ABUNDANCE, IDENTIFICATION AND EFFECTIVENESS OF INDIGENOUS AMF SPECIES ASSOCIATED WITH AGROFORESTRY SYSTEMS IN THE SEMI-ARID AND HUMID AGROECOLOGIES OF RWANDA

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Declaration

This thesis is my original work and has not been submitted to any other university for examination.

Signature ……………………… Date ……………………………….

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This thesis report has been submitted for examination with our approval as University supervisors.

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Dedication

To my family

Abstract

Arbuscular Mycorrhizal Fungi (AMF) form symbiotic association with the roots of plants. AMF help to facilitate mobilization of nutrients, especially phosphorus, from soil to plant. The study was carried out in sub-humid Rubavu and semi-arid Bugesera districts in Rwanda. We hypothesized that the presence of tree species in farming systems enhances mycorrhizal fungal density. The occurrence and abundance of AMF in the soil around main agroforestry tree species in the two regions were therefore studied. The tree species in Rubavu included *Alnus acuminata*, *Markhamia lutea, Grevillea robusta* and *Eucalyptus* sp. while those in Bugesera were *Acacia polyacantha, Senna spectabilis, Grevillea robusta* and *Eucalyptus* sp. AMF spores were isolated from soil samples collected under trees and outside the trees canopy. Results showed that AMF spores abundance in the soil varied with the distance from the tree trunk but not consistently. A significant difference in spore density was recorded between the tree species. Soil samples from under *Alnus acuminata* of Rubavu gave a significantly greater density of AMF spores compared to the soils collected around *Markhamia lutea* (p=0.001), *Grevillea robusta* (p=0.018) and *Eucalyptus sp.* (p=0.011). In Bugesera, soils collected around *Sena spectabilis* showed a significantly greater density of AMF spores than soils around *Grevillea robusta* and *Eucalyptus* sp. (p=0.0002 and 0.030 respectively); and spores densities around *Acacia polyacanta* and *Eucalyptus* sp. were significantly greater than the density around *Grevillea robusta* (p=0.022 and 0.043, respectively). Based on major differences in spore morphological appearance, four different AMF genera were detected from the agroforestry systems in the semi-arid and humid agroecologies of Rwanda. They were identified as *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora.* All spore types were found in all rhizosphere soil samples and *Glomus* was the predominant taxonomic group. Genetic characterization was done using nested PCR amplification targeting different regions of the rDNA. The first amplification step was done using the universal primers NS5 and ITS4. The 2nd step of PCR amplification was done with specific primers ARCH1311, GLOM1310 and LETC1670 along with ITS4. Due to the little amount of DNA per single spore, only

32.35 % of PCR amplifications gave successful results on agarose gel; three of them were sequenced successfully and only *Gigaspora margarita* was identified at species level with bioinformatics tools. All the maize roots in the treatments with AMF were colonized by AMF, with morphotype AMF2 performing better than morphotypes AMF1 and AMF3. The mean percentage colonization varied between 40% and 70%.

The present work is the initial phase to study the AMF communities associated with some common tree species in agroforestry systems in Rwanda. Further studies are proposed relating mycorrhizal diversity in the agroforestry systems to performance and yields of crops.

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List of Abbreviations

ICRAF: International Center for Research in Agroforestry

DNA: Deoxyribonucleic Acid

PCR: Polymerase Chain Reaction

rDNA: Ribosomal Deoxyribonucleic Acid

sdd: Sterile double distilled

bp: Base Pairs

RPM: Revolutions Per Minute

CHAPTER 1: GENERAL INTRODUCTION

Crop productivity is declining in Rwanda mainly due to declining soil fertility and other challenges like high population density, water scarcity, land degradation, land fragmentation and deforestation notwithstanding. Most options to improve productivity involve the use of expensive inputs that inherently increase risks that farmers are often unwilling or unable to bear. The government of Rwanda is interested in eco-efficient means to raise land productivity that farmers can afford to adopt like scaling up the adoption of farm trees in Rwanda. The bigger project under which this research is based targets two key agroecologies mainly the semi-arid and sub-humid districts in Rwanda and Ethiopia with a view of scaling out successes to Uganda and Burundi. One of the objectives of the project is to study the impacts of tree species and management on crop productivity, water resources and nutrients at field, farm and landscape scales to inform scaling up to improve food security and reduce climate risk. This study therefore focused on understanding the impact of trees on soil nutrients with specific emphasis on mychorhiza and their influence on maize productivity.

Mycorrhizas are mutualistic symbiotic associations formed between many plant roots and certain fungi. The association is characterized by the movement of plant produced carbon to the fungus and fungal acquired nutrients to the plant. The term mycorrhiza, which literally means fungus root, was first applied to fungus tree associations described in 1885 by the German forest pathologist A.B. Frank (Dhami, 2005). Bidirectional nutrient transfer is the main basis for mutualism. The fungi depend on a supply of photosynthate from the plants and at the same time enable the plant to increase uptake of mineral nutrients particularly phosphorus from the soil (Smith and Read, 1997). Hence both members of the association can benefit, but whereas symbiosis is obligate for the fungi, this is not the case for the plants, which vary in their responsiveness to fungal colonization. There are many types of mycorrhizal associations (Harley and Smith, 1983), of which the endomycorrhizal

association of the arbuscular mycorrhiza type are the most widespread geographically as well as within the plant kingdom.

Arbuscular Mycorrhizal Fungi (AMF) are root-inhabiting soil fungi which form obligate symbiotic associations with over 80% of terrestrial plant families (Smith and Read, 2008; Van Hoewyk *et al*., 2001; Harley and Smith, 1983). They are ubiquitous in almost all plant communities in both natural and managed ecosystems, but the number has decreased due to tillage, removal of topsoil, erosion, fumigation and over-fertilization (Rajah and Tang, 2005).They are widespread in tropical soils and associate with a wide variety of plant species, including most commercial crops (Sieverding, 1991) and trees (Atayese, *et al.,* 1993; Adjoud-Sadadou and Halli-Hargas, 2000). These very important organisms form an interface between soils and plant roots (Power and Mills, 1995; Ingleby, 2007) increasing the absorptive surfaces of the roots (Manjunath and Habte, 1988). Extra-radical hyphae of the AMF extend beyond the root and act as extensions of the root system in acquiring nutrients from the soil (Rhodes and Gerdemann, 1975). AMF can therefore absorb mineral nutrients from soil through their extended intricate hyphal network and deliver them to their host plants in exchange for carbohydrates (Oehll *et al.,* 2003). AMF can also enhance tolerance of abiotic stresses such as drought and metal toxicity (Meharg and Cairney, 2000).

