MYCORRHIZAL STATUS OF LEGUMINOUS PLANTS (FABACEAE) GROWING IN SOUTH AFRICAN GRASSLAND BIOME

BY

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DECLARATION

By submitting this thesis to the University of Johannesburg, I declare that the entire work contained therein is my own and that I have not previously submitted it for any other degree or professional qualification at this university or any other institution.

_____ Date: 24/03/2022

AFOLAKEMI ABIBAT ALIMI

DEDICATION

I dedicate this thesis to my loving parents, MR. KASALI AYODEJI ALIMI and MRS. MODINAT OMOLOLA ALIMI, whose unwavering support enabled me to attain the highest level of academic education. Thank you, dear parents, for not cutting my wings; thank you for letting me fly!

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LIST OF ABBREVIATIONS

%: Percentage

°C: Degree Celsius

°E: Degrees East

°S: Degrees South

µL: Microliter

mL: Mililiter

µm: Micrometer

AM: Arbuscular mycorrhizal

ANOVA: Analyses of variance

BD: Bulk density

C: Carbon

CCA: Canonical correspondence analysis

Chao1: Estimated richness

Cl: Clay

ClLm: Clay Loam

cm: Centimetre

Cu: Copper

D: Simpson Dominance index of species diversity

DNA: Deoxyribonucleic acid

e.g: For example

ERH: Extra-radical hyphae

g: Gram(s)

h: Hour(s)

H': Shannon-Wiener index of species diversity

HCl: Hydrochloric acid

IF: Isolation frequency

J: Pielou evenness index of species proportionality

K: Available potassium

KOH: Potassium hydroxide

Lm: Loam

LmSa: Loamy Sand

m: Metre

m: Minute(s)

mg: Milligram

mm: Millimetre

Mn: Manganese

ng: Nanogram

NH_4: Ammonia

NO_3: Nitrate

OTUs: Operation taxonomic units

PVLG: Polyvinyl-lactic acid-glycerol

P: Phosphorus/Available phosphorus

PCoA: Principal coordinate analysis

PCR: Polymerase chain reaction

PERMANOVA: Permutational analysis of variance

PPA: Pre-penetration apparatus

RA: Relative abundance

rpm: Revolutions per minute

rRNA: Ribosomal ribonucleic acid

SaCl: Sandy Clay

SaClLm: Sandy Clay Loam

SaLm: Sandy Loam

SD: Spore density

SEM: Standard Error of the Mean

SLs: Strigolactones

SR: Species richness

SSU rRNA: Small subunit ribosomal ribonucleic acid

v/v: Volume to volume

VT: Virtual taxa

w/v: Weight to volume

Zn: Zinc

ABSTRACT

Arbuscular mycorrhizal (AM) fungi play a significant role in the adaptation of plants to semi-arid environments. However, no information exists regarding the diversity of AM fungi in indigenous legumes of South Africa. Hence, for the first time, this study identified the AM fungal communities in the roots, topsoils, and rhizosphere soils of selected leguminous species from the Gauteng and Mpumalanga Provinces in South Africa, using morphological and molecular approaches. In addition, the influence of soil conditions on the diversity and community composition of AM fungi associated with the roots, topsoils, and rhizosphere soils was investigated. The colonisation of roots by AM fungi was assessed by staining and microscopic observation. Morphological diversity of AM fungal communities in topsoils was examined by spore-based identification, while the molecular diversity of AM fungal communities in roots and rhizosphere soils were explored by Illumina Miseq sequencing of the partial small subunit ribosomal ribonucleic acid (SSU rRNA) gene.

Microscopic assessments showed that the roots of all the studied legumes were colonised by AM fungi and the levels of colonisation were high. One hundred and seventy-two operational taxonomic units (OTUs) were found in the roots. The OTUs were categorised into eight AM fungal genera; *Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Diversispora*, *Glomus*, *Paraglomus*, and *Scutellospora*, with *Glomus* being the dominant genus. The community composition of root-colonising AM fungi differed amongst the studied plants within and between provinces. Correlation analysis showed that AM fungal OTU richness and Shannon-Wiener index of diversity were significantly correlated with available potassium, copper, manganese, zinc, and silt content. Canonical correspondence analysis indicated that nitrates, pH, manganese, and organic carbon were the main soil properties that considerably influenced AM fungal community composition in the roots of the legumes studied.

Twenty AM fungal species belonging to ten genera; *Acaulospora*, *Claroideoglomus*, *Entrophospora*, *Funneliformis*, *Gigaspora*, *Glomus*, *Rhizophagus*, *Scutellospora*, *Septoglomus*, and *Sieverdingia* were morphologically identified in the topsoils. *Glomus* was the dominant genus. The AM fungal spore density, species richness, and Shannon-Wiener index of diversity differed considerably among legumes within provinces, but no noteworthy variation was found between provinces. Correlation analysis revealed that spore density and diversity indices of AM fungi were significantly related to soil pH, nitrates, available phosphorus, available potassium, and bulk density. Canonical correspondence analysis showed that available phosphorus, available potassium, bulk density, zinc, and ammonium have significant effects on the composition of AM fungal communities in the topsoils of legumes.

A total of 322 and 335 OTUs were detected in the rhizosphere soils of legumes in Gauteng and Mpumalanga Provinces, respectively. The OTUs were grouped into eight genera; *Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Diversispora*, *Glomus*, *Paraglomus*, and *Scutellospora*, with *Glomus* being the predominant genus. The AM fungal OTU richness and Shannon-Wiener index of diversity varied significantly among legumes within province, but no significant difference was observed in all the diversity indices between provinces. Correlation analysis showed no significant relationship between soil properties and alpha diversity indices of AM fungi. However, canonical correspondence analyses indicated that available phosphorus and soil texture (sand and clay contents) were the significant drivers of AM fungal community composition in the rhizosphere soils of legumes.

This study found that the two approaches used for characterising AM fungal diversity produced different results regarding the communities (genera) detected. While the genera detected by Illumina Miseq sequencing were similar for roots and rhizosphere soils, only four out of the ten genera identified in the topsoils using the morphological technique were confirmed by the

Illumina Miseq sequencing. In spite of the inherent limitations of the morphological identification method, it provided significant insights into the diverse AM fungal communities (spores) that have the potential of colonising the legumes. The detection capacity of the Illumina Miseq sequencing technique was, however, more comprehensive and reliable. Overall, this study provides a valuable contribution to the biodiversity of AM fungi associated with indigenous legumes of South Africa and highlights the roles of soil environmental factors in shaping AM fungal diversity and community composition of legumes in semi-arid habitats.

Key words: Arbuscular mycorrhiza, Arbuscular mycorrhizal fungi, Indigenous legumes, Colonisation, Spore density, Diversity, Community composition, Illumina Miseq sequencing, Nested polymerase chain reaction, Operational taxonomic units.

OUTLINE OF THESIS

This thesis has seven chapters.

Chapter 1 provides a background to the study by describing AM symbiosis as an adaptive mechanism of plants to stressed ecosystems. This chapter also includes the problem statement, aims, and objectives.

Chapter 2 provides a review of the literature on mycorrhizal associations, AM symbiosis, establishment of AM symbiosis, classification and phylogeny of AM fungi, methods used in studying AM fungal diversity, and drivers of AM fungal diversity and community composition.

Chapter 3 provides the general methodology used in this study.

Chapter 4 depicts the AM status and root colonisation percentages, characterised the diversity of root-colonising AM fungal communities using Illumina Miseq sequencing of the partial SSU rRNA gene, and investigated the relationships between soil physico-chemical properties and AM fungal diversity and community composition.

Chapter 5 shows the isolated and quantified AM fungal spore population in the topsoils, identified AM fungal diversity using spore morphological features, and examined the relationships between soil physico-chemical properties and AM fungal diversity and community composition.

Chapter 6 presents the molecular diversity of AM fungal communities in the rhizosphere soils of legumes using Illumina Miseq sequencing of the partial SSU rRNA gene and explores the correlations between soil physico-chemical properties and AM fungal diversity and community composition.

Chapter 7 provides a summary of the previous chapters and concludes with recommendations for future research regarding the indigenous legume-AM fungal symbiosis in South Africa.

CHAPTER 1

INTRODUCTION

Plants require an adequate supply of mineral nutrients for survival, growth, and reproduction (Marschner, 1995). However, in semi-arid ecosystems, plants often face various challenges such as soil mineral nutrients deficiency and unfavourable conditions caused by biotic and abiotic factors. Most plants overcome these limitations by depending on mycorrhizal associations that allow them to maximize their nutrient acquisition abilities (Averill et al., 2019). The most prevalent of these mycorrhizal associations is the arbuscular mycorrhizal (AM) symbiosis, which is an ancient mutualism between nutrient-acquiring AM fungi and roots of most terrestrial plants. The AM association plays a crucial role in the nutritional adaptation of plants by enhancing the nutrient uptake of plants from nutrient-deficient soils and improving the overall fitness of plants to environmental stress situations (Karandashov and Bucher, 2005; Bonfante and Genre, 2008).

Fabaceae is the third-largest family of flowering plants (Graham and Vance, 2003). The family contains about 770 genera and 19,500 species divided into six subfamilies: Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioidea, and Papilionoideae (Azani et al., 2017). The cosmopolitan family, characterised by its distinct legume fruit, includes trees, shrubs, herbs, vines, and woody lianas (Schrire et al., 2005). Legumes are an important food source for man and fodder in livestock production (Reyes-Moreno and Paredes-Lopez, 1993; Yahara et al., 2013). Legumes are also noteworthy for their unique ecological role in nitrogen (N) fixation, an ability attributable to their symbiotic relationship with N-fixing soil bacteria, rhizobia (Postgate, 1998). Legumes are host plants for mycorrhizal fungi, and colonisation levels are generally high (Frioni et al., 1999; Oba et al., 2001). Legumes form arbuscular mycorrhizal and ectomycorrhizal associations (Sprent and James, 2007), although the former

is usually the most frequent type formed (Trappe, 1987; Berliner et al., 1989). Generally, the AM fungi enhance the phosphorus (P) nutrition of legumes, which consequently improves nodulation and N fixation (Francesco and Kerstin, 2004). This premise forms the basis of the high reliance of legumes on the AM symbiosis compared to other plant families (Albrecht et al., 1999; Muleta, 2010).

1.1 Problem statement

South Africa is a semi-arid country marked by drought, acidity, and low soil nutrient concentrations, especially P (Barnard and du Preez, 2004; Hawkins et al., 2005). However, the country hosts highly diverse indigenous legumes that are widespread in different biomes, including the Central Bushveld Bioregion, Savanna, Fynbos, and Grassland Biomes. With 24 tribes, 118 genera, and 1662 species of legumes indigenous to South Africa, including Lesotho and Swaziland (Trytsman et al., 2011), it is envisaged that the ability of these legumes to adapt to the low soil nutrient conditions may be, partly, a consequence of their association with AM fungi. However, unlike the indigenous legume-rhizobia symbiosis, which has been extensively explored in this country (Dagutat, 1995; Le Roux, 2003; Lindeque, 2005; Pérez-Fernández et al., 2008; Pule-Meulenberg et al., 2010; Lemaire et al., 2015; Beukes et al., 2019), there is little information on the legume-AM fungal symbiosis. Previous studies have only assessed the AM status of few indigenous legumes in the Cape Floristic Region and the forest biome of the Eastern Cape Province (Laughton, 1964; Hoffman and Mitchell, 1986; Allsopp and Stock, 1993; Hawley and Dames, 2004). So far, there is no known study conducted to establish the identity of AM fungal biodiversity in these legumes, especially for AM fungal species that perform symbiotic functions in the roots of these plants. This study is now necessary as about 30% of native leguminous plants of South Africa are currently at risk of becoming extinct or are of conservation concern (Yahara et al., 2013).

Given the significant ecological roles of AM fungi, knowledge about the identity of the AM fungal symbionts is essential to deepen our understanding of the adaptation mechanism of indigenous legumes of South Africa, and consequently, facilitate the exploitation of these fungi for future management and conservation of legumes. Besides, this information will add to the existing body of knowledge on the diversity of AM fungi indigenous to South Africa. Furthermore, understanding the impacts of soil environmental factors on AM fungal diversity and community composition will be valuable when selecting complementary plant-fungus combinations for specific ecological situations.

1.2 Aims and Objectives

This study aimed to characterise and compare the diversity and community composition of AM fungi in eleven indigenous leguminous plants from the Gauteng and Mpumalanga Provinces in South Africa based on morphological and molecular identification techniques, and to examine the influence of soil environmental factors on the diversity and composition of AM fungal communities in the studied plants.

The objectives of this study were to:

- assess the AM status and levels of root colonisation by AM fungi and describe the molecular diversity of root-colonising AM fungal communities in legumes within and between provinces.
- determine AM fungal spore population and compare the morphological diversity of AM fungal communities in topsoils of legumes within and between provinces.
- investigate and compare the molecular diversity of AM fungal communities in the rhizosphere soils of legumes within and between provinces.

- evaluate the effects of soil physico-chemical properties on the diversity and community composition of AM fungi present in the roots, topsoils, and rhizosphere soils.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycorrhizal associations

The symbiotic association between fungi and roots was discovered in *Monotropa hypopitys* L. by Kamienski (1881). However, the term 'mycorrhiza' which originates from the Greek words 'mykes' and 'rhiza' and literally translates to 'fungus-root', was coined four years later by Frank (1885). Mycorrhizal associations simply refer to the mutually symbiotic relationships between specialised soil fungi referred to as mycorrhizal fungi and plant roots, where fungal hyphae-scavenged soil nutrients are exchanged for plant-fixed carbon (Smith and Read, 2008). Generally, mycorrhizal symbioses involve fungal members of the phyla Ascomycota, Basidiomycota, and Glomeromycota and a wide range of land plants (Wang and Qui, 2006). Although most mycorrhizae occur in roots of higher plants, they can also be formed in the subterranean stems of some plants, thalli of bryophytes and pteridophytes, as well as sporophytes of most pteridophytes (Read et al., 2000). Based on their morphology, potential reciprocal benefits, phylogenetic relatedness, and involvement of specific fungi, these associations are broadly categorised into four. These are arbuscular mycorrhizal, ectomycorrhizal, ericoid mycorrhizal, and orchid mycorrhizal (Brundrett and Tedersoo, 2018).

So far, the majority (ca. 85.5%) of the vascular plants assessed for potential mycorrhizal associations are reported to be mycorrhizal, of which 1.5% are ericoid mycorrhizal (ERM), 2% are ectomycorrhizal (ECM), 10% are orchid mycorrhizal (OM), and 72% are arbuscular mycorrhizal (AM). Only 8% are completely non-mycorrhizal (NM), 7% are shown to have inconsistent NM-AM associations, while the remaining 0.5% represent the mycorrhizal assignment error rate in surveys of mycorrhizal plants (Brundrett and Tedersoo, 2018). Mycorrhizal symbioses are mutually beneficial for both partners; based on the evolutionary

success of the interaction (Kiers et al., 2011). Nonetheless, in conditions where soil nutrient level is high, or the mycorrhizal fungus is aggressive, the association may have detrimental effects on the host plant, possibly by competing with the host for nutrients or by interfering with other vital interactions (Johnson et al., 1997; Jones and Smith, 2004; Garrido et al., 2010). Mycorrhizal associations are characterised by reciprocal nutrient exchanges across the plant-fungus interface of living cells (Pfeffer et al., 2001). These exchange structures are either formed outside (ectomycorrhizal) or inside (endomycorrhizal) the root epidermal cells. In this way, other plant-fungus associations differ from mycorrhizae mainly due to the lack of specialised structures for exchange of resources (Brundrett, 2004; Adeleke et al., 2019).

The mycorrhizal fungi enhance the host's mineral nutrient acquisition through various mechanisms. The extraradical hyphae (ERH) of the fungus, which serve as the link between the host plant and soil, increase the absorptive surface area of roots for nutrients, even beyond the depletion zone (Smith and Read, 2008). Some mycorrhizal fungi also have a saprophytic ability that enables them to enzymatically digest and mobilise nutrients from organic substrates and transport them through the ERH to colonised roots (Warner, 1984; Koide et al., 2008). Furthermore, the ERH can cross soil air gaps and penetrate pores, down to large ultra-micropores to exchange water between soil pockets and host plants (Allen, 2007). Accordingly, water and nutrients are more efficiently mobilised into the roots of mycorrhizal plants compared to non-mycorrhizal plants (Mukerji et al., 1991). In some mycorrhizal associations, the fungal partners may connect multiple host plants together by common mycorrhizal networks (CMNs) that facilitate the long-distance transfer of signals, water, carbon, and other nutrients from the soil to interconnected plants (Teste et al., 2009; Bingham and Simard, 2011; Simard et al., 2012; Bücking et al., 2016).

2.2 Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizal symbiosis is evolutionarily the most ancient of all the mycorrhizal associations (Humphreys et al., 2010). Fossils and molecular records revealed that the rhizomes of first land plants (which appeared on land about 450 myr ago), were colonised by fungal structures (such as arbuscules, hyphae, and spores) that strikingly resemble the present-day arbuscular mycorrhizal (Simon et al., 1993; Remy et al., 1994). Subsequent morphological and molecular studies that followed these pioneering observations demonstrated that the earliest land plants developed in association with AM fungi and co-evolved with them to build up the mycorrhizal root systems of extant land plants (Brundrett, 2002). Based on these extensive analyses, it was concluded that terrestrial plants have a long-standing relationship with AM fungi, and that the evolution of ancestral plants on land would probably have not been possible without the nutrient-acquiring AM fungi (Pirozynski and Malloch, 1975, Malloch et al., 1980). The other types of mycorrhizal associations and incidence of non-mycorrhization emerged much later in plant lineages (Honrubia, 2009).

The AM association is formed in about 72% of land plants, making it the most predominant plant-microbe symbiosis on earth (Brundrett and Tedersoo, 2018). This association is widespread across diverse ecosystems including tropical and temperate forests (Lovelock et al., 2003; Saks et al., 2014), alpine (Zhang et al., 2016), dunes (Rodríguez-Echeverría and Freitas, 2006; Jobim and Goto, 2016), deserts (Zhang et al., 2011), grasslands (Lugo and Cabello, 2002; Van Geel et al., 2018), aquatic (Radhika and Rodrigues; 2007; Moora et al., 2016), arid and semi-arid (Uhlmann et al., 2004, Barea et al., 2011), and agroecosystems (Douds and Millner, 1999; Oehl et al., 2003).

The AM association is characterised by intra- and intercellular penetration of the obligate biotrophic AM fungi into the root cortex of host plants, producing highly branched tree-like

structures referred to as arbuscules (Smith and Read, 2008). While arbuscules are the main diagnostic structure of the AM association, other structures including vesicles, intraradical hyphal coils, intercellular hyphae, and spores can also be produced in the host's roots (Dickson, 2004). The formation of these structures is dependent on the type of AM fungi colonising the host roots. For instance, many of the species of the genus *Glomus* frequently produce spores inside the roots (Rodrigues and Rodrigues, 2020).

The arbuscules are believed to be the functional site of nutrient exchange between the symbionts (Cox and Tinker, 1976), although resources can also be exchanged through the intraradical hyphal coils (Johnson and Gehring, 2007). Arbuscules are ephemeral structures, and their lifespan varies in different plant species (Toth and Miller, 1984; Alexander et al., 1988). Vesicles are lipid-rich balloon-like structures formed within roots (Smith and Read, 2008). They function primarily as storage organs of the fungus but can also serve as infective propagules (Biermann and Linderman, 1983). The ERH absorb mineral nutrients and water from the soil and transport them to the host plant through the intercellular hyphae (Jakobsen et al., 1992; Finlay, 2008). Spores are multi-nucleate and may be formed singly or in clusters called sporocarps (Morton, 1988). The spores function as propagules, resting stages, and storage structures for presymbiotic growth (Morton, 1993). Auxiliary cells are swollen structures produced terminally by extraradical hyphae. They are formed by some species within the families Gigasporaceae, Pacisporaceae, and Scutellosporaceae (Bianciotto and Bonfante, 1999). While the biological functions of auxiliary cells remain speculative, studies have shown that they could function in reproduction, nutrition, and storage (Jabaji-Hare et al., 1988; Pons and Gianinazzi-Pearson, 1985; Morton and Benny, 1990; Pearson and Schweiger, 1993; de Souza and Declerck, 2003).

The principal function of the AM symbiosis is to enhance mineral nutrients uptake, particularly P, of host plants (Smith and Read, 2008). In addition to nutritional benefits, AM fungi can enhance plant health through protection against soil-borne pathogens (Lewandowski et al., 2013; Delavaux et al., 2017) and improved tolerance to environmental stresses such as drought (Augé, 2001), salinity (Evelin et al., 2009; Porcel et al., 2012), and heavy metal toxicity (Javaid, 2011; Doubková et al., 2012). As a reciprocal reward for the symbiotic services, host plants deposit a substantial amount (up to 20%) of photosynthetically fixed carbon into their rhizosphere, thereby nourishing and influencing the growth and reproduction of AM fungal symbionts (Parniske, 2008). AM fungi have also been reported to influence plant diversity and community structure (O'Connor et al., 2002), play vital roles in nutrient cycling (Bender et al., 2015), and improve soil aggregate stability (Rillig, 2004a). These multi-functional roles of AM fungi make them potential bio-inoculants, bio-protectants, and bio-control agents for environmentally sustainable agriculture and ecological restorations of degraded habitats (Cameron, 2010; Abiala et al., 2013; Berruti et al., 2016).

It is noteworthy that plant responsiveness to AM, dependency of plants on AM, and the AM status of plants are distinctive traits (Janos, 2007; Moora, 2014). Plant responsiveness to AM, also referred to as a measure of AM fungus effectiveness, connotes the difference in growth between plants with and without AM at any specified level of soil fertility i.e., P availability. Arbuscular mycorrhizal dependency describes the lowest level of P availability at which plants can grow without the AM association. Thus, plants can be obligately dependent (plants that are consistently colonised by AM fungi), facultatively dependent (plants that are colonised by AM fungi under some soil conditions but not others), or non-dependent on AM (plants whose roots are highly resistant to colonisation by AM fungi) (Brundrett, 2002). The AM status of plants, on the other hand, indicates the presence or absence of AM fungal colonisation in plants and can provide information about plant reliance on the symbiosis (Moora, 2014). This reliance is

presumed to be low among non-mycorrhizal plants, intermediate among facultative mycorrhizal plants, and high in obligate mycorrhizal plants (Menzel et al., 2018).

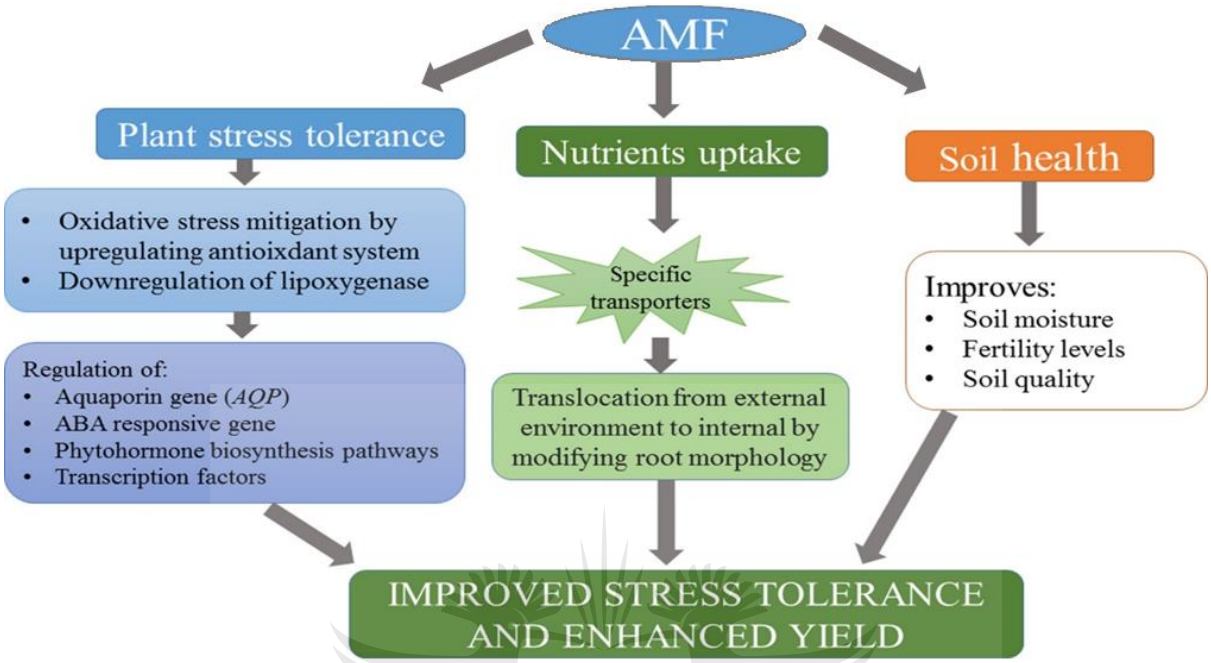


Figure 2.1: Schematic representation of the multi-functional roles of arbuscular mycorrhizal fungi in the ecosystem (Begum et al., 2019).

2.3 Establishment of arbuscular mycorrhizal symbiosis

Under optimal water and temperature conditions, spores of AM fungi can germinate and produce hyphae independent of a host plant (asymbiotic hyphal growth) using their triacylglyceride reserves (Buée et al., 2010). Nevertheless, root colonisation is indispensable for AM fungi, as this provides the only means by which they can complete their life cycle and produce the next generation of spores (Bonfante and Genre, 2010). Colonisation of new host roots by AM fungi can arise from three main sources of inoculum: spores (which are considered the most important source of inoculum), colonised root fragments, and extraradical hyphae of an already established AM association. These are collectively referred to as propagules (Smith and Read, 2008). AM fungal propagules are dispersed by wind, water, small animals, and

human activities (Warner et al., 1987; Janos and Sahley, 1995; Mangan and Adler, 2002), and each propagule exhibits different colonisation capabilities (Klironomos and Hart, 2002).

The development of the AM association is preceded by a complex molecular dialogue (perception and exchange of signals) that keeps the symbiotic partners informed about their proximity (Bucher et al., 2009). This interaction begins when plant roots exude carotenoid-derived plant hormones identified as strigolactones (SLs) into the rhizosphere (Besserer et al., 2006; Parniske, 2008). The perception of strigolactones by AM fungi stimulates spore germination and hyphal branching (Akiyama et al., 2005). With this event, the pre-symbiotic growth of AM fungi is initiated.

In response to SLs, AM fungi secrete diffusible fungal signalling molecules referred to as "Myc-factors" (Maillet et al., 2011; Genre et al., 2013). The presence of Myc-factors activates calcium oscillations (plant symbiosis-related genes) in root epidermal cells that reprogram the root for colonisation (Kosuta et al., 2003). Once this chemical contact has been established between the fungus and the root, the pre-symbiotic phase of the AM interaction terminates in a physical encounter between the symbiotic partners, wherein the fungi hyphal tip touches the root surface (Bonfante and Genre, 2010). The hypha may wander for several centimetres along the root surface in search of the best location to initiate penetration. Once found, the hypha penetrates the root and branches between the root epidermis to form AM-specific appressorium referred to as hyphopodium on the root surface (Genre et al., 2005; Parniske, 2008). The formation of an appressorium is one of the first morphological signs that recognition between the plant and the fungus has occurred. This stage marks the symbiotic growth of the AM fungus (Van Buuren et al., 1999).

When the hyphopodium becomes tightly adhered to the root surface, a pre-penetration apparatus (PPA) is produced by the plant epidermal cells. The PPA guides the mature hypha

that extends from the hyphopodium towards the root cortex (Parniske, 2008). Immediately the hypha enters the cortical cells, it spreads within the cortex to form either arbuscules (Arum-type morphology) or intraradical hyphal coils (Paris-type morphology) (Smith and Read, 2008). Although the determining factors (whether the host plant, fungus, or environment) defining the two different morphologies are still poorly understood, co-occurrence of both morphological types has been observed (Dickson et al., 2007). Upon getting nourished through the arbuscules or hyphal coils, the AM fungus develops an extensive ERH, which grow out of the root to explore the soil for nutrients and new hosts. The life cycle of AM fungus is completed after the formation of spores on the ERH or inside the roots.

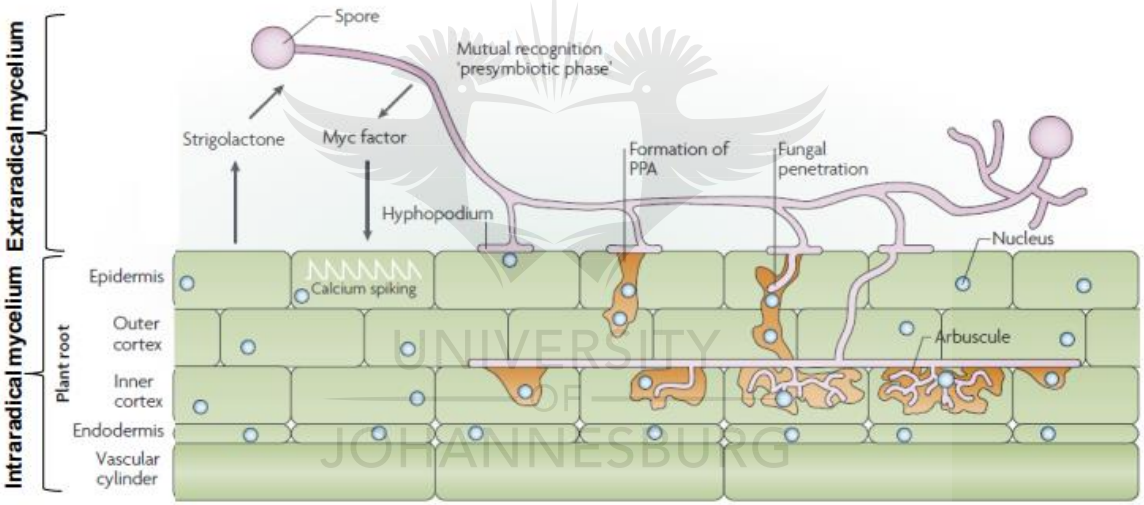


Figure 2.2: Schematic illustration of the development of arbuscular mycorrhizal symbiosis (Parniske, 2008).

2.4 Classification and phylogeny of arbuscular mycorrhizal fungi

The AM fungi belong to the phylum Glomeromycota (Schüßler et al., 2001). Before the AM fungi became a monophyletic phylum, it was classified in the phylum Zygomycota, family Endogonaceae (Thaxter, 1922). This was due to an observational error in a sporocarp

containing spores of both *Endogone* and *Glomus*, and, by their seeming morphological resemblance, one was assumed to be an anamorph of the other (Thaxter, 1922). After a thorough examination of the differences in spore characteristics of AM fungi and zygospores of members of the order Endogonales, coupled with the recognition of the asexual and obligate symbiotic nature of AM fungi (as opposed to sexual reproduction in Endogone species), a new order, the Glomales, was separated from the Endogonales. Nonetheless, Glomales was still retained in the Zygomycota because of the homology of their aseptate hyphae (Morton and Benny, 1990).

Schüßler et al. (2001) detected the relationship between AM fungi and other fungi using molecular analyses of the small subunit ribosomal RNA (SSU rRNA) gene sequences. AM fungi were finally removed from the polyphyletic Zygomycota and reclassified into a new monophyletic fungal phylum, the Glomeromycota. The resulting phylogenetic tree showed that the Glomeromycota are a closely related and sister clade to the Basidiomycota and the Ascomycota (Figure 2.3). To eliminate confusion and provide a robust systematics of the Glomeromycota, Redecker et al. (2013) published an evidence-based consensus for the classification of the phylum Glomeromycota. The classification grouped the phylum into one class; Glomeromycetes; 4 orders, 11 families, and 26 genera, out of which 21 are supported by sufficient evidence (Figure 2.4). So far, there are 341 morphologically-defined species of AM fungi (http://www.amf-phylogeny.com/amphylo_species.html, accessed 5 December 2021). Molecular based studies of ribosomal DNA sequences from environmental samples have, however, shown that AM fungi are highly diverse (Moora et al., 2011; Öpik et al., 2013).

