. M. & Bending, G.D. (2014). Diversity of fungi associated with hair roots of ericaceous plants is affected by land use. FEMS microbiology ecology, 87(3) 586-600.

Hewitt, E. J., 1966. Sand and Water Culture Methods Used in the Study of Plant Nutrition. Technical Communication No. 22. Commonwealth Bureau, London.

Hodkinson, B. P., & Grice E. A. (2015). Next-Generation Sequencing: A Review of Technologies and Tools for Wound Microbiome Research. Advances in wound care.

Hosoe, T., Nozawa, K., Lumley, T. C., Currah, R. S., Fukushima, K., Takizawa, K..... & Kawai, K. I. (1999). Tetranorditerpene lactones, potent antifungal antibiotics for human pathogenic yeasts, from a unique species of *Oidiodendron*. Chemical and Pharmacetical Bulletin, 47(11), 1591-1597.

Huang, X., & Madan, A. (1999). CAP3: A DNA sequence assembly program. Genome research, 9(9), 868-877.

Hutton, B. J., Sivasithamparam, K., Dixon, K. W., & Pate, J. S. (1996). Pectic zymograms and water stress tolerance of endophytic fungi isolated from Western Australian heaths (Epacridaceae). Annals of Botany, 77(4), 399-404.

Ishida, T. A., & Nordin, A. (2010). No evidence that nitrogen enrichment affect fungal communities of Vaccinium roots in two contrasting boreal forest types. Soil Biology and Biochemistry, 42(2), 234-243.

Jansa, J., & Vosátka, M. (2000). In vitro and post vitro inoculation of micropropagated Rhododendrons with ericoid mycorrhizal fungi. Applied Soil Ecology, 15(2), 125-136.

Johnson, N. C., Graham, J. H., & Smith, F. A. (1997). Functioning of mycorrhizal associations along the mutualism–parasitism continuum*. New phytologist, 135(4), 575-585.

Joseph, J., Cole, G., Head, E., and Ingram, E., 2009. Nutrition, brain aging, and neurodegeneration. Journal of Nueroscience, 29(41): 12795-801.

Jumpponen, A. (2001). Dark septate endophytes–are they mycorrhizal? Mycorrhiza, 11(4), 207-211.

Jumpponen, A. R. I., & Trappe, J. M. (1998). Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytologist, 140(2), 295-310.

Kajula, M., Tejesvi, M. V., Kolehmainen, S., Mäkinen, A., Hokkanen, J., Mattila, S., & Pirttilä, A. M. (2010). The siderophore ferricrocin produced by specific foliar endophytic fungi *in vitro*. Fungal biology, 114(2), 248-254.

Kauserud, H., Kumar, S., Brysting, A. K., Nordén, J. & Carlsen, T. (2012). High consistency between replicate 454 pyrosequencing analyses of ectomycorrhizal plant root samples. Mycorrhiza, 22(4) 309-315.

Kerley, S. J., & Read, D. J. (1995). The biology of mycorrhiza in the Ericaceae. New Phytologist, 131(3), 369-375.

Kjøller, R., Olsrud, M., & Michelsen, A. (2010). Co-existing ericaceous plant species in a subarctic mire community share fungal root endophytes. Fungal Ecology, 3(3), 205-214.

Koca, I., & Karadeniz, B. (2009). Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. Scientia Horticulturae, 121(4), 447-450.

Koske, R., Gemma, J. & Englander, L. (1990).Vesicular-arbuscular mycorrhizae in Hawaiian Ericales. American Journal of Botany, pp. 64-68.

Kõljalg, U., Larsson, K. H., Abarenkov, K., Nilsson, R. H., Alexander, I. J., Eberhardt, U., ... & Vrålstad, T. (2005). UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist, 166(3), 1063-1068.

Krebs, S. L., & Wilson, M. D. (2002). Resistance to Phytophthora root rot in contemporary rhododendron cultivars. HortScience, 37(5), 790-792.

Kron, K. A., Fuller, R., Crayn, D. M., Gadek, P. A., & Quinn, C. J. (1999). Phylogenetic relationships of Epacrids and Vaccinioids (Ericaceae) based on matK sequence data. Plant Systematics and Evolution, 218(1-2), 55-65.

Kron, K. A., Judd, W. S. Stevens, P. F. Crayn, D. M. Anderberg, A. A. Gadek, P. A. Quinn, C. J.& Luteyn J. L. (2002). Phylogenetic classification of Ericaceae: molecular and morphological evidence. The Botanical Review, 68(3), 335-423.