AMF are not host-specific (Ingleby, 2007). Because of this, the same fungi can associate with tree and crop species and therefore have the potential to enhance both tree and crop growth in agroforestry systems. In this situation, the tree species can act as a ‘reservoir’ of AM fungi, from which roots of germinating crop seedlings can quickly form mycorrhizal associations (Ingleby, 2007).

Arbuscular mycorrhizal associations are characterized by structures called arbuscules and vesicles which are produced inside the host plant root cells together with asexual spores which they produce in the soil (Ingleby, 2007). Though hyphal networks, dead root fragments and other organic material occupied by fungal structures are important, AMF spores have traditionally been considered to be the most important propagules of AMF (Chandrasekara *et al.,* 2005; Brundrett and Abbott, 1994).

Therefore, analysis of spore populations in soils is currently the most used method to assess the species density and diversity of AM fungal communities (Chandrasekara *et al.*, 2005). However, the interpretation of these results remains conditional as AMF species vary greatly in spore production; some species produce spores copiously, while others rarely or never sporulate (Chandrasekara *et al.*, 2005).
All the soils harbor AMF spores despite the different structural and chemical differences of the cropping fields (Don-Rodrgue *et al*., 2013). However, the major factors affecting their diversity, abundance and distribution in agro-ecosystems are soil pH, availability of phosphorous (P), nitrogen (N), organic matter and water. These factors could also affect the crop production in different agro-ecosystems (Porass-Soriano *et al.,* 2009).

The relationship between plants and AMF species abundance and diversity is not completely understood for most natural ecosystems. Even though species richness of mycorrhizal fungal communities has been correlated with the species richness of plant communities in temperate grasslands and tropical agroecosystems (Nancy and David, 1997), agricultural soils have a low density and diversity of AMF compared to natural ecosystems. When a soil is put to agricultural use, it undergoes a series of physical changes like tillage and fertilizer use, which can negatively affect microorganism population (Bellgard, 1994).
Since AMF form symbiotic associations with a large number of terrestrial plant species where AMF contribute to the plant growth by increasing plant access to phosphorous, improving soil quality, enhancing absorption of nutrients and water from the soil, and providing an increased resistance against soil pathogens, they can be used in improving crop production and therefore enhancing food security.

The ability to take advantage of the natural resources in crop production is a major step towards economic prosperity for countries like Rwanda where majority of the population depend on agriculture. This is because use of chemical fertilizers for crop production is expensive, as a result of shortfall in availability and lead to environmental pollution. Currently, more efforts are directed on the development of

alternative technology to minimize the dependence on chemical fertilizers and encourage the use of bio-fertilizers on a large scale by farmers. Furthermore, taking advantage of the indigenous micro-organism for bio-fertilizers rather than exotic species is promoted. Indigenous species are inexpensive and well adapted to local environment.

Maize, as one of the most important crops in Rwanda, was identified as a priority crop by the government of Rwanda within the context of the National Crop Intensification Programme. It plays an important role in food security and income generation for the majority of Rwandese. It grows in most parts of Rwanda but it requires substantial inputs of nutrients to produce maximum yield.

This study therefore intends to determine the abundance of indigenous AMF species in Rwanda, to identify them and investigate their colonization potential on maize. The investigated AMF species were isolated from agroforestry systems of Rubavu and Bugesera districts representing the sub-humid and semi-arid agroecological zones of Rwanda, respectively.

1.1 Statement of the problem

It has been reported that there is greater soil biota in agroforestry systems than in agriculture systems (without trees) with greater biodiversity generally reported near the trees but the effect varies with tree species (Barrios *et al.,* 2010). AMF are undoubtedly of extraordinary importance in plant production, plant and soil ecology and play a key role in sustainable agriculture. Infection of crop roots with AMF can improve their uptake of nutrients, particularly of phosphorus and increase crop production (Joner, 2000; Atimanav and Adholeya, 2002). AMF play a central role in many microbiological and ecological processes, influencing soil fertility, decomposition, cycling of minerals and organic matter, as well as plant health and nutrition (Finlay, 2008). Variation in the population of these fungi and their symbiosis with plant roots is related to both soil properties and host plant (Hayman, 1982a).

Thus the distribution of AMF in different ecological regions and their relations to soil properties and plants have been investigated by several researchers.

Trees are being incorporated in the agricultural lands in Rwanda to provide products, e.g. timber, firewood, fruits and furniture and through provision of ecosystem services like water regulation. It is therefore critical to understand tree –crop-nutrient- water interactions in these systems and their effect on crop productivity. This information is important in informing the right tree for the right place and management for optimized crop yields. Past agroforestry studies in Rwanda have concentrated on water and nutrient aspects in either dry or humid agroecologies separately with no focus on the role of AMF in productivity systems. Therefore, there is a need to document the AMF around trees in these systems as a preparatory phase to understand their contribution in these systems as they provide several free ecosystem services. Furthermore, a study on their potential to colonize roots of crop plants is an initial phase to quantify their contribution to crop growth and yields at large. This will provide a cheap, readily available and sustainable option of enhancing agricultural production to the majority rural poor.

This study focused on AMF in both semi-arid and humid agroecologies which makes the findings and those from other complementary studies in the bigger project useful in helping to generalize results across agroecologies using a combination of tree-crop models at field scale and tree-crop-livestock models at farm and landscape scales.

1.2 Justification

The investigation of indigenous AMF species found within the agroforestry systems in the semi-arid and humid agroecologies of Rwanda leads to improved knowledge about mycorrhizal associations with common tree species in the agroforestry systems. The inoculation of maize with these indigenous AMF helps to assess their potential which is an important step in taking advantage of the indigenous AMF communities. Use of AMF rather than chemical fertilizers for crop production is inexpensive, assured availability, and cause less environmental pollution. The characterization of different mycorrhiza in the rhizosphere under different tree species from the two

districts in Rwanda has contributed to basic knowledge in the world of science whose application will help address a critical problem for the rural poor in Rwanda.

1.3 Hypotheses

1. The agroforestry systems in the semi-arid and humid agroecologies of Rwanda do not have AMF organisms.
2. The indigenous AMF species of the semi-arid and humid agroecologies of Rwanda do not have potential to colonize maize.