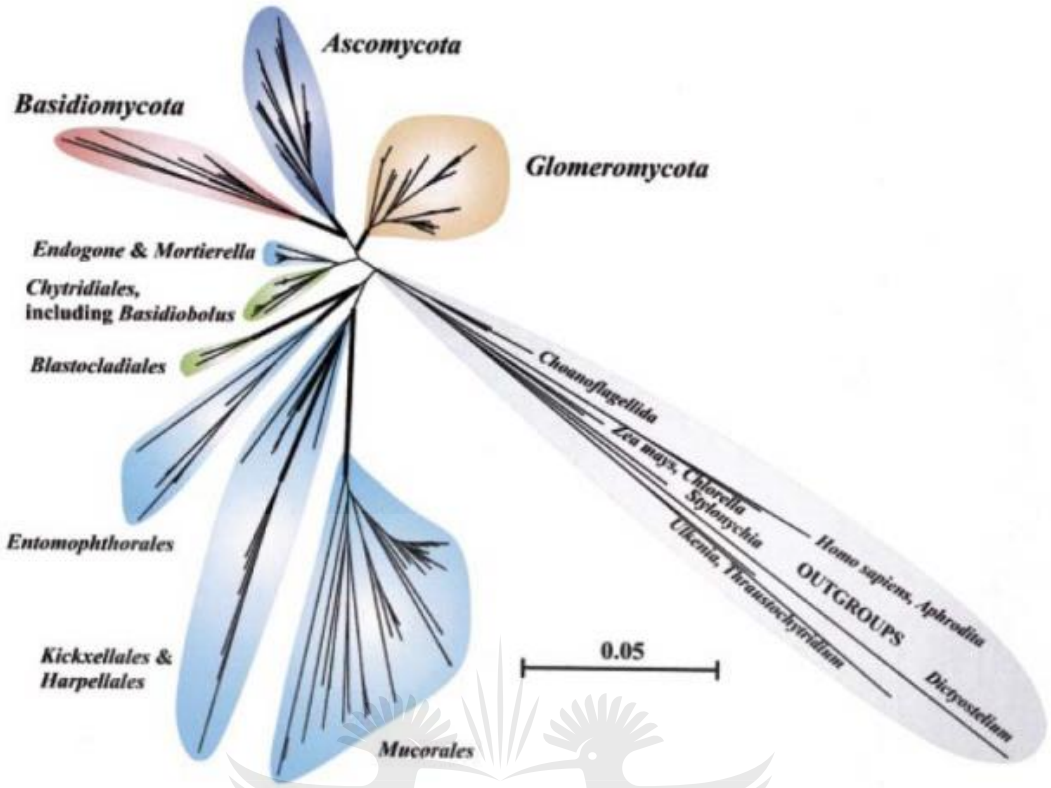


Figure 2.3: Phylogeny of arbuscular mycorrhizal fungi based on SSU rRNA sequences (Schüßler et al., 2001).

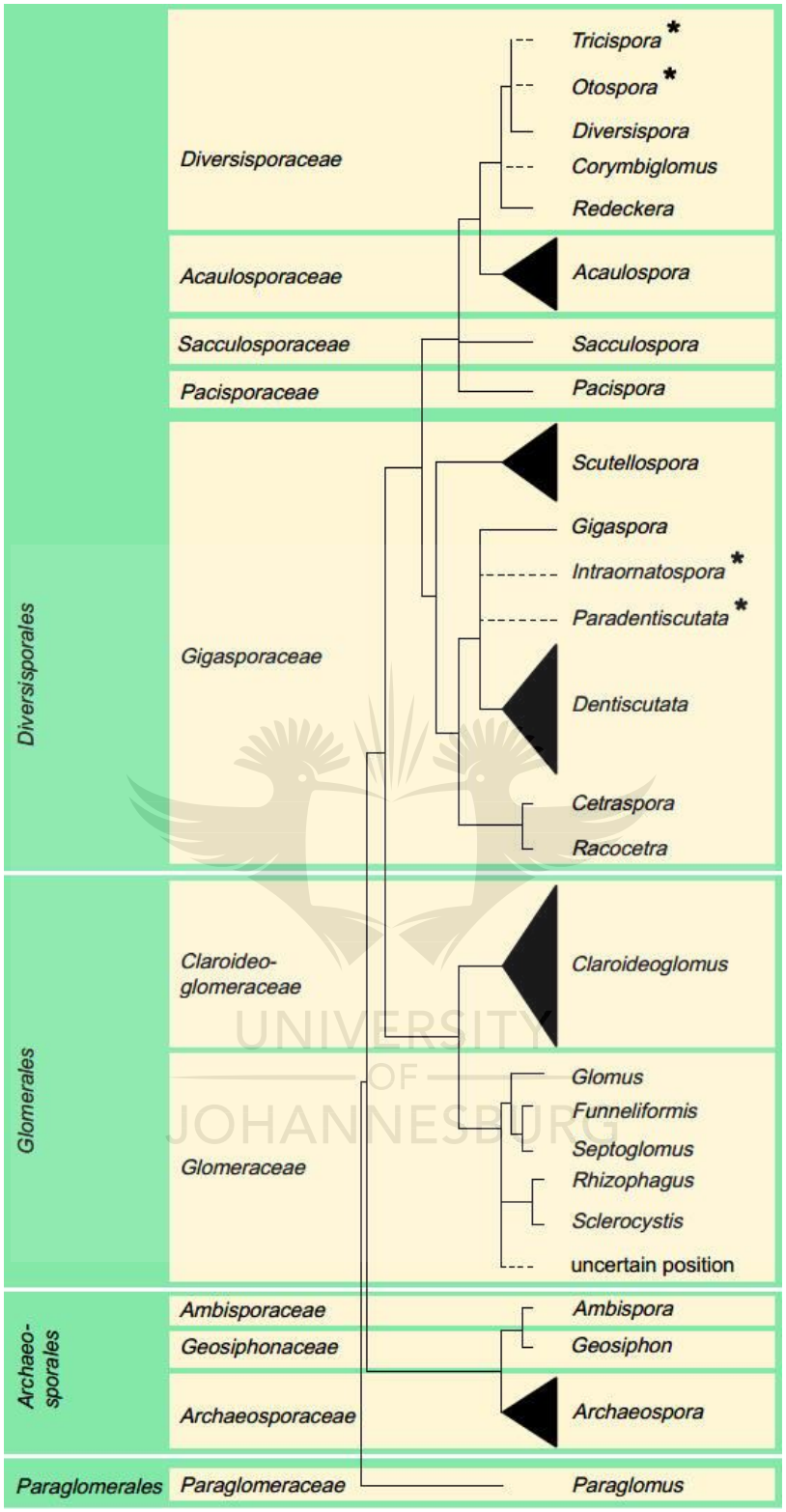


Figure 2.4: Consensus classification for the arbuscular mycorrhizal fungi (Glomeromycota). Dashed lines indicate genera of uncertain position, genera marked by asterisks are questionable with respect to data used for description and/or with respect to phylogenetic position, inverted

triangles indicate taxa that were already rejected in previous publications but now reinstated (Redecker et al., 2013).

2.5 Methods used in studying arbuscular mycorrhizal fungal diversity

Different techniques have been employed for the identification and diversity analyses of AM fungi (Krishnamoorthy et al., 2017). The conventional approach to AM fungal taxonomy is based primarily on the morphology and ontogeny of spores extracted and/or trapped from soils (Wetzel et al., 2014). In this method, families and genera were distinguished mainly by the mode of spore formation, while species are delineated using spore morphological features such as colour, shape, size, texture, surface ornamentation, spore contents, and spore-wall properties (Morton, 1988; Schenck and Perez, 1990). However, this technique has been faulted due to limitations and bias in its detection capacity (Sanders, 2004). Sometimes, it is difficult to distinguish between species in distantly related genera using spore morphology. For instance, some AM fungal genera (*Archaeospora* and *Paraglomus*) share very similar morphological features but were found to be phylogenetically distant (Morton and Redecker, 2001). Also, the formation of dimorphic spores in some AM fungal species (e.g., *Archaeospora leptoticha*, *Glomus dimorphicum*) creates confusion during identification.

Moreover, alteration in spore morphological characters as a result of biotic and environmental influences make accurate identification of spores in field samples quite challenging (Sanders, 2004). A supplementary strategy used for identifying field-collected spores is by setting up trap cultures. With trap cultures, species occurring in the field are propagated using soil samples from the field site and a suitable host plant to obtain many healthy spores containing all morphological traits for accurate identification (Vieira et al., 2020). However, the host species used in trap cultures may influence which AM fungal species are detected (Jansa et al., 2002).

Additionally, several AM fungal taxa/lineages do not stain or stain weakly using standard staining techniques, thus impeding microscopic observations and correct identification (Redecker et al., 2000). Besides the formation of spores, plant roots are also colonised by fungal structures, but morphological analysis of root colonisation using intraradical structures can only allow identification to family level (Merryweather and Fitter, 1998). Hence, identification of AM fungi based solely on spore morphology may underestimate the true AM fungal diversity, since spore populations in the soil do not reflect a symbiotically active AM fungal community in roots (Clapp et al., 1995) and cryptic AM fungal species that were not sporulating in field conditions cannot be detected by spores (Rosendahl, 2008).

Different biochemical markers such as glomalin-related soil protein (Lovelock et al., 2004; Rosier et al., 2006), fatty acids (Graham et al., 1995; Ngosong et al., 2012), and isozymes (Hepper et al., 1986; Rosendahl et al., 1989; Rosendahl and Sen, 1992) have also been analysed for AM fungal identification, but their usage as AM fungal markers is quite limited.

Molecular identification approaches have helped to circumvent the analytical difficulties associated with spore-based identification and have revolutionised the ecological studies of AM fungi. The application of diverse deoxyribonucleic acid (DNA)-based techniques has enabled the characterisation of AM fungi in plant roots, revealed several cryptic taxa in the soil, and have transformed the understanding of the taxonomy and phylogeny, ecology, genetics, evolution, and functional diversity of these fungi (Sharmah et al., 2010). A further breakthrough that has led to a significant improvement in AM fungal community profiling was the introduction of the high throughput sequencing technologies such as the 454-pyrosequencing, Illumina Miseq, Ion Torrent, and PacBio SMRT sequencing (Margulies et al., 2005). These sequencing technologies have been used to efficiently characterise the AM fungal communities in environmental samples in space and time (Öpik et al., 2009; Schlaeppi et al.,

2016; Xu et al., 2017). The Illumina Miseq is the most widely adopted sequencing platform due to its high-throughput, high-quality read cover, lower rate of erroneous sequences, and cost-effectiveness (Lindahl et al., 2013).

Since the emergence of molecular identification methods, three nuclear encoded ribosomal rRNA regions, i.e., the partial small subunit (SSU) rRNA gene, the internal transcribed spacers (ITS1, 5.8S and ITS2), and the partial large subunit (LSU) rRNA gene are the commonly used molecular markers for ecological studies of AM fungi (Clapp et al., 2003; Sharmah et al., 2010). This genome is ubiquitous and composed of highly conserved as well as variable domains that can show evolutionary relationship among AM fungal lineages and distinguish taxa at many different levels. It is important to note that the choice of rRNA region is vital because each rRNA region differs in its ability to distinguish closely related AM fungal species (intra and inter-species resolution power), coverage of taxonomic diversity (due to PCR primer specificity and efficiency), and in the extent to which well-determined sequences are represented in public reference sequence databases (Rodriguez et al., 2004; Stockinger et al., 2010). Nevertheless, the SSU rRNA gene has been the most frequently amplified locus (Helgason et al., 1999; Öpik et al., 2008; Zeng et al., 2019). This is because primer pairs that amplify most known AM fungal families exist for this region, hence providing a wider view of the AM fungal community. In addition, the reference sequence database contains more sequences from this region compared to the other genomic regions (Öpik et al., 2010), thus facilitating easy comparison between studies.

2.6 Drivers of arbuscular mycorrhizal fungal diversity and community composition

The AM fungal diversity and community composition vary considerably within regional and global landscapes (Öpik et al., 2006). This variation is influenced by both deterministic and stochastic processes (Chaudhary et al, 2008; Dumbrell et al., 2010). Deterministic forces such

as host plants (Johnson et al., 2004; Pivato et al., 2007), climate (Dumbrell et al., 2011; Gai et al., 2012; Shi et al., 2014), and soil characteristics (Coughlan et al., 2000; Moebius-Clune et al., 2013; Camenzind et al., 2014; Sheldrake et al., 2017) can directly influence the available habitat for a given AM fungal species. This ultimately affects its ability to colonise and exist in each location. Similarly, stochastic processes (intrinsic properties of AM fungal species) such as sporulation rate and dispersal ability can determine whether AM fungal species will be present in each location, hence its abundance and distribution (Lekberg et al., 2012; Chaudhary et al., 2014). Studies have also reported that soil disturbances and land-use type can impact the diversity and composition of AM fungal communities (Xiang et al., 2014; Lekberg et al., 2012; Stover et al., 2018).

CHAPTER 3

GENERAL METHODOLOGY

3.1 Description of study areas

The plants were collected from the Gauteng and Mpumalanga Provinces of South Africa. These provinces form part of the grassland biome of South Africa and receive summer rainfall (Rutherford and Westfall, 1986). The vegetation of the study areas is predominated by grasses (Poaceae), but there are also a variety of forbs, and woody species are restricted to specific areas (Mucina et al., 2006; Lötter et al., 2014). The soil of the study areas is characterised into fourteen groups, i.e., organic, humic, vertic, melanic, silicic, calcic, duplex, podzolic, plinthic, oxidic, gleyic, cumulic, lithic, and anthropic (Fey, 2010). Gauteng is positioned on latitude 26.2708°S and longitude 28.1123°E, with the median elevations of 1,512 m above sea level. The average lowest and highest temperature range from 10.2–24.8 °C, and average annual rainfall is 771 mm per year (SAWB, 1997). The Mpumalanga Province is located on latitude 29.8129°S and longitude 30.6364°E. There are three distinct physiographic regions in the province; the Highveld in the west, where the altitude ranges from 1,200–1,800 m above sea level; the forested Drakensberg mountains in the east, where the altitude exceeds 2,300 m above sea level; and the lowland Lowveld in the northeast. In the Mpumalanga Province, temperatures fluctuate according to elevation, from a mean of 10 °C in the Highveld and an average of 23 °C in the subtropical Lowveld. The annual precipitation increases from west to east, averaging 341–1933 mm (SAWB, 1997).

3.2 Field sampling and processing of samples

Plant collection took place in February 2019. Plant collecting permits were sourced from the appropriate authorities before collection. Eleven species from the family Fabaceae were

sampled from their natural habitats in Gauteng and Mpumalanga Provinces (Figure 3.1). The distinctive fruit and floral morphology were used as diagnostic characters for correct identification of the plants in the field. Correct identification of the species names was later authenticated at the University of Johannesburg herbarium (JRAU), where voucher specimens were also deposited. In each province, three replicate plants were randomly collected for each legume species. Replicates were collected at more than 10 m apart to ensure the independence of samples. The entire legume root systems were gently excavated, and the soils bound to the surface of roots were carefully scraped with a clean brush. The scraped soils were labelled the rhizosphere soil samples for DNA extraction.

Topsoils (0–20 cm depth) were collected around each replicate plant from different points with a sterile soil auger (Eijkelkamp Soil & Water, EM Giesbeek, Netherlands). Additionally, tubular samples of soil were collected per replicate using a bulk density sampler (Eijkelkamp Soil & Water, EM Giesbeek, Netherlands) for soil bulk density calculation. All samples were collected in sterile Ziplock bags and delivered in freezing boxes to the laboratory. Fine roots of each replicate plant per legume species were carefully cleaned with water to remove the soil, then dried with tissue paper, and cut into 1 cm long pieces. A subsample of the roots was preserved in 50% ethanol prior to examining the roots for colonisation by AM fungi, while the remaining fraction was stored at −80 °C until processing for DNA extraction. Similarly, the rhizosphere soil sample of each replicate plant was stored at −80 °C until processing for DNA extraction. An aliquot of topsoils of each replicate plant was stored at 4 °C before the spores were isolated, while the other fraction was air-dried, sifted through a 2 mm sieve, and used for the assessment of soil physico-chemical properties.

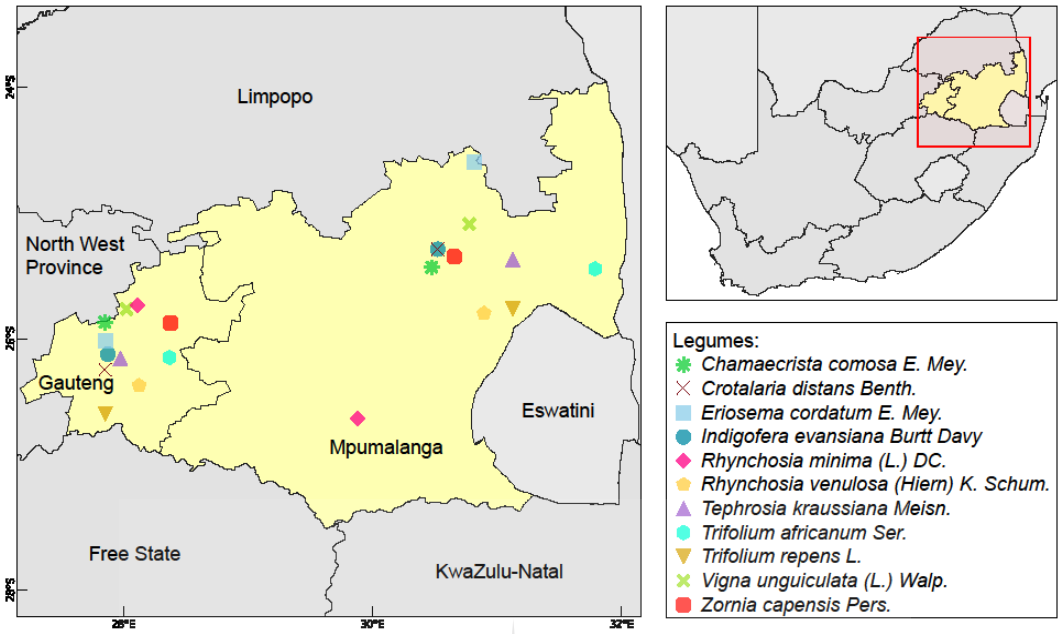


Figure 3.1: Map of South Africa showing the points where legumes were sampled in Gauteng and Mpumalanga Provinces.

3.3 Soil analyses

Thirteen different soil properties; pH, available potassium (K), organic carbon (OC), available phosphorus (P), nitrate (NO_3^--N), ammonium (NH_4^+-N), copper (Cu), manganese (Mn), Zinc (Zn), particle size distribution (sand, silt, and clay), and bulk density (BD) were analysed at the Agricultural Research Council-Institute for Industrial Crops, South Africa, using standard methods described below.

3.3.1 Soil pH

Soil pH was measured from a 1:2.5 soil suspension in 1N potassium chloride (KCl). 25 mL of KCl was added to 10 g of air-dried soil weighed into a 100 mL bottle. The bottle was capped and shaken occasionally for 1 hr on a reciprocal shaker. Then, the glass electrode of the pH

meter was immersed into the soil suspension and pH was recorded when the reading became stable.

3.3.2 Available Potassium (K)

Available K was determined by the ammonium acetate ($NH_4CH_3CO_2$) method of Schollenberger and Simon (1945). 50ml of 0.5M $NH_4CH_3CO_2$ (pH 7) was added to 10 g of soil. The mixture was shaken for 30 m and filtered. Therefater, the available K in the filtrate was determined by Atomic Absorption Spectrophotometer after dilution with Lanthanum Chloride against standard of known concentration.

3.3.3 Organic Carbon (OC)

The quantification of OC was based on the Walkley-Black chromic acid wet oxidation method (Walkley and Black, 1934). 5 g of soil was treated with 10 mL of 1N potassium dichromate (K_2Cr_2O7) solution and then mixed with 20 mL of concentrated sulphuric acid (H_2SO_4). The mixture was heated at 170–180 °C for 5 m and cooled at room temperature. The solution was transferred into a 250 mL flask, and unreacted K_2Cr_2O7 was determined by titrating with 0.2 M ferrous sulfate ($FeSO_4$). Thereafter, OC was calculated from the difference in $FeSO_4$ used between the blank and the soil solution.

3.3.4 Available phosphorus (P)

Available P was assessed by the Bray 1 method (Bray and Kurtz, 1945). 2 g of soil was shaken manually with Bray 1 solution for 60 s. The total phosphate concentration was then determined by automated colorimetric analysis at 660 nm.

3.3.5 Nitrate (NO_3^--N)

Nitrate was assessed using Sonneveld and Van den Ende's (1971) protocol. 5 g of soil was reacted with 0.1N ammonium chloride colour reagent and reduced by copper cadmium

reduction column. The solution produced a pink compound and NO_3^--N was measured by Segmented Flow Analayzer at at 520 nm.

3.3.6 Ammonium nitrogen (NH_4^+-N)

Ammonium nitrogen was calculated using the ammonia-selective electrode method (Banwart et al., 1972). 10 g of soil was treated with ammonium colour reagent and sodium hypochlorite. The solution produced a blue compound and NH_4^+-N was measured using Segmented Flow Analayzer at at 660 nm.

3.3.7 Extractable Copper (Cu), Manganese (Mn), and Zinc (Zn)

Concentrations of extractable Cu, Mn, Zn were obtained by acid digestion of soil (Jackson, 1958). 50 mL of 0.1N HCl solution was added to10 g of soil. The mixture was shaken for 15 m on a reciprocal shaker and filtered. Then Cu, Mn, Zn were determined by Atomic Absorption Spectrophotometer at 324 nm, 213 nm, and 280 nm, respectively.

3.3.8 Particle size distribution

The Bouyoucos hydrometer procedure was used to determine soil particle size distribution (Bouyoucos, 1962). 25 g of soil was weighed into a dispersing cup, and 100 mL of 5% dispersing solution (Calgon 33:7) was added. The dispersing cup was attached to a mixer and mixed for 60 s. The solution was transferred into a 1000 mL cylinder and allowed to stand overnight to equilibrate. Then, the plunger was inserted into the suspension and mixed gently for 30 s until a uniform suspension was obtained. The plunger was removed, the hydrometer was gently inserted into the suspension, and the reading recorded at 40 s.

3.3.9 Bulk Density (BD)

Bulk density (BD) was measured by drying soil samples at 105 °C for 48 h (ISO, 2017). Then, BD was expressed as:

$$BD\ (g/cm^3) = \frac{\text{Oven-dried weight of soil (g)}}{\text{volume of core } (cm^3)}$$

CHAPTER 4

MOLECULAR DIVERSITY OF ROOT-COLONISING ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES (This chapter has been published in Rhizosphere 19, 100405)

4.1 Introduction

Semi-arid ecosystems are harsh environments for plants to grow in (Alguacil et al., 2016; Oyediran et al., 2018). The reason is that these habitats are characterised by abiotic stresses (low soil nutrient content, drought, and salinity) that limit the establishment, development, and productivity of plants (Martínez-García et al., 2011; Zhao et al., 2017). Most plants form a mutualistic symbiosis with AM fungi as a crucial adaptation and survival strategy in such extreme environments (Mohammad et al., 2003; Barea et al., 2011). The AM fungi are a specialised category of valuable soil-dwelling microorganisms that form obligate symbiotic interactions with roots of many terrestrial plants in almost all ecosystems (Chen et al., 2018; Wang et al., 2019). These microbes play essentail role in the health and fitness of host plants by enhancing the uptake and transfer of soil nutrients and improving resistance to pathogen infections and environmental challenges (Barea et al., 2002; Jung et al., 2012). In addition, the AM fungi impact several critical ecosystem functions such as nutrient cycling, soil aggregate stability, and plant diversity and succession, thus highlighting the importance of these fungi in ecosystem sustainability (Rillig, 2004a; Van Der Heijden et al., 2006; Jansa et al., 2011).

Until the last two decades, determining the identity of AM fungi within plant roots seemed practically impossible (Simon et al., 1992). The traditional method of studying AM fungal diversity involves morphological inspection of fungal structures within plant roots, as well as the determination and identification of spore morphotypes in soils (Bencherif et al., 2016; Vilcatoma-Medina et al., 2018; Baltruschat et al., 2019; Wang et al., 2021). However, the

morphology of hyphae can only determine the presence of AM fungi in the roots and identifying the AM fungal populations using these intraradical structures below the family level is not possible (Merryweather and Fitter, 1998). Moreover, various Glomeromycotan taxa exhibit differential sporulating patterns depending on biotic and abiotic circumstances (Sanders, 2004). As a result, spore richness does not always imply a functionally active AM fungal population colonising plant roots, and therefore, may underestimate the overall AM fungal diversity (Clapp et al., 1995). Remarkably, the introduction of molecular identification methods, particularly high-throughput sequencing technologies (Margulies et al., 2005), has considerably changed our understanding of AM fungal ecology by revealing an unusually high diversity of these fungi in field root samples (Öpik et al., 2013). High-throughput sequencing approaches are now frequently used in molecular profiling of AM fungal communities in environmental root samples in many ecosystems (Xu et al., 2017; Zeng et al., 2019; Ezeokoli et al., 2020).

Based on previous research (Deepika and Kothamasi, 2015; Alguacil et al., 2016; Casazza et al., 2017; Sarkodee-Addo et al., 2020), soil factors could shape the diversity and community make-up of AM fungi inhabiting roots at various sites or in different habitats. In addition, there is functional diversity among AM fungi, and a single root segment can be colonised by multiple AM fungal species at the same time (Leake et al., 2004; Helgason et al., 2007). Therefore, to determine the ecological impact of AM fungi on plant communities, researchers must first characterise the AM fungal communities present in plant roots and identify the possible factors that control these communities and their associations with host plants. Such information could eventually strengthen plans aimed at the management of plant conservation. Therefore, the current study utilised the Illumina Miseq sequencing of the partial SSU rRNA gene to assess the diversity and composition of active AM fungal communities associated with the roots of

eleven indigenous legume species in Gauteng and Mpumalanga Provinces, and then examined the effect of soil physico-properties on AM fungal diversity and community composition.

4.2 Materials and methods

4.2.1 Assessment of root colonisation by AM fungi

Root colonisation by AM fungi was studied utilising the procedure described by Phillip and Hayman (1970). Preserved roots of each replicate plant per legume species per province were rinsed thoroughly under running water to get rid of the ethanol. The roots were then cleared in 10% potassium hydroxide (KOH) by incubating in a microwave for 2 m (Dalpé and Séguin, 2013). Roots that remained dark after clearing in KOH were bleached in a freshly prepared 30% (v/v) solution of alkaline hydrogen peroxide (H_2O_2) at room temperature for 60 m. Cleared roots were rinsed several times and then acidified in 2% hydrochloric acid (HCl) solution at room temperature overnight to increase staining efficiency. The HCl was decanted, roots were stained overnight with 0.05% trypan blue-lactic acid solution (Sigma Aldrich, USA), and de-stained by transferring into a freshly prepared 1:1 (v/v) lactoglycerol solution for 48 h. Stained root fragments (six slides; five root pieces per slide) were mounted and observed at 40× magnification under a light microscope (Olympus CX23). Roots were scored for colonisation by AM fungi when the following structures were observed: arbuscules, vesicles, hyphal coils, intra- or extraradical hyphae, and spores. Photos of AM fungal structures were taken using a microscope-mounted 5.0-megapixel digital camera (Leica DFC480, Cambridge, UK).

4.2.2 Estimation of percentage of root colonisation by AM fungi

The percentage of root colonisation by AM fungi was estimated by the gridline intersect method described by Giovannetti and Mosse (1980). Thirty stained root segments of each replicate plant per legume species per province in lactoglycerol were randomly dispersed in a

9-cm diameter Petri dish with 1-cm square inch inscribed grid lines. Horizontal and vertical grid lines were scanned under a stereo-microscope (Olympus SZX16). The total number of roots intersecting grid lines and the total number of intersections having colonised roots were recorded. Percentage of root colonised by AM fungi was then calculated as:

$$\text{Percentage of root colonisation (\%)} = \frac{\text{No of root segments colonised}}{\text{Total no of root segments observed}} \times 100$$

4.2.3 DNA extraction and polymerase chain reaction (PCR) amplification of the partial SSU rRNA gene

The preserved (at –80 °C) fraction of roots from each replicate plant of each legume species per province were combined into one composite sample per legume species per province in order to have sufficient material for DNA extraction. Then, genomic DNA was extracted using the DNeasy plant mini kit (Qiagen, Hilden, Germany). Summarily, 70 mg of roots was pulverized with liquid nitrogen in a chilled mortar and pestle. The powdered samples were collected into 2 ml tubes, and 400 µL Buffer AP1 and 4 µL RNase A were added. The mixture was vortexed for 10 s and incubated at 65 °C for 10 m. Thereafter, 130 µL of Buffer P3 was added to the solution, mixed gently, and incubated on ice for 5 m. The lysate was centrifuged at 20,000 ×g for 5 m. The lysate was collected into a spin column placed in a 2 ml collection tube and centrifuged at 20,000 ×g for 2 m. The supernatant was transferred into a new tube, 1.5 v of Buffer AW1 was added and mixed by pipetting.

Then, 650 µL of the mixture was transferred into a spin column placed in a 2 ml collection tube, centrifuged at 6000 ×g for 1 m, and the flow-through was discarded. The spin column was placed into a new 2 ml collection tube, 500 µL of Buffer AW2 was added, and centrifuged at 6000 ×g for 1 m. The flow-through was discarded, 500 µL of Buffer AW2 was added, and centrifuged at 20,000 ×g for 2 m. The spin column was transferred to a new 2 ml

microcentrifuge tube and DNA was eluted by adding 100 µL of Buffer AE, incubated at 25°C for 5 m, and centrifuged at 6000 ×g for 1 m. DNA concentration was determined using a NanoDropTM Spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA), and DNA quality was verified on a 1% agarose gel (Sigma Aldrich, USA). The extracted DNA was stored at –20°C until further processing.

A nested PCR approach was used to amplify the partial SSU rRNA gene. Nested PCR was conducted to improve the specificity and sensitivity of amplification. The first PCR step was performed using AM fungal primer pairs AML1 (5´-ATCAACTTTCGATGGTAGGATAGA-3') and AML2 (5´-GAACCCAAACACTTTGGTTTCC-3') (Lee et al., 2008). The reaction mixture for the first PCR was prepared in a final volume of 25 µL containing 12.5 µL of Q5® High-Fidelity 2X Master Mix (New England BioLabs, Inc., Ipswich, Massachusetts, USA), 1.25 µL of each primer (0.5 µM), 1 µL DNA template (approximately 10 ng), and 9 µL of PCR-grade water. The PCR conditions were an initial denaturation at 98 °C for 3 m, 30 cycles of denaturation at 98 °C for 30 s, primer annealing at 58 °C for 30 s, extension at 72 °C for 1 m, and a final extension step of 72 °C for 10 m. PCR amplicons were verified on 1 % agarose gel and amplicon size was checked with a Thermo Scientific GeneRuler 1 kb DNA Ladder (yielded approximately 800 bp amplicons).

The reaction components for the second PCR step was similar to the first PCR, except that 2 µL of the first PCR products (diluted with PCR-grade water to 1:10) was used as the template and AM fungal primer sets AMV4.5NF (5´-AAGCTCGTAGTTGAATTTCG-3') and AMDGR (5´-CCCAACTATCCCTATTAATCAT-3') (Sato et al., 2005) were used with the following cycling conditions: 98 °C for 3 m, 28 cycles at 98 °C for 30 s, 60 °C for 40 s, 72 °C for 1 m, and 72 °C for 10 m. PCR amplicons were checked and amplicons size yielded approximately 260 bp amplicons. All primers contained forward and reverse Illumina MiSeq

overhang adapters for sequencing (Illumina Inc., San Diego, CA, USA). All PCR reactions were run in an Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific, Wilmington, DE, USA).

4.2.4 Illumina Miseq sequencing and bioinformatics analyses

PCR amplicons were purified using the Agent Court AMPure XP beads (Beckman Coulter, Brea, CA, United States). The libraries were amplified with a limited-cycle PCR program (12 cycles) to add the index 1 (i7) and index 2 (i5) adapters, containing sequences required for cluster generation of the Illumina flow cell. The resulting DNA fragments' quality and sizes were evaluated on a 1.5% (w/v) agarose gel. The libraries were quantified with a fluorometer (Qubit, Life Technologies) and normalised to 4 nM using a standard dilution method. Subsequently, the amplicon libraries were pooled, and paired-end sequencing was done on an Illumina MiSeq platform using a MiSeq Reagent Kit V3 600 cycles (Illumina, San Diego, CA, United States).

Demultiplexed paired-end reads obtained from the sequencer were quality-checked using FastQC software version 0.11.5 (Babraham Institute, United Kingdom). Based on the FastQC report, no trimming of barcodes and low-quality sequence reads at the 5' and 3'- ends were required. Reads were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the DADA2 denoiser (Callahan et al., 2016) implemented in the Quantitative Insight Into Microbial Ecology version 2 (QIIME2) environment (Bolyen et al., 2019). OTU clustering was done using an open reference strategy in which representative sequences were first aligned against the AM fungal sequences from the MaarjAM database (Öpik et al., 2010), and a subsequent *de novo* clustering of sequences that failed to hit the AM fungal virtual taxa (VT) reference sequences. OTU clustering was done using the VSEARCH (Rognes et al., 2016). Taxonomic assignment of OTUs against the MaarjAM database was performed using

the Scikit-learn feature classifier at 0.7 default confidence threshold (Pedregosa et al., 2011; Bokulich et al., 2018). Singletons (i.e., reads occurring once in all dataset) were removed before computing alpha diversity and beta diversity indices in QIIME2.