Krüger, M., Stockinger, H., Krüger, C., & Schüßler, A. (2009). DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytologist, 183(1), 212-223.

Kühdorf, K., Münzenberger, B., Begerow, D., Karasch-Wittmann, C., Gómez-Laurito, J., & Hüttl, R. F. (2014). Sebacina sp. is a mycorrhizal partner of Comarostaphylis arbutoides (Ericaceae). Mycological Progress, 13(3), 733-744.

Lacap, D. C., Hyde, K. D., & Liew, E. C. Y. (2003). An evaluation of the fungal 'morphotype'concept based on ribosomal DNA sequences. Fungal Divers, 12, 53-66.

Larach, A., Besoain, X., & Salgado, E. (2009). Crown and root rot of highbush blueberry caused by *Phytophthora cinnamomi* and *Phytophthora citrophthora* and cultivar susceptibility. Ciencia Investigación Agraria, 36(3), 433-442.

Leake, J. R., & Miles, W. (1996). Phosphodiesterase as mycorrhizal P sources. New Phytologist, 132(3), 435-443.

Leake, J. & Read, D. (1990). Proteinase Activity in Mycorrhizal Fungi .1. The Effect of Extracellular pH on the Production and Activity of Proteinase by Ericoid Endophytes from Soils of Contrasted pH. New Phytologist, 115(2), 243-250.

Lin, L., Lee, M. & Chen, J. (2011). Decomposition of organic matter by the ericoid mycorrhizal endophytes of *Formosan rhododendron* (*Rhododendron formosanum* Hemsl.). Mycorrhiza, 21(5), 331-339.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J. & Kauserud, H. (2013). Fungal community analysis by

high-throughput sequencing of amplified markers–a user's guide. New Phytologist, 199(1), 288-299.

Machuca, A., & Milagres, A. M. F. (2003). Use of CAS-agar plate modified to study the effect of different variables on the siderophore production by *Aspergillus*. Letters in applied microbiology, 36(3), 177-181.

Mandyam, K. & Jumpponen, A. (2005). Seeking the elusive function of the root-colonising dark septate endophytic fungi. Studies in Mycology 53(1), 173-189.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M.S., Chen, Y. & Chen, Z. (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature, 437(7057), 376-380.

Marschner, H., & Dell, B. (1994). Nutrient uptake in mycorrhizal symbiosis. Plant and soil, 159(1), 89-102.

Martino, E., Murat, C., Vallino, M., Bena, A., Perotto, S. & Spanu, P. (2007). Imaging mycorrhizal fungal transformants that express EGFP during ericoid endosymbiosis. Current genetics, 52 (2) 65-75.

Marx, D. H. (1969). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology, 59, 153-163.

Massicotte, H., Melville, L. & Peterson, R. (2005). Structural characteristics of root fungal interactions for five ericaceous species in eastern Canada. Canadian journal of botany, 83(8), 1057-1064.

McGuire, A. F., & Kron, K. A. (2005). Phylogenetic relationships of European and African *Erica*s. International journal of plant sciences, 166(2), 311-318.

Meyer, H. J., & Prinsloo, N. (2003). Assessment of the potential of blueberry production in South Africa. Small Fruits Review, 2(3), 3-21.

Midgley, D. J., Chambers, S. M. & Cairney, J. W. (2004). Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighbouring Ericaceae plants in the field. Plant and Soil, 259(1-2), 137-151.

Midgley, D. J., Jordan, L. A., Saleeba, J. A. & McGee, P. A. (2006). Utilisation of carbon substrates by orchid and ericoid mycorrhizal fungi from Australian dry sclerophyll forests. Mycorrhiza, 16(3) 175-182.

Milagres, A. M., Machuca, A., & Napoleão, D. (1999). Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. Journal of microbiological methods, 37(1), 1-6.

Monreal, M., Berch, S. M., & Berbee, M. (2000). Molecular diversity of ericoid mycorrhizal fungi. Canadian Journal of Botany, 77(11), 1580-1594.

Nafar, F. (1998). Studies of interactions among cultured ericoid mycorrhizal fungi of salal (*Gaultheria shallon* pursh) (Doctoral dissertation, University of British Columbia).

Narisawa, K., Ohki, K. T., & Hashiba, T. (2000). Suppression of clubroot and *Verticillium* yellows in Chinese cabbage in the field by the root endophytic fungus, *Heteroconium chaetospira*. Plant Pathology, 49(1), 141-146.

Narisawa, K., Usuki, F., & Hashiba, T. (2004). Control of *Verticillium* yellows in Chinese cabbage by the dark septate endophytic fungus LtVB3. Phytopathology, 94(5), 412-418.