1.4 Objectives of the study

1.4.1 General objective

To investigate the indigenous AMF species associated with agroforestry systems in the semi-arid and humid agroecologies of Rwanda and analyze their potential to colonize maize.

1.4.2 Specific objectives

1. To isolate and compare AMF spore abundance among the four most common tree species of agroforestry systems in the semi-arid and humid agroecologies of Rwanda.
2. To characterize both morphologically and genetically AMF species of agroforestry systems in the semi-arid and humid agroecologies of Rwanda.
3. To compare AMF species among the four most common tree species of agroforestry systems in the semi-arid and humid agroecologies of Rwanda.
4. To determine maize response in terms of root colonization to inoculation with AMF spores of agroforestry systems in the semi-arid and humid agroecologies of Rwanda.

CHAPTER 2: LITERATURE REVIEW

Arbuscular mycorrhizal fungi form close symbiotic associations with majority of land plants (Smith and Read, 1997). Plant diversity and productivity in ecosystems are significantly influenced by AM fungal diversity in the soil (Van der Heijden *et al.,* 1998). Worldwide, interest in AMF has now reached a point wherein any discussion of agricultural biotechnology that does not include their role in plant productivity can hardly be considered complete (Habte and Osorio, 2001). Many individuals and organizations concerned with managing native plant species, restoring natural ecosystems, and producing agronomic, horticultural, and forest plants with minimal chemical inputs are interested in applying AMF technology. But a major recurring challenge to large scale utilization of AMF is the lack of availability of large quantities of high-quality AMF inoculum. The problem is largely due to the fact that AM fungi are obligate symbionts; they require the presence of actively growing plants during their reproduction. They therefore cannot be cultured on laboratory media in the same manner as other beneficial soil microorganisms such as *Rhizobium* bacteria (Habte and Osorio, 2001). However there are several documented benefits of AMF.

2.1 AMF functions

2.1.1 AMF and plant nutrition

AMF absorb and translocate N, P, K, Ca, S, Fe, Mn, Cu, and Zn and make them available to associated plants (Mac Donald and Lewis, 1978). However, the most prominent and consistent nutritional effect of AMF is in the improved uptake of immobile nutrients, particularly P, Cu, and Zn (Pacovsky, 1986; Manjunath and Habte, 1988). The fungi enhance immobile nutrient uptake by increasing the absorptive surfaces of the root (Manjunath and Habte, 1988). Extra-radical hyphae of the AMF extend up to 8 cm beyond the root and act, in effect, as extensions of the root system in acquiring nutrients from the soil (Rhodes and Gerdemann, 1975).

AMF are also very useful to plant species that inherently lack either morphological or physiological mechanisms for efficient P uptake (Koide and Schreiner, 1992; Manjunath and Habte, 1991). Consequently, enhancement of growth of plants associated with AMF is explained in most instances by improved P nutrition (Bolan, 1991; Ingleby, 2007).

Another advantage to associated plants is improved maintenance of a balanced supply of nutrients. This occurs because plants grown in association with AMF can grow with only a fraction of the P required for growth by plants lacking a mycorrhizal association. Moreover, when P is applied at high concentrations, as is commonly done when growing plants in soil where AMF are absent, it can cause nutritional disorders because of its antagonistic interactions with other nutrients, or because it inhibits mycorrhizal formation (Lambert *et al*., 1979).

The ability of AMF to reduce plants' external P requirement has also an important environmental benefit. High levels of P in soils can result in pollution of bodies of water when eroded soil rich in P is deposited in them. P enrichment of water bodies causes eutrophication (Sharpley *et al*., 1992) due to excessive development of algae, cyanobacteria, and aquatic plants, and this condition impairs the usefulness of these waters. When plants rely on AMF association rather than heavy P fertilization, risks to water quality are reduced. Arbuscular mycorrhizal fungi, therefore, are an important component of nutrient management programs that aim to reduce environmental pollution (Habte and Osorio, 2001).

2.1.2 AMF and soil structure

Soil structure refers to pore space as well as to aggregates, and the number and dimension of the pore spaces between soil particles. These are important in functional considerations of soil structure, especially from the standpoint of soil water relations (Hamblin, 1985).

There is increasing evidence that hyphal networks of AMF contribute significantly to the development of soil aggregates, and hence to soil conservation (Miller and Jastrow, 1990). AMF symbiosis has been linked to changes in soil structure in both

pot and field experiments (Rillig, 2004). AMF hyphae grow into the soil matrix and create the skeletal structure that holds primary soil particles together via physical entanglement. They create conditions conducive to formation of micro-aggregates, and they chemically enmesh and stabilize microaggregates (Miller and Jastrow, 2000).

2.1.3 AMF and its role as a soil enhancer

AMF form symbiotic associations between plant and soil fungi playing an essential role in plant growth and protection (Meharg and Cairney, 2000) and soil quality (Miller and Jastrow, 2000). They form gossamer-like formation in the root zone, very much smaller than the plant root hairs. When these thread-like bodies are unraveled, they can be of several miles in length creating more surface area than the hairs of plant roots. With this mechanism, absorption of soil nutrients becomes higher. The fungal hyphae enter the roots of the plants, allowing therefore exchange of soil nutrients to the plant cells (Rhodes and Gerdemann, 1975). As soil enhancer, AMF release a mixture of powerful chemicals that release tightly bound chemicals such as phosphorus (P), zinc (Zn) and copper (Cu) and transform them into a form that can be easily absorbed by plants (Rajah and Tang, 2005).

2.1.4 AMF and plant water relations

Even though many reports have compared water relations of AM and non AM plants (Augé, 2001), the influence of AM symbiosis on the water relations and moisture retention properties of soils remains not fully studied. However, as AM fungi affect soil structure, it seems logical to expect that AM colonization of a soil might affect its moisture retention properties and, in turn, the behaviors of plants growing in the soil, particularly when soil is relatively dry (Tadesse, 2006).

Mycorrhizal plants show physiological responses linked to increased drought resistance. AMF often alter rates of water influx and efflux in host plants, thus affecting tissue water content and leaf physiology (Auge´, 2001).