4.2.5 Statistical analyses

Statistical analyses were performed using R statistical software version 4.0.0. (www.r-project.org/index.html). The data were checked for normality before analysis of variance. Test for differences in soil physico-chemical parameters and root colonisation percentages among plants within and between locations were determined using Two-way analysis of variance (ANOVA). The Tukey's honest significant difference post hoc test was used for mean separations at $P < 0.05$. To determine the similarities in AM fungal community composition among legumes, cluster analysis was performed on the Bray-Curtis dissimilarities using the unweighted pair-group method of arithmetic averages (UPGMA). The relationship between soil physico-chemical properties and alpha diversity of AM fungi (richness and Shannon-Wiener index) was determined using Spearman rank correlation. To obtain insights into the potential influence of soil properties on the AM fungal communities, a canonical correspondence analysis (CCA) was performed. For the CCA analysis, soil and AM fungal community data were log-transformed using an automatic forward and backward stepwise model ("ordistep ()" function) in the vegan package of R software. Test for significance of the environmental (constraining) variables was determined using a permutation test. Multicollinear constraining variables (variance inflation factors > 10) were removed from the final CCA plot. The contribution of soil properties to the AM fungal community composition was then explained by chi-square-based partitioning in the vegan package of R software.

4.2.6 Data availability

Paired-end sequence reads for the partial SSU rRNA gene generated from this study have been deposited in the Sequence Read Archives of the National Centre for Biotechnological Information under the BioProject ID PRJNA690541 (Accession Nos. SRX9811346–SRX9811419).

4.3 Results

4.3.1 Soil physico-chemical properties

The physico-chemical properties of soils of legumes in both provinces are presented in table 4.1. Generally, the soils were acidic (pH < 7), with a mean range of 4.38–5.87 in Gauteng and 4.48–5.84 in Mpumalanga. The soil textural classification in Gauteng was largely sandy-loam, whereas in Mpumalanga, the soils were characterised by a broad spectrum of textural types, including sandy-loam, sandy clay, sandy clay loam, loam, clay, loamy sand, and clay loam. Apart from bulk density, the quantified soil properties differed significantly ($F_{1,94} = 1.18$; $P < 0.05$) among legumes both within and between provinces.

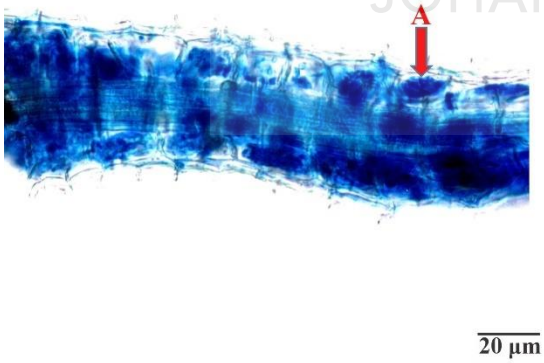
Table 4.1: Physico-chemical properties of soils of legumes in Gauteng and Mpumalanga Provinces

Gauteng		*Chamaecrista*	*Crotalaria*	*Eriosema*	*Indigofera*	*Rhynchosia*	*Rhynchosia*	*Trifolium*	*Tephrosia*	*Trifolium*	*Vigna*	*Zornia*
	Soil properties	*comosa*	*distans*	*cordatum*	*evansiana*	*minima*	*venulosa*	*africanum*	*kraussiana*	*repens*	*unguiculata*	*capensis*
	pH (KCl)	5.40 ± 0.09^{ab}	5.48 ± 0.11^{ab}	4.38 ± 0.19^{b}	4.85 ± 0.34^{ab}	4.95 ± 0.29^{ab}	5.35 ± 0.12^{ab}	5.38 ± 0.11^{ab}	5.41 ± 0.17^{ab}	5.58 ± 0.12^{ab}	5.87 ± 0.58^{a}	5.52 ± 0.34^{ab}
	NO_3 (mg kg^{-1})	8.26 ± 0.05^{c}	8.68 ± 0.10^{c}	16.10 ± 0.12^{b}	6.45 ± 0.12^{d}	0.29 ± 0.03^{h}	3.77 ± 0.12^{f}	5.14 ± 0.07^{e}	28.40 ± 0.42^{a}	8.51 ± 0.09^{c}	8.48 ± 0.26^{c}	1.46 ± 0.06^{g}
	NH_4 (mg kg^{-1})	1.35 ± 0.02^{f}	1.96 ± 0.02^{d}	5.79 ± 0.11^{a}	1.16 ± 0.02^{fg}	0.96 ± 0.02^{g}	3.61 ± 0.09^{b}	1.73 ± 0.01^{e}	2.65 ± 0.02^{c}	2.53 ± 0.03^{c}	2.18 ± 0.01^{d}	3.64 ± 0.01^{b}
	P (mg kg^{-1})	1.14 ± 0.03^{gh}	3.33 ± 0.03^{e}	4.77 ± 0.02^{d}	2.46 ± 0.06^{f}	1.24 ± 0.03^{gh}	7.12 ± 0.02^{b}	1.53 ± 0.24^{g}	5.44 ± 0.04^{c}	1.02 ± 0.02^{h}	9.30 ± 0.14^{a}	2.07 ± 0.01^{f}
	Organic C (%)	0.96 ± 0.02^{h}	1.00 ± 0.01^{h}	3.26 ± 0.01^{c}	1.50 ± 0.01^{g}	2.27 ± 0.01^{e}	2.66 ± 0.01^{d}	3.45 ± 0.01^{b}	0.73 ± 0.01^{i}	4.04 ± 0.04^{a}	0.65 ± 0.02^{j}	2.00 ± 0.01^{f}
	K (mg kg^{-1})	94.40 ± 0.25^{h}	130.00 ± 0.02^{f}	175.00 ± 0.41^{d}	224.00 ± 3.21^{c}	188.00 ± 0.26^{d}	233.00 ± 0.13^{c}	282.00 ± 7.24^{b}	155.00 ± 5.77^{e}	394.00 ± 3.25^{a}	113.00 ± 0.12^{g}	153.00 ± 0.05^{e}
	Cu (mg kg^{-1})	3.37 ± 0.01^{a}	0.97± 0.01^{g}	1.18 ± 0.04^{f}	0.25 ± 0.02^{i}	1.75 ± 0.02^{e}	0.77 ± 0.03^{h}	1.85 ± 0.01^{d}	2.06 ± 0.02^{c}	2.26 ± 0.02^{b}	0.81 ± 0.02^{h}	1.15 ± 0.02^{f}
	Mn (mg kg^{-1})	84.40 ± 0.25^{a}	40.10 ± 0.07^{e}	35.60 ± 0.63^{h}	11.40 ± 0.03^{j}	46.30 ± 0.35^{c}	36.50 ± 0.30^{gh}	42.30 ± 0.06^{d}	37.80 ± 0.10^{f}	52.50 ± 0.28^{b}	27.20 ± 0.05^{i}	37.80 ± 0.07^{fg}
	Zn (mg kg^{-1})	4.72 ± 0.03^{e}	3.93 ± 0.02^{f}	8.58 ± 0.02^{c}	0.76 ± 0.01^{i}	1.95 ± 0.02^{h}	12.30 ± 0.05^{b}	4.05 ± 0.02^{f}	7.44 ± 0.03^{d}	12.00 ± 0.03^{b}	3.45 ± 0.02^{g}	14.50 ± 0.26^{a}
	BD (gcm^{-3})	1.45 ± 0.05^{ns}	1.55 ± 0.04^{ns}	1.53 ± 0.06^{ns}	1.51 ± 0.02^{ns}	1.50 ± 0.03^{ns}	1.59 ± 0.09^{ns}	1.59 ± 0.08^{ns}	1.45 ± 0.12^{ns}	1.53 ± 0.10^{ns}	1.51 ± 0.01^{ns}	1.53 ± 0.07^{ns}
	Sand (%)	74.00 ± 3.06^{ac}	76.00 ± 3.00^{ac}	40.00 ± 3.61^{d}	73.00 ± 0.44^{ac}	78.00 ± 1.25^{a}	74.00 ± 1.53^{ac}	65.00 ± 2.52^{c}	38.00 ± 1.73^{d}	74.00 ± 1.15^{ac}	66.00 ± 1.73^{bc}	77.00 ± 1.83^{ab}
	Silt (%)	14.00 ± 1.15^{ab}	10.00 ± 1.15^{b}	12.00 ± 1.53^{ab}	15.00 ± 1.53^{ab}	8.00 ± 1.53^{b}	13.00 ± 1.53^{b}	20.00 ± 2.65^{a}	12.00 ± 2^{ab}	14.00 ± 2.08^{ab}	13.00 ± 2.00^{ab}	10.00 ± 1.53^{b}
	Clay (%)	12.00 ± 1.53^{b}	14.00 ± 1.53^{b}	48.00 ± 4.16^{a}	12.00 ± 1.15^{b}	14.00 ± 3.06^{b}	13.00 ± 1.15^{b}	15.00 ± 2.52^{b}	50.00 ± 2.89^{a}	12.00 ± 2.65^{b}	21.00 ± 3^{b}	13.00 ± 1.53^{b}
	Textural Class	SaLm	SaLm	Cl	SaLm	SaLm	SaLm	SaLm	SaCl	SaLm	SaClLm	SaLm
Mpumalanga	pH (KCl)	5.84 ± 0.20^{a}	5.61 ± 0.10^{ab}	5.39 ± 0.17^{ac}	5.35 ± 0.13^{ac}	5.39 ± 0.17^{ac}	4.64 ± 0.28^{bc}	5.63 ± 0.23^{ab}	4.61 ± 0.25^{bc}	4.61 ± 0.05^{bc}	4.48 ± 0.22^{c}	5.37 ± 0.36^{ac}
	NO_3 (mg kg^{-1})	4.31 ± 0.13^{g}	6.28 ± 0.16^{e}	11.10 ± 0.08^{c}	10.90 ± 0.05^{c}	5.37 ± 0.05^{f}	8.99 ± 0.16^{d}	3.22 ± 0.06^{h}	19.50 ± 0.28^{a}	3.68 ± 0.07^{gh}	13.90 ± 0.18^{b}	0.60 ± 0.13^{i}
	NH_4 (mg kg^{-1})	3.46 ± 0.01^{b}	2.60 ± 0.03^{c}	17.80 ± 0.13^{a}	3.47 ± 0.01^{b}	1.28 ± 0.01^{ef}	2.73 ± 0.10^{c}	1.50 ± 0.01^{de}	1.23 ± 0.01^{f}	1.42 ± 0.01^{df}	1.25 ± 0.02^{f}	1.67 ± 0.01^{d}
	P (mg kg^{-1})	3.06 ± 0.02^{f}	4.45 ± 0.02^{e}	2.02 ± 0.02^{g}	1.04 ± 0.03^{h}	5.21 ± 0.04^{d}	11.20 ± 0.03^{a}	2.33 ± 0.06^{g}	8.64 ± 0.12^{b}	2.14 ± 0.05^{g}	6.20 ± 0.18^{c}	3.04 ± 0.01^{f}
	Organic C (%)	1.55 ± 0.02^{e}	2.59 ± 0.03^{c}	5.34 ± 0.02^{a}	5.35 ± 0.02^{a}	1.54 ± 0.02^{e}	4.77 ± 0.01^{b}	1.89 ± 0.01^{d}	1.57 ± 0.01^{e}	1.00 ± 0.03^{g}	1.96 ± 0.05^{d}	1.36 ± 0.01^{f}
	K (mg kg^{-1})	131.00 ± 0.42^{e}	163.00 ± 0.10^{d}	233.00 ± 0.06^{ac}	247 ± 3.31^{ab}	135.00 ± 14.60^{e}	163.00 ± 0.05^{d}	223.00 ± 2.81^{bc}	218.00 ± 8.77^{c}	258.00 ± 3.43^{a}	103.00 ± 0.52^{f}	138.00 ± 0.06^{de}
	Cu (mg kg^{-1})	0.28 ± 0.01^{h}	1.77 ± 0.01^{e}	3.73 ± 0.03^{b}	1.06 ± 0.03^{g}	11.60 ± 0.03^{a}	3.18 ± 0.03^{c}	1.57 ± 0.01^{f}	0.21 ± 0.02^{h}	0.24 ± 0.02^{h}	2.44 ± 0.07^{d}	1.03 ± 0.04^{g}
	Mn (mg kg^{-1})	25.40 ± 0.03^{e}	53.00 ± 0.06^{c}	84.40 ± 0.12^{b}	131.00 ± 0.42^{a}	25.60 ± 0.03^{e}	29.40 ± 0.05^{d}	25.40 ± 0.06^{e}	1.08 ± 0.11^{i}	13.10 ± 0.06^{h}	16.10 ± 0.02^{g}	18.10 ± 0.09^{f}
	Zn (mg kg^{-1})	2.09 ± 0.04^{e}	6.11 ± 0.04^{c}	13.40 ± 0.05^{a}	6.15 ± 0.02^{bc}	1.34 ± 0.01^{g}	3.68 ± 0.02^{d}	6.26 ± 0.02^{b}	0.67 ± 0.01^{i}	1.07 ± 0.01^{h}	1.48 ± 0.01^{f}	1.46 ± 0.01^{fg}
	BD (gcm^{-3})	1.49 ± 0.12^{ns}	1.51 ± 0.03^{ns}	1.59 ± 0.04^{ns}	1.55 ± 0.02^{ns}	1.58 ± 0.06^{ns}	1.60 ± 0.03^{ns}	1.48 ± 0.05^{ns}	1.54 ± 0.07^{ns}	1.57 ± 0.09^{ns}	1.53 ± 0.06^{ns}	1.60 ± 0.08^{ns}
	Sand (%)	75.00 ± 0.84^{ab}	75.00 ± 1.73^{ab}	52.00 ± 1.73^{c}	68.00 ± 0.49^{b}	67.00 ± 2.30^{b}	36.00 ± 1.53^{d}	68.00 ± 3.32^{b}	50.00 ± 2.89^{c}	83.00 ± 2.31^{a}	34.00 ± 2.39^{d}	33.00 ± 1.33^{d}
	Silt (%)	11.00 ± 1.15^{cd}	13.00 ± 2.52^{cd}	10.00 ± 1.53^{cd}	14.00 ± 2.31^{cd}	10.00 ± 2.00^{cd}	42.00 ± 3.06^{a}	20.00 ± 4.62^{bc}	8.00 ± 1.53^{cd}	6.00 ± 1.15^{d}	27.00 ± 3.61^{b}	19.00 ± 2.00^{bc}
	Clay (%)	14.00 ± 2.00^{b}	12.00 ± 1.15^{b}	38.00 ± 3.00^{a}	18.00 ± 2.08^{b}	23.00 ± 1.73^{b}	22.00 ± 2.31^{b}	12.00 ± 2.00^{b}	42.00 ± 1.15^{a}	11.00 ± 1.53^{b}	39.00± 4.04^{a}	48.00 ± 4.16^{a}
	Textural Class	SaLm	SaLm	SaCl	SaLm	SaClLm	Lm	SaLm	Cl	LmSa	ClLm	Cl

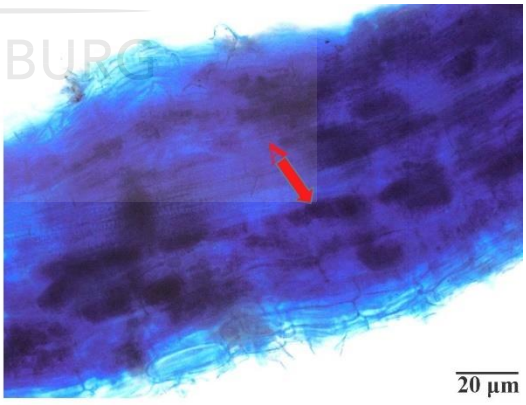
Values are given as means ± SEM. N = 3. Means with a letter in common are not significantly different (P < 0.05) according to the the Tukey test.
ns, not significant; NO_3, nitrate; NH_4, ammonia; P, available phosphorus; K, available potassium; Cu, copper; Mn, manganese; Zn, zinc; BD, Bulk density; Texture: SaLm, Sandy Loam; Cl, Clay; SaCl, Sandy Clay; SaClLm, Sandy Clay Loam; Lm, Loam; ClLm, Clay Loam; LmSa; Loamy Sand.

4.3.2 Root colonisation by AM fungi

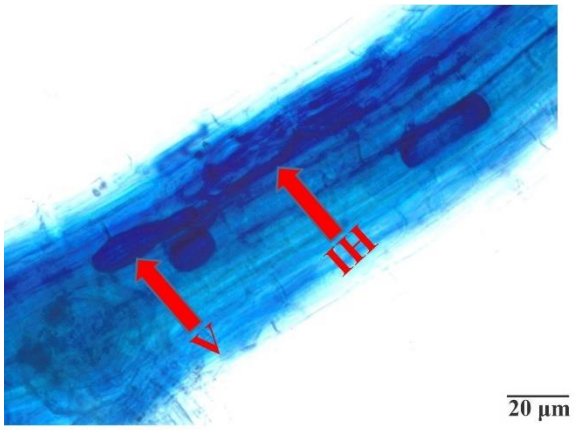
Microscopic observation of roots revealed that all the legume species in both provinces were colonised by AM fungi (Figure 4.1). Typical AM fungal structures such as arbuscules, vesicles of various shapes and sizes, intra- and extraradical hyphae, hyphal coils, and intra- and extraradical spores were observed in the roots of the examined plants, though not necessarily in the same root segment. AM fungal colonisation pattern varied among legume species; hyphal coils, vesicles, and intraradical hyphae were the most frequently observed structures, while arbuscules, extraradical hyphae, and spores were less common. The average percentage of root colonisation ranged from 71.3–98% in Gauteng and 65–98.3% in Mpumalanga (Figure 4.2). *Trifolium* species and *Tephrosia kraussiana* exhibited the highest and lowest rate of root colonisation in both provinces, respectively. Significant differences ($F_{1,94} = 0.99$; $P < 0.05$) were found in root colonisation percentage among legumes within provinces, whereas root colonisation percentage of the same plant did not vary significantly ($F_{1,94} = 0.85$; $P > 0.05$) between provinces.



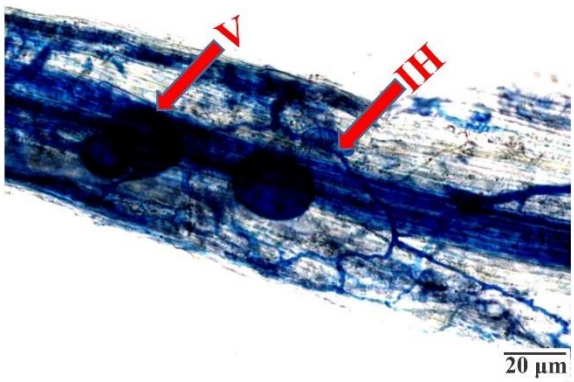
Chamaecrista comosa (Gauteng)



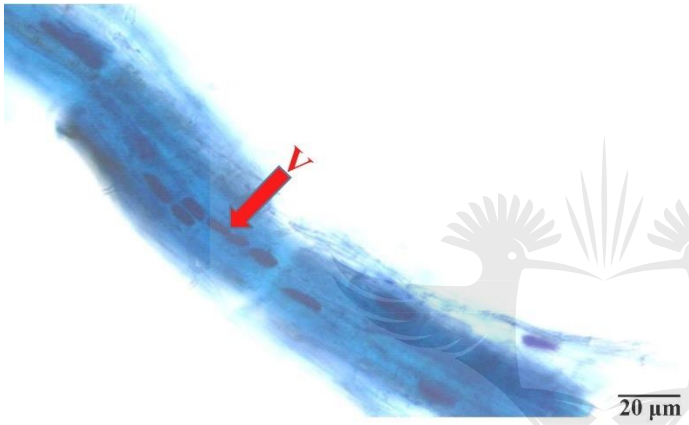
Chamaecrista comosa (Mpumalanga)



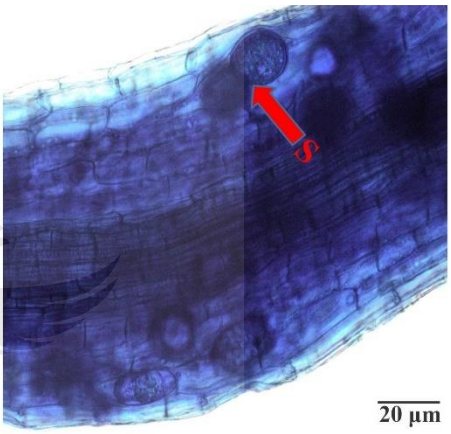
Crotalaria distans (Gauteng)



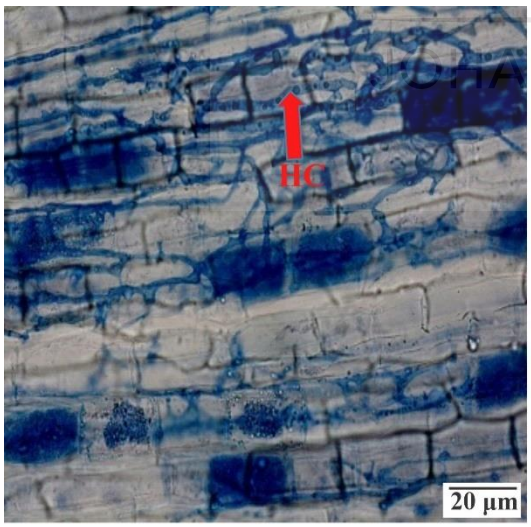
Crotalaria distans (Mpumalanga)



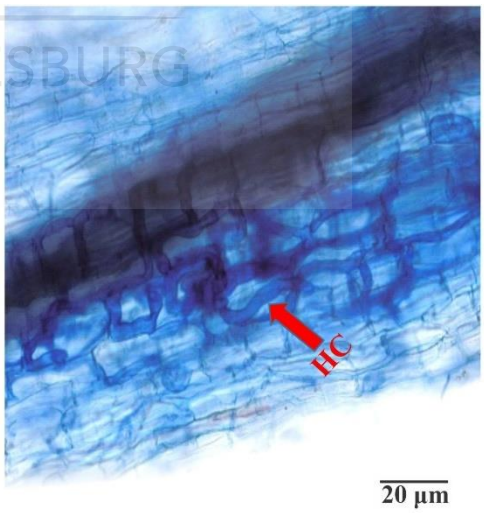
Eriosema cordatum (Gauteng)



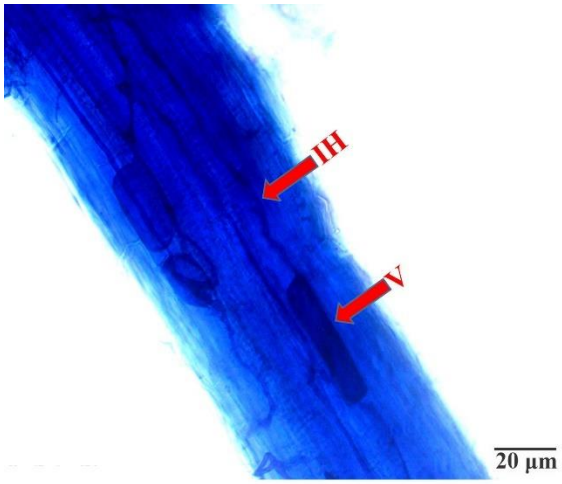
Eriosema cordatum (Mpumalanga)



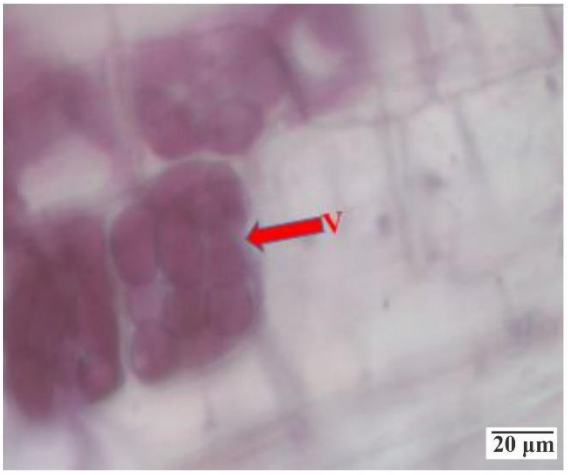
Indigofera evansiana (Gauteng)



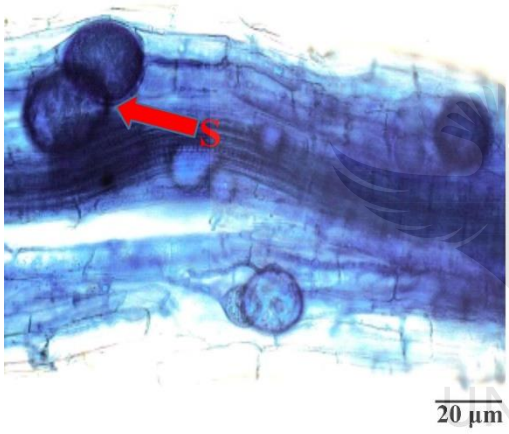
Indigofera evansiana (Mpumalanga)



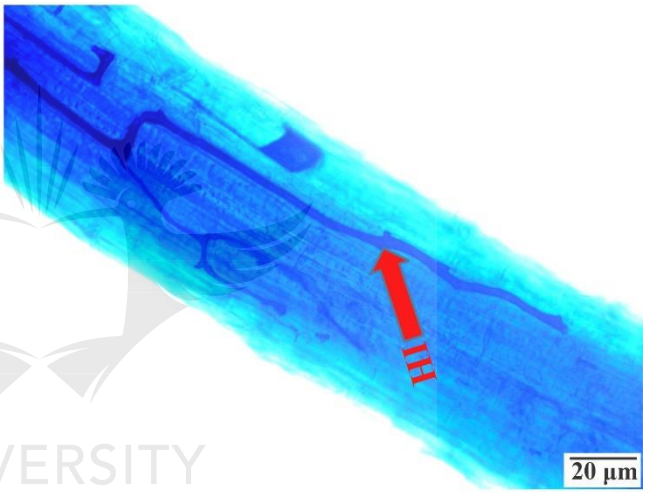
Rhynchosia minima (Gauteng)



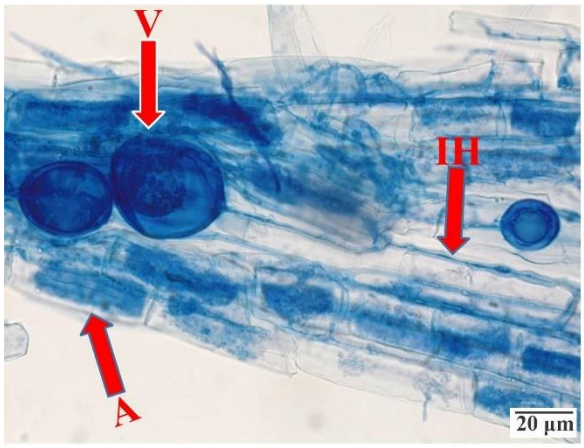
Rhynchosia minima (Mpumalanga)



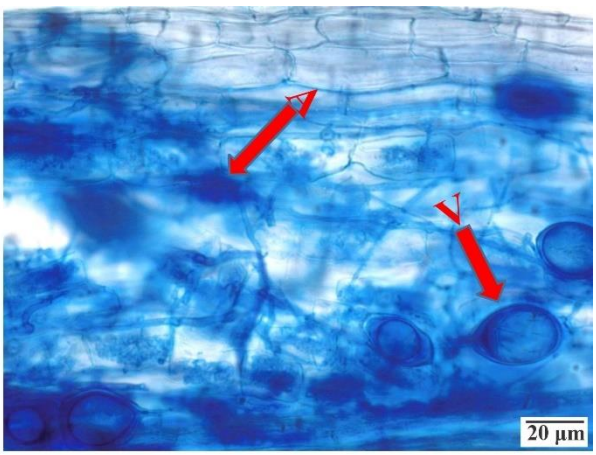
Rhynchosia venulosa (Gauteng)



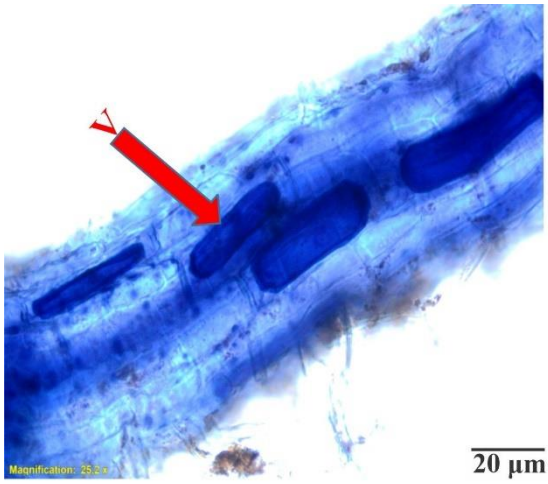
Rhynchosia venulosa (Mpumalanga)



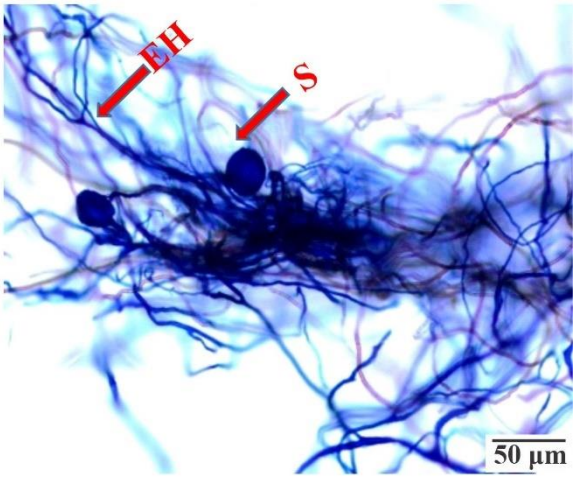
Trifolium africanum (Gauteng)



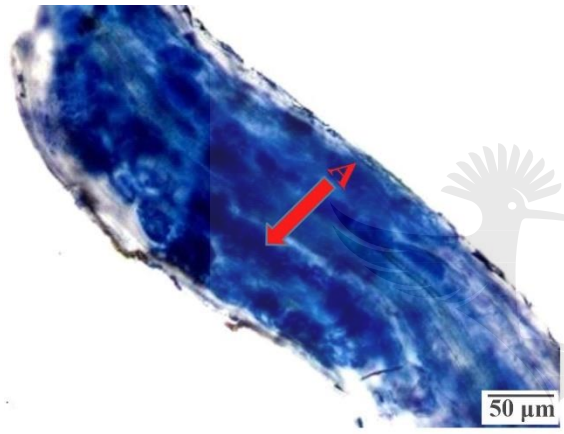
Trifolium africanum (Mpumalanga)



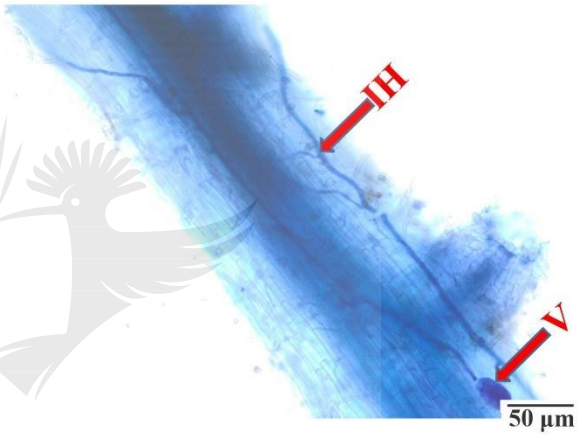
Tephrosia kraussiana (Gauteng)



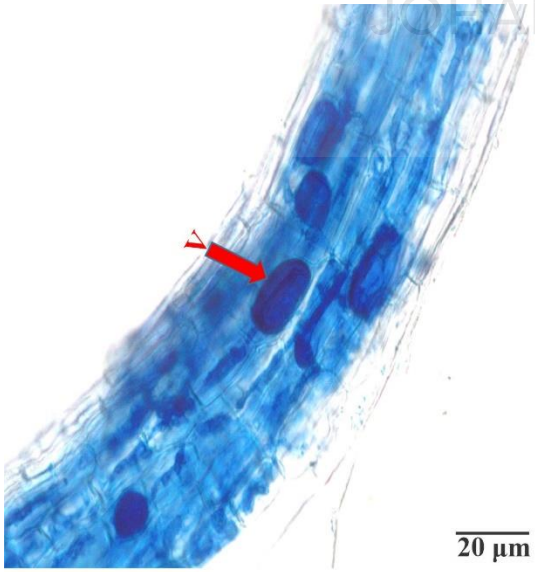
Tephrosia kraussiana (Mpumalanga)



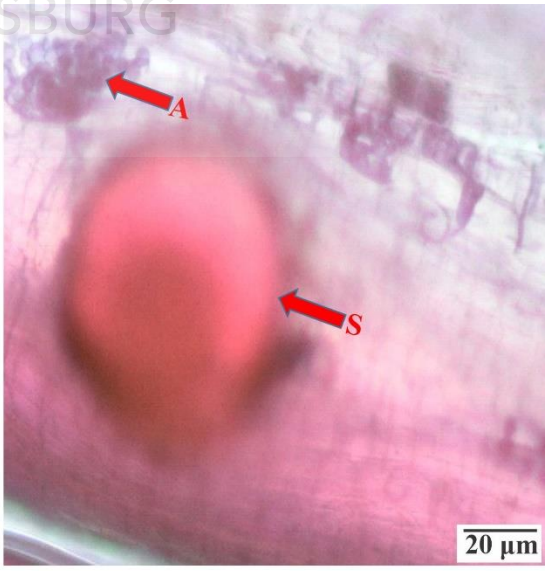
Trifolium repens (Gauteng)



Trifolium repens (Mpumalanga)



Vigna unguiculata (Gauteng)



Vigna unguiculata (Mpumalanga)

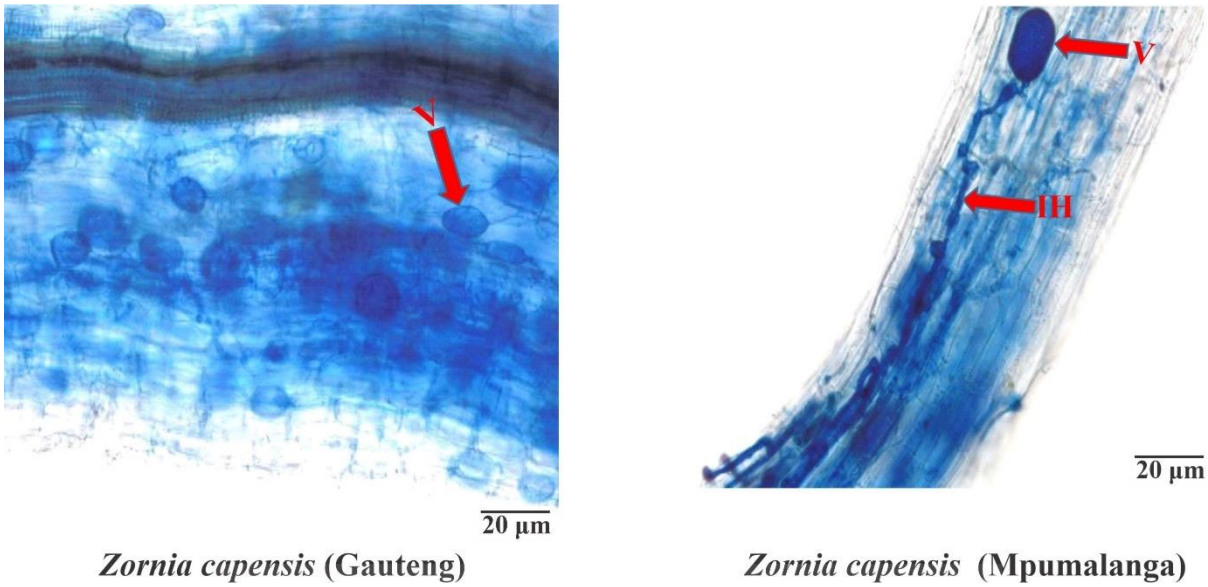


Figure 4.1: Photographs of the different AM fungal structures observed in the roots of studied legumes within and between provinces. A, Arbuscule; HC, Hyphal coil; V, Vesicle; IH, Intraradical hyphae; EH, Extraradical hyphae; ES, Extraradical spore; IS, Intraradical spore.