Neilands, J. B. (1995). Siderophores: structure and function of microbial iron transport compounds. Journal of Biological Chemistry, 270(45), 723-726.

Nguyen, H. D. T., & Seifert, K. A. (2008). Description and DNA barcoding of three new species of *Leohumicola* from South Africa and the United States. Persoonia: Molecular Phylogeny and Evolution of Fungi, 21, 57.

Nilsson, R. H., Abarenkov, K., Veldre, V., Nylinder, S., De Wit, P., Brosché, S., Alfredsson, J. F., Ryberg, M. & Kristiansson, E. (2010). An open source chimera checker for the fungal ITS region. Molecular Ecology Resources, 10(6), 1076-1081.

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N., & Larsson, K. H. (2008). Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evolutionary bioinformatics online, 4, 193.

Nilsson, R. H., Ryberg, M., Abarenkov, K., Sjökvist, E. & Kristiansson, E. (2009). The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. FEMS microbiology letters, 296(1), 97-101.

Nurfadilah, S., Swarts, N. D., Dixon, K. W., Lambers, H. & Merritt, D. J. (2013). Variation in nutrient-acquisition patterns by mycorrhizal fungi of rare and common orchids explains diversification in a global biodiversity hotspot. Annals of botany, 111(6), 1233-1241.

Obase, K., Matsuda, Y., & Ito, S. I. (2013). *Enkianthus campanulatus* (Ericaceae) is commonly associated with arbuscular mycorrhizal fungi. Mycorrhiza, 23(3), 199-208.

Ohtaka, N., & Narisawa, K. (2008). Molecular characterization and endophytic nature of the root-associated fungus *Meliniomyces variabilis* (LtVB3). Journal of General Plant Pathology, 74(1), 24-31.

Ojeda, F. (1998). Biogeography of seeder and resprouter *Erica* species in the Cape Floristic Region—Where are the resprouters? Biological Journal of the Linnean Society, 63(3), 331-347.

Ojeda, F., Brun, F. G., & Vergara, J. J. (2005). Fire, rain and the selection of seeder and resprouter life-histories in fire-recruiting, woody plants. New Phytologist, 168(1), 155-165.

Oliver, E. G. H. (1967). Three new species of *Erica.* Journal of South African Botany, 33(3), 197-204.

Oliver, I., & Oliver, E. G. H. (2000). Field guide to the Ericas of the Cape Peninsula. Cape Town: Protea Atlas Project, National Botanical Institute, ISBN, 1919684239.

Palmer, T. (2004). Vegetation of Makana. Makana LEAP: Comprehensive Environmental Audit: Vegetation of Makana.

Paris, F., Dexheimer, J., & Lapeyrie, F. (1993). Cytochemical evidence of a fungal cell wall alteration during infection of Eucalyptus roots by the ectomycorrhizal fungus Cenococcum geophilum. Archives of microbiology, 159(6), 526-529.

Pearson, V., & Read, D. J. (1973). The biology of mycorrhiza in the Ericaceae: I. The isolation of the endophyte and synthesis of mycorrhizas in aseptic culture. New Phytologist, 72(2), 371-379.

Perotto, S., Actis-Perino, E., Perugini, J., & Bonfante, P. (1996). Molecular diversity of fungi from ericoid mycorrhizal roots. Molecular Ecology, 5(1), 123-131.

Perotto, S., Coisson, J. D., Perugini, I., Cometti, V., & Bonfante, P. (1997). Production of pectin-degrading enzymes by ericoid mycorrhizal fungi. New Phytologist, 135(1), 151-162.

Perotto, S., Peretto, R., Faccio, A., Schubert, A., Bonfante, P., & Varma, A. (1995). Ericoid mycorrhizal fungi: cellular and molecular bases of their interactions with the host plant. Canadian Journal of Botany, 73(S1), 557-568.

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. & Fernández, F. (2007). O-CAS, a fast and universal method for siderophore detection. Journal of Microbiological Methods, 70(1), 127-131.

Peterson, R. L. & Massicotte, H. B. (2004). Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Canadian Journal of Botany, 82(8), 1074-1088.

Piercey, M., Thormann, M., & Currah, R. (2002). Saprobic characteristics of three fungal taxa from ericalean roots and their association with the roots of *Rhododendron groenlandicum* and *Picea mariana* in culture. Mycorrhiza, 12(4), 175-180.

Porter, T. M., & Golding, G. B. (2012). Factors that affect large subunit ribosomal DNA amplicon sequencing studies of fungal communities: classification method, primer choice, and error. PloS one, 7(4), e35749.