Measured reductions in soil moisture content (SMC) indicate that root systems of AM plants often dry soils at a faster rate and more thoroughly than root systems of non-AM plants. This effect likely results from either the greater evaporative surface area (i.e. larger above-ground biomass) or more extensive root systems observed in AM relative to non-AM plants. However, it may also result from the adherence of AM hyphae to soil particles, thus improving contact with the soil solution (Auge´, 2001). Enhanced drying by AM plants may also be associated with the access of hyphae to small pore spaces inaccessible to host roots and root hairs (Ruiz-Lozano, 2003) and the subsequent uptake of water by AM mycelia for the maintenance of physiological activities during water stress (Sa´nchez-Dı´az and Honrubia, 1994). A summary of other AMF advantages is shown in Table1.

Table 1: Advantages of AMF in agricultural systems

(Rajah and Tang, 2005)

Field	**Advantages**	**Benefits**
Agriculture	Agro-system stability	Improved plant roots, soil microflora and the abiotic geochemical soil matrix
	Enhanced nutrient uptake	**Improves the absorption of phosphate along with other macro- and micronutrients in addition to better Cation Exchange Capacity (CEC) in the root rhizosphere**
	Plant production	**Increased shoot and root biomass**
	Stress resistance	Drought, cold and pollution
	Resistance to pathogens	Crop protection, reduced use of pesticides, fungicides, improvement in animal, plant, human health and product quality
	Value added to product	Increased synthesis of primary and secondary metabolites
Plant physiology	Improvement in nutrition	**Reduced chemical fertilizers (10-30%)**
	Carbohydrate level	Improved photosynthetic activity
	Tolerance to water stress	**Cultivation of arid soils or soils unfit for agriculture**
	Resistance to low temperatures	Diversity of crops in inhospitable areas
Plant Morphology	Transformation of root system and root architecture	**Adaptation to stress, increased resistance to erosion, fixation of soils, increased numerous and branched roots, root apices and improved production with certain root crops**
Plant Community	Micro-organism diversity in the subsoil	**Re-establishment of soil microflora and improvement of soil quality**
	Habitat restoration	Stabilizes biogeochemical cycling
	Survival of partners	Improvement in yields, better acclimatization at transplanting and diversity of plant cover
Micropropa-gation plants	High value plants	Uniform size, better growth, shortened nursery period

2.2 Biology of AMF

2.2.1 AMF structure

AMF comprise intra- and extraradical structures. Intraradical hyphae penetrate the outer cell wall of root and grow between or inside of the root cell wall and plasma membrane where they develop the intraradical structures, arbuscules and vesicles. Extraradical structures are hyphae and spores that develop outside of the roots in the soil (Figure 1) (From http://www.ffp.csiro.au/research/mycorrhiza/method.html).

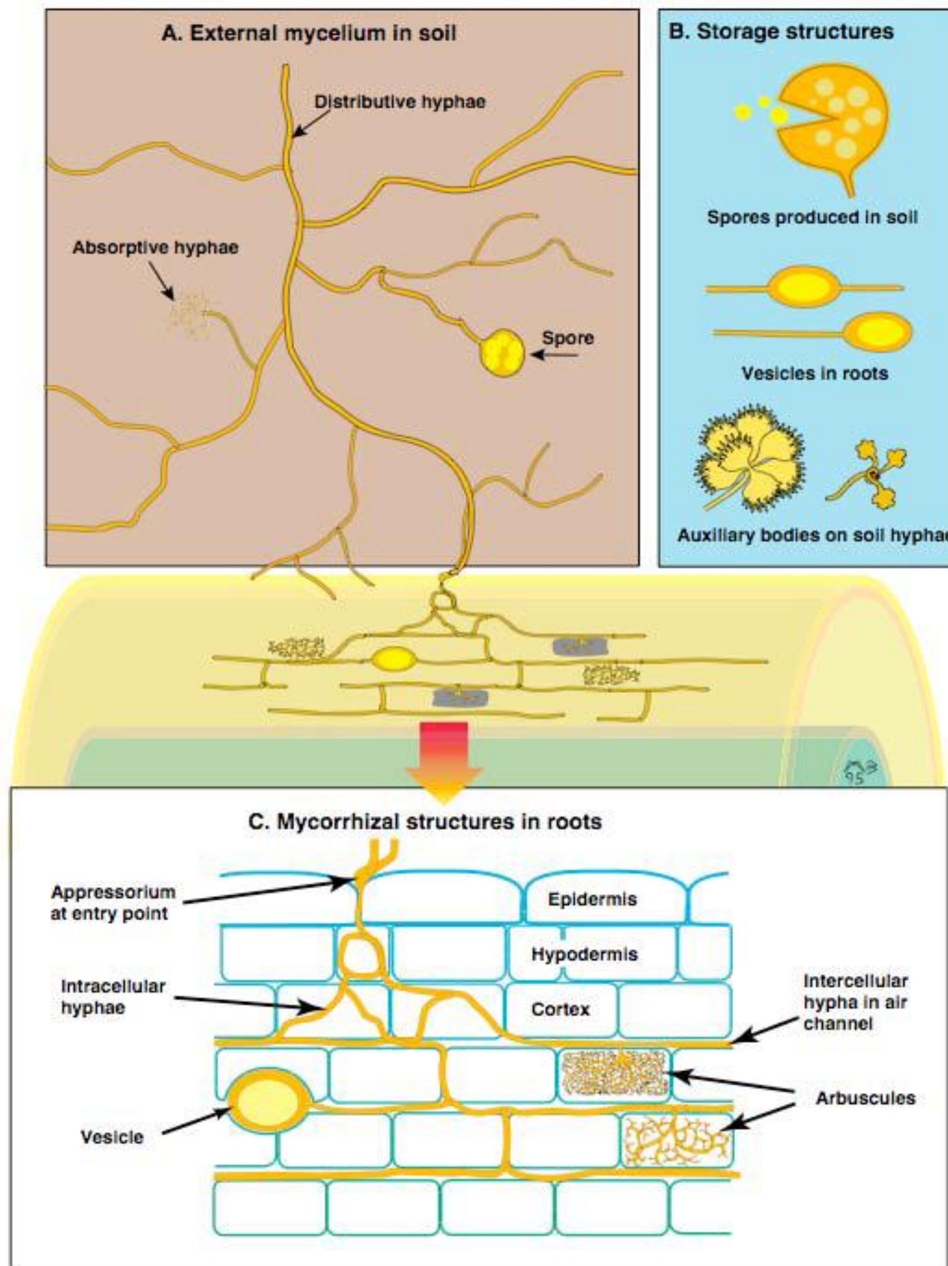

Figure 1: Structures of arbuscular mycorrhiza

Spores (greek: *spore* = germ) are formed as swellings on subtending hyphae in the soil or in roots (Fig 1 C). They contain cytoplasm, numerous nuclei (576 to 35000 depending on species) (Hosny *et al*., 1998; Viera and Glenn, 1990) and storage lipids as an energy source for presymbiotic growth. Spores are storage structures, resting stages and propagules. They are the only plant-independent phase of AMF. Their size

varies strongly among the species (Dalpé *et al*., 2005). Spores usually develop thick walls with more than one layer but colour, morphology and the composition differ depending on species (Błaszkowski *et al*., 2002). This was the reason why they were the most important structures used so far in the classification of arbuscular fungi until rRNA sequencing methods appeared.