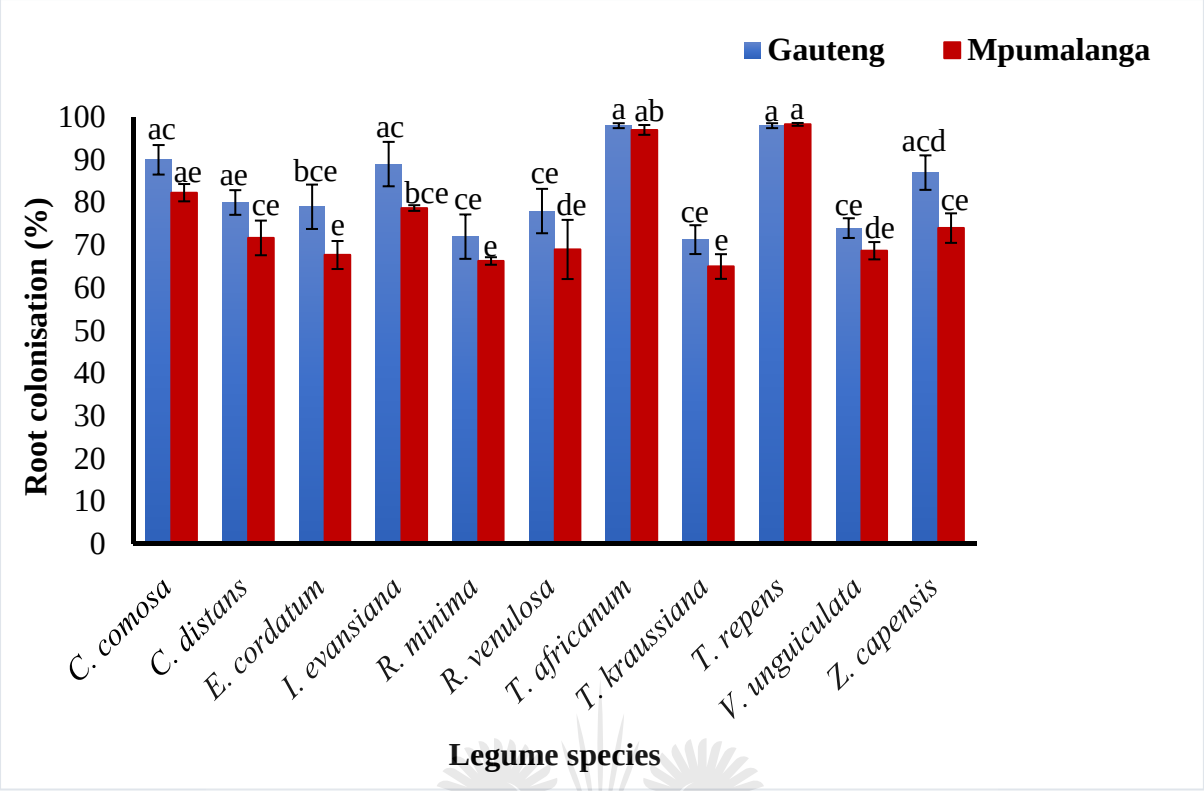


Figure 4.2: Percentage of root colonisation of different legume species within and between provinces. *N* = 3. Bars represent standard errors. Means with a letter in common are not significantly different ($P < 0.05$) according to the the Tukey test.

4.3.3 Sequencing information and sampling intensity

The Illumina sequencing generated 4,586,345 and 1,770,599 raw sequences for Gauteng and Mpumalanga, respectively, with an average sequence length of 245.6 bp. After quality check and removal of non-target and chimeric sequences, a total of 225, 149 and 90, 995 high-quality Glomeromycotan partial SSU rRNA sequences were obtained for Gauteng and Mpumalanga, respectively. These sequences were assigned into 172 OTUs. The OTUs that could be classified were affiliated with eight genera, namely *Glomus* (117 OTUs), *Claroideoglomus* (15), *Paraglomus* (10), *Diversispora* (9), *Scutellospora* (6), *Acaulospora* (5), *Archaeospora* (3), and *Ambispora* (1), while the remaining OTUs that could not be classified to the genus level were grouped as unclassified at the various taxonomic levels. These are unclassified

Glomeromycetes (4), unclassified Glomerales (1), and unclassified Diversisporales (1). To determine whether the number of sequences analysed was sufficient to capture the AM fungal diversity in roots, rarefaction curves were constructed. The results show that all the curves reached the plateau, indicating that the sequencing intensity provided a reasonable coverage of OTU diversity in each legume species (Figure 4.3).

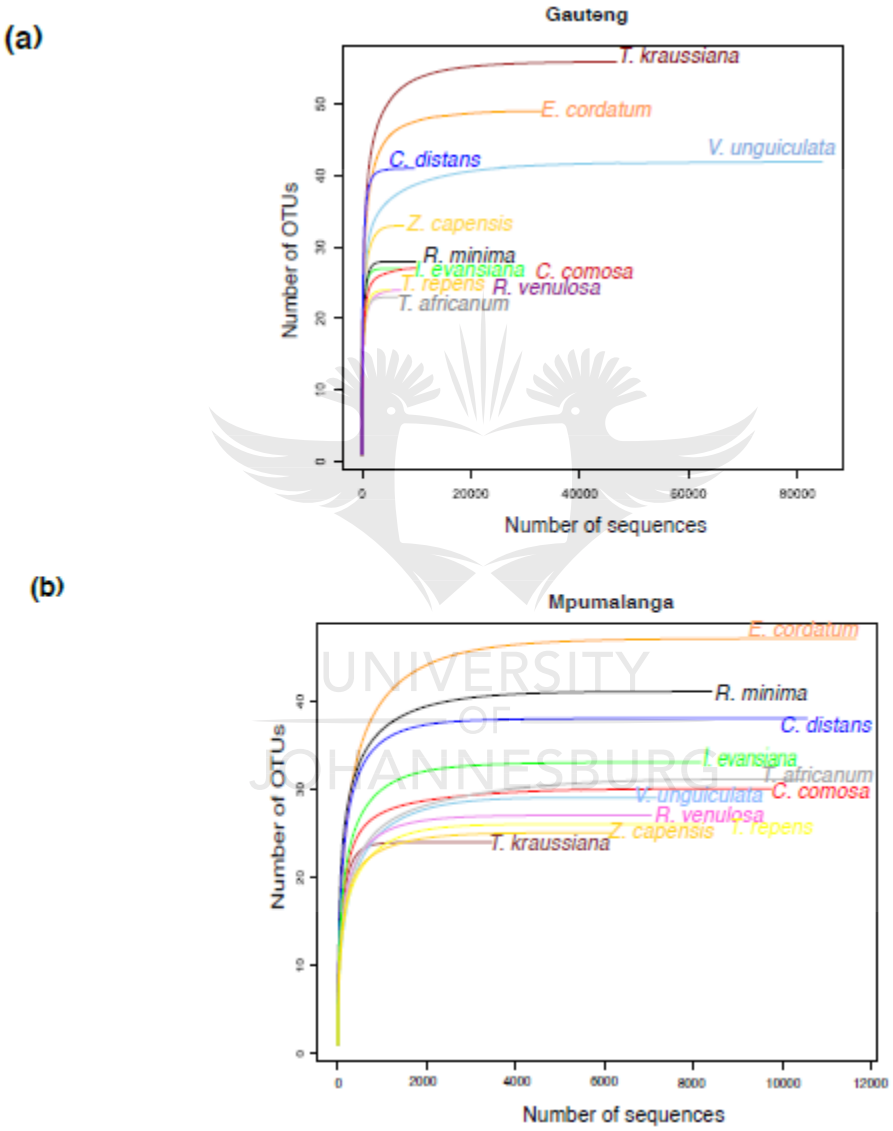


Figure 4.3: Rarefaction curves of OTU numbers in roots of each legume species (a) Gauteng. (b) Mpumalanga.

4.3.4 AM fungal diversity

The diversity of AM fungi in roots of legumes within and between provinces is presented in table 4.2. In Gauteng, the maximum and minimum OTU richness was observed in *Tephrosia kraussiana* and *Trifolium africanum*, respectively, whereas in Mpumalanga, *E. cordatum* possessed the highest and *T. kraussiana* had the lowest. Furthermore, in Gauteng, *Z. capensis* and *T. repens* exhibited the highest and lowest Shannon-Wiener index of species diversity (*H'*) respectively. In Mpumalanga, *R. minima* showed the highest *H'* while *I. evansiana* and *T. repens* recorded the lowest. In Gauteng, Simpson Dominance index of species diversity (*D*) was maximum in *R. venulosa* and minimum in *Z. capensis*, while in Mpumalanga, *D* was highest in *T. repens* and lowest in *R. minima*. In Gauteng, the highest Pielou evenness index of species proportionality *(J')* was found in *Z. capensis* and the lowest was oserved in *R. venulosa* and *T. repens*. Meanwhile, in Mpumalanga, the highest and lowest *J'* were found in *R. minima* and *T. repens*, respectively. Since roots of replicate plants were pooled into one composite sample per legume species per province, it was impossible to compare (statistically), the differences in alpha diversity indices of AM fungi among legumes within and between provinces.

Table 4.2: Alpha diversity indices of AM fungi in the roots of different legumes in Gauteng and Mpumalanga Provinces

Provinces	Diversity indices	*C. comosa*	*C. distans*	*E. cordatum*	*I. evansiana*	*R. minima*	*R. venulosa*	*T. africanum*	*T. kraussiana*	*T. repens*	*V. unguiculata*	*Z. capensis*
Gauteng	OTU richness	27.00	41.00	49.00	27.00	28.00	24.00	23.00	56.00	24.00	42.00	33.00
	Chao1	27.00	41.00	49.00	27.00	28.00	24.00	23.00	56.00	24.00	42.00	33.00
	H'	2.95	3.75	3.52	3.08	3.36	2.70	3.14	3.56	2.68	3.41	3.91
	D	0.20	0.12	0.15	0.17	0.14	0.24	0.17	0.13	0.23	0.15	0.08
	J	0.62	0.70	0.63	0.65	0.70	0.59	0.69	0.61	0.59	0.63	0.78
Mpumalanga	OTU richness	30.00	38.00	47.00	33.00	41.00	27.00	31.00	24.00	26.00	29.00	25.00
	Chao1	30.00	38.00	47.00	33.00	41.00	27.00	31.00	24.00	26.00	29.00	25.00
	H'	3.36	3.77	3.76	2.75	4.21	3.39	3.13	3.42	2.75	3.06	2.98
	D	0.15	0.12	0.12	0.18	0.08	0.13	0.16	0.13	0.23	0.17	0.21
	J	0.68	0.72	0.68	0.66	0.79	0.71	0.63	0.75	0.59	0.63	0.64

Chao1, *H'*, *D*, and *J* are the Estimated richness, Shannon-Wiener index, Simpson Dominance index, and Pielou's evenness index, respectively.

4.3.5 AM fungal community composition

The composition and relative abundance of AM fungal communities varied among legumes within and between provinces. At the genus taxonomic rank, *Glomus* and *Claroideoglomus* occurred in all samples in both provinces; *Glomus* dominated the AM fungal communities, whereas *Ambispora* was the least dominant (Figure 4.4). At the OTU level, *Ambispora leptoticha* was only associated with *V. unguiculata* (Gauteng) and *C. comosa* (Mpumalanga). The most frequent OTU which appeared in all the legume species in both provinces was *Claroideoglomus clo27*. Again, since roots of replicate plants were combined into one composite sample per legume species per province, it was impossible to compare (statistically), the differences in the community composition of AM fungi among legumes within and between provinces.

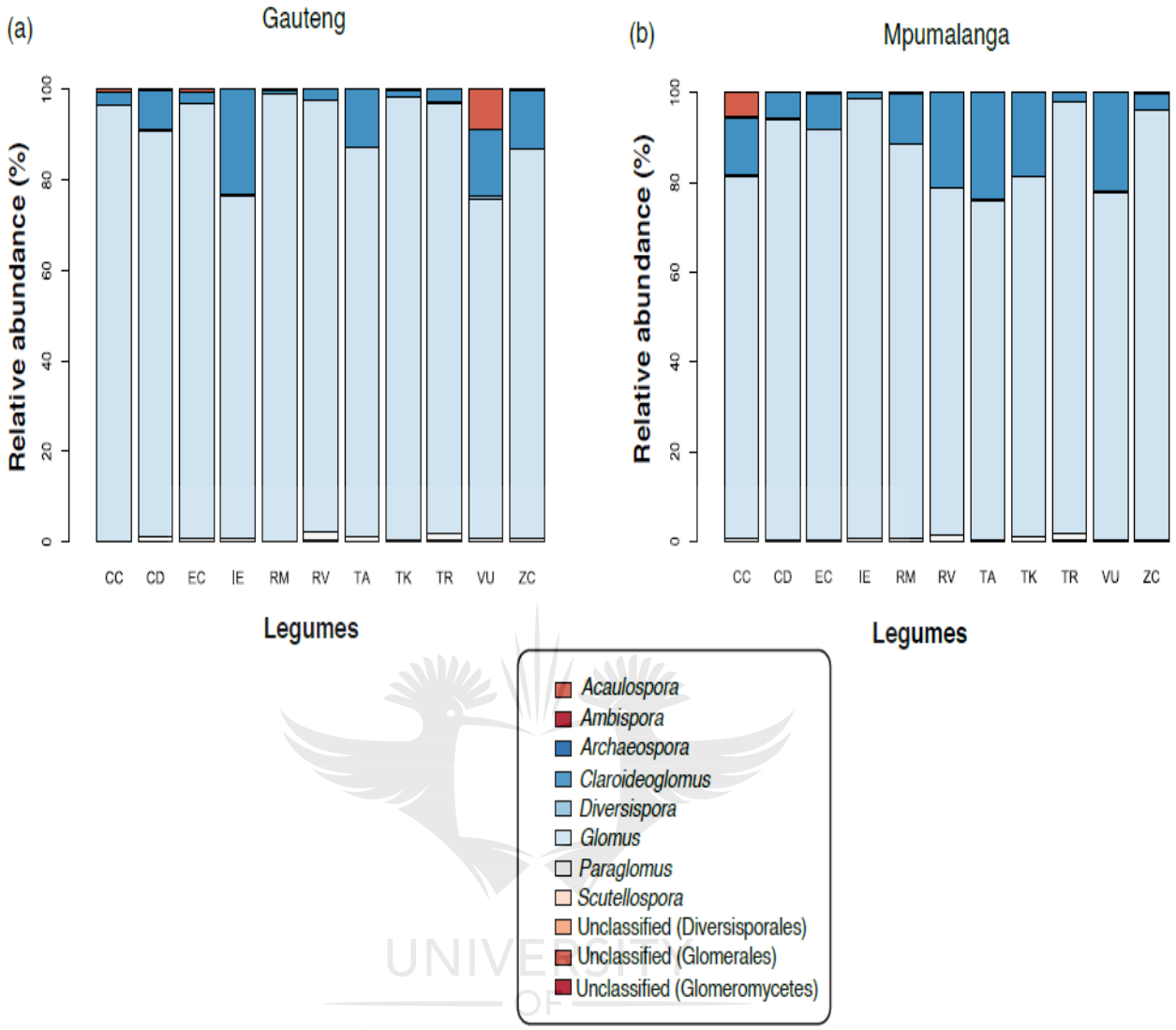


Figure 4.4: AM fungal genera in the roots of legumes (a) Gauteng. (b) Mpumalanga. CC, *Chamaecrista comosa*; CD, *Crotalaria distans*; EC, *Eriosema cordatum*; IE, *Indigofera evansiana*; RM, *Rhynchosia minima*; RV, *Rhynchosia venulosa*; TA, *Trifoilum africanum*; TK, *Tephrosia kraussiana*; TR, *Trifolium repens*; VU, *Vigna unguiculata*; ZC, *Zornia capensis*.

4.3.6 Cluster analysis of AM fungal communities

The similarities and clustering of AM fungal communities in legumes in each province is illustrated by a cluster dendrogram (Figure 4.5). In Gauteng, *R. venulosa* and *T. repens* exhibited the highest similarities in AM fungal communities (Figure 4.5a), while in

Mpumalanga, *E. cordatum* and *C. distans* have the most similar AM fungal communities (Figure 4.5b). Nevertheless, some AM fungi are unique to certain legumes. For example, in Gauteng, the AM fungal communities in *Z. capensis* and *T. africanum* are more similar than in *Z. capensis* and *C. distans* or in *T. africanum* and *C. distans*. In Mpumalanga, *C. distans* and *E. cordatum* showed a higher degree of similarities in AM fungal communities than *C. distans* and *I. evansiana* or *E. cordatum* and *I. evansiana*.

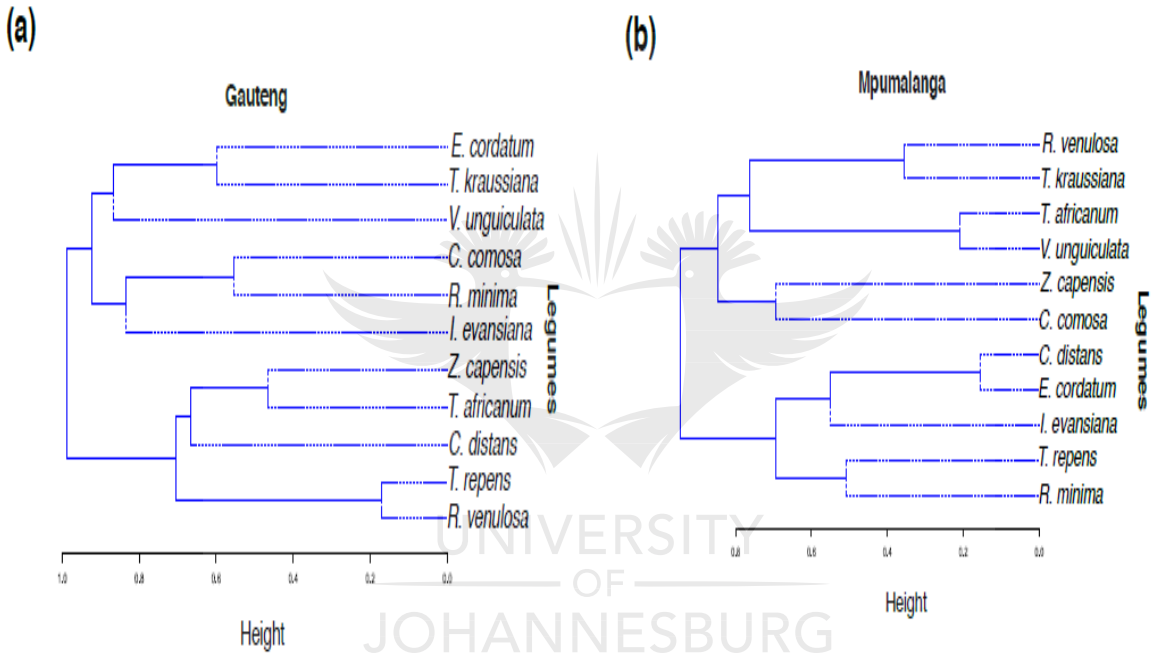


Figure 4.5: Cluster analysis of AM fungal communities among legumes in (a) Gauteng (b) Mpumalanga. Dendrogram was constructed based on UPGMA of the Bray-Curtis dissimilarities between AM fungal community composition in legumes.

4.3.7 Relationship between soil properties and AM fungal diversity and community composition

Analysis of relationship between alpha diversity indices of AM fungi and soil physico-chemical properties showed that in Gauteng, OTU richness was significantly correlated with

K (rho = -0.63, *P* = 0.04) and silt content (rho = -0.62, *P* = 0.04), and *H´* was significantly correlated with silt content (rho = -0.67, *P* = 0.023) (Figure 4.6a, e, f). Conversely, in Mpumalanga, OTU richness was positively associated with Cu (rho = 0.71, *P* = 0.019), Mn (rho = 0.79, *P* = 0.004), and Zn (rho = 0.66, *P* = 0.031) (Figure 4.6b, c, d).

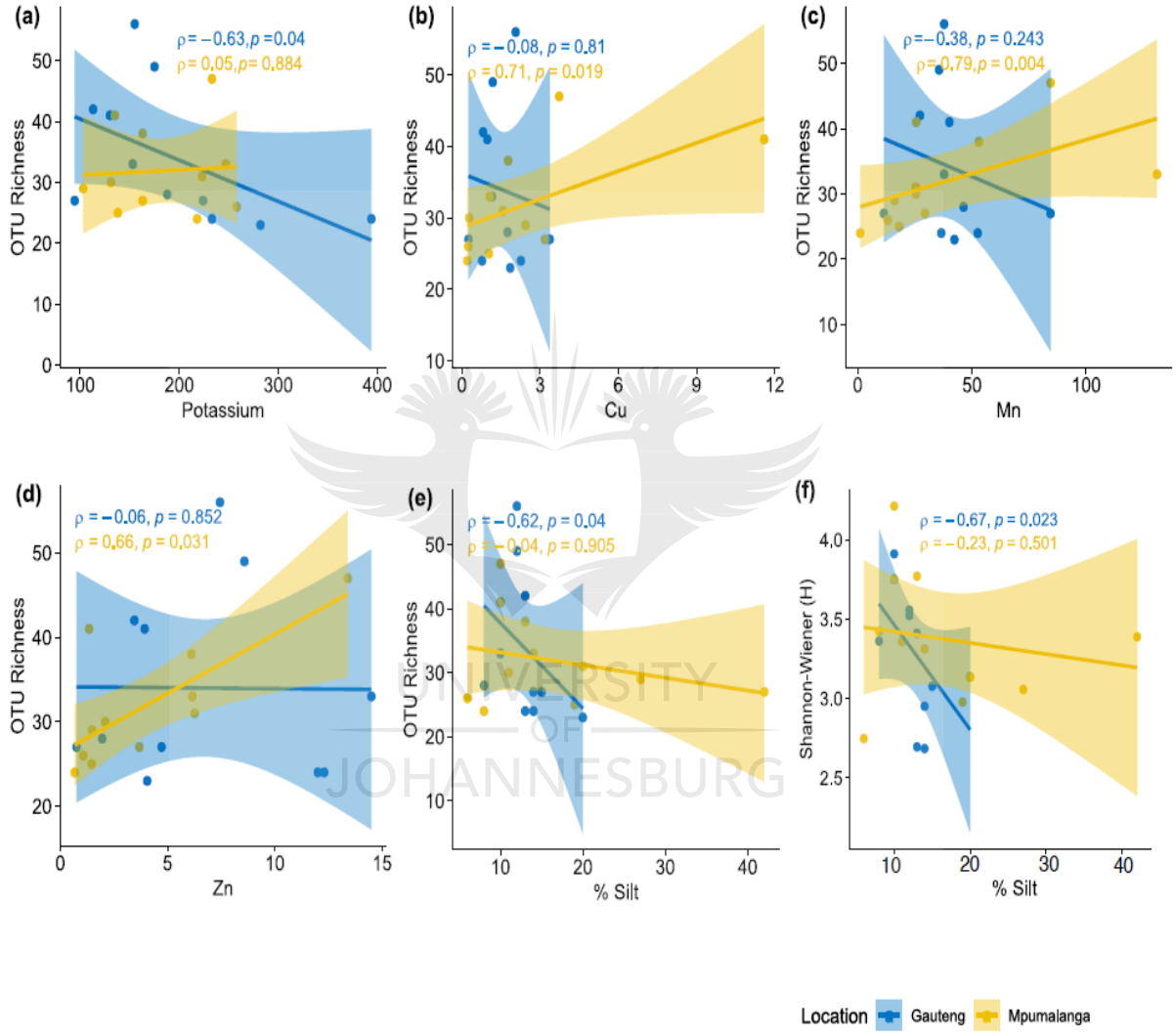


Figure 4.6: Correlations between AM fungal diversity indices and soil physico-chemical properties in both provinces.

The stepwise model for the CCA triplot showed that soil environmental variables had a significant (*P* = 0.001) effect on the composition of AM fungal communities (Figure 4.7). Precisely, 72.13% of the overall variation in AM fungal community composition was explained

by soil variables, as indicated by the first and second CCA axes. Significance of environmental terms fitted into the stepwise CCA model showed that NO_3 (R^2 = 0.40891, *P* = 0.018), pH (R^2 = 0.51379, *P* = 0.011), Mn (R^2 = 0.59794, *P* = 0.026), and organic C (R^2 = 0.66454, *P* = 0.032) were the soil properties significantly influencing AM fungal community composition.

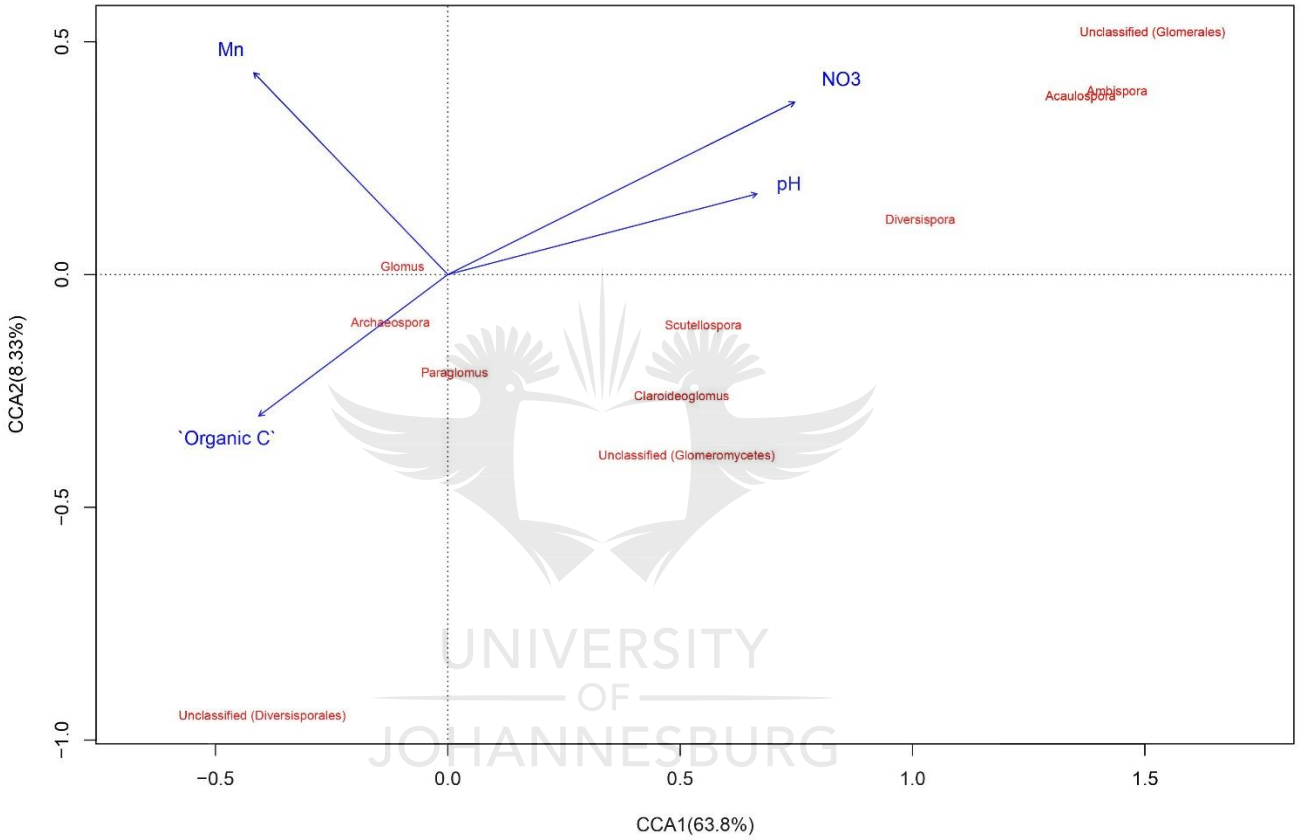


Figure 4.7: Canonical correspondence analysis (CCA) showing the relationship between AM fungal community composition and soil physico-chemical properties. The first (CCA1) and second (CCA2) canonical axes of the CCA plot are significant at *P* < 0.05.

4.4 Discussion

The degree to which plants rely on mycorrhizal symbiosis under nutrient-deficient soil conditions can be related to their root system architecture (Hetrick, 1991). Generally, legumes have coarse, deep root systems, low plasticity in root: shoot ratio, and fewer fine roots

(Pregitzer, 2008; Liu et al., 2013; Fort et al., 2015). Such root systems negatively affect the capability of the plants to acquire the necessary nutrients, resulting in a greater reliance on the AM association (Baylis, 1975; Siqueira and Saggin-Júnior, 2001; Carrenho et al., 2007). In this study, all the legume species from both provinces were found to be colonised by AM fungi. These observations agree with the evidence that species of the legume family are highly mycotrophic and that the AM interaction may be crucial for the establishment and survival of legumes in semi-arid habitats (Duponnois et al., 2001; Tao and Zhiwei, 2005).