Popa, R., Popa, R., Mashall, M. J., Nguyen, H., Tebo, B. M. & Brauer, S. (2009). Limitations and benefits of ARISA intra-genomic diversity fingerprinting. Journal of microbiological methods, 78(2), 111-118.

Postma, J., Olsson, P. A., & Falkengren-Grerup, U. (2007). Colonisation of arbuscular mycorrhizal, fine and dark septate endophytic fungi in forbs of acid deciduous forests. Soil Biology and Biochemistry, 39, 400-408.

Prodorutti, D., Pertot, I., Giongo, L., & Gessler, C. (2007). Highbush blueberry: cultivation, protection, breeding and biotechnology. The European journal of plant science and biotechnology, 1(1), 44-56.

R Development Core Team. (2014). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2012. Open access available at: http://cran. r-project. org.

Rahman, M. H., Shovan, L. R., Hjeljord, L. G., Aam, B. B., Eijsink, V. G., Sørlie, M. & Tronsmo, A. (2014). Inhibition of Fungal Plant Pathogens by Synergistic Action of Chito-Oligosaccharides and Commercially Available Fungicides. PloS one, 9(4), e93192.

Raimondo, D., Staden, L. V., Foden, W., Victor, J. E., Helme, N. A., Turner, R. C., Kamundi, D.A. & Manyama, P. A. (2009). Red list of South African plants 2009. South African National Biodiversity Institute.

Rains, K. C., Nadkarni, N. M., & Bledsoe, C. S. (2003). Epiphytic and terrestrial mycorrhizas in a lower montane Costa Rican cloud forest. Mycorrhiza, 13(5), 257-264.

Rath, M., Grolig, F., Haueisen, J., & Imhof, S. (2014). Combining microtomy and confocal laser scanning microscopy for structural analyses of plant–fungus associations. Mycorrhiza, 24(4), 293-300.

Read, D. (1996). The structure and function of the ericoid mycorrhizal root. Annals of Botany, 77(4), 365-374.

Read, D. J. (1974). *Pezizella ericae* sp. nov., the perfect state of a typical mycorrhizal endophyte of ericaceae. Transactions of the British Mycological Society, 63(2), 381-IN22.

Read, D. & Perez-Moreno, J. (2003). Mycorrhizas and nutrient cycling in ecosystems–a journey towards relevance? New Phytologist, 157(3), 475-492.

Redecker, D., Kodner, R., & Graham, L. E. (2000). Glomalean fungi from the Ordovician. Science, 289(5486), 1920-1921.

Richter, B. S., Ivors, K., Shi, W. & Benson, D. (2011). Cellulase activity as a mechanism for suppression of Phytophthora root rot in mulches. Phytopathology, 101(2), 223-230.

Riihinen, K., Jaakola, L., Kärenlampi, S., & Hohtola, A. (2008). Organ-specific distribution of phenolic compounds in bilberry (*Vaccinium myrtillus*) and 'northblue' blueberry (*Vaccinium corymbosum* x *V. angustifolium*). Food chemistry, 110(1), 156-160.

Rossi, M., Giussani, E., Morelli, R., Scalzo, R. L., Nani, R. C., & Torreggiani, D. (2003). Effect of fruit blanching on phenolics and radical scavenging activity of highbush blueberry juice. Food Research International, 36(9), 999-1005.

Sadowsky, J., Hanson, E. & Schilder, A. (2012). Root Colonization by Ericoid Mycorrhizae and Dark Septate Endophytes in Organic and Conventional Blueberry Fields in Michigan. International Journal of Fruit Science, 12(1-3), 169-187.

Scagel, C. F. (2005). Inoculation with ericoid mycorrhizal fungi alters fertilizer use of highbush blueberry cultivars. HortScience, 40(3), 786-794.

Scagel, C. F., Wagner, A., & Winiarski, P. (2005). Frequency and intensity of root colonization by ericoid mycorrhizal fungi in nursery production of blueberry plants. Small fruits review, 4(4), 95-112.

Scagel, C. F., Wagner, A., & Winiarski, P. (2005b). Inoculation with ericoid mycorrhizal fungi alters root colonization and growth in nursery production of blueberry plants from tissue culture and cuttings. Small fruits review, 4(4), 113-135.

Scagel, C. F., & Yang, W. Q. (2005). Cultural variation and mycorrhizal status of blueberry plants in NW Oregon commercial production fields. International journal of fruit science, 5(2), 85-111.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., ... & Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology, 75(23), 7537-7541.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen W. & Griffith, G. W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a

universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences, 109(16), 6241-6246.