Extraradical hyphae increase the absorptive area of roots (Fig 1 A).They form hyphal bridges transferring nutrients from soil to host plants (Bücking and Shachar-Hill, 2005; Govindarajulu *et al*., 2005) and bind soil particles causing improvement of the soil aggregation (Rillig & Mummey, 2006). **Inter- and intracellular hyphae** in roots contain storage material and contribute in transporting the substances absorbed from the soil to plants and sugars from plants to the fungi (Solaiman and Saito, 1997).

Arbuscules (tree shape structures) are formed inside the host root cortical cell wall and indicate active mycorrhizae (Bago *et al*., 1998) (Fig 1 C). Arbuscules are the main sites of nutrient, and perhaps signal exchange between the plant host and a fungus (Parniske, 2008). Arbuscules which are the key sites for nutrient exchange remain active for only 4 - 15 days (Goltapeh *et al*., 2008).
Vesicles originate from the swelling of intraradical hyphae and are localized inter- or intracellularly (Pawlowska *et al*., 1999) depending on the AMF species (Fig 1B). They are filled with lipids, glycolipids and organelles required for autonomous growth (Dalpé *et al*., 2005).

2.2.2 AMF life cycle

AMF has three growth phases in their life cycle: asymbiotic, pre-symbiotic and symbiotic (Figure 2). The asymbiotic mycelium stage includes spore germination and germ tube growth for about 2 - 3 weeks without physical contact with roots or root exudates. During this phase, the fungus is living mostly from its reserves. The second phase is called pre-symbiotic. During this phase, signals from plant root exudates (strigolactones) induce AMF hyphal branching (Parniske, 2008). It takes one to several weeks to establish contact between the root and fungal hyphae (Declerck *et al*., 1998). Once the root-fungi contact is established, the fungal morphology and

metabolism change radically which allows the fungus to enter into the symbiotic stage (Besserer *et al.,* 2006).

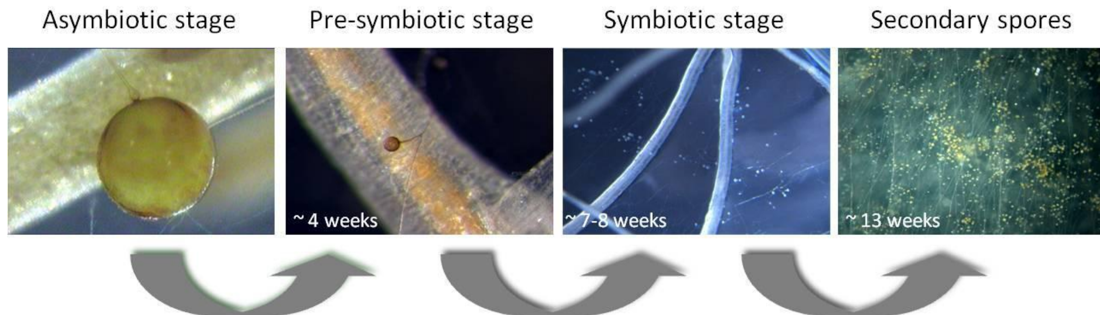

Figure 2: Life cycle stages of an arbuscular mycorrhizal fungus belonging to *G. irregulare* species.

In the symbiotic stage, the fungus penetrates the surface of root and colonizes the root cortex. The plant cell responds to this contact by developing a pre-penetration apparatus (PPA). The PPA is a subcellular membrane structure that predetermines the fungal growth pathway through the plant cell and seems to form a tunnel (Bonfante and Genre, 2008; Parniske, 2008). However, the signals that initiate the development of PPA are not yet known. The fungus subsequently enters root cortical cells and branches to form an appressorium, also called hyphopodium. Two structural types of colonization may develop: the Arum-type, in which hyphae grow intercellularly and form intracellular arbuscules, and the Paris-type, which exhibits intracellular growth and forms hyphal coil cells from which small arbuscules can originate (Smith and Smith, 1997).

Current studies have shown several intermediate colonization forms exist (Dickson, 2004). Factors influencing the colonization type are however not yet well known. Except species from the genera *Scutellospora* and *Gigaspora*, all endomycorrhizal fungi able to form arbuscules will later also form terminal or intercalary ellipsoid vesicles in the root apoplast (Hause and Fester, 2005).

Following the colonization of roots, extraradical hyphae that grow out into the soil in search of mineral nutrients are produced. These hyphae can also inhabit other

available roots. Within soil, the AMF hyphal network may also connect plants of different species as a result of lack of host specificity (Goltapeh *et al*., 2008; Requena *et al*., 2007). New spores, called secondary spores, can be typically synthesized outside of the plant root at the leading tip of individual fungal hyphae (Dalpé *et al*., 2005). The fungal life cycle is completed after asexual spores are formed on the external mycelium.

2.3 AMF and host specificity

Traditionally, AMF have been considered to be generalists with regard to the hosts that they infect. They have also been considered to be functionally equivalent in their effects on a host. These beliefs are based largely on the fact that most AMF can successfully infect a wide range of plant species when grown experimentally in monocultures. However, when different plants and fungi are grown together, AMF growth and species composition is host specific (Bever *et al*., 1996; Douds and Millner, 1999; Eom *et al*., 2000; Kiers *et al*., 2000). Consequently, some AMF species are more beneficial to a host plant than are others, because of genetic and/or physiological incompatibilities between an AMF and its host (Van der Heijden *et al*.,1998) . In addition, AMF species differ in the services that they provide for host plants (e.g. nutrient uptake, protection against pathogens, and water uptake) (Klironomos, 2000).