While arbuscules (Arum-type morphology) are a key distinguishing structure for plants with functional AM association (Giovannetti et al., 1994), they may be difficult to find under natural conditions due to their short life span (Alexander et al., 1989; Smith and Read, 2008). This could explain why arbuscules are rare in the roots of the plants examined. On the other hand, the results revealed the notable presence of hyphal coils (Paris-type morphology), supporting the observations from other studies in which hyphal coils were found to be common (Hawley and Dames, 2004; Becerra et al., 2007; Harikumar et al., 2015). The fact that hyphal coils are the dominant structures may be due to their ecological importance; since they are long-lived, they are more beneficial to the plants growing under unfavourable environments such as the semi-arid ecosystems, than arbuscules (McGee, 1989; Imhof and Weber, 1997; Becerra et al., 2007). Other factors such as host plants, the type (species) of AM fungi colonising the roots, and most likely environmental conditions such as moisture, soil temperature, and light intensity, could also impact the occurrence or dominance of either or both morphological types (Hawley and Dames, 2004; Dickson et al., 2007). In addition, hyphae and vesicles were commonly detected, which concurs with previous studies (Muthukumar and Udaiyan, 2000; Muthukumar et al., 2003; Gai et al., 2006). According to Klironomos and Hart (2002), the prevalence of vesicles suggests that most of the root-colonising AM fungi belong to the suborder Glomineae.

Plants growing in phosphorus-deficient soils tend to apportion significant carbon to AM fungi, which in turn stimulates the establishment of mycorrhizae for the purpose of P acquisition (Johnson et al., 2010; Kowalska et al., 2015). It is therefore possible that the high levels of colonisation observed in the current study could be due to the low amounts of accessible P in the soils of the studied plants. The extent of root colonisation differed considerably between legume species. Previous studies have also reported differential colonisation levels in different plant genera, species, and cultivars (Sathiyadash et al., 2010; Abdullahi et al., 2014; Rios-Ruiz et al., 2019; Wang et al., 2019). These differences may be a result of various factors such as the types and proportion of root exudates released by different legumes, variations in colonisation capabilities of different AM fungal taxa, as well as climatic and soil conditions (Steinkellner et al., 2007; Smith and Read, 2008).

A total of 172 OTUs were found in the roots of all the legumes evaluated, with most of them belonging to the genus *Glomus*. The prevalence of *Glomus* in various legumes has been implicated in other studies as well (Scheublin et al., 2004; Li et al., 2010; Johnson et al., 2016). Their widespread distribution and high ecological resilience have also been established (Rodrıguez-Echeverrıa et al., 2017; Muneer et al., 2019). The predominance of this genus could be due to their capability to generate more propagules (spores, hyphae, and colonised root fragments) that can colonise plant roots extensively (Avio et al., 2006; Zhao et al., 2017). Notwithstanding the similarities in AM fungal communities between the legumes, *Ambispora leptoticha* was present only in the species *V. unguiculata* and *C. comosa*, suggesting host preference. Preferential association between certain host plants and AM fungal species has been previously reported (Torrecillas et al., 2012a; Muneer et al., 2019). On the other hand, *Claroideoglomus clo27* was found to be common in all the legume species in both provinces. This indicates that the AM fungal species does not have a preferred host and is probably well adapted to the environmental conditions of their leguminous hosts. In both provinces, the

Shannon-Wiener diversity index *H´* was high for all legumes. These results coincide with other studies illustrating those plants growing in natural conditions had a high diversity of AM fungi in their roots (Öpik et al., 2006; Li et al., 2010; Torrecillas et al., 2012a).

The impact of soil environmental variables on AM fungal diversity and community composition has been extensively studied (Liu et al., 2015; Alguacil et al., 2016; Xu et al., 2017). Soil quality impacts nutrient retention, which affects the species diversity of AM fungi (Zhao et al., 2017; Ezeokoli et al., 2020). The significant negative relationship found between AM fungal diversity (*H´*) and silt quantity suggests that the higher levels of nutrients in silty soils resulted in less species diversity. Furthermore, fine-textured soils are less porous, and this may have created inadequate aeration necessary for optimal AM fungal dvelopment (Saif, 1981). Soil K is known to have stimulatory effects on AM fungi in drought stress conditions (Garcia and Zimmermann, 2014). Nevertheless, Ardestani et al. (2011) found lower root colonisation rates at higher levels of soil K. Thus, the significant negative relationship observed between OTU richness and K could possibly be ascribed to the high content of available K in the soils of the legumes investigated. A significant positive relationship was found between OTU richness and Cu, Mn, and Zinc levels. The presence of AM fungi can improve the uptake of these micronutrients, which are critical for nitrogen fixation (Weisany et al., 2013; Lehmann and Rillig, 2015).

The AM fungal community composition could be significantly influenced by soil pH (Jansa et al., 2014), Mn (Xu et al., 2017, Alguacil et al., 2016), nitrogen (VAN Diepen et al., 2011; Avio et al., 2013), and organic C (Yang et al., 2011; Luo et al., 2019). Soil N may bring about changes in the composition of AM fungal communities by suppressing or enhancing AM fungal sporulation and abundance (Treseder and Allen, 2002). Soil pH influences AM fungal community composition by regulating nutrients and ion availability for plant uptake (da Silva

et al., 2014). Soil organic C serves as energy source for hyphal proliferation and spore production, which could directly affect AM fungal community composition in the soil (Zhu et al., 2020). These features may explain why NO_3, pH, Mn, and organic C affected the composition of AM fungal communities associated with the roots of studied legumes.

In conclusion, the study has shown that the high levels of root colonisation and the diverse AM fungal communities harboured by the legumes indicates the ecological significance of AM fungi on the indigenous legumes. The results also indicated that *Glomus* dominated the AM fungal communities and that soil K, Cu, Mn, Zn, NO3, pH, organic C, and silt content were significant drivers of AM fungal diversity and community composition in roots of the legumes studied.

CHAPTER 5

MORPHOLOGICAL DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES (This chapter has been published in BIODIVERSITAS 22(5), 2466–2476)

5.1 Introduction

In terms of economic importance, the legume family is the second only after the grass family (Poaceae) and contributes considerably to world food and nutrition security (Graham and Vance, 2003). Furthermore, legumes are regarded as indispensable service providers for natural and agro-ecosystems due to their ability to increase soil fertility through biological nitrogen fixation (Peoples and Craswell, 1992; Cleveland et al., 1999). Legumes form symbiotic associations with soil microorganisms that greatly impact their establishment and adaptation to nutrient-deficient soils (Sugiyama and Yazaki, 2012). An example of such microorganisms are the AM fungi, which assist legumes in meeting their nutritional demands for phosphorus from P-deficient soils (Chalk et al., 2006), and augment their tolerance to a variety of biotic and abiotic stresses (Smith et al., 2010). Such AM fungal-mediated supply of P is important in maintaining the high P-demanding legume-rhizobia symbiosis, thus making legumes highly dependent on the AM symbiosis (Javaid, 2010).

The AM fungi are common in terrestrial environments, where they associate with many land plants, including legumes (Allsopp and Stock, 1993; Brundrett and Tedersoo, 2018; Choosa-Nga et al., 2019). Nonetheless, reports have shown that soil properties could influence the diversity and composition of AM fungal communities in the soil (Santos-González et al., 2011; Liu et al., 2015).

The use of indigenous AM fungi from the soil has been emphasized as a promising approach for the propagation and management of indigenous plant communities (Hawley and Dames, 2004; Fitzsimons and Miller, 2010). Indigenous AM fungi improve host plants' absorption of nutrients from the soil, enhance soil aggregate stability through the secretion of glomalin into the soil, and alleviate drought stress by collecting water from soil particles. In addition, the AM fungi are well adapted to other biotic and abiotic stress conditions typical of the local environments of their hosts (Mena-Violante et al., 2006; Maltz and Treseder, 2015; de Oliveira et al., 2017). The combinations of these traits can help plants grow, perform, and survive in stressful situations, which could aid long-term conservation efforts (Powell et al., 2009; Graham et al., 2013).

Accordingly, the information about the indigenous AM fungal diversity in the soil is an essential step towards the understading of their functional roles in semi-arid environments (Souza et al., 2010). Such knowledge would assist in determining and selecting the effective AM fungal species or species combinations that can be utilised as local inoculants for the conservation of plants in both nursery and field conditions (Soka and Ritchie, 2014; Chen et al., 2018). Therefore, this study aimed to assess the diversity of AM fungal communities present in the topsoils of eleven indigenous legumes, using morphological characters. The objectives are to describe and compare the morphological diversity of AM fingal communities in the topsoils of legumes within and between provinces and to examine the effects of soil properties on the diversity and community structure of AM fungi.

5.2 Materials and Methods

5.2.1 Isolation, quantification, and identification of AM fungal spores

Spores of AM fungi were independently isolated from each replicate topsoil sample of each legume species per province using the modified wet sieving and decanting method (Brundrett

et al., 1996) as well as the sucrose density gradient centrifugation method (Daniels and Skipper, 1982). Briefly, 1000 ml of water was added to 100 g of air-dried soil. The mixture was stirred with a magnetic rod to break apart soil aggregates and dissociate spores from clumps and heavy soil particles. The mixture was left to settle for 2 m and the supernatant was decanted through a series of 750, 250, 100, and 38 µm sieves arranged in decreasing order of mesh sizes. Washing and decanting were repeated several times until the supernatant was clear. The materials retained in the 750 µm sieve was checked for large spores and sporocarps but were later discarded as they were root pieces and coarse debris. Soil materials retained in the 250, 100, and 38 µm sieves were washed into separate centrifuge tubes. The tubes were centrifuged at 4,000 rpm for 5 m and the supernatants were carefully decanted. Thereafter, 50% (w/v) sucrose solution was added to the pellets, mixed gently, and centrifuged at 3,000 rpm for 1 m. Then, the supernatant from each tube was decanted into the 38 µm sieve and rinsed with distilled water several times to remove the sucrose solution. Recovered spores and sporocarps were washed into sterile petri dishes and counted under the dissecting microscope at 50× magnification. Spore density (SD) was expressed as the total number of spores and/or sporocarps in 100 g of soil.

For identification, five spores and/or sporocarps of each AM fungal species were picked under the dissecting microscope with an extruded 9-inch glass micropipette, mounted on slides with polyvinyl-lactic acid-glycerol (PVLG) or PVLG mixed with Melzer's reagent (1:1 (v/v) and observed under the light microscope at 100–400× magnification. The spores were identified to the species level on the basis of shape, colour, size, number of spore walls, and presence or absence of subtending hyphae using the taxonomic criteria described by Schenck and Perez (1990), together with the descriptions of reference cultures from the International Culture Collection of (vesicular) arbuscular mycorrhizal fungi INVAM (1997).

5.2.2 AM fungal community composition and diversity analyses

Species richness (SR), isolation frequency (IF), and relative abundance (RA) were used to evaluate the composition of AM fungal communities in the topsoil of different legumes. These indices were computed as follows: Species richness (SR) was measured as the total number of identified AM fungal species per soil sample. Isolation frequency (IF) = (the number of samples in which a particular AM fungal species was observed/the total number of samples) × 100, where AM fungal species were then classified into the following groups according to Zhang et al. (2004): dominant (IF > 50%), most common (IF 31%–50%), common (IF 10%–30%), and rare (IF < 10%). Relative abundance (RA) = (the number of spores of a particular genus)/total number of identified spores) × 100. The AM fungal species diversity in different legumes was assessed by the Shannon-Wiener index, *H′* (Shannon, 1948) using the formula, $H' = -\Sigma^{k}_{i}=1(Pi \ln Pi)$; Pi is the relative abundance of each identified AM fungal species per sample and is calculated by the formula $Pi = ni/N$, where ni represents the number of individuals of a species and N is the total number of individuals of all species. Species evenness was obtained by Pielou evenness index, *J* (Pielou, 1966) using the formula, J = *H′*/log(S), where *H′* is the value obtained for Shannon-Wiener index and S is the species richness.

5.2.3 Statistical analyses

Differences in SD, SR, *H′*, and *J*, among legumes within and between provinces were tested using Two-way ANOVA. Significant means were separated using the Tukey's honest significant difference post hoc test at $P < 0.05$. The data were tested for normality and transformation was attempted, if necessary, prior to analysis of variance. Pearson correlation analysis was used to test for the relationship between spore density, diversity indices, and soil physico-chemical properties. The canonical correspondence analysis (CCA) was employed to evaluate the effect of soil parameters on the AM fungal community composition. The CCA

was done on log-transformed soil and AM fungal community data using an automatic forward and backward stepwise model ("ordistep ()" function) in the vegan package. Test for significance of the environmental (constraining) variables were checked using a permutation test. Multicollinear environmental variables (Variance inflation factor > 10) were removed from the final CCA plot. The contribution of soil properties to the AM fungal community composition was then explained by variance partitioning (chi-square-based partitioning) in the vegan package of R software.

5.3 Results

5.3.1 Soil physico-chemical properties

The soils of the studied plants in both provinces were acidic (pH < 7). Soil texture was largely sand-loam in Gauteng, whereas Mpumalanga has different textural types. Except for bulk density, all the soil properties determined varied significantly ($F_{1,94} = 1.18$; $P < 0.05$) among legumes within and between provinces (Please refer to section 4.3.1 and Table 4.1 for details).

5.3.2 Spore density

The mean spore density isolated from the soil of studied plants ranged from 306–812 per 100g of soil in Gauteng and 284–759 per 100g of soil in Mpumalanga (Figure 5.1). In both provinces, the highest and lowest average SD was observed in *Trifolium repens* and *Tephrosia kraussiana*, respectively. Spore density differed significantly ($F_{1,94} = 0.83$; $P < 0.05$) among legume species within provinces, but no significant difference ($F_{1,94} = 1.15$; $P > 0.05$) was observed in SD of the same legume species between provinces.

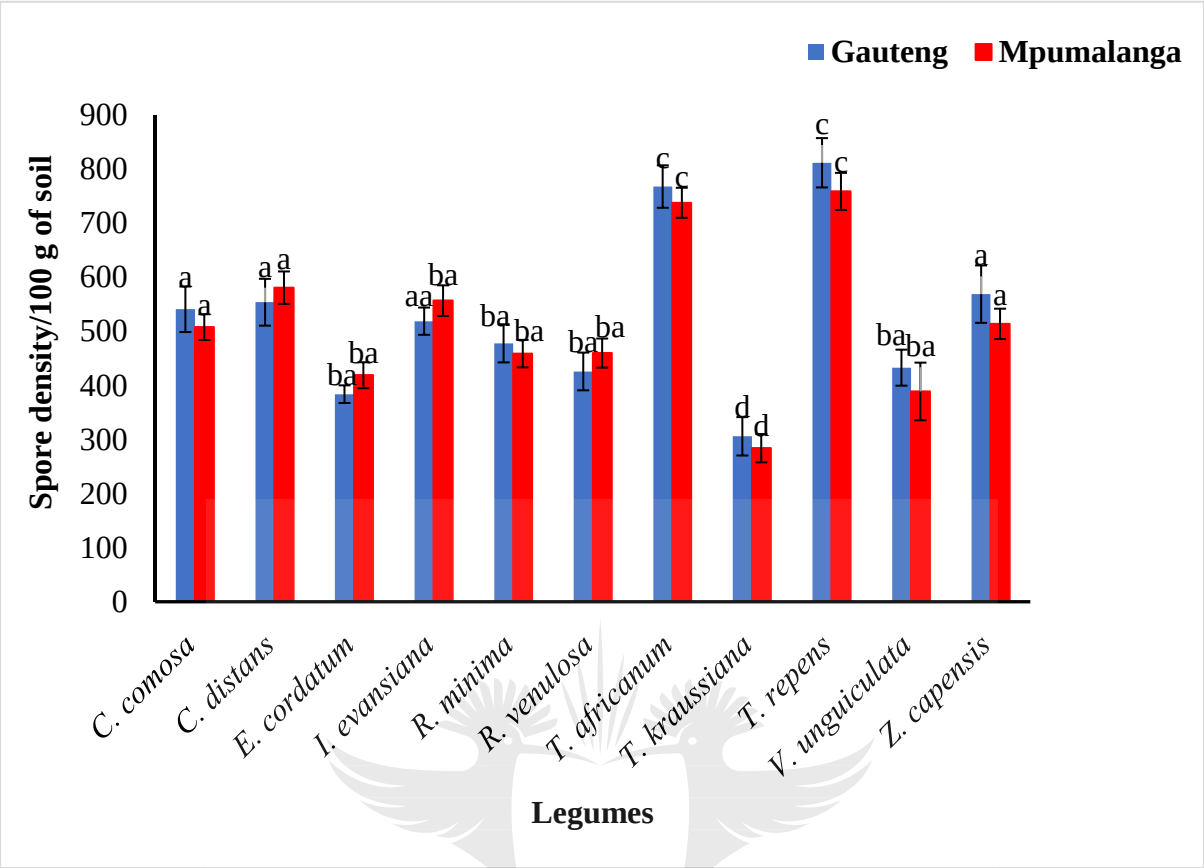


Figure 5.1: Spore density of AM fungi in the topsoil of different legumes within and between provinces. *N* = 3. Bars represents standard errors. Means with a letter in common are not significantly different ($P < 0.05$) according to the the Tukey test.

5.3.3 Descriptions of identified spores

Twenty species of AM fungi were identified in the topsoils of the studied legumes (Figure 5.2A-T). These species are distributed within 10 genera and 6 families (Glomeraceae, Acaulosporaceae, Claroideoglomeraceae, Entrophosporaceae, Diversporaceae, and Gigasporaceae). The genus *Glomus* was represented by 10 species, making it the predominant genus; followed by *Acaulospora* with 3 species, *Rhizophagus* and *Septoglomus* with 2 species each, while *Claroideoglomus*, *Entrophospora*, *Funneliformis*, *Sieverdingia*, *Gigaspora*, and *Scutellospora* recorded 1 species each.

A. *Glomus magnicaule* Hall

Spores are brown, globose, 128 µm in diameter. Spore wall composed of two layers; an outer brown and finely laminated layer swL1, and a colourless to light brown laminated inner layer, swL2. Subtending hypha (sh) is slightly flared at the point of attachment. Plug (p) of the wall-like material gradually build up on the inner wall of subtending hypha till pore occlude completely at maturity (Figure 5.2A).

B. *Glomus delhiense* Mukerji, Bhattacharjee & Tewari

Spores are brown, globose, 120 µm in diameter. It has two layers of spore wall; the outer layer, swL1 is yellowish-brown, laminate, and slightly roughened, while the inner layer, swL2 is hyaline. Subtending hypha (sh) is slightly flared at the point of attachment (Figure 5.2B).

C. *Glomus ambisporum* Smith & Schenck

Spores are black, globose (83 µm in diameter) to sub-globose (92 µm in diameter). The spore wall is composed of three layers; the outer subhyaline layer swL1 that extends to the entire length of the hyphal attachment to the center of sporocarp, the dark finely adherent laminated middle layer swL2 that is confluent with hyphal attachment, and the thin and flexible innermost layer swL3, which is a continuation of swL2. Subtending hypha (sh) is cylindrical at the spore base (Figure 5.2C).

D. *Glomus tubaeforme* Tandy

Spores are white, sub-spherical, 25 µm in diameter. Spore wall forms one thick highly refractive layer, swL1. The spores are borne on a straight subtending hypha (sh), which are at first thin-walled, later becoming very thick-walled and similar in appearance to the spore wall (Figure 5.2D).

E. *Glomus rubiforme* (Gerdemann & Trappe) Almeida & Schenck

Spores are dark-brown, obovoid, 28 µm in diameter. Sporocarp consists of a single layer of chlamydospore (cs) surrounding a central plexus of hyphae, resembling a miniature blackberry. Spore wall is one-layered (swL1), laminate, dark-brown, and with thick perforated projections on the inner surface. A variable stalk-like projections protrudes near the base of some spores (Figure 5.2E).

F. *Glomus sinuosum* (Gerdemann & Bakshi) Almeida & Schenck

Spores are orange-brown, pulvinate, with irregular surface due to protruding spores surrounded by a dense layer of tightly interwoven hyphae known as peridia, 22 µm in diameter. There is an orange-brown single layer of spore wall, swL1. Subtending hyphae (sh) is cylindrical but are sometimes hard to detect due to profuse side branching connected to the central peridia hyphae (Figure 5.2F).

G. *Glomus taiwanense* (Wu & Chen) Almeida & Schenck ex Yao

Spores are reddish-brown, sub-globose, 200 µm in diameter. Spore wall is composed of one layer, swL1. Chlamydospores (cs) are enclosed in a thin network of tightly appressed hyphae (Figure 5.2G).

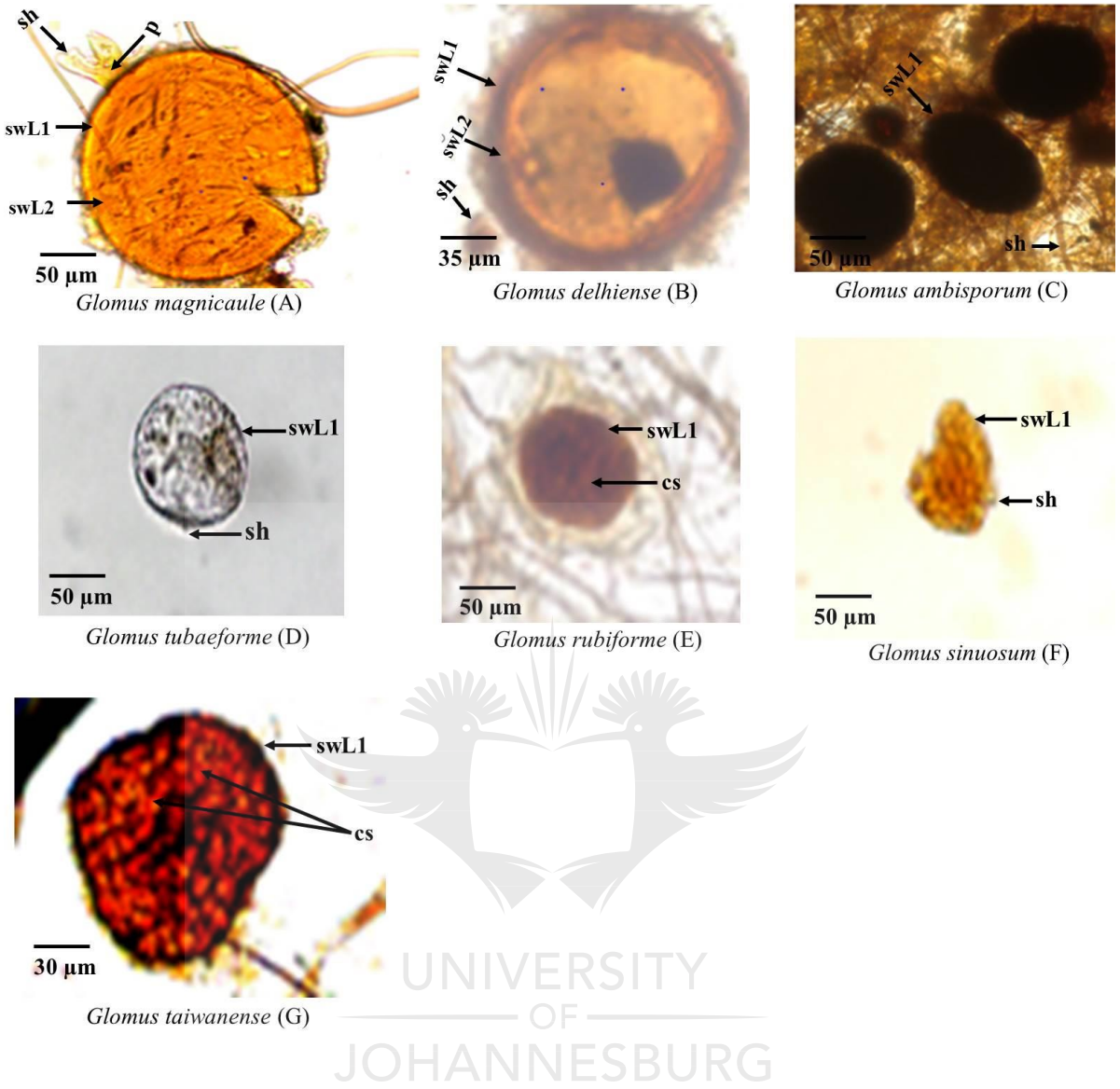


Figure 5.2(A-G): Micrographs of spores of *Glomus* species identified in the topsoils of legumes in both provinces. sh, subtending hypha; p, plug; cs, chlamydospore; swL1, Layer one of spore wall; swL2, Layer two of spore wall; swL3, Layer three of spore wall.

H. *Acaulospora colombiana* (Spain & Schenck) Kaonongbua, Morton & Bever (INVAM reference accession CL356)

Spores are golden-brown, globose, 130 µm in diameter. Spore wall consists of three layers; a hyaline outer layer swL1 that is continuous with the wall of the saccule neck; darker yellow-brown laminated second inner layer swL2 that originates from swL1 as the spore expands, and

a third single inner layer swL3, which is difficult to determine due to thinness. Spore has a germination shield (gs) and a cicatrix (c) that remains from the connection between spore and saccule neck (Figure 5.2H).

I. *Acaulospora mellea* Spain & Schenck (INVAM reference accession BR983A)

Spores are dark orange-brown, globose, 124 µm in diameter. Spore wall consists of three layers; swL1, swL2, and swL3. swL1 is hyaline, thin, flexible, and sloughs in many spores, particularly those collected from the field or stored pot cultures. When this layer does not slough, it produces numerous folds on the spore surface and appears "rugose". swL2 is an orange-yellow finely adherent sublayers whose surface appear smooth if the outer layer has sloughed; swL3 is a yellow-brown, slightly flexible laminated thin layer. Spore has a germination shield (gs) and a cicatrix (c) that shows region of contact between spore and saccule neck during spore synthesis (Figure 5.2I).

J. *Acaulospora tuberculata* Janos & Trappe (INVAM reference accession VZ103E)

Spores are light yellowish-brown, globose, 145 µm in diameter. Spore surfaces are covered with tubercles (t). There are three layers of spore wall; an outer hyaline layer, swL1 that remains after tubercles on swL2 have formed, a tightly adherent middle layer, swL2 that thickens by formation of yellowish-brown sub-layers, and a yellow-brown, swL3, which can be completely adherent to swL2, and hence undetected in some spores. Two flexible hyaline germinal inner walls (iw1 and iw2) are present (Figure 5.2J).

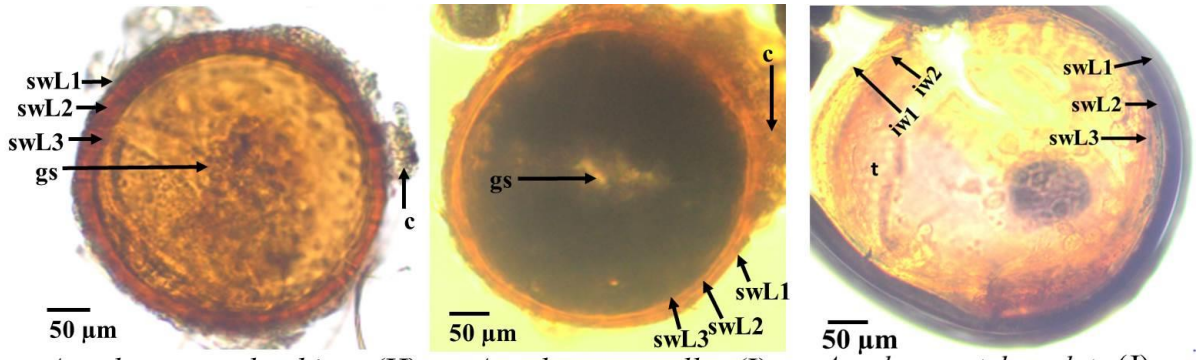


Figure 5.2(H-J): Micrographs of spores of *Acaulospora* species identified in the topsoils of legumes in both provinces. swL1, Layer one of spore wall; swL2, Layer two of spore wall; swL3, Layer three of spore wall; iw1, germinal inner wall one; iw2, germinal inner wall two; gs, germination shield; c, cicatrix; t, tubercules.

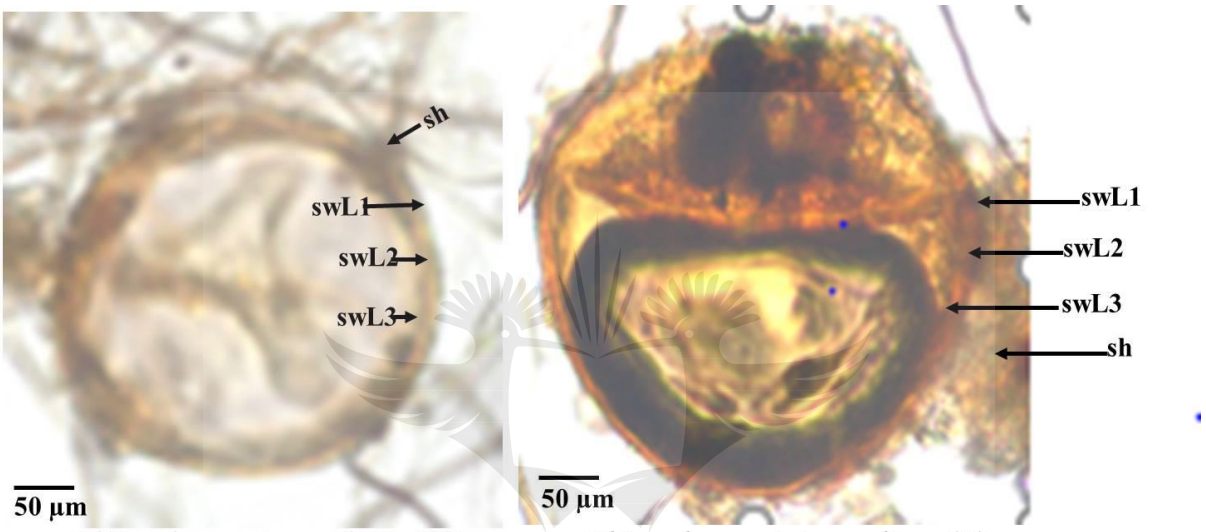
K. *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) Schüßler & Walker (INVAM reference accession PL112)

Spores are yellowish-brown, globose, 125 µm in diameter. There are three layers of spore wall; the outermost hyaline layer, swL1 is intact in young spores and disintegrates at maturity. The middle layer, swL2 is hyaline and adherent to the outer layer swL1 when intact in young spores. With age, this layer degrades at a similar rate to swL1 and appears granular or sloughs in patches. swL3 is a yellow-brown laminate layer. Subtending hypha (sh) is cylindrical (Figure 5.2K).

L. *Rhizophagus intraradices* (Schenck & Smith) Walker & Schüßler (INVAM reference accession UT126)

Spores are yellowish-brown, elliptical, 86 µm diameter in size. Spore wall composed of three layers swL1, swL2, and swL3. The outermost layer swL1 is hyaline, mucilagenous, and present only in juvenile spores. When spores mature, this layer degrades naturally and from the action

of microorganisms. swL2 is hyaline and adherent to the mucilagenous outer layer in young spores but degrades concomitantly with swL1 at maturity. L3 is a layer that consists of pale yellow-brown sublayers that either remain adherent or separate when pressure is applied. Degree of separation among sublayers varies considerably among spores, and is often influenced by age, degree of parasitism, or the amount of pressure applied after mounting. Subtending hypha (sh) is slightly flared (Figure 5.2L).



Rhizophagus irregularis (K) *Rhizophagus intraradices* (L)

Figure 5.2(K-L): Micrographs of spores of *Rhizophagus* species identified in the topsoils of legumes in both provinces. sh, subtending hypha; swL1, Layer one of spore wall; swL2, Layer two of spore wall; swL3, Layer three of spore wall.

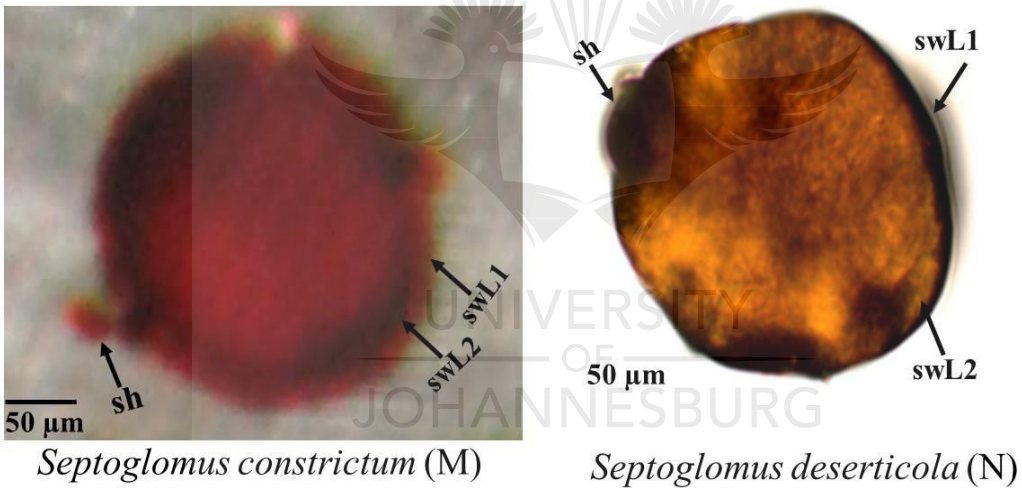
M. *Septoglomus constrictum* (Trappe) Sieverd., Silva & Oehl (INVAM reference accession KS890)

Spores are reddish-brown with shiny-smooth spore surface, globose, 153 µm in diameter. Spore wall is one-layered or occasionally seeming two-layered. The outer hyaline layer, swL1 is adherent until it degrades and sloughs in older spores, no reaction in Melzer's reagent. The

inner layer swL2 is a dark red-black laminate layer, which is continuous with the inner layer of a persistent subtending hypha. Subtending hypha (sh) is slightly flared (Figure 5.2M).