Schulz, B. (2006). Mutualistic interactions with fungal root endophytes. In Microbial root endophytes (pp. 261-279). Springer Berlin Heidelberg.

Schulz, B., Boyle, C., Draeger, S., Römmert, A. K., & Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research, 106(09), 996-1004.

Schumann, D., & Kirsten, G. (1992). Ericas of South Africa. Vlaeberg, S. Africa: Fernwood Press 272p.-col. illus. ISBN, 958315434.

Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry, 160(1), 47-56.

Selosse, M., Setaro, S., Glatard, F., Richard, F., Urcelay, C. & Weiß, M. (2007). Sebacinales are common mycorrhizal associates of Ericaceae. New Phytologist, 174(4), 864-878.

Setaro, S., Kottke, I., & Oberwinkler, F. (2006a). Anatomy and ultrastructure of mycorrhizal associations of neotropical Ericaceae. Mycological Progress, 5(4), 243-254.

Setaro, S., Suárez, J. P., Herrera, P., Cruz, D., & Kottke, I. (2013). Distinct but closely related Sebacinales form mycorrhizae with coexisting Ericaceae and Orchidaceae in a Neotropical mountain area. In *Piriformospora indica* (pp. 81-105). Springer Berlin Heidelberg.

Setaro, S., Weiß, M., Oberwinkler, F. & Kottke, I. (2006b). Sebacinales form ectendomycorrhizas with Cavendishia nobilis, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytologist, 169(2), 355-365.

Sharples, J. M., Chambers, S. M., Meharg, A. A., & Cairney, J. W. G. (2000). Genetic diversity of root-associated fungal endophytes from *Calluna vulgaris* at contrasting field sites. New Phytologist, 148(1), 153-162.

Shaw, G., Leake, J., Baker, A. & Read, D. (1990). The biology of mycorrhiza in the Ericaceae. New Phytologist, 115(2), 251-258.

Silva, A., Patterson, K., Rothrock, C. & Moore, J. (2000). Growth promotion of highbush blueberry by fungal and bacterial inoculants. HortScience, 35(7), 1228-1230.

Smith, B. J. (2012). Survival of Southern Highbush Blueberry Cultivars in *Phytophthora* Root Rot-Infested Fields in South Mississippi. International Journal of Fruit Science, 12(1-3), 146-155.

Smith, B. J. (2000). Susceptibility of southern highbush blueberry cultivars to *Phytophthora* root rot. VII International Symposium on *Vaccinium* Culture 574, pp. 75.

Smith, S. E., & Dickson, S. (1991). Quantification of active vesicular-arbuscular mycorrhizal infection using image analysis and other techniques. Functional Plant Biology, 18(6), 637-648.

Smith, M. A. L., Marley, K. A., Seigler, D., Singletary, K. W., & Meline, B. (2000). Bioactive properties of wild blueberry fruits. Journal of Food Science, 65(2), 352-356.

Smith, S. E., & Read, D. J. (2008). Mycorrhizal symbiosis. 3rd Edn.Academic press.

Specht, R. L., & Rundel, P. W. (1990). Sclerophylly and foliar nutrient status of Mediterranean-climate plant communities in southern Australia. Australian Journal of Botany, 38(5), 459-474.

Starrett, M. C. (2002, August). Initial shoot growth and development of micropropagated blueberry plants following inoculation with an ericoid mycorrhizal isolate. In XXVI International Horticultural Congress: Berry Crop Breeding, Production and Utilization for a New Century 626 (pp. 191-197).

Steinkellner, S., Hage-Ahmed, K., García-Garrido, J. M., Illana, A., Ocampo, J. A., & Vierheilig, H. (2012). A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. lycopersici. Mycorrhiza, 22(3), 189-194.

Straker, C. J. (1996). Ericoid mycorrhiza: ecological and host specificity. Mycorrhiza, 6(4), 215-225.

Straker, C. J., & Mitchell, D. T. (1986). The activity and characterization of acid phosphatases in endomycorrhizal fungi of the Ericaceae. New Phytologist, 104(2), 243-256.

Strik, B. C. & Yarborough, D. (2005). Blueberry production trends in North America, 1992 to 2003, and predictions for growth. HortTechnology, 15(2), 391-398.

Sun, X. & Guo, L. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. Mycology, 3(1), 65-76.

Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., Bahram, M., Bechem, E., Chuyong, G. & Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. New Phytologist, 188(1), 291-301.

Tian, W., Zhang, C. Q., Qiao, P. & Milne, R. (2011). Diversity of culturable ericoid mycorrhizal fungi of *Rhododendron decorum* in Yunnan, China. Mycologia, 103(4), 703-709.