2.4 Identification, phylogeny, systematics and diversity of AMF

2.4.1 Systematics and phylogeny of AMF

Previously, AMF were placed into the order Glomales and to Zygomycota phylum (Morton and Benny 1990). However, based on their obligate symbiotic habit, the apparent lack of zygospores and the rDNA phylogeny, Schüßler *et al.* (2001) defined the phylum Glomeromycota as a sister clade of Basidio- and Ascomycota.

Currently, Glomeromycota comprise approximately 200 described species distributed among ten genera, most of which were defined primarily based on the morphology of their spores or spore-bearing structures (http://www.lrz-

muenchen.de/~schuessler/amphylo/). These spores can be analyzed under the microscope and the spore wall structures can be stained. The way the spore is formed on the hypha ("mode of spore formation") has been important to define genera and families; and the number of the walls, their layer structure as well as ornamentation to distinguish species. However, these characteristics can differ according to some factors like environmental conditions and stage of the spore; spores may look different, even if they belong to the same species. Moreover, to distinguish and classify the spores by their morphology is quite difficult and requires expert knowledge (Morton, 1988).

Recently, DNA sequences have also been used to describe AMF taxa and their phylogenetic relationships (Schwarzott *et al.*, 2001; Redecker and Raab, 2007). Molecular techniques allow the identification of spores as well as the AMF community currently colonizing plant roots or growing in the soil at any given time. At the present time, the phylogeny of all genera of AMF is based entirely on analyses of the nuclear small subunit RNA gene (Redecker and Raab, 2007).

Four orders, containing eight families and ten genera have been delimited within the Glomeromycota (Figures 3 and 4).

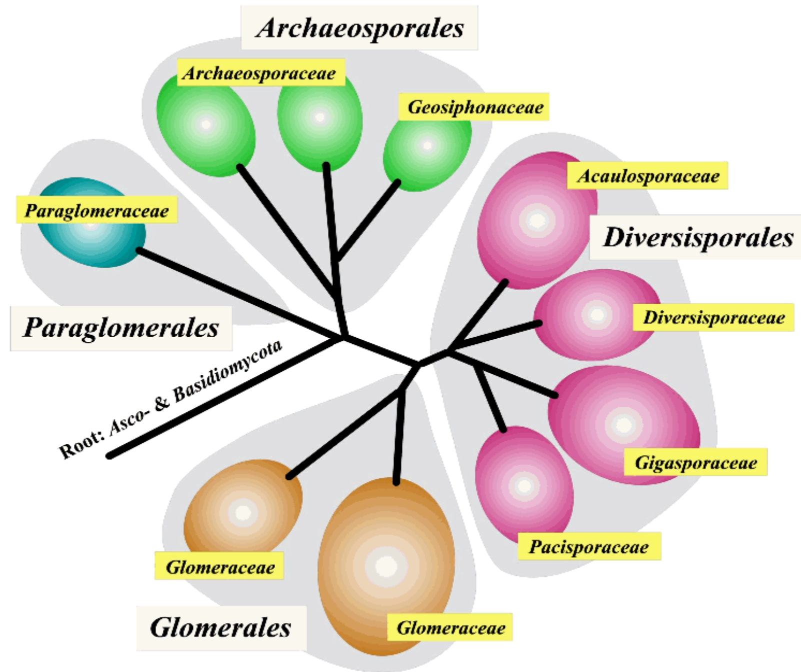

Figure 3: Phylogenetic tree implementing recent changes in the taxonomy of the Glomeromycota.

The tree shows the glomeromycotan taxa before the establishment of the families Appendicisporaceae and Entrophosporaceae. From http://www.lrz-muenchen.de/~schuessler/amphylo/.

Glomus is the largest genus within this phylum. This genus was divided into the following five genera: *Glomus, Paraglomus, Archaeospora* (Morton and Redecker, 2001), *Pacispora* (Oehl and Sieverding, 2004) and *Diversispora* (Walker *et al.*, 2004). The remaining species of the genus *Glomus* are further divided into three clades A, B and C (Schwarzott *et al.*, 2001). Other glomeromycotan genera are *Scutellospora* and *Gigaspora*, which form the family Gigasporaceae; and *Acaulospora* and *Entrophospora,* belonging previously to the family Acaulosporaceae (Figure 4). However, just recently, the genera *Archaeospora* and *Entrophospora* have been revised by several groups of authors (Sieverding and Oehl, 2006; Walker *et al.*, 2007). Based on the mode of spore formation, spore wall structures, trypan blue staining intensity of the fungal intraradical structures as well as

DNA analyses, two new families have been established: Appendicisporaceae and Entrophosporaceae.

Glomus groups A and B form a monophyletic clade (Figure 4). Their spores are formed by budding from a hyphal tip and typically have a layered wall structure. The sporogenic hyphae often remain attached to the mature spore. This glomoid mode of spore formation occurs in all other glomeromycotean families with exception of the Acaulosporaceae, Entrophosporaceae and Gigasporaceae (http://invam.caf.wvu.edu/fungi/taxonomy/classification.htm). Members of the *Glomus* group A are the dominant and most diverse AMF in many field sites (Öpik *et al.*, 2006). This group also includes the ubiquitous species *Glomus intraradices*. *Glomus* group B contains several AMF species like *Glomus etunicatum, Glomus claroideum* and *Glomus lamellosum,* which are difficult to distinguish (Rodriguez *et al.*, 2005). *Glomus* group C (recently defined as a new family Diversisporaceae; Walker *et al.* (2004) is more closely related to the Acaulosporaceae than to *Glomus* groups A and B based on phylogenies of nuclear-encoded ribosomal genes (Schwarzott *et al.*, 2001) and also contains tropical species forming large sporocarps, e.g. *Glomus fulvum* (Redecker *et al.*, 2007).