N. *Septoglomus deserticola* (Trappe, Bloss & Menge) Silva, Oehl & Sieverding (INVAM reference accession CA113)

Spores are orange-brown with a smooth spore surface, sub-globose, 85 µm in diameter. There are two spore wall layers; a hyaline outer layer, swL1 that is adherent until it degrades and sloughs in aged spores (does not react in Melzer's reagent), and an orange-brown laminate layer swL2, which is continuous with the inner layer of a persistent subtending hypha. Subtending hypha (sh) is slightly flared (Figure 5.2N).



Septoglomus constrictum (M) *Septoglomus deserticola* (N)

Figure 5.2(M-N): Micrographs of spores of *Septoglomus* species identified in the topsoils of legumes in both provinces. sh, subtending hypha; swL1, Layer one of spore wall; swL2, Layer two of spore wall.

O. *Claroideoglomus etunicatum* (Becker & Gerdemann) Walker & Schüßler (INVAM reference accession NE108A)

Spores are orange, globose (roughened from decomposition of outer wall and adherent debris), 120 µm in diameter. Spore wall is made up of two layers; the outer mucilaginous wall layer swL1 that degrades and sloughs as spore age to develop a granular appearance, stains pink to reddish purple in Melzer's reagent. The inner wall layer, swL2 consists of light orange-brown thin adherent sub-layers. Subtending hypha (sh) is cylindrical (Figure 5.2O).

P. *Entrophospora infrequens* (Hall) Ames & Schneider (INVAM reference accession AZ237)

Spores are black, globose, 148 µm in diameter. There are four layers of spore wall, L1, L2, L3, and L4. The swL1-L3 are continuous with the wall of the neck of the parent sporiferous saccule (ss), while the inner wall, swL4 forms de novo (Figure 5.2P).

Q. *Funneliformis geosporum* (Nicolson & Gerdemann) Walker & Schüßler (INVAM reference accession CA112)

Spores are black, ellipsoidal, 138 µm in diameter. Spores consists of three layers of spore wall swL1, swL2, and swL3 that form consecutively as the spore wall differentiates. The outer hyaline layer, swL1 degrades and forms a sloughing granular layer, does not react in Melzer's reagent. The middle spore wall layer, swL2 is an orange-brown rigid layer consisting of adherent sublayers, while the inner spore wall layer, swL3 is a semi-rigid to rigid layer, often adherent to swL2, but usually resolved by slightly darker colour. Subtending hypha (sh) is cylindrical (Figure 5.2Q).

R. *Gigaspora decipiens* Hall & Abbott (INVAM reference accession AU102)

Spores are yellowish-brown, globose, 280 µm in diameter. Spore consists of three layers of spore wall; swL1 is an outer permanent rigid layer, smooth, and adherent to sublayers of swL2. swL2 layer is pale yellow to yellow in newly formed spores, becoming darker brownish-yellow with age and storage. swL3 is a "germinal" layer that is concolorous and adherent with the laminate layer. Subtending hypha (sh) is flared (Figure 5.2R).

S. *Scutellospora biornata* (Spain, Sieverding & Toro) Sieverding, Souza & Oehl

Spores are orange-brown, sub-globose, 260 µm in diameter. Spore wall is distinctly two-layered; the brown outermost layer swL1 with blunt tapering projections on the surface, and the orange-brown inner layer swL2 with numerous finely adherent sublayers. Spore has one flexible germinal inner wall (iw1) and an ovoid, brown-coloured germination shield (gs) (Figure 5.2S).

T. *Sieverdingia tortuosa* (Schenck & Smith) Błaszk., Niezgoda & Goto

Spores are light yellow, globose, 125 µm in diameter. Most mature spores, whether single or arranged in clusters, are surrounded individually by a hyphal mantle (hm). Spore consists of a single pale-yellow to light-yellow laminated wall layer, swL1. Subtending hypha (sh) can be cylindrical, curved, or flared, but the shape is usually difficult to detect because it is covered with the hyphal mantle (Figure 5.2T).

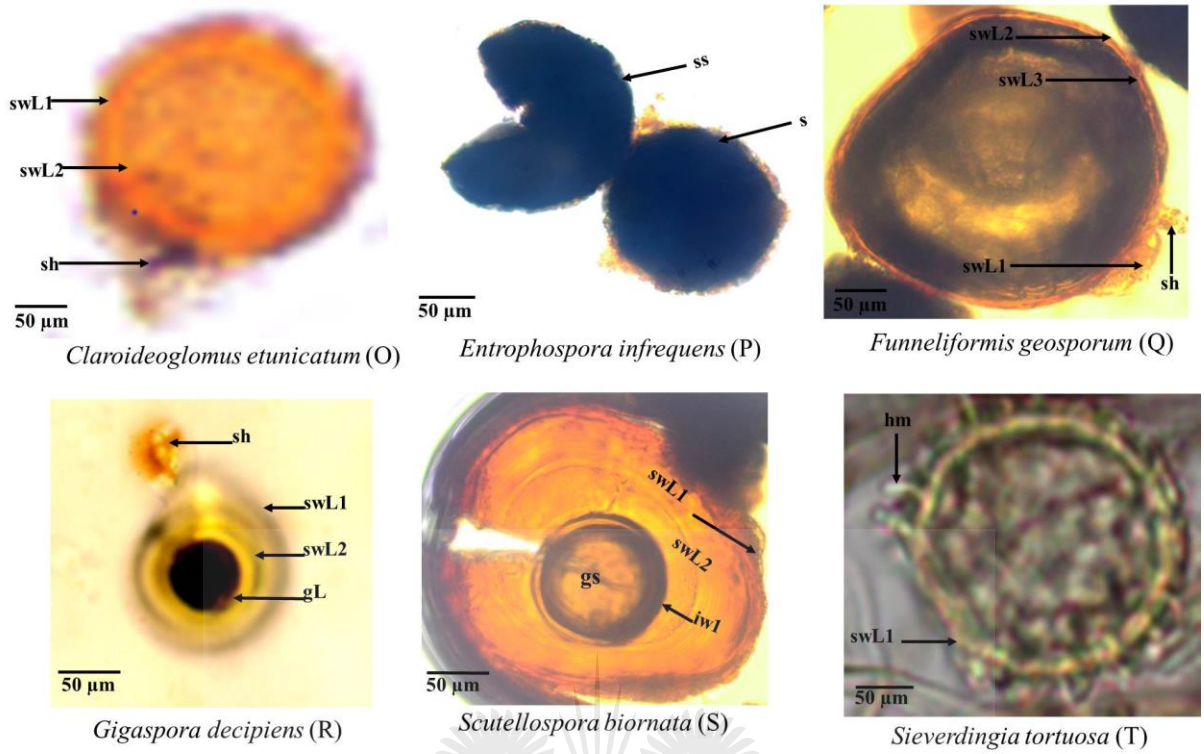


Figure 5.2(O-T): Micrographs of spores of *Claroideoglomus etunicatum*, *Entrophospora infrequens*, *Funneliformis geosporum*, *Gigaspora decipiens*, *Scutellospora biornata*, *Sieverdingia tortuosa* identified in the topsoils of legumes in both provinces. sh, subtending hypha; swL1, Layer one of spore wall; swL2, Layer two of spore wall; swL3, Layer three of spore wall; iw1, germinal inner wall one; gs, germination shield; ss, sporiferous saccule; s, spore; gL, germinal layer; hm, hyphal mantle.

5.3.4 AM fungal diversity

The average species richness ranged between 4–18 species in Gauteng and 4–15 species in Mpumalanga (Table 5.1). The mean range of *H'* in Gauteng was 1.38–2.72, while in Mpumalanga, *H'* ranged from 1.31–2.50. In Gauteng, the average range of *J'* was 0.87–0.99, whereas in Mpumalanga, *J'* ranged between 0.95–0.99. According to two-way ANOVA, only

SR and *H'* differed significantly ($F_{1,94} = 0.92$; $P < 0.05$) among legumes within provinces, but no significant difference ($F_{1,94} = 0.79$; $P > 0.05$) was detected between provinces.

Table 5.1: Alpha diversity indices of AM fungi in the topsoils of different legumes in Gauteng and Mpumalanga Provinces

Provinces	Diversity indices	*C. comosa*	*C. distans*	*E. cordatum*	*I. evansiana*	*R. minima*	*R. venulosa*	*T. africanum*	*T. kraussiana*	*T. repens*	*V. unguiculata*	*Z. capensis*
Gauteng	SR	8.00± 1.31^{a}	9.00 ± 1.15^{a}	6.00 ± 1.35^{ac}	11.00 ± 1.73^{a}	5.00 ± 1.43^{ac}	8.00 ± 2.31^{a}	12.00 ± 1.05^{b}	4.00 ± 0.98^{ac}	18.00 ± 2.89^{b}	7.00 ± 1.55^{a}	8.00 ± 1.73^{a}
	H′	1.82± 0.59^{a}	2.06± 0.54^{a}	1.70± 0.52^{a}	2.33± 0.30^{a}	1.57± 0.55^{ab}	1.99± 0.58^{a}	2.32± 0.20^{ac}	1.38± 0.54^{b}	2.72± 1.10^{c}	1.89± 0.57^{a}	2.06± 0.27^{a}
	J′	0.87± 0.08^{ns}	0.94± 0.05^{ns}	0.95± 0.03^{ns}	0.97± 0.01^{ns}	0.98 ± 0.01^{ns}	0.98± 0.02^{ns}	0.93± 0.02^{ns}	0.99± 0.01^{ns}	0.94± 0.03^{ns}	0.97± 0.02^{ns}	0.99± 0.06^{ns}
Mpumalanga	SR	6.00± 1.53^{a}	6.00 ± 0.58^{a}	4.00 ± 0.76^{a}	7.00 ± 1.15^{a}	4.00 ± 0.53^{a}	8.00 ± 2.31^{a}	15.00 ± 1.27^{b}	5.00 ± 0.58^{a}	14.00 ± 2.31^{b}	9.00 ± 0.99^{a}	10.00 ± 2.08^{a}
	H′	1.75± 0.44^{a}	1.76± 0.56^{a}	1.37± 0.57^{ab}	1.93± 0.58^{a}	1.31± 0.49^{ab}	2.02± 0.56^{a}	2.61± 0.18^{c}	1.54± 0.55^{ab}	2.50± 0.29^{c}	2.20± 0.44^{a}	2.20± 0.42^{a}
	J′	0.98± 0.05^{ns}	0.98± 0.09^{ns}	0.99± 0.04^{ns}	0.99± 0.02^{ns}	0.95± 0.02^{ns}	0.97± 0.01^{ns}	0.96± 0.01^{ns}	0.96± 0.03^{ns}	0.95± 0.01^{ns}	0.96± 0.09^{ns}	0.95± 0.01^{ns}

Values are means ± SEM. *N* = 3. Means with a letter in common are not significantly different (P < 0.05) according to the the Tukey test.
ns, not significant; SR, *H′*, and *J′* are the species richness, Shannon-Wiener index, and Pielou's evenness index, respectively.

5.3.5 AM fungal community composition

The AM fungal community composition (genus taxa level) differed among legumes within and between provinces (Figure 5.3). Similarly, the relative abundance of each genus varied among the legumes within and between provinces. Precisely, in Gauteng, *Glomus* was the most abundant in *C. distans*, *I. evansiana*, *T. africanum*, *T. repens*, and *Z. capensis*; *Acaulospora* exhibited higher abundance in *C. comosa*, *R. minima*, and *V. unguiculata*; *Septoglomus* was the most abundant genus in *E. cordatum* and *R. venulosa*, and genus *Funneliformis* had higher abundance in *T. kraussiana*. In Mpumalanga, *Glomus* was the most abundant genus in *C. comosa*, *R. minima*, and *T. africanum*, whereas genus *Acaulospora* was the most abundant in *C. distans*, *E. cordatum*, *I. evansiana*, *R. venulosa*, *T. krausiana*, *T. repens*, *V. unguiculata*, and *Z. capensis*.

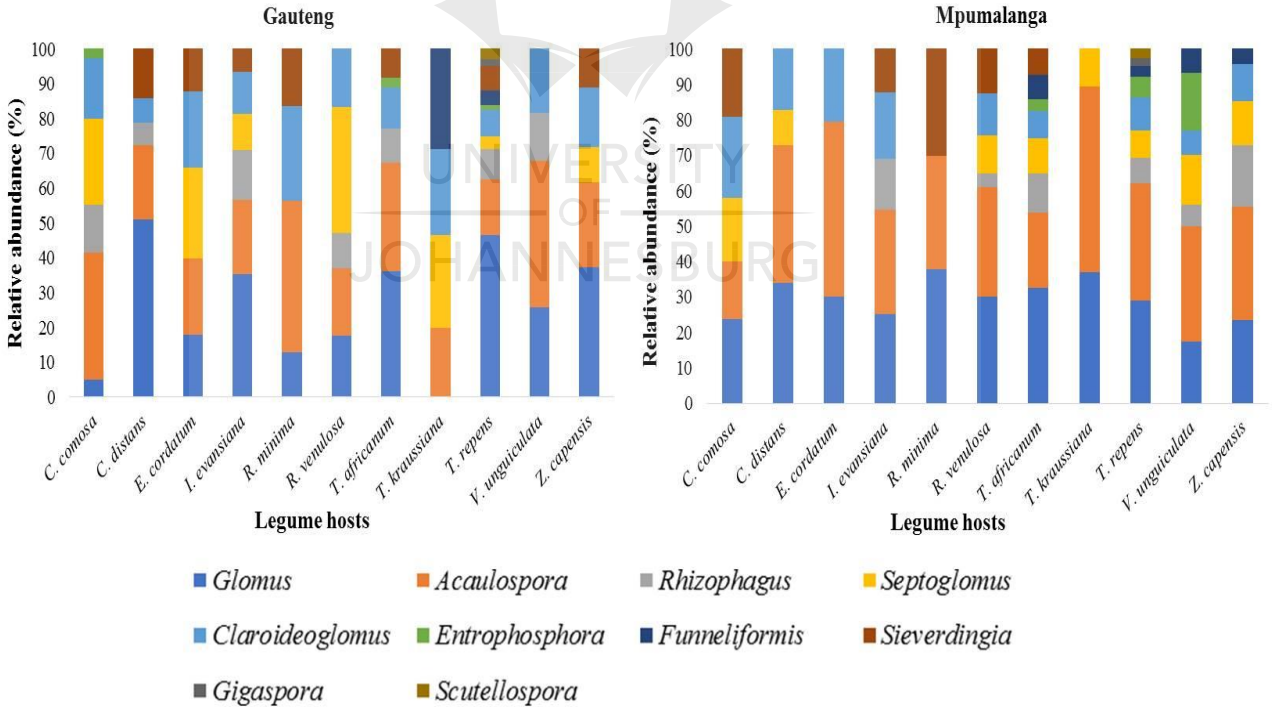


Figure 5.3: AM fungal genera in the topsoil of different legume in Gauteng and Mpumalanga provinces.

Based on IF, *Glomus ambisporum*, *Acaulospora colombiana*, *A. mellea*, *Rhizophagus intraradices*, *Claroideoglomus etunicatum*, and *Sieverdingia tortuosa* were the dominant species in Gauteng, whereas *G. magnicaule*, *G. ambisporum*, *A. colombiana*, *A. mellea*, *Septoglomus constrictum*, and *C. etunicatum* were the dominant species in Mpumalanga (Table 5.2). *Acaulospora colombiana*, *A. mellea*, and *C. etunicatum* occurred in all samples in both provinces and had the highest IF of 100%. *Gigaspora decipiens* and *Scuttelospora biornata* were the rare species in both provinces and were found only in *T. repens*.

Table 5.2: Isolation frequency (IF) of AM fungi in Gauteng and Mpumalanga Provinces

AM fungal species	IF (%)	
	Gauteng	Mpumalanga
Glomus magnicaule	45.45 (MC)	72.73 (D)
Glomus delhiense	36.36 (MC)	18.18 (C)
Glomus ambisporum	54.55 (D)	81.82 (D)
Glomus tubaeforme	45.45 (MC)	18.18 (C)
Glomus rubiforme	36.36 (MC)	18.18 (C)
Glomus sinuosum	27.27 (C)	18.18 (C)
Glomus taiwanense	18.18 (C)	18.18 (C)
Acaulospora colombiana	100 (D)	63.64 (D)
Acaulospora mellea	72.73 (D)	100 (D)
Acaulospora tuberculata	45.45 (MC)	27.27 (C)
Rhizophagus intraradices	54.55 (D)	27.27 (C)
Rhizophagus irregularis	36.36 (MC)	45.45 (MC)
Septoglomus deserticola	36.36 (MC)	18.18 (C)
Septoglomus constrictum	36.36 (MC)	54.55 (D)
Claroideoglomus etunicatum	100 (D)	100 (D)
Entrophospora infrequens	27.27 (C)	27.27 (C)
Funneliformis geosporum	18.18 (C)	36.36 (MC)
Sieverdingia tortuosa	63.64 (D)	36.36 (MC)
Gigaspora decipiens	9.09 (R)	9.09 (R)
Scutellospora biornata	9.09 (R)	9.09 (R)

D, dominant (IF > 50%), MC, most common (IF 31% - 50%); C, common (IF 10% - 30%); R, rare (IF < 10%)

5.3.6 Relationship between soil properties and AM fungal spore density, diversity, and community composition

Pearson correlation analysis showed that SD, SR, *H'*, and *J'* were positively correlated with soil pH; SD was negatively correlated with NO_3 but positively correlated with Mn; SD and SR were

negatively correlated with P but positively correlated with K (Table 5.3). Also, a significant positive correlation was found between SR, *H'*, *J'*, and BD. The CCA triplot revealed that there was a significant correlation (*P* = 0.001) between soil properties and AMF community composition (Figure 5.4). The first and second CCA axes showed that more than 50% of the total variability in AMF community composition was attributed to soil variables and/or host. Test of significance of the soil parameters fitted into the step-wise CCA model indicated that P (R^2 = 3.1714 , *P* = 0.001), K (R^2 = 4.9343, *P* = 0.001), Zn (R^2 = 5.8443, *P* = 0.001), NH_4 (R^2 = 4.9675, *P* = 0.001), and BD (R^2 = 3.6818, *P* = 0.001) were the important soil drivers of AMF community composition.

Table 5.3: Correlation analysis between AM fungal spore density, diversity indices, and soil physio-chemical properties

	SD	SR	*H'*	*J'*
pH	0.32*	0.35**	0.44***	0.41**
NO_3	-0.4***	-0.23	-0.13	0.10
NH_4	-0.07	-0.17	-0.09	0.20
P	-0.59***	-0.30*	-0.12	0.05
Organic C	0.13	0.05	0.01	0.07
K	0.54***	0.49***	0.20	0.01
Cu	-0.09	-0.18	-0.18	-0.05
Mn	0.25*	-0.14	-0.13	0.12
Zn	0.11	-0.01	-0.02	0.14
BD	0.03	0.31*	0.5***	0.38**
Sand	0.13	-0.08	-0.02	0.13
Silt	-0.2	-0.04	0.03	0.05
Clay	0.01	0.15	0.14	0.01

NO_3, nitrate; NH4, ammonia; P, available phosphorus; K, available potassium; Cu, copper; Mn, manganese; Zn, zinc; BD, Bulk density; SD, spore density; SR, species richness; *H'*, Shannon-Wiener diversity index; *J'*, Pielou's evenness index; *Significant at *P* < 0.05; **Significant at *P* < 0.01; *** Significant at *P* < 0.001.

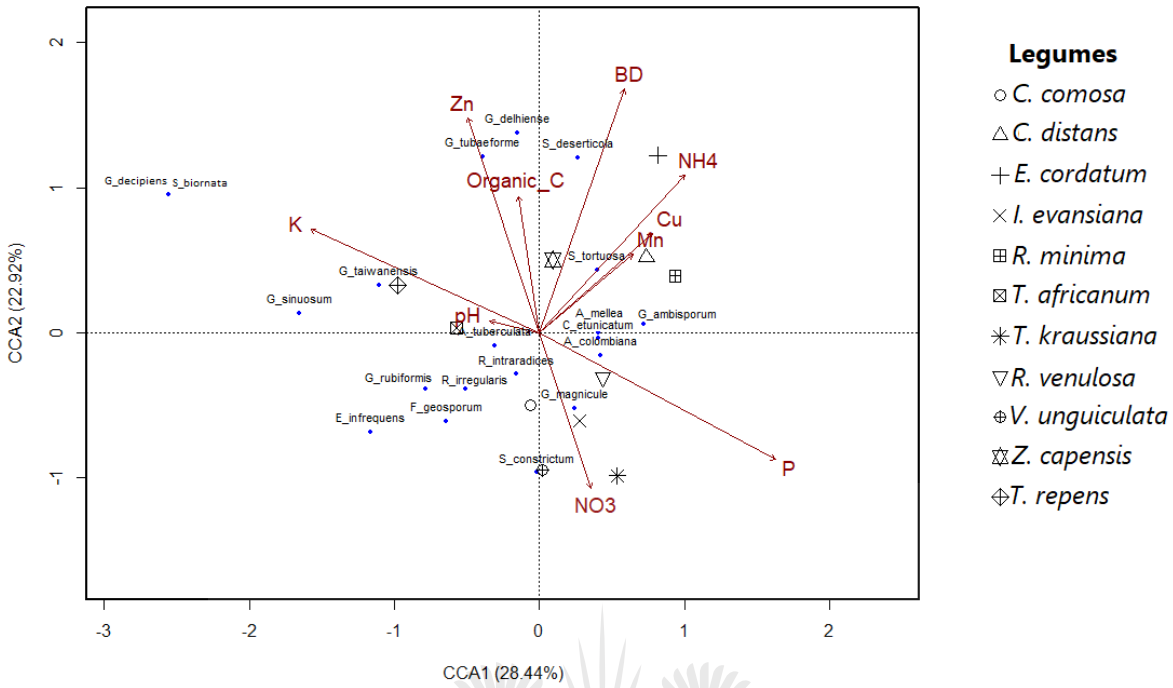


Figure 5.4: The canonical correspondence analysis (CCA) showing the relationship between AM fungal community composition and soil physico-chemical properties. The first (CCA1) and second (CCA2) canonical axes of the CCA plot are significant at $P < 0.05$. NO_3, nitrate; NH_4, ammonia; P, available phosphorus; K, available potassium; Cu, copper; Mn, manganese; Zn, zinc; BD, Bulk density.

5.4 Discussion

Previous studies have shown that most members of the family Fabaceae form AM symbioses, although the responsiveness of different legume species to inoculation by AM fungi are variable (Trappe, 1987; Allsopp and Stock, 1993; Janos, 2007). In this study, SD, SR, and *H'* varied significantly among legumes, suggesting host plant influence. This influence may be mediated by the composition and proportion of root exudates secreted into the soil by different plants (Carrenho et al., 2002; Jones et al., 2004). Legumes secrete different types and amounts of exudates, which act as energy sources for AM fungal growth, and ultimately influence spore

population, species richness, and diversity (D'arcy-Lameta, 1988; Steinkellner et al., 2007). Other factors like differences and seasonal variability in sporulation patterns among AM fungal taxa and soil environmental influences can equally contribute to the variation observed (Jamiołkowska et al., 2018).

Arbuscular mycorrhizal fungal spores possess key morphological features for identification to species level, and many studies have inferred the diversity of AM fungal communities based on spore morphological traits (Charoenpakdee et al., 2010; Ambili et al., 2012; Abdullahi et al., 2014; Wang et al., 2019). In this study, twenty glomeromycotan species were identified in the topsoils of examined legumes. This diversity is higher than what was found in other host plants in South Africa and in different legumes from other semi-arid regions. Previous studies from South Africa identified four species in *Vangueria infausta* (Gaur et al., 1999) and eight species in *Manihot esculenta* (Straker et al., 2010). Dalpé et al. (2000) isolated five species from *Faidherbia albida* in the northern semi-arid region of Senegal. Feitosa de Souza (2016) detected 18 species in *Mimosa tenuiflora* in semi-arid region of Brazil.

The results revealed that the dominant taxon was *Glomus,* followed by *Acaulospora*. These genera have been reported to predominate in other species of Fabaceae. For example, Songachan and Kayang (2013) isolated 61 species of AM fungi from natural and cultivated sites of *Flemingia vestita* Benth. ex Baker., with 35 species belonging to genus *Glomus* and 12 to *Acaulospora*. Choosa-Nga et al. (2019) identified 14 species in three tree species (*Dalbergia cochinchinensis, Pterocarpus macrocarpus*, and *Xylia xylocarpa*), out of which six species belong to *Glomus* and three to *Acualospora*. The abundance of *Glomus* could be attributable to the ease with which they sporulate in the soil (Vieira et al., 2020). More so, species of this genus often produce abundant spores, which are considered the most resilient propagules under severe environmental conditions (Lennon and Jones, 2011). On the other

hand, *Acaulospora* has better ability to thrive in acidic environments (Chagnon et al., 2013; Veresoglou et al., 2013). Thus, the low soil pH recorded in this study may justify the abundance of *Acaulospora* in the examined plants.

In this study, the genera *Gigaspora* and *Scutellospora* (family, Gigasporaceae) were present in low numbers. This finding is consistent with those of Dalpé et al. (2000) and Feitosa de Souza (2016), who recorded low numbers for these genera in *Faidherbia albida* in the semi-arid region of Senegal and in *Mimosa tenuiflora* in the semi-arid region of Brazil, respectively. Members of the family Gigasporaceae propagate mainly through mycelial fragments (Hart and Reader, 2002; Brito et al., 2012). In addition, several studies have shown that members of this family are common in sandy soils such as dunes (Lee and Koske, 1994; Lekberg et al., 2007; Chaudhary et al., 2014). Therefore, the low fitness of these genera compared to other AM fungal genera in other ecosystems may explain the scarcity of these taxa. The occurrence of these genera solely in *T. repens* may imply host preference. Reports have shown that some glomeromycotan species show preference for certain host plants under field conditions (Castillo et al., 2016; Wang et al., 2019). Furthermore, *A. colombiana*, *A. mellea*, and *C. etunicatum* were found to be the most frequent AM fungal species. Other studies have shown that *Acaulospora* species can tolerate harsh environmental conditions and *C. etunicatum* is considered a cosmopolitan species (Stutz et al., 2000; Straker et al., 2010; Jefwa et al., 2006). It is possible that the capability of these species to adapt well in a wide range of environments is the reason for their presence in all the legume species studied.

Soil factors are key regulators of AM fungal diversity and community composition (Jansa et al., 2014; Alguacil et al., 2016). In the current study, a significant positive correlation was observed between SD, SR, *H´*, *J*, and soil pH. pH is an important soil abiotic variable that influences AM fungal diversity by controlling the availability of soil nutrients and ions for

absorption by plants (Coughlan et al., 2000; Kawahara et al., 2016). Given that AM associations are mainly activated under nutrient-deficient conditions, the low concentrations of P and N in the soils could justify the significant inverse relationship between SD, SR, and P and N.

Soil compactions alter the physical properties of the soil, leading to poor aeration and reduced nutrient availability for plants and microbial populations (Nadian et al., 1998; Li et al., 2002). One of the most widely used metrics of soil compaction is bulk density. Although the threshold bulk density value for impeding root growth and microbial activities varies with soil type and across different regions (Houlbrooke et al., 1997; McKenzie et al., 2004), the bulk density values recorded in this study could have created a favourable condition for AM fungal sporulation and may account for the significant positive correlation between SR, *H´*, *J*, community composition, and BD.

Manganese and zinc play crucial roles in the metabolic process of legumes, in particular nitrogen fixation. Therefore, the significant positive relationship between SD, SR, community composition, and Mn and Zn amounts indicates that the absorption of these micronutrients may be enhanced by AM fungi (Weisany et al., 2013; Lehmann and Rillig, 2015). AM fungal SD, SR, and community composition were positively correlated with soil K. Reports have shown that soil K has stimulatory effects on AM fungal attributes under drought stress (Furlan et al., 1989; Ouimet et al., 1996). Therefore, the high concentration of K in soils of examined plants might have stimulated spore production, and as a result, improved SD, and community structure.

To conclude, this study showed that the diversity of AM fungal communities in the topsoils of the legume species varied significantly within provinces. This study also showed the prevalence of the genus *Glomus* in the topsoils of the legumes. *Trifolium* species had the

highest spore density and species richness, demonstrating that their soils could be a major source of inoculum. Finally, the study indicated that soil pH, NO_3, P, K, BD, Zn, and NH_4 have significant effects on the diversity and community composition of AM fungi in the topsoil of legumes.

CHAPTER 6

MOLECULAR DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES IN RHIZOSPHERE SOILS

6.1 Introduction

Fungi are highly diverse and vital components of soil microbial communities. They are potential regulators of critical ecosystem processes, including plant productivity and community dynamics (Van der Heijden et al., 2008; Mommer et al., 2018), decomposition of soil organic matter (Strickland et al. 2009; Baumann et al., 2013), biogeochemical cycling (Schimel and Schaeffer 2012; Philippot et al., 2013), and carbon sequestration (Clemmensen et al., 2013; Zak et al., 2019). Among the various soil fungi, AM fungi are particularly renowned for their profound impacts on plant mineral nutrition (Smith and Smith, 2011; Averill et al., 2019), plant protection (Aroca et al., 2007; Smith et al., 2010; Wang et al., 2018), and ecosystem functions (Rillig, 2004b; Frac et al., 2018).

The considerable ecological roles of AM fungi have invigorated studies of the diversity and biogeography of these fungi, as well as the potential drivers of their distribution and community assemblages (Rodrıguez-Echeverrıa et al., 2017; Zhao et al., 2017; Šmilauer et al., 2020; Zhang et al., 2021). There is clear evidence that biotic and environmental factors are predictors of AM fungal diversity and community composition (Dumbrell et al., 2010; That and Sijam, 2012; Martínez-García et al., 2015). Reports have shown that soil properties are important environmental variables that could shape the abundance and composition of AM fungal communities in soils, especially at landscape scales (Jansa et al., 2014; Alguacil et al., 2016; Xu et al., 2017). Huang et al. (2019) found that pH, available P, and Cu significantly influenced the AM fungal communities in pear orchard soils. Luo et al. (2019) reported that SOC had a marked effect on the AM fungal communities in soils of *Cynodon dactylon*. Zhu et al. (2020)

demonstrated that organic C, N, P, and pH shaped the AM fungal diversity and community composition in the cropland black soils of China.

Assessing the diversity of AM fungi in the rhizosphere is essential to understanding the ecological impacts of AM fungi, as this provides information on the AM fungal communities with the potential to colonise plant roots (Antunes et al., 2011; Song et al., 2019). Traditionally, investigating the diversity of AM fungal communities in field soils relied on spore quantification and identification (Sale et al., 2015; Mahecha-Vásquez et al., 2017; Wang et al., 2019; Melo et al., 2020). This method, however, suffers from some significant drawbacks that render it unreliable (Sanders, 2004; Taylor et al., 2013). Specifically, alterations in morphological features of field-collected spores can lead to misidentification, and insufficient morphological characters make it difficult to discriminate between some AM fungal taxa (Morton and Redecker, 2001; Redecker and Raab, 2006). Moreover, spore production dynamics vary in different AM fungal taxa depending on environmental conditions and the physiological status of the fungus, and some taxa rarely sporulate, if at all (Oehl et al., 2009). Accurate identification is crucial in AM fungal community studies, which increasingly rely on molecular-based techniques. With the development of various high-throughput sequencing technologies (Margulies et al., 2005), an in-depth analysis of the diversity and structure of soil microbial communities is now possible. In recent years, the Illumina Miseq sequencing platform has gained increasing application in AM fungal diversity research and has provided a robust approach to identify the AM fungi in field soils and explore the drivers of community composition (Zhao et al., 2017; Luo et al., 2019; Zhang et al., 2021).

Therefore, the current study analysed the AM fungal communities in the rhizosphere soils of eleven indigenous legume species, using Illumina MiSeq sequencing of the partial SSU rRNA gene. The objectives are to characterise and compare the diversity and community composition

of AM fungi in the rhizosphere soils of legumes within and between provinces and to determine the influence of soil physico-chemical properties on the diversity and composition of AM fungal communities.