Tibbett, M. (2002). Considerations on the use of the *p*-nitrophenyl phosphomonoesterase assay in the study of the phosphorus nutrition of soil borne fungi. Microbiological research, 157(3), 221-231.

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of Ascomycetes and Basidiomycetes in environmental samples. PloS one, 7(7), e40863.

Tortora, M. L., Díaz-Ricci, J.C. & Pedraza, R. O. (2011). *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum*. Archives of Microbiology, 193(4), 275-286.

Trappe, J. M. (1987). Phylogenetic and ecologic aspects of mycotrophy in the angiosperms from an evolutionary stand point.

Upson, R., Read, D. J., & Newsham, K. K. (2007). Widespread association between the ericoid mycorrhizal fungus Rhizoscyphus ericae and a leafy liverwort in the maritime and sub-Antarctic. New Phytologist, 176(2), 460-471.

Urcelay, C. (2002). Co-occurrence of three fungal root symbionts in *Gaultheria poeppiggi* DC in Central Argentina. Mycorrhiza, 12(2), 89-92.

Usuki, F., & Narisawa, K. (2007). A mutualistic symbiosis between a dark septate endophytic fungus, *Heteroconium chaetospira*, and a nonmycorrhizal plant, Chinese cabbage. Mycologia, 99(2), 175-184.

Usuki, F. & Narisawa, K. (2005). Formation of structures resembling ericoid mycorrhizas by the root endophytic fungus *Heteroconium chaetospira* within roots of *Rhododendron obtusum* var. kaempferi. Mycorrhiza, 15(1), 61-64.

Valbuena, L., Tárrega, R., & Luis-Calabuig, E. (2000). Seed banks of *Erica australis* and *Calluna vulgaris* in a heathland subjected to experimental fire. Journal of Vegetation Science, 11(2), 161-166.

Vano, I., Sakamoto, K., & Inubushi, K. (2011). Phylogenetic relationship among non-pathogenic isolates of dark septate endophytes from Ericaceae plants. Hort Research, 65, 41-47.

Vega, A. R., Garciga, M., Rodríguez, A., Prat, L., & Mella, J. (2009). Blueberries mycorrhizal symbiosis outside of the boundaries of natural dispersion for ericaceous plants in Chile. Acta Hort, 810, 665-672.

Verdaguer, D., & Ojeda, F. (2005). Evolutionary transition from resprouter to seeder life history in two Erica (Ericaceae) species: insights from seedling axillary buds. Annals of Botany, 95(4), 593-599.

Verdú, M. (2000). Ecological and evolutionary differences between Mediterranean seeders and resprouters. Journal of Vegetation Science, 11(2), 265-268.

Villarreal-Ruiz, L., Anderson, I. & Alexander, I. (2004). Interaction between an isolate from the *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*. New Phytologist, 164(1), 183-192.

Villarreal-Ruiz, L., Neri-Luna, C., Anderson, I. C. & Alexander, I.J. (2012). In vitro interactions between ectomycorrhizal fungi and ericaceous plants. Symbiosis, 56(2), 67-75.

Vohník, M. & Albrechtová, J. (2011). The Co-occurrence and Morphological Continuum between Ericoid Mycorrhiza and Dark Septate Endophytes in Roots of Six European *Rhododendron* Species. Folia Geobotanica, 46(4), 373-386.

Vohník, M., Albrechtová, J., & Vosátka, M. (2005). The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C: N ratio and root biomass distribution in *Rhododendron* cv. Azurro. Symbiosis, 40(2), 87-96.

Vohník, M., Fendrych, M., Albrechtová, J., & Vosátka, M. (2007). Intracellular colonization of *Rhododendron* and *Vaccinium* roots by *Cenococcum geophilum*, *Geomyces pannorum* and *Meliniomyces variabilis*. Folia microbiologica, 52(4), 407-414.

Vohník, M., Lukančič, S., Bahor, E., Regvar, M., Vosátka, M., & Vodnik, D. (2003). Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. Folia Geobotanica, 38(2), 191-200.

Vohník , M., Sadowsky, J. J., Lukesova, T., Albrechtová, J. & Vosatka, M. (2012). Inoculation with a ligninolytic Basidiomycete, but not root symbiotic ascomycetes, positively affects growth of highbush blueberry (Ericaceae) grown in a pine litter substrate. Plant and Soil, 355(1-2), 341-352.

Vrålstad, T., Fossheim, T., & Schumacher, T. (2000). Piceirhiza bicolorata–the ectomycorrhizal expression of the Hymenoscyphus ericae aggregate? New Phytologist, 145(3), 549-563.