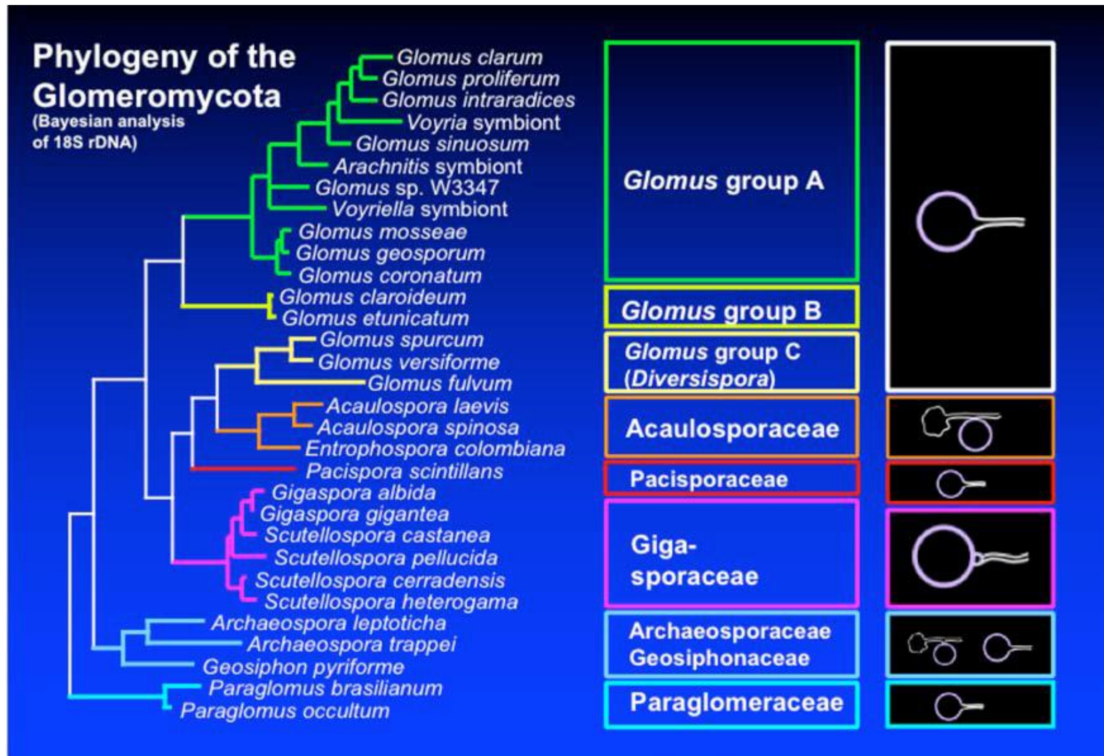

Figure 4: Phylogenetic tree based on analysis of ribosomal small subunit sequences

The tree gives *Glomus* subgroups as defined by Schwarzott *et al.* (2001). The tree shows the glomeromycotan taxa before the establishment of the families Appendicisporaceae and Entrophosporaceae. The boxes on the right hand side show the delimitation of glomeromycotan families or subgroups and the mode of the spore formation in each group.

The Acaulosporaceae form spores laterally next to “sporiferous saccules”, (Figure 5) which collapse or completely disappear after the spore maturation. A germ tube of the Acaulosporaceae emerges from a spherical "germination orb". The Gigasporaceae do not form vesicles within the roots but form so-called “auxiliary cells” on the extraradical mycelium (Figure 1). Their spores are are generally larger than 200µm in diameter at maturity and are formed from a morphologically specialized bulbous sporogenous cell formed terminally on a fertile hypha (http://invam.caf.wvu.edu/fungi/taxonomy/classification.htm). The genus

Scutellospora possesses a "germination shield", a membraneous structure that is used during the spore germination to penetrate the spore wall. In contrast, the genus *Gigaspora* lacks this germination shield and as well as the flexible inner spore wall. *Pacispora* species form spores terminally on the hyphae like members of the genus *Glomus* but have flexible inner walls and germinate by means of a germination shield (Walker *et al.*, 2004). The genus *Pacispora* was recently established by Oehl and Sieverding (2004).

The Archaeosporaceae and Paraglomeraceae are thought to be the basal members of the Glomeromycota. This conclusion is based on phylogenetic studies of the nuclear rDNA by Morton and Redecker (2001) and the possession of unique fatty acids which could not be found in other glomeromycotan fungi (Graham *et al.,* 1995). Their intraradical structures stain very faintly and they do not seem to form vesicles. Some members of the Archaeosporaceae form dimorphic spores – the acaulosporoid type formed similarly to the *Acaulospora* on the neck of a sporiferous saccule that is formed terminally on a fertile hypha, and the glomoid type. Paraglomeraceae form their spores like members of the genus *Glomus* (Spain *et al.,* 2006).

The new family Appendicisporaceae contains e.g. *Appendicispora gerdemanni, Ap. appendicula, Ap. fennica*, which were tranferred from the family Archaeosporaceae. *Appendicispora* is a dimorphic genus, forming both acaulosporoid and glomoid spores. This genus may form vesicles in the roots and the fungal intraradical structures stain pale with trypan blue (Sieverding and Oehl, 2006; Walker *et al.* 2007). *Geosiphon pyriformis* is a glomeromycotan species belonging to the order Archaeosporales. It is the only fungus in the Glomeromycota currently known to form a symbiosis with a cyanobacterium: it produces bladders that harbor symbiotic *Nostoc punctiforme* (Schüßler *et al.,* 1994). Despite its different morphology and life strategy, molecular phylogenetic analysis has shown that *Geosiphon* is a member of the Glomeromycota (Schwarzott *et al.,* 2001).

2.4.2 Molecular identification of AMF using PCR-based techniques

Molecular methods enable the identification of AMF in the host plant roots or directly in the soil. PCR-based methods using AMF-specific primers enable the amplification even of very small amounts of template DNA from the fungi growing in the soil or roots. The specificity of the primers is essential, as a single root or soil sample may be simultaneously colonized by glomeromycotan fungi as well as by numerous fungal pathogens and saprophytes (Sykorová, 2007).

During the past ten years, nuclear-encoded ribosomal DNA (rDNA) has been well established for molecular identification and phylogeny of AMF. These genes are present in multiple copies and contain conserved coding (small subunit - SSU and large subunit - LSU) as well as variable non-coding parts (internal transcribed spacers - ITS) (Sykorová, 2007).Thus, they are useful to distinguish taxa at many different levels (Redecker, 2006). The ITS region evolves faster than the conserved regions and therefore provides more information about close relationships, whereas the conserved regions enable primer construction and taxa resolution on the genus and family level. A comprehensive molecular phylogeny has been based largely on sequences of the small subunit (18S) rDNA, as these sequences were available first (Simon *et al.*, 1993). Different authors have tried to develop AMF-specific primers (Figure 5) targeting different regions of the small subunit of the rDNA (18S).