6.2 Materials and Methods

6.2.1 DNA extraction and PCR amplification of the partial SSU rRNA gene

Total genomic DNA was independently extracted from each replicate rhizosphere soil sample of each legume species per province using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Summarily, 60 µL of solution C1 was added to powerbead tubes containing 0.25 g of soil. The tubes were vortexed at 14, 000 ×g for 10 m and centrifuged at 10,000 ×g for 30 s. Thereafter, 500 µL of the supernatant was transferred to a clean 2 ml collection tube, 250 µL of solution C2 was added and vortexed briefly for 5 s. The tubes were incubated at 8 °C for 5 m and centrifuged at 10,000 ×g for 1 m. Then, 600 µL of the supernatant was transferred to a 2 ml collection tube, 200 µL of solution C3 was added, vortexed briefly, incubated at 8 °C for 5 m, and centrifuged at 10,000 ×g for 1 m. 750 µL of the supernatant was transferred to a 2 ml collection tube, 1200 µL of solution C4 was added and vortexed for 5 s. Thereafter, 675 µL of the supernatant was loaded onto the spin column, centrifuged at 10,000 ×g for 1 min, and the flow-through was discarded. This step was repeated twice.

Then, DNA was cleaned by adding 500 µL of solution C5 to the supernatant and centrifuged at 10,000 ×g for 30 s. The flow-through was discarded and the tubes were centrifuged at 10,000 ×g for 1 m. DNA was eluted by adding 100 µL of solution C6 and centrifuging at 10,000 ×g for 30 s. Final DNA concentrations were determined by NanoDropTM Spectrophotometer ND-1000 and DNA quality was checked on a 1% agarose gel. DNA samples were stored at −20°C for further processing.

The partial SSU rRNA gene was amplified by a nested PCR reaction. The primer pairs AML1 and AML2 were utilised in the first PCR step, while the primer sets AMV4.5F and AMDGR were employed in the second PCR reaction. The first PCR cocktail was prepared in a total volume of 25 µL containing 12.5 µL of Q5® High-Fidelity 2X Master Mix (New England BioLabs, Inc., Ipswich, Massachusetts, USA), 1.25 µL of each primer (0.5 µM), 1 µL DNA template (approximately 10 ng), and 9 µL of PCR-grade water. The PCR conditions were an initial denaturation at 98°C for 3 m, 35 cycles at 98°C for 45 s, 58°C for 30 s, 72°C for 1 m, and 72°C for 10 m. PCR amplicons were verified on 1 % agarose gel and amplicon size was yielded approximately 800 bp amplicons. For the second PCR, 2 µL of 1:10 dilution of the first PCR products was used as the DNA template with the following conditions: 98°C for 3 m, 30 cycles at 98°C for 30 s, 58°C for 30 s, 72°C for 1 m, and 72°C for 10 m. The PCR products were checked on 1 % agarose gel and amplicons size was approximately 260 bp amplicons.

6.2.2 Illumina MiSeq sequencing and bioinformatics analyses

The PCR products were purified. Then, the libraries were amplified, quantified, normalized, pooled, and paired-end sequenced on an Illumina MiSeq platform (Please refer to chapter 4.2.3 for details). Raw paired-end sequences were demultiplexed and quality-filtered. Reads were then clustered into OTUs at 97% sequence identity. The resulting OTU count table was depleted of singletons, and sub-sampled to an even depth across samples before computing alpha- and beta diversities in QIIME2.

6.2.3 Statistical analyses

Statistical tests for differences in alpha diversity indices among legumes within and between provinces were determined by the non-parametric Kruskal-Wallis H and Mann-Whitney U tests, respectively. The Fisher's least significant difference post hoc test was used for mean separations at $P < 0.05$. For analyses of variability in AM fungal community composition (beta-

diversity) among legumes, relative count data were log_{10} transformed [log_{10} (x)+1, where x > 0] using the "decostand ()" function in the vegan package of R software. Comparisons of beta-diversity were based on both unweighted (presence or absence) and weighted (relative abundance) Bray-Curtis dissimilarity matrices. The dissimilarity matrices were further examined by principal coordinate analyses. Differences in beta-diversity among legumes within and between provinces were tested using the Two-way permutational multivariate analysis of variance (PERMANOVA). Furthermore, permutational test of homogeneity of multivariate dispersions (PERMDISP) was performed to test the homogeneity of multivariate dispersions in AM fungal community composition among legumes. Post hoc test for significant PERMANOVA ($P<0.05$) was performed in the vegan package using the "pairwiseAdonis ()" function.

To determine the similarities in AM fungal community composition among legumes, cluster analysis was performed on the Bray-Curtis dissimilarities using the unweighted pair-group method of arithmetic averages (UPGMA). An indicator species analysis was done using the *multipatt* function of the *indicspecies* package in R, to identify OTUs that are specific to any legume or province. The indicator species analysis assigns an indicator value (between 0 and 1) to each OTU in the group based on the product of the relative abundance and relative frequency of the OTU in the group. Tests for significance of the indicator value were further determined through permutation (probability) tests. Venn diagrams were drawn using the Venn package in R, to show the OTUs that are unique to legumes in each province or that are shared between provinces.

The relationship between soil physico-chemical properties and AM fungal diversity and community composition were assessed by Spearman rank correlation and by canonical correspondence analysis, respectively. The CCA was performed on the log-transformed

environmental and AM fungal community data by using an automatic forward and backward stepwise model ("ordistep ()" function) in the vegan package of R software. Test for significance of the environmental (constraining) variables was determined using a permutation test. Multicollinear environmental variables (Variance inflation factor > 10) were discarded from the final CCA plot. The contribution of soil properties to the AM fungal community composition was further elucidated by variance partitioning (chi-square-based partitioning) in the vegan package of R software.

6.2.4 Data availability

Paired-end sequence reads for the partial SSU rRNA gene generated from this study have been deposited in the Sequence Read Archives of the National Centre for Biotechnological Information under the BioProject ID PRJNA690541 (Accession Nos. SRR14740571–SRR14768778).

6.3 Results

6.3.1 Soil physico-chemical properties

The soils of the examined plants in both provinces were acidic (pH < 7). The textural classification of soil in Gauteng was predominantly sandy-loam, while in Mpumalanga, the soils were classified into seven textural types. Excluding bulk density, all the soil properties determined differed significantly ($F_{1,94} = 1.18$; $P < 0.05$) among legumes within and between provinces (Please refer to section 4.3.1 and Table 4.1 for details).

6.3.2 Sequencing information and sampling intensity

Illumina sequencing generated a total of 4,116,057 and 2,951,885 raw sequences for Gauteng and Mpumalanga, respectively, with average sequence length of 259.6 bp. After quality filtering, a total of 630,412 and 701,477 high-quality partial Glomeromycotan SSU rRNA

sequences were obtained for Gauteng and Mpumalanga, respectively. Based on 97% sequence similarity, these sequences clustered into 322 and 335 OTUs for Gauteng and Mpumalanga, respectively. The OTUs that could be classified belong to 8 AM fungal genera, while those that could not be classified to the genus level were assigned as unclassified.

The genus *Glomus* had the highest number of OTUs (223 and 232 OTUs for Gauteng and Mpumalanga, respectively), whereas, the contributions of other genera were lower: *Claroideoglomus* (26 and 32 OTUs for Gauteng and Mpumalanga, respectively), *Paraglomus* (27 OTUs for each province), *Diversispora* (14 and 11 OTUs for Gauteng and Mpumalanga, respectively), *Acaulospora* (2 and 3 OTUs for Gauteng and Mpumalanga, respectively), *Scutellospora* (7 and 10 OTUs for Gauteng and Mpumalanga, respectively), *Ambispora* (1 and 3 OTUs for Gauteng and Mpumalanga, respectively), *Archaeospora* (2 OTUs in each province), unclassified Glomeromycetes (15 and 9 OTUs for Gauteng and Mpumalanga, respectively), and unclassified Glomerales (5 and 6 OTUs for Gauteng and Mpumalanga, respectively). In both provinces, the rarefaction curves showed saturation at an even depth of 10,000 sequences per sample, indicating that the sampling intensity was sufficient to describe the OTU diversity in each legume species (Figure 6.1a-k).

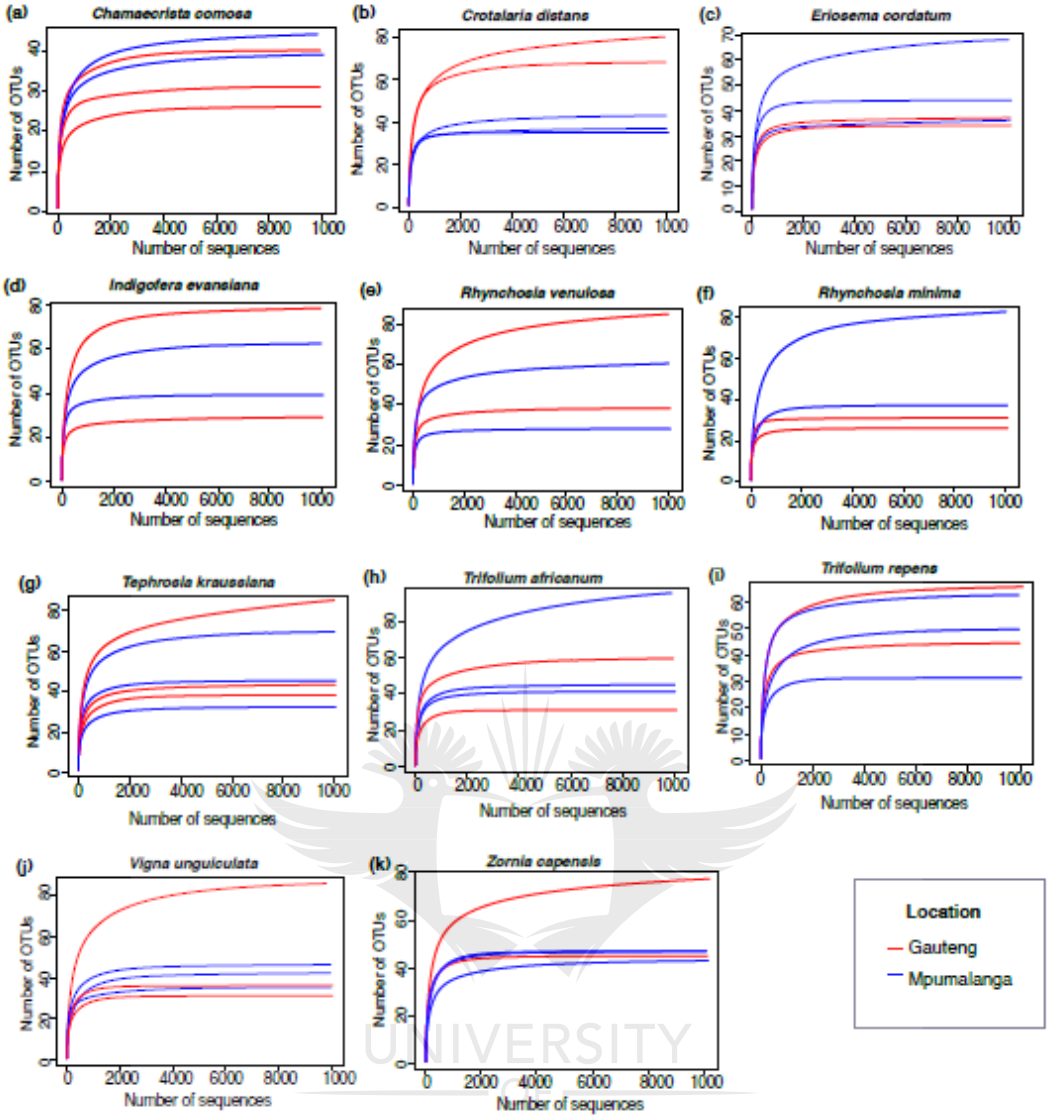


Figure 6.1a-k: Rarefaction curves of subsampled SSU sequences of each legume species between provinces.

6.3.3 AM fungal diversity

The mean OTU richness ranged from 28.50–74.00 in Gauteng and 36.00–58.00 in Mpumalanga (Table 6.1). Moreover, the average Chao1 ranged between 28.50–76.10 and 36.00-63.50 in Gauteng and Mpumalanga, respectively. The average *H'* ranged from 3.51–4.24 in Gauteng and 3.52-4.09 in Mpumalanga. In Gauteng, the mean range of *D* was 0.11–0.14 and 0.10–0.14 in Mpumalanga. The mean *J'* was 0.68–0.77 in Gauteng and 0.65–0.78 in

Mpumalanga. Of all the alpha diversity indices, only OTU richness (observed and Chao1) and *H'* significantly differ ($P < 0.05$) among legumes within province, but no significant difference ($P > 0.05$) was observed in all the diversity indices between provinces.

Table 6.1: Alpha diversity indices of AM fungi in different legumes in Gauteng and Mpumalanga Provinces

Provinces	Diversity indices	*C. comosa*	*C. distans*	*E. cordatum*	*I. evansiana*	*R. minima*	*R. venulosa*	*T. africanum*	*T. kraussiana*	*T. repens*	*V. unguiculata*	*Z. capensis*
Gauteng	OTU richness	32.33±7.09^{cd}	74.00±6.00^{a}	35.50±1.50^{bcd}	53.54±24.50^{abcd}	28.50±2.50^{d}	56.00±28.00^{abc}	45.00±14.00^{abcd}	55.00±25.24^{abc}	54.50±10.50^{a}	55.67±25.89^{abc}	61.00±16.00^{ab}
	Chao1	32.33±7.09^{cd}	76.10±8.10^{a}	35.50±1.50^{bcd}	55.00±26.00^{abc}	28.50±2.50^{d}	57.75±29.75^{abc}	45.50±14.50^{abcd}	58.06±30.51^{abc}	54.63±10.63^{abc}	56.17±26.74^{abc}	62.50±17.50^{ab}
	H'	3.51±0.39^{b}	4.23±0.19^{a}	3.73±0.16^{b}	4.09±0.37^{ab}	3.71±0.29^{b}	3.89±0.10^{ab}	3.93±0.67^{ab}	3.77±0.54^{ab}	3.93±0.10^{ab}	4.04±0.38^{ab}	4.24±0.10^{a}
	D	0.14±0.03^{a}	0.13±0.03^{ab}	0.12±0.02^{ab}	0.09±0.01^{ab}	0.12±0.03^{ab}	0.13±0.03^{ab}	0.12±0.05^{ab}	0.14±0.03^{a}	0.14±0.01^{a}	0.11±0.03^{ab}	0.11±0.01^{ab}
	J	0.70±0.03^{a}	0.68±0.04^{ab}	0.72±0.02^{ab}	0.74±0.03^{ab}	0.77±0.04^{ab}	0.71±0.08^{ab}	0.71±0.07^{ab}	0.66±0.03^{a}	0.68±0.02^{a}	0.71±0.05^{ab}	0.72±0.03^{ab}
Mpumalanga	OTU richness	38.33±6.03^{ab}	41.33±3.79^{ab}	51.50±16.02^{ab}	44.00±16.09^{ab}	56.00±23.30^{ab}	46.50±11.82^{ab}	58.00±31.58^{ab}	57.83±12.09^{a}	50.67±10.60^{ab}	36.00±5.57^{b}	45.67±2.31^{ab}
	Chao1	38.44±6.19^{b}	41.33±3.79^{ab}	51.79±15.98^{ab}	44.00±16.09^{ab}	63.50±34.87^{ab}	47.56±13.50^{ab}	59.50±34.14^{ab}	61.33±14.15^{a}	50.67±10.60^{ab}	36.00±5.57^{b}	45.67±2.31^{ab}
	H'	3.52±0.19^{b}	4.00±0.44^{a}	4.03±0.21^{a}	3.95±0.53^{ab}	3.96±0.49^{ab}	4.09±0.51^{a}	4.08±0.48^{a}	3.92±0.14^{a}	3.82±0.37^{ab}	3.67±0.44^{b}	3.95±0.48^{ab}
	D	0.14±0.03^{a}	0.10±0.04^{a}	0.12±0.03^{a}	0.11±0.04^{a}	0.11±0.04^{a}	0.10±0.03^{a}	0.10±0.02^{a}	0.13±0.02^{a}	0.12±0.03^{a}	0.12±0.05^{a}	0.11±0.04^{a}
	J	0.67±0.03^{abc}	0.75±0.08^{ab}	0.70±0.04^{abc}	0.73±0.07^{abc}	0.65±0.00^{abc}	0.78±0.01^{a}	0.71±0.01^{abc}	0.69±0.03^{abc}	0.68±0.05^{abc}	0.71±0.09^{abc}	0.72±0.08^{abc}

Values are given as means ± SEM. *N* = 3. Means with a letter in common are not significantly different (P < 0.05) according to the Fisher's test.
Chao1, *H'*, *D*, and *J* are the estimated OTU richness, Shannon-Wiener index, Simpson Dominance index, and Pielou's evenness index, respectively

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6.3.4 AM fungal community composition

The composition and relative abundance of AM fungal communities (genus taxa level) varied among legumes within and between provinces (Figure 6.2). The genera *Glomus* and *Claroideoglomus* were present in all legumes in both provinces; with *Glomus* being the dominant genus and accounting for 69.3% of the total AM fungal communities. In Gauteng, the genus *Ambispora* was the least dominant (0.62%) and was only detected in *T. kraussiana*. Meanwhile in Mpumalanga, *Archaeospora* was the least dominant (0.89%) and was exclusive to *E. cordatum*, *T. africanum*, *Z. capensis*, *R. venulosa*, and *R. minima*. However, the indicator species analysis showed that no significant ($P > 0.05$) indicator species were detected across legumes and between provinces (Appendix I).

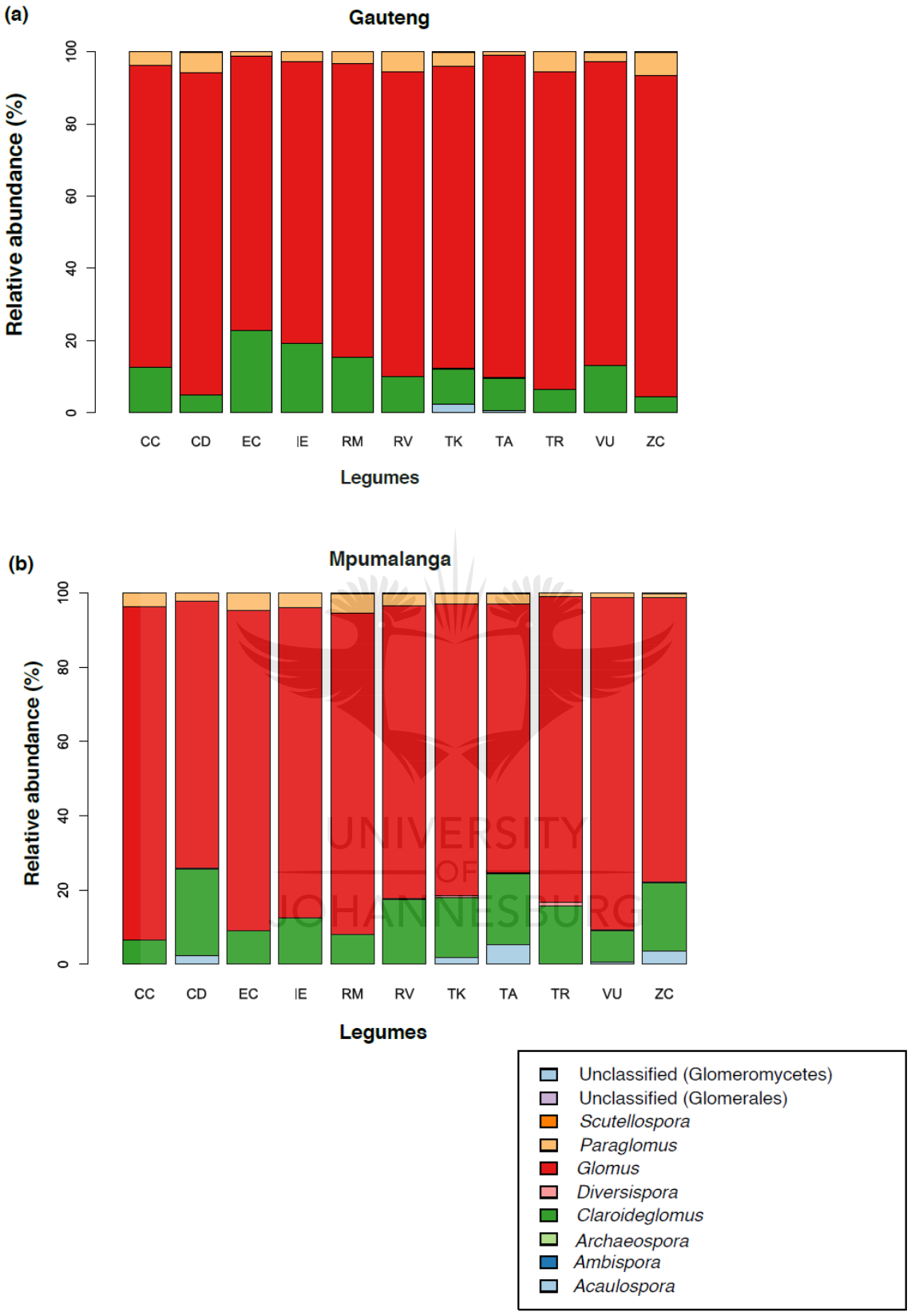

Figure 6.2: AM fungal genera in the rhizosphere soil of legumes (a) Gauteng (b) Mpumalanga. CC, *Chamaecrista comosa*; CD, *Crotalaria distans*; EC, *Eriosema cordatum*; IE, *Indigofera*

evansiana; RM, *Rhynchosia minima*; RV, *Rhynchosia venulosa*; TA, *Trifoilum africanum*; TK, *Tephrosia kraussiana*; TR, *Trifolium repens*; VU, *Vigna unguiculata*; ZC, *Zornia capensis*.

At the OTU level, the PCoA analysis based on Bray-Curtis dissimilarity revealed that there was variation in AM fungal community composition among legumes. In Gauteng, 40.9% of the total variation in AM fungal communities among legumes was explained by the first and second principal component axes (Figure 6.3a). Whereas in Mpumalanga, 52.1% of the overall variation was explained by the first and second principal component axes (Figure 6.4a). However, PERMANOVA and PERMDISP showed that the variation in AM fungal community composition and distribution observed in multivariate space was not significantly different both within province (Gauteng; PERMANOVA R^2 = 0.35521, P = 0.20; PERMDISP P = 0.988; Mpumalanga; PERMANOVA R^2 = 0.3945, P = 0.062; PERMDISP P = 0.946) and between provinces (PERMANOVA R^2 = 0.17822, P = 0.515). The clustering of AM fungal communities in legumes was further depicted by dendrograms (Figures 6.3b and 6.4b). The dendrograms revealed that the legumes, notably replicates of the same legume species, clustered together in each province, indicating similarity in the fungal communities.

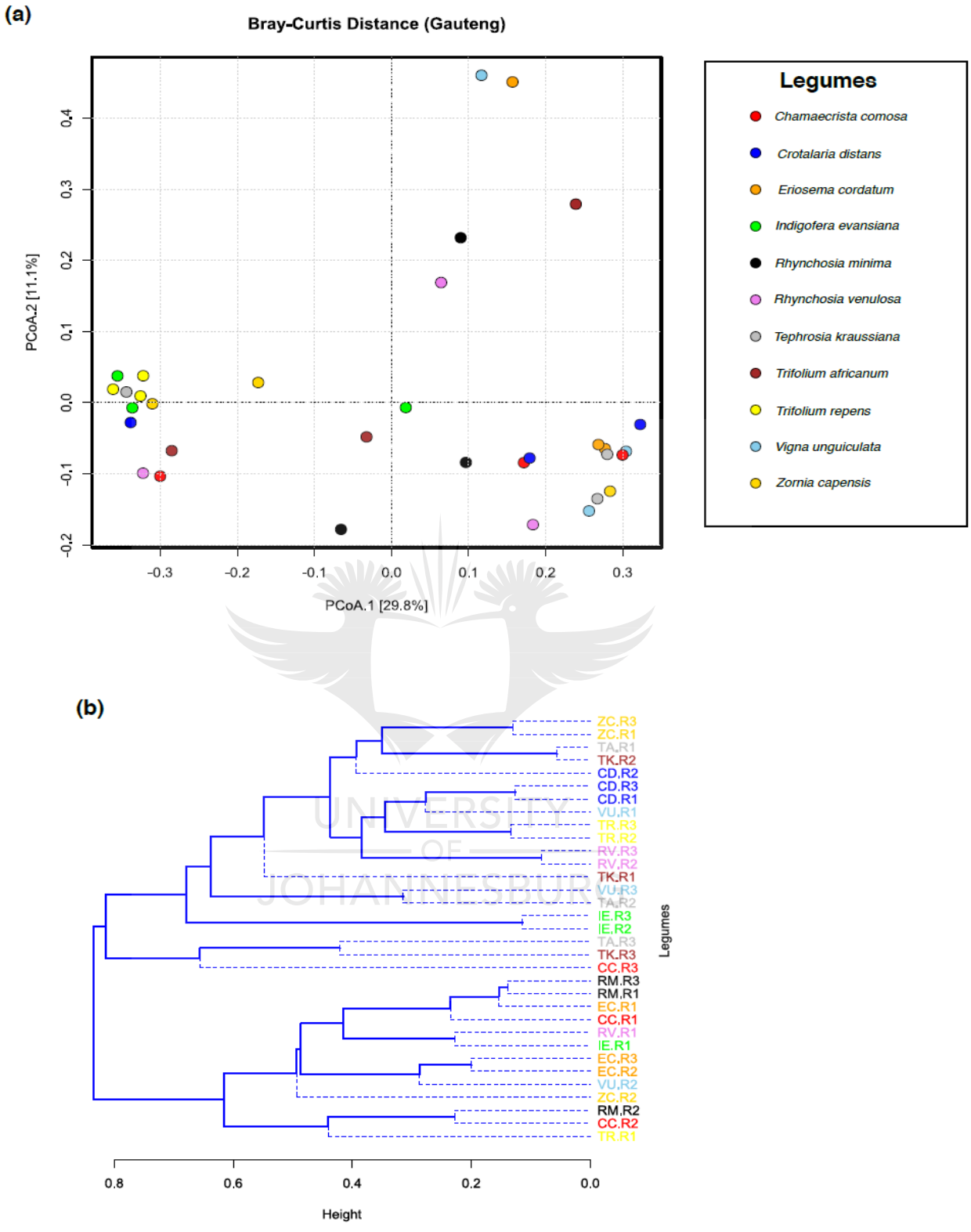

Figure 6.3: AM fungal community differentiation among legumes in Gauteng**.** (a) Principal coordinates analysis (PCoA) plot. (b) UPGMA denrogram. PCoA and the UPGMA were based on the Bray-Curtis dissimilarities between samples. CC, *Chamaecrista comosa*; CD,

Crotalaria distans; EC, *Eriosema cordatum*; IE, *Indigofera evansiana*; RM, *Rhynchosia minima*; RV, *Rhynchosia venulosa*; TA, *Trifoilum africanum*; TK, *Tephrosia kraussiana*; TR, *Trifolium repens*; VU, *Vigna unguiculata*; ZC, *Zornia capensis*. R1, Replicate 1; R2, Replicate 2; R3, Replicate 3.

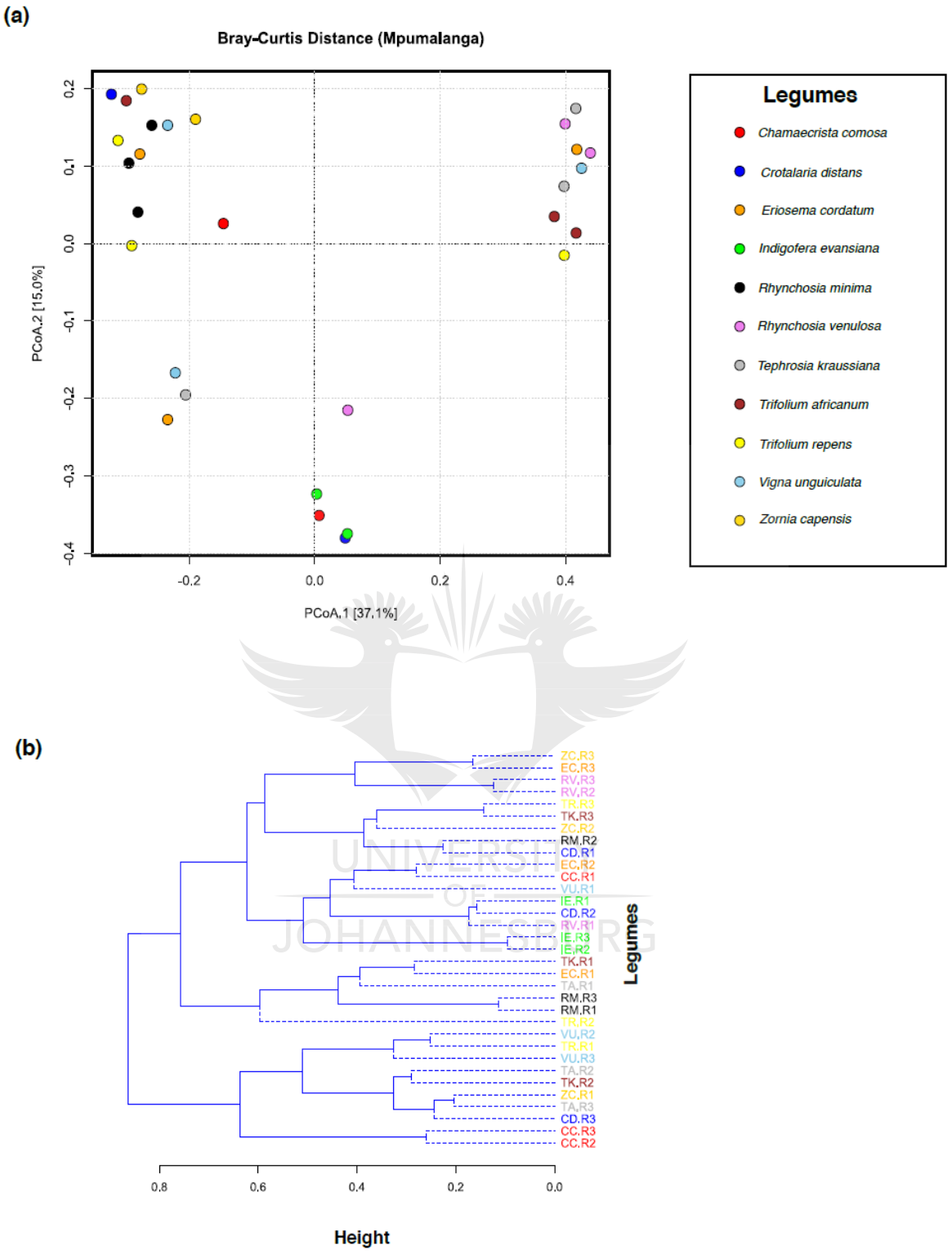

Figure 6.4: AM fungal community differentiation among legumes in Mpumalanga**.** (a) Principal coordinates analysis (PCoA) plot. (b) UPGMA dendrogram. PCoA and the UPGMA were based on the Bray-Curtis dissimilarities between samples. CC, *Chamaecrista comosa*;

CD, *Crotalaria distans*; EC, *Eriosema cordatum*; IE, *Indigofera evansiana*; RM, *Rhynchosia minima*; RV, *Rhynchosia venulosa*; TA, *Trifoilum africanum*; TK, *Tephrosia kraussiana*; TR, *Trifolium repens*; VU, *Vigna unguiculata*; ZC, *Zornia capensis*. R1, Replicate 1; R2, Replicate 2; R3, Replicate 3.

6.3.5 Unique and shared OTUs

The OTUs that are unique to legumes or shared between provinces are presented in Figure 6.5. The Venn diagram showed that each legume had different number of OTUs and the abundance of the shared OTUs varied between the two provinces. In total, 165 and 152 OTUs were unique to legumes in Mpumalanga and Gauteng, respectively, with 170 OTUs shared between all legumes from the two provinces. In Gauteng, the highest number of unique OTUs was found in *V. unguiculata* (73), whereas the lowest was observed in *R. minima* (7). Meanwhile, in Mpumalanga, *Trifolium africanum* (79) and *V. unguiculata* (34) recorded the highest and lowest number of unique OTUs, respectively. *Tephrosia kraussiana* had the maximum number of shared OTUs (72) between the two provinces, while the minimum was found in *C. comosa* (32).