Vrålstad, T., Schumacher, T., & Taylor, A. F. (2002). Mycorrhizal synthesis between fungal strains of the *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. New Phytologist, 153(1), 143-152.

Walker, J. F., Aldrich-Wolfe, L., Riffel, A., Barbare, H., Simpson, N. B., Trowbridge, J., & Jumpponen, A. (2011). Diverse Helotiales associated with the roots of three species of Arctic Ericaceae provide no evidence for host specificity. New Phytologist, 191(2), 515-527.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and environmental microbiology, 73(16), 5261-5267.

Wang, B., & Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza, 16(5), 299-363.

Waters, E. K., Scagel, C. F., & McLean, C. (2008). A Survey of *Vaccinium* Cultural Practices in Australia Emphasizing Implications for Mycorrhizal Infection. International journal of fruit science, 8(1-2), 109-124.

Whipps, J. M. (2004). Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Canadian Journal of Botany, 82(8), 1198-1227.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18, 315-322.

Whittaker, S. P. & Cairney, J. W. G. (2001). Influence of amino acids on biomass production by ericoid mycorrhizal endophytes from *Woollsia pungens* (Epacridaceae). Mycological Research, 105(1), 105-111.

Williamson, J. G., & Miller, E. P. (2009). Effects of fertilizer rate and form on vegetative growth and yield of southern highbush blueberry in pine bark culture. HortTechnology, 19(1), 152-157.

Yamamoto, S., Sato, H., Tanabe, A. S., Hidaka, A., Kadowaki, K. & Toju, H. (2014). Spatial Segregation and Aggregation of Ectomycorrhizal and Root-Endophytic Fungi in the Seedlings of Two *Quercus* Species. PloS one 9(5), e96363.

Yang, W. Q., Goulart, B. L., & Demchak, K. (1998). Mycorrhizal infection and plant growth of highbush blueberry in fumigated soil following soil amendment and inoculation with mycorrhizal fungi. HortScience, 33(7), 1136-1137.

Yang, W., Goulart, B. & Demchak, K. (1996). The effect of aluminium and media on the growth of mycorrhizal and non-mycorrhizal highbush blueberry plantlets. Plant and Soil, 183(2) 301-308.

Yang, W., Goulart, B., Demchak, K. & Li, Y. (2002). Interactive effects of mycorrhizal inoculation and organic soil amendments on nitrogen acquisition and growth of highbush blueberry. Journal of the American Society for Horticultural Science, 127(5), 742-748.

Zhang, C., Yin, L., & Dai, S. (2009). Diversity of root-associated fungal endophytes in *Rhododendron fortunei* in subtropical forests of China. Mycorrhiza, 19(6), 417-423.

Zhang, Y. H., & Zhuang, W. Y. (2004). Phylogenetic relationships of some members in the genus *Hymenoscyphus* (Ascomycetes, Helotiales). Nova Hedwigia, 78(3-4), 475-484.

Zhuang, X., & Chen, Y. (1996). Investigation of plant mycorrhizae in secondary forests of Hong Kong. Chinese Biodiversity, 5(4), 287-292.

Zijlstra, J. D., Van't Hof, P., Baar, J., Verkley, G. J., Summerbell, R. C., Paradi, I., ... & Berendse, F. (2005). Diversity of symbiotic root endophytes of the Helotiales in ericaceous plants and the grass, *Deschampsia flexuosa*. Studies in Mycology, 53, 147-162.

454 Life Sciences Corp 2011. 454 sequencing system guidelines for amplicon experimental design. 454 Life Sciences Corp., Roche, Branford, CT.

Ryan Davies. Email: ryan@amatholeberries.co.za

Professor Dames Email: j.dames@ru.ac.za

Appendices

Appendix 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

B1. 70% ethanol

700 ml ethanol

300 ml distilled water

B2. Maize meal agar

Constituent components	g L^{-1}
Corn meal	17
Agar	15

B3. Modified Melin Norkrans

Constituent components	g L^{-1}
Glucose	20
Potassium di-hydrogen orthophosphate	1
Magnesium sulphate hexahydrate	0.5
Ammonium nitrate	0.5
1% Ferric citrate	0.5 ml
1% Zinc solution	0.5 ml
Thiamine	50 µg
Bacteriological agar	15

Media adjusted to pH 5

B4. Modified Fontana media (Fontana, 1963)

Constituent component	g L^{-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