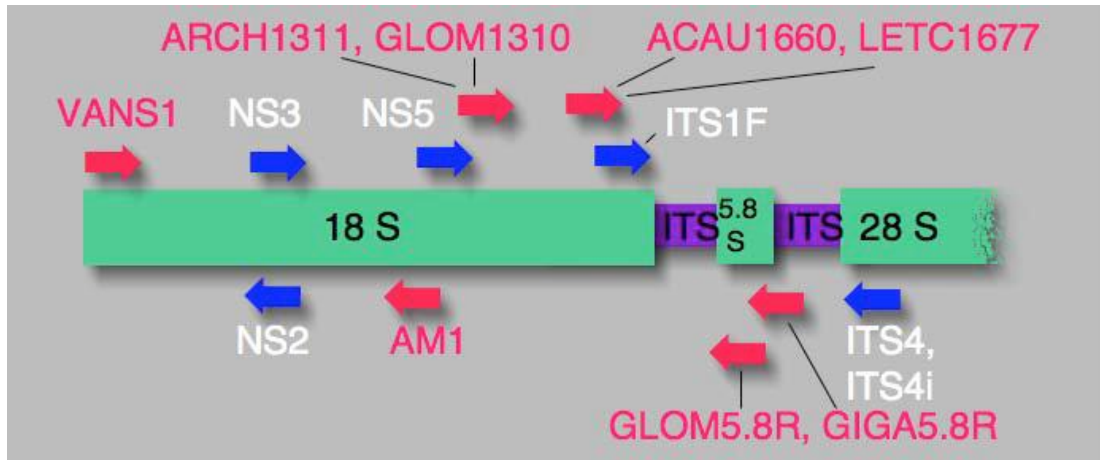

Figure 5: Ribosomal DNA structure and annealing sites of different primers used in AMF community studies

In red AMF-specific primers, in white universal primers (Sykorová, 2007).

2.4.3 Ecology of AMF

A global survey of root-colonizing AMF communities ranked different ecosystems according to their AMF species richness (Öpik *et al.,* 2006). The species-richest habitats were the tropical forests with eighteen AMF taxa/host plant species; followed by the plant-species-rich non-disturbed grasslands. Approximately eight AMF taxa were associated with a single host plant species here. In the temperate forests and habitats under strong antropogenic influence like arable fields and polluted sites, approximately five AMF taxa associated with a single host plant species. In European grasslands and woodlands, using both molecular and morphological approaches, AMF species richness was also reported to be higher in diverse natural plant communities compared to arable fields (Daniell *et al.*, 2001; Oehl *et al.,* 2004; Öpik *et al.,* 2006). However, evidence for a relatively high AMF diversity in arable sites with low-input agriculture was presented by Hijri *et al.,* (2006). Ten to 24 AMF phylotypes were detected in the roots of two to 18 plant species growing in different temperate grasslands (Gollotte *et al.,* 2004; Börstler *et al.*, 2006).

Using molecular and morphological methods, some evidence for ecological specialization of different AMF species was found. For instance, *Acaulospora alpina* was described in plant species-rich grasslands of the Swiss Alps at altitudes between 1800 and 2700 m above sea level (Oehl *et al.*, 2006) and *Glomus badium* was found in grasslands, grassintercropped vineyards or olive fields, or non-tilled arable lands in Central Europe (Oehl *et al.,* 2005a). In contrast, *G. intraradices* has been reported as a ubiquitous AMF species with global distribution (Öpik *et al.*, 2006) occurring in many different native (Appoloni, 2006) and human-influenced ecosystems (Hijri *et al.*, 2006). *Glomus mosseae, G. caledonium, G. claroideum, G. intraradices* and *G. etunicatum* were frequently found in intensively managed tilled arable soils from different sites in Europe (Daniell *et al.*, 2001; Hijri *et al.*, 2006). These species were also found in trap cultures from several agroecosystems already after two to four months, in some cases after six months, as the first sporulating ones (Oehl *et al.*, 2003; Oehl *et al.,* 2004). However, further molecular and morphological investigations are necessary to elucidate the ecological preferences of AMF (Sykorová, 2007)

2.5 Phosphorus and Mycorrhizas

2.5.1 Plant phosphorus status on mycorrhizal association

Higher P concentration in plant tissues reduces the production of spores (De Miranda and Harris, 1994) and of external hyphae (Bruce *et al.,* 1994). Signal molecules from host plant roots exudation that encourage hyphal branching is enhanced by low level of phosphorus in host roots (Nagahashi and Douds, 2000). Therefore, increasing P level in host roots may reduce the secretion of these signal molecules, reducing therefore hyphal branching and mycorrhizal association. Phosphorus level of the root may affect membrane phospholipids, thus influencing membrane permeability and the release from the roots of carbohydrates that nourish the fungi (Schwab *et al.*, 1991). Therefore, where P concentration in the plant is low, carbohydrate exudation will encourage mycorrhizal association (Grant *et al.,* 2004).

2.5.2 Impact of soil phosphorus levels on mycorrhizal association

Mycorrhizal association tends to decrease with increasing P concentration within plant tissues. If plant-available P increases and consequently tissue P concentration increases, mycorrhizal association can negatively be affected (Grant *et al.,* 2004).
P supply to crops is largely influenced by the concentration of available P in the soil solution and the speed of replenishment of the solution. Thus, factors reducing P concentration in soil solution can influence plant P level and mycorrhizal associations. The effect of P level in the soil on mycorrhizal association appears to be indirect, through its influence on plant tissue P concentration rather than directly on the soil fungi (Menge *et al.,* 1978). This suggests that P content in soil may not affect colonization of juvenile plants before they begin to absorb significant amounts of soil P; in corn, for example, this would be near the three-leaf stage (Grant *et al.,* 2004).

2.5.3 Impact of Phosphorus Application and other Nutrients

Phosphorus fertilizers can depress mycorrhizal association since they can increase the P concentration in plant tissue (Kahiluoto *et al.,* 2000; Liu *et al*., 2000). Both mycorrhizal colonization and length of extraradical hyphae can be reduced by P fertilization (Liu *et al.*, 2000). However, P fertilization does not always reduce mycorrhizal association. If the available P in the soil is very low, mycorrhizal colonization and spore production may be restricted and mycorrhizal associations may be increased by P application (Grant *et al.,* 2004).

Source of P fertilization may also influence mycorrhizal development, this being primarily related to the solubility