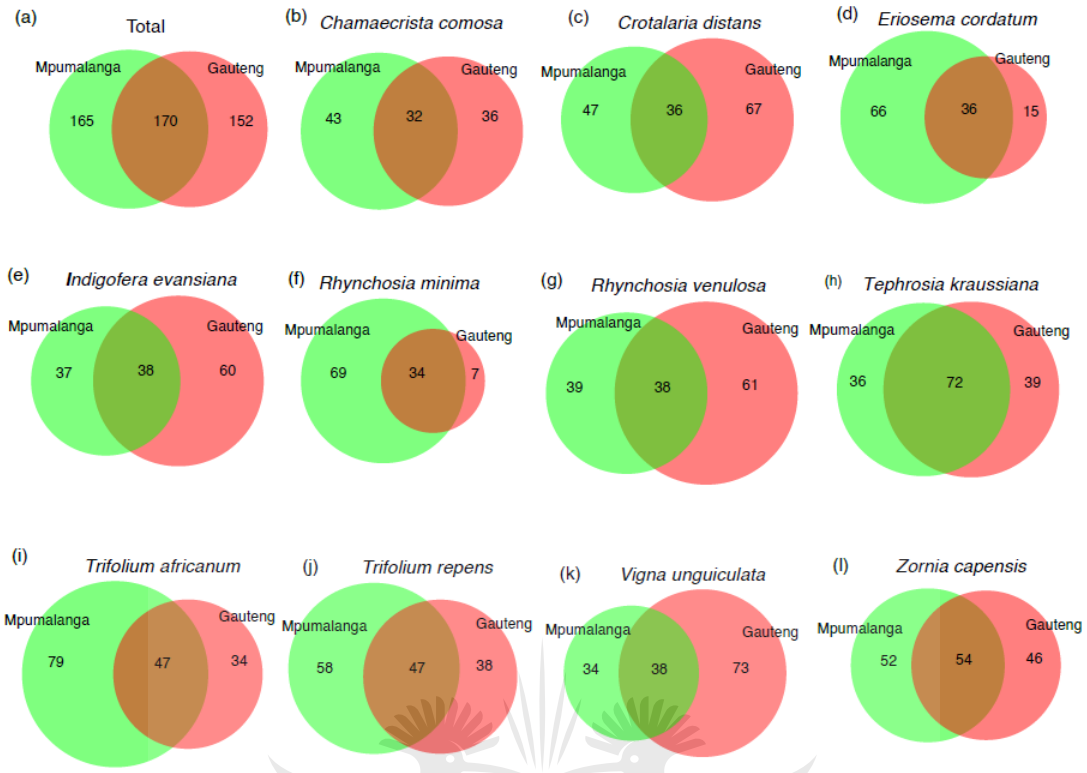

Figure 6.5: Venn diagram showing the number of OTUs that are unique or shared in legumes between provinces.

6.3.6 Relationship between soil properties and AMF diversity and community composition

Correlation analysis showed no significant relationship ($P > 0.05$) between alpha diversity metrics of AM fungi and soil physico-chemical properties (Appendix II). Nevertheless, the stepwise model for the CCA triplot indicated that soil environmental parameters had a significant correlation ($P = 0.009$) with AM fungal community composition (Figure 6.6). Exactly 71.60 % of the overall variation in AM fungal community composition was explained by soil variables, as revealed by the first and second CCA axes. Test of significance of the soil parameters fitted into the step-wise CCA model showed that available P ($R^2 = 2.8040$, $P = 0.017$), sand ($R^2 = 5.1146$, $P = 0.001$), and clay ($R^2 = 2.4573$, $P = 0.033$) were the soil variables

that significantly influenced the community composition of AM fungi in the rhizosphere of studied legumes.

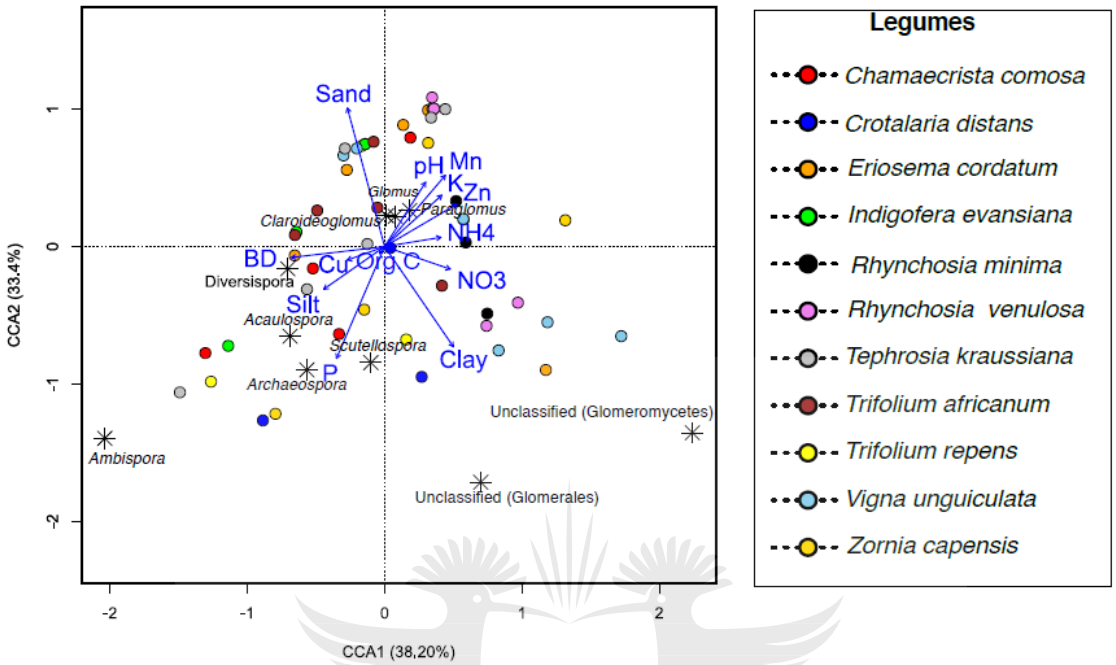

Figure 6.6: Canonical correspondence analysis (CCA) showing the relationship between soil physico-chemical properties and AM fungal community composition in rhizosphere soil. The first (CCA1) and second (CCA2) canonical axes of the CCA plot are significant at $P < 0.05$.

6.4 Discussion

Arbuscular mycorrhizal fungi are integral components of soil ecosystems, especially in semi-arid environments, where plants' access to soil nutrients is limited (Requena et al., 2001; Caravaca et al., 2003). Assessing the diversity of AM fungi in the rhizosphere is essential to understanding the AM fungal communities with the potential to colonise plant roots. This study explored the molecular diversity of AM fungal communities in the rhizosphere soils of studied legumes. The result showed that OTU richness and *H'* were significantly different among legumes, suggesting both the effect of host plants and the effect of the plant-to-environment interaction on the rhizospheric composition of AM fungal communities. Similar observations

have been found in other reports (Singh et al., 2007; Hannula et al., 2010; Song et al., 2019). Host plants shape microbial communities in the rhizosphere through their root exudation (Paterson et al., 2007; Hartmann et al., 2009; Wei et al., 2017; Olanrewaju et al., 2019). Root exudates are highly dynamic in composition, and plants vary considerabley in the type and amount released into their rhizosphere, thereby influencing the abundance and dynamics of microbial communities (Bais et al., 2006; Uren et al., 2007; Badri and Vivanco, 2009; Badri et al., 2013).

Reports from natural ecosystems (Li et al., 2010; Torrecillas et al., 2012a) and experimental studies (Alguacil et al., 2011; Zheng et al., 2016) have shown that the community composition of AM fungi is host-specific. However, in this study, there was no significant variation in AM fungal community composition among legumes at both genus and OTU levels, suggesting no host specificity. Indeed, it has been shown that strict host specificity is low in plant-AM fungal symbiosis due to the ability of AM fungi to associate with a broad range of hosts in different ecosystems (Smith and Read 2008; Lee et al., 2013). Regardless of the overall similarity in community composition, some AM fungal OTUs were still unique to certain legumes in this study. This implies that to a certain extent, AM fungi showed host preferences. Similar findings have been reported in other studies. For example, Lugo and Cabello (2002) reported that some AM fungal species were exclusive to *B. subarista* and *P. stuckertii*, while *Glomus* species only occurred in P. *stuckertii*. Similarly, Muneer et al. (2019) studied the species diversity and community composition of AM fungi associated with three dominant plant species in Inner Mongolia and found that most of the AM fungi had no host specificity, but few species exhibited some level of host preference.

The result demonstrated that genus *Glomus* dominated the AM fungal communities, which is consistent with other studies from various ecosystems, such as semi-arid (Alguacil et al. 2011;

da Silva et al. 2014; Zhao et al., 2017), forest (Qin et al., 2017; Zhang et al., 2021), grassland (Li et al., 2015; Zhang et al., 2019), coastal sand dunes (Kawahara and Ezawa, 2013), agricultural (Dai et al., 2014; Zhu et al., 2020), and polluted soils (Zarei et al., 2010; Hassan et al., 2014; Yang et al., 2015). *Glomus* exhibits wide ecological amplitude and propagates easily in the soil (Liu et al., 2016; Zhu et al., 2016; Powell and Rillig, 2018). These factors may justify the dominance of *Glomus* in the rhizosphere soils of the examined plants.

Similar to other landscape studies (Jansa et al., 2014; Xu et al., 2017; Ma et al., 2018; Ezeokoli et al., 2020), soil factors shaped the composition of rhizosphere AM fungal communities. The significant effect of available P on AM fungal community composition accords with reports from other researchers (Sheng et al., 2013, De Beenhouwer, 2015; Liu et al., 2016; Ji and Bever, 2016; Ceulemans et al., 2019; Zhu et al., 2020). Soil available P mediates the formation and functioning of AM symbiosis and AM fungal abundance (Treseder, 2004; Johnson, 2010). Therefore, given the relationship between soil available P and mycorrhization, the influence of available P on AM fungal communities is not un-expected. Soil texture has been identified as driving factors of AM fungal community assemblages. Montes-Borrego et al. (2014) found that clay and sand contents were among the soil properties that strongly influenced the community composition of AM fungi in the rhizosphere soils of orchids. Elsewhere, de Carvalho et al. (2012) demonstrated that the structure of AM fungal communities in Brazilian rupestrian fields was more correlated with coarse sand, gravel, and silt than with soil chemical properties. Moebius-Clune et al. (2013) showed that sand and clay influenced the structure of AM fungal communities associated with maize fields across eastern New York State. Xiang et al. (2014) reported that silt proportion was the strongest predictor of the AM fungal community composition in an ecotone between pastures and agricultural fields. Zhao et al. (2017) studied the species diversity of AM fungal communities in a semi-arid mountain region in China and found that silt and sand were part of the key soil properties that affected the fungal communities

in soils. In this study, soil texture i.e., sand and clay, were significantly related to AM fungal communities. Sandy soils are highly porous, well-aerated, and less fertile, which is advantageous for optimum AM fungal development (Torrecillas et al., 2014; Vieira et al., 2020). Also, the nutrient-rich clay fraction could have promoted the growth and community composition of AM fungi. These may explain why soil texture affected the AM fungal communities in the rhizosphere of studied plants.

In conclusion, this study showed that the composition of AM fungal communities was similar in the rhizosphere soils of the legumes both within and between provinces. The genus *Glomus* predominated the communities. Furthermore, the study indicated that available P and soil texture (sand and clay contents) were the soil properties that significantly affected the community composition of AM fungi in the rhizosphere soils of studied legumes.

CHAPTER 7

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Arbuscular mycorrhizal fungi are crucial for the survival of plants in harsh, nutrient-limited environments, such as semi-arid ecosystems. This study explores the AM symbiosis as a probable adaptation mechanism of indigenous legumes to the semi-arid conditions of South Africa. The overall goal of this study was to identify and compare the diversity of AM fungal communities in the roots, topsoils, and rhizosphere soils of eleven indigenous legume species within and between Gauteng and Mpumalanga Provinces of South Africa, using morphological (spore-based identification) and molecular (Illumina Miseq sequencing) techniques. Additionally, the study examined the relationships between soil environmental properties and AM fungal diversity and community composition in the roots, topsoils, and rhizosphere soils of the studied plants. Findings from this study will provide baseline information that may be desirable when making decisions on the sustainable conservation of indigenous legumes, vis-a-vis the use of indigenous AM fungal biotechnology.

The results showed that the examined legumes were not only colonised by AM fungi but also sustained highly diverse AM fungal communities in their roots and soils, as revealed by microscopic assessment and Illumina miseq sequencing. A clear differentiation was observed in AM fungal structures, percentage colonisation, spore density, richness, diversity, and community composition among legumes within and between provinces. Soil environmental factors had significant effects on the morphological and molecular diversities of AM fungal communities. However, the soil properties influencing the diversity and community composition of AM fungi in each compartment (roots, topsoils, and rhizosphere soils) are variable. Unfortunately, since the design of the study is unable to allow the independent determination of host effects alongside soil properties effect, the influence of soil properties on

the AM fungal community composition of the legumes is likely not exclusive to soil properties alone but also due to host effects. Thus, the constrained ordination analysis only provided an insight into the potential effect of soil environmental properties on the AM fungal communities in roots, topsoils, and rhizosphere soils of the studied legumes.

The two methods used for characterising AM fungal communities produced different results regarding the communities (genera) detected. While the genera detected by Illumina Miseq sequencing were similar for roots and rhizosphere soils, only four out of the ten genera identified in topsoils using the morphological approach were confirmed by the Illumina Miseq sequencing. This observation shows that complementing morphological analysis with molecular technique is essential for a reliable and comprehensive description of AM fungal communities in field samples. Despite the differences in detection capacity, both approaches detected *Glomus* as the predominant genus in the roots and soils of all legumes in both provinces. The genus *Glomus* is a generalist AM fungal taxon (Öpik et al., 2009) and is considered the most tolerant to stress conditions (Zhao et al., 2017; Mosbah et al., 2018; Mahmoudi et al., 2019). These traits could facilitate the survival of *Glomus* in the semi-arid region of South Africa. Accordingly, it could be the most promising genus for use as local inoculants when investigating the ecological impacts of the symbiotic interactions between AM fungi and the studied legumes.

Some studies (Hijri et al., 2006; Santos et al., 2006; Hempel et al., 2007) reported difficulties in detecting sequences from the families Archaeosporaceae and Paraglomeraceae, probably owing to the amplification of different molecular markers with primers that exhibit variations in specificity and efficiency across AM fungal taxa (Kohout et al., 2014; Van Geel et al., 2014). Intriguingly, sequences from these two rarely amplified families were detected in this study, implying that the primer combinations demonstrated complementary specificity, which

resulted in a broad spectrum of detection of Glomeromycotan lineages. Other studies have shown the specificity and efficiency of primer pairs AML1 and AML2 and AMV4.5NF and AMDGR or their combinations in AM fungal community profiling (Lee et al., 2008; Lumini et al., 2010; Alguacil et al., 2011; Van Geel et al., 2014).

Although molecular identification methods have revolutionalised AM fungal community analyses, the power of molecular markers to discriminate AM fungi at intra and inter-specific levels remains a major challenge in ecological studies of AM fungi. In this study, some AM fungi in roots and rhizosphere soils were not identifiable at higher and lower taxonomic ranks. This may be due to the use of the SSU rRNA gene, which cannot resolve closely related species and may also not satisfactorily define taxa at higher levels (Walker et al., 2007; Gamper et al., 2009; Redecker et al., 2013). To uncover the functional roles of distinct AM fungal communities associated with each legume and deepen our understanding of legume-AM fungal preferences, identifying AM fungi to the lowest (species) taxonomic rank is indispensable. The amplification of the entire rDNA regions (SSU–ITS–LSU), the use of longer-length read sequencing technologies such as PacBio single-molecule real-time and nanopore sequencing platforms, availability of broader database for taxonomic alignment, and the use of MALDI-TOF-MS biotyping could potentially help in delineating AM fungi at different taxonomic levels (Krüger et al., 2012; Crossay et al., 2017; Bruns et al., 2018; Kolaříková et al., 2021).

Studies have shown that the AM fungal communities in the rhizosphere soils were significantly different from those in the roots, suggesting that soils are the species pool from which a fraction is recruited by plants (Mirás-Avalos et al., 2011; Torrecillas et al., 2012b; Saks et al., 2014; Alguacil et al., 2016; Xu et al., 2017; Ji et al., 2020). However, the limitation in the present study was the inability to compare the molecular diversity of AM fungal communities in the roots of the studied legumes with those in their corresponding rhizosphere soils. This is because

the data regarding the diversity of AM fungal communities in roots are not suitable for statistical comparison, as replicates have been combined into one composite sample per legume species per province. Since a statistical comparison cannot be made between the AM fungal diversity in the roots and rhizosphere soils, there was no research question related to this in this study. Nonetheless, from a superficial comparison, the detected communities (genera) in the roots and rhizosphere soils were similar, suggesting that the rhizosphere soils contained propagules (spores and extraradical hyphae) of the AM fungi colonising the roots. Given that this study did not examine the seasonal variation in AM fungal propagules in the soils and root colonisation, the effect that this may have on the molecular profiling of AM fungal diversity, particularly within the roots, is unknown.

Given the multiple positive effects of locally adapted AM fungi on plant establishment, growth, fitness, as well as soil quality, indigenous AM fungal biotechnology is becoming a center of interest in plant management and conservation programs (Estrada et al., 2013; Manaut et al., 2015; Koziol et al., 2018; Drumonde-Melo et al., 2020). In this context, it is recommended that future studies establish a connection between the AM fungal communities (especially species of the genus *Glomus*) and their functional roles on the studied legumes. A step forward in this direction is by conducting greenhouse studies to investigate the symbiotic effectiveness and legumes' growth responses to inoculation by different AM fungal isolate or combinations. Observations from these studies will enable the selection of suitable symbiotic partners. Consequently, the dynamics and influence of these fungi on legumes under field conditions can be predicted. Furthermore, more indigenous legumes (particularly the endangered species) from other biomes should be assessed for their AM fungal diversity. Such knowledge will enhance the understanding and facilitate the ecological exploitation of the legume-AM fungal symbiosis in South Africa.

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APPENDICES

Appendix I: Indicator species analysis in rhizosphere soils of legumes (at genus level)

Amongst legume

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soil_indval <- indval(species_relab, soil_meta$Legume)
> summary(soil_indval)
[1] cluster        indicator_value probability
<0 rows> (or 0-length row.names)

Sum of probabilities                =  7.565

Sum of Indicator Values             =  1.02

Sum of Significant Indicator Values  =  0

Number of Significant Indicators     =  0

Significant Indicator Distribution
< table of extent 0 >
```

Between locations

```
> soil_indval <- indval(species_relab, soil_meta$Location)
> summary(soil_indval)
[1] cluster        indicator_value probability
<0 rows> (or 0-length row.names)

Sum of probabilities                =  3.77

Sum of Indicator Values             =  3.21

Sum of Significant Indicator Values  =  0

Number of Significant Indicators     =  0

Significant Indicator Distribution
< table of extent 0 >
```

Appendix II: Correlation analysis between alpha diversity indices of AM fungi in rhizosphere soils and soil properties

	Coefficient of correlation						P-values					
	observed_otus	chao1	shannon	dominance	equitability	goods_coverage	observed_otus	chao1	shannon	dominance	equitability	goods_coverage
pH	0.16	0.17	0.09	0.03	-0.13	-0.15	0.1863	0.1638	0.482	0.8307	0.2825	0.2441
NO3	-0.06	-0.08	0.05	-0.09	0.07	-0.04	0.6226	0.5484	0.6781	0.4972	0.5865	0.742
NH_4	0.1	0.09	0.09	0	0.03	-0.18	0.4338	0.4654	0.4649	0.9892	0.8302	0.1377
P	0.04	0.05	0.08	-0.15	0.08	-0.21	0.7199	0.677	0.5169	0.2197	0.5395	0.0865
Organic_C	-0.08	-0.08	0	0	0.15	0.01	0.5065	0.5082	0.9804	0.995	0.2366	0.9482
K	0.16	0.15	0.06	0.02	0.01	-0.01	0.2028	0.2267	0.6054	0.8911	0.9259	0.9333
Cu	-0.14	-0.12	-0.06	0.07	-0.07	0.06	0.2744	0.3316	0.6469	0.5749	0.5926	0.631
Mn	-0.12	-0.13	-0.02	0.12	0.04	0.09	0.3461	0.3164	0.8906	0.3394	0.7553	0.495
Zn	0.07	0.07	0.06	0.13	0.01	-0.1	0.5535	0.5546	0.6578	0.2875	0.9537	0.4414
BD	-0.05	-0.05	-0.06	-0.07	-0.07	0.03	0.6839	0.7186	0.6374	0.6038	0.5796	0.8101
Sand	0	-0.01	0.06	-0.01	0.09	-0.06	0.9952	0.9664	0.6555	0.9481	0.4925	0.6306
Silt	-0.12	-0.13	-0.04	-0.08	0.15	0.09	0.3425	0.3073	0.7743	0.5286	0.2168	0.462
Clay	-0.07	-0.06	-0.17	0.18	-0.17	0.06	0.6021	0.64	0.1834	0.1592	0.1841	0.61

Appendix 111: Metrics of sequencing analysis in the roots of legumes in Gauteng Province

Sample-ID	Input	Filtered	Percentage of input passed filter	Denoised	Merged	Percentage of input merged	Non-chimeric	Percentage of input non-chimeric
#q2:types	numeric	numeric	numeric	numeric	numeric	numeric	numeric	numeric
C. comosa	213885	10969	5.13	10865	10215	4.78	9748	4.56
C. distans	173461	11396	6.57	11333	10822	6.24	10553	6.08
E. cordatum	214968	13564	6.31	13383	12455	5.79	11651	5.42
I. evansiana	158703	11090	6.99	10880	9858	6.21	8156	5.14
R. minima	154590	9869	6.38	9641	9015	5.83	8415	5.44
R. venulosa	167515	8133	4.86	7969	7457	4.45	7042	4.2
T. africanum	197585	11876	6.01	11697	11038	5.59	10504	5.32
T. kraussiana	80466	3892	4.84	3798	3632	4.51	3470	4.31
T. repens	158437	10966	6.92	10755	9733	6.14	8139	5.14
V. unguiculata	1889180	97232	5.15	96739	92001	4.87	85083	4.5
Z. capensis	121655	7601	6.25	7435	6623	5.44	6151	5.06

Appendix 1V: Metrics of sequencing analysis in the roots of legumes in Mpumalanga Province

Sample-ID	Input	Filtered	Percentage of input passed filter	Denoised	Merged	Percentage of input merged	Non-chimeric	Percentage of input non-chimeric
#q2:types	numeric	numeric	numeric	numeric	numeric	numeric	numeric	numeric
C. comosa	213885	10969	5.13	10865	10215	4.78	9748	4.56
C. distans	173461	11396	6.57	11333	10822	6.24	10553	6.08
E. cordatum	214968	13564	6.31	13383	12455	5.79	11651	5.42
I. evansiana	158703	11090	6.99	10880	9858	6.21	8156	5.14
R. minima	154590	9869	6.38	9641	9015	5.83	8415	5.44
R. venulosa	167515	8133	4.86	7969	7457	4.45	7042	4.2
T. africanum	197585	11876	6.01	11697	11038	5.59	10504	5.32
T. kraussiana	80466	3892	4.84	3798	3632	4.51	3470	4.31
T. repens	158437	10966	6.92	10755	9733	6.14	8139	5.14
V. unguiculata	1889180	97232	5.15	96739	92001	4.87	85083	4.5
Z. capensis	121655	7601	6.25	7435	6623	5.44	6151	5.06

Appendix V: Metrics of sequencing analysis in the rhizosphere soils of legumes in Gauteng Province

Sample-ID #q2:types	Input numeric	Filtered numeric	Percentage of input passed filter numeric	Denoised numeric	Merged numeric	Percentage of input merged numeric	Non-chimeric numeric	Percentage of input non-chimeric numeric
C. comosa R1	41280	24273	58.8	23557	20281	49.13	11462	27.77
C. comosa R2	108151	69080	63.87	67675	57601	53.26	35547	32.87
C. comosa R3	171955	111449	64.81	109446	97190	56.52	62360	36.27
C. distans R1	157121	75216	47.87	74137	65769	41.86	33852	21.55
C. distans R2	54948	26175	47.64	25705	22238	40.47	13055	23.76
C. distans R3	96657	45805	47.39	45144	39833	41.21	22307	23.08
E. cordatum R1	173933	111640	64.19	109352	93961	54.02	58106	33.41
E. cordatum R1	143508	90371	62.97	88653	76956	53.62	41577	28.97
E. cordatum R1	83825	51870	61.88	50509	42155	50.29	27913	33.3
I. evansiana R1	62031	34868	56.21	34245	30178	48.65	16010	25.81
I. evansiana R2	80324	44944	55.95	44052	39283	48.91	21271	26.48
I. evansiana R3	42056	24588	58.46	24074	20022	47.61	9234	21.96
R. minima R1	53286	29902	56.12	29219	26045	48.88	15566	29.21
R. minima R1	52201	29175	55.89	28637	25532	48.91	14205	27.21
R. minima R1	41138	24306	59.08	23720	19671	47.82	10138	24.64
R. venulosa R1	81343	52077	64.02	50852	42918	52.76	27214	33.46
R. venulosa R2	57657	30494	52.89	29746	24045	41.7	13028	22.6
R. venulosa R3	59099	34085	57.67	33282	29235	49.47	14908	25.23
T. africanum R1	67045	43207	64.44	42247	36629	54.63	25343	37.8
T. africanum R2	129073	58739	45.51	57877	50589	39.19	27500	21.31
T. africanum R3	130912	80720	61.66	79588	72285	55.22	38472	29.39
T. kraussiana R1	97982	48450	49.45	47933	42108	42.98	23797	24.29
T. kraussiana R2	49620	28800	58.04	28204	23967	48.3	11675	23.53
T. kraussiana R3	93446	46736	50.01	45832	38699	41.41	21083	22.56
T. repens R1	133189	68941	51.76	67662	57835	43.42	30614	22.99
T. repens R2	147672	90102	61.01	88240	76198	51.6	30324	20.53
T. repens R3	98067	46944	47.87	45701	35077	35.77	15632	15.94
V. unguiculata R1	115863	75907	65.51	74571	66465	57.37	47897	41.34
V. unguiculata R2	91363	43079	47.15	42271	36659	40.12	20012	21.9
V. unguiculata R3	158703	103686	65.33	101728	89659	56.49	55694	35.09
Z. capensis R1	134282	71555	53.29	69916	58843	43.82	28358	21.12
Z. capensis R2	115133	67387	58.53	66148	57878	50.27	28032	24.35
Z. capensis R3	52636	32848	62.41	32020	27451	52.15	16306	30.98

R1, R2, R3 are replicates 1, 2, and 3, respectively.

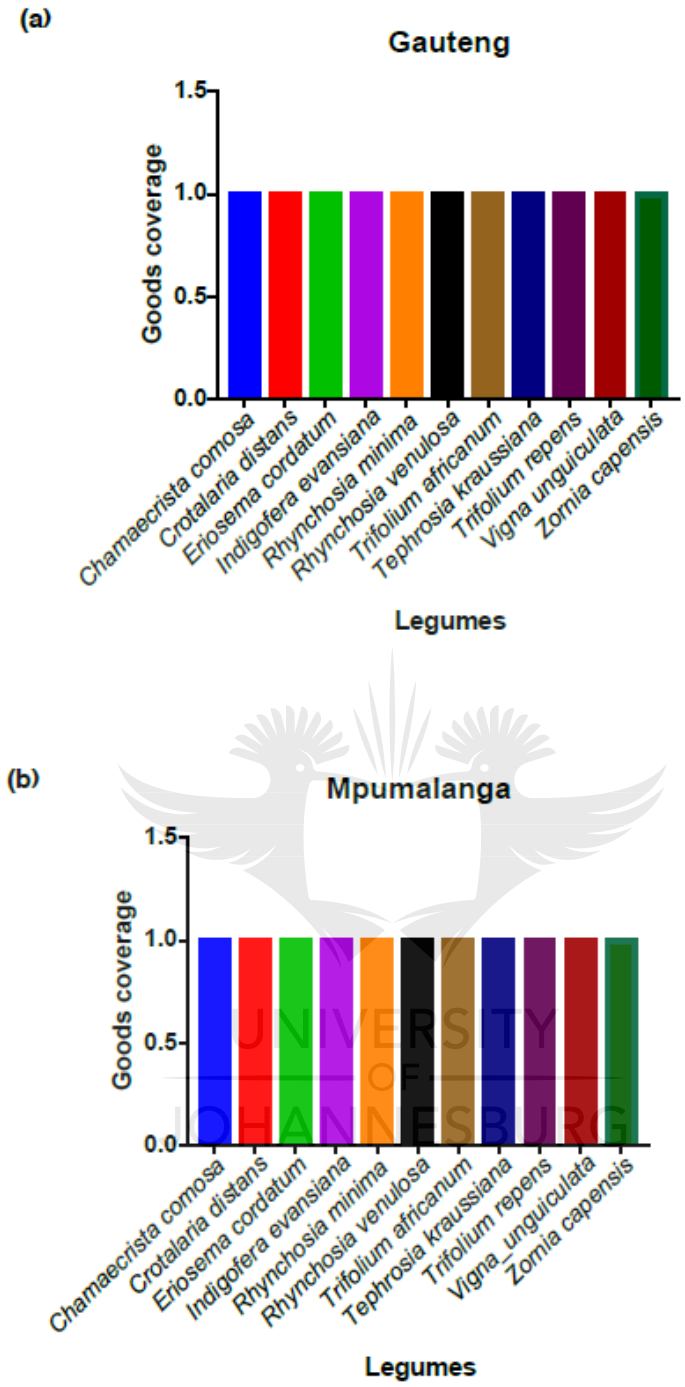
Appendix VI: Metrics of sequencing analysis in the rhizosphere soils of legumes in Mpumalanga province

Sample-ID #q2:types	Input numeric	Filtered numeric	Percentage of input passed filter numeric	Denoised numeric	Merged numeric	Percentage of input merged numeric	Non-chimeric numeric	Percentage of input non-chimeric numeric
C. comosa R1	53736	32621	60.71	31656	26674	49.64	17599	32.75
C. comosa R2	90856	44723	49.22	43784	36136	39.77	20676	22.76
C. comosa R3	111356	64031	57.5	62405	50563	45.41	24671	22.16
C. distans R1	104498	59944	57.36	58304	46893	44.87	21889	20.95
C. distans R2	115116	72637	63.1	71389	61316	53.26	24112	20.95
C. distans R3	164965	100866	61.14	98890	84564	51.26	29363	17.8
E. cordatum R1	46685	27092	58.03	26646	23083	49.44	11856	25.4
E. cordatum R1	41853	23805	56.88	23362	20476	48.92	11267	26.92
E. cordatum R1	45647	25714	56.33	25104	22194	48.62	12040	26.38
I. evansiana R1	46949	27498	58.57	26850	22383	47.68	11557	24.62
I. evansiana R2	82177	51896	63.15	50629	43258	52.64	29506	35.91
I. evansiana R3	86022	24033	27.94	23790	21603	25.11	16906	19.65
R. minima R1	86210	23056	26.74	22609	19982	23.18	17021	19.74
R. minima R1	29718	7032	23.66	6837	5287	17.79	5287	17.79
R. minima R1	87910	23327	26.54	23050	21458	24.41	18434	20.97
R. venulosa R1	75359	16840	22.35	16628	15170	20.13	13761	18.26
R. venulosa R2	69567	20055	28.83	19781	17880	25.7	14041	20.18
R. venulosa R3	59856	13013	21.74	12860	11527	19.26	10190	17.02
T. africanum R1	38950	10715	27.51	10469	8773	22.52	8032	20.62
T. africanum R2	58586	13732	23.44	13510	12158	20.75	9966	17.01
T. africanum R3	81653	21295	26.08	20714	18048	22.1	15283	18.72
T. kraussiana R1	309815	19235	6.21	18953	17321	5.59	16571	5.35
T. kraussiana R2	279531	16383	5.86	16021	13026	4.66	6929	2.48
T. kraussiana R3	184438	11098	6.02	10784	8344	4.52	4426	2.4
T. repens R1	194292	12571	6.47	12413	12014	6.18	11621	5.98
T. repens R2	174597	10855	6.22	10705	9983	5.72	9381	5.37
T. repens R3	695405	31505	4.53	31288	29485	4.24	27898	4.01
V. unguiculata R1	148995	9397	6.31	9228	8604	5.77	8313	5.58
V. unguiculata R2	47173	2037	4.32	2010	1680	3.56	1680	3.56
V. unguiculata R3	38042	2111	5.55	1994	1744	4.58	1680	4.42
Z. capensis R1	118225	7479	6.33	7439	6866	5.81	6450	5.46
Z. capensis R2	19757	4183	21.17	4079	3531	17.87	3382	17.12
Z. capensis R3	104504	27960	26.75	27663	25976	24.86	21609	20.68

R1, R2, R3 are replicates 1, 2, and 3, respectively.

Appendix VII: Goods coverage of AM fungi in the roots of legumes in both provinces

	Gauteng	Mpumalanga
C. comosa	1.0	1.0
C. distans	1.0	1.0
E. cordatum	1.0	1.0
I. evansiana	1.0	1.0
R. minima	1.0	1.0
R. venulosa	1.0	1.0
T. africanum	1.0	1.0
T. krraussiana	1.0	1.0
T. repens	1.0	1.0
V. unguiculata	1.0	1.0
Z. capensis	1.0	1.0



Appendix V1II: Goods coverage of AM fungi in the rhizosphere soils of legumes (a) Gauteng (b) Mpumalanga