B5. 2% Malt extract agar

Constituent components	g L^{-1}
Malt extract	30
Soy peptone	5.0
Agar	15

Appendix C

C1. 1% agarose gel

1 g agarose powder

100 ml distilled water

C2. TE (Tris/ EDTA) Buffer pH 8

Tris/ HCI pH 8 10 mM

EDTA pH 8 10 mM

Preparation

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

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

To make make 1X TBE Buffer working solution

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

Appendix D

D1. PCR Primers

Primer	Sequence 5'- 3'	Tm ˚C
ITS1F	- CTTGGTCATTTAGAGGAAGTAA-	49.7
ITS3F	-GCATCGATGAAGAACGCACG-	57
ITS4	-TCCTCCGCTTATTGATATGC-	52.1

D2. 454 Pyrosequencing fusion primers

Primer	Primer Description	Sequence of fusion Primer
ERM1F	Primers for 454 FLX pyrosequencing (MID1)	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTGCATCGATGAAGAACGCACG – 3'
ERM2F	Primers for 454 FLX pyrosequencing (MID2)	5'-CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGCATCGATGAAGAACGCACG – 3'
ERM3F	Primers for 454 FLX pyrosequencing (MID3)	5'-CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGCATCGATGAAGAACGCACG – 3'
ERM4F	Primers for 454 FLX pyrosequencing (MID4)	5'-CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGGCATCGATGAAGAACGCACG – 3'
ERM5F	Primers for 454 FLX pyrosequencing (MID5)	5'-CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGCATCGATGAAGAACGCACG – 3'
ERM6F	Primers for 454 FLX pyrosequencing (MID6)	5'-CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGCATCGATGAAGAACGCACG – 3'
ERM1R	Primers for 454 FLX pyrosequencing (MID1)	5' – CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTTCCTCCGCTTATTGATATGC– 3'
ERM2R	Primers for 454 FLX pyrosequencing (MID2)	5' – CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACATCCTCCGCTTATTGATATGC– 3'
ERM3R	Primers for 454 FLX pyrosequencing (MID3)	5' – CTATGCGCCTTGCCAGCCCGCTCAGAGACGCACTCTCCTCCGCTTATTGATATGC– 3'
ERM4R	Primers for 454 FLX pyrosequencing (MID4)	5' – CTATGCGCCTTGCCAGCCCGCTCAGAGCACTGTAGTCCTCCGCTTATTGATATGC– 3'
ERM5R	Primers for 454 FLX pyrosequencing (MID5)	5' – CTATGCGCCTTGCCAGCCCGCTCAGATCAGACACGTCCTCCGCTTATTGATATGC– 3'
ERM6R	Primers for 454 FLX pyrosequencing (MID6)	5' – CTATGCGCCTTGCCAGCCCGCTCAGATATCGCGAGTCCTCCGCTTATTGATATGC– 3'

Appendix E

Bacteriological agar PIPES (L^{-1})

- Add 15 g of bacteriological agar to 900 ml of distilled H_2O
- Adjust medium pH to 6.8 (adding NaOH).
- Add 30g of PIPES.
- Add NaOH pellet by pellet until pH is 6.8 while stirring.
- Autoclave at 121ºC for 15 minutes

CAS 10X (Chrome Azurol S) solution

Solution 1 (CAS):
Add 0.121g CAS in 100 ml distilled H_2O

Solution 2 (1 mM $FeCl_3 \cdot 6H_2O$, 10 mM HCl)
0.003gr $FeCl_3.6H_2O$ + 10 ml H_2O + 10 µl HCl (32%)

Solution 3 (use a bottle with enough room to add the other solutions)
0.146g HDTMA + 80 ml H_2O

To prepare CAS solution:

- To solution 3 (80mL)
- Add 100 mL of solution 1
- add 20mL of solution 2
- Autoclave

Preparation of medium.

After autoclaving add let the solutions cool down.

Add CAS solution to the bacteriological agar/PIPES.

Mix and pour on Petri dishes.

The medium will be dark blue after plates set.

Appendix F

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

Stock A

Chemical	**Quantity in L^{-1} 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 L^{-1} distilled water**
FeEDTA	3.0 g

Stock C

Chemical	**Quantity in L^{-1} distilled water**
$CaCl_2$	50 g

Stock D

Chemical	**Quantity in L^{-1} distilled water**
K_2SO_4	21.7 g

Stock E

Chemical	Quantity in L^{-1} distilled water
$(NH_4)_2SO_4$	105.0 g

Stock F

Chemical	Quantity in L^{-1} distilled water
$NaH_2PO_4.2H_2O$	2.5 g

Stock solutions were stores at 4°C.

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 up to 1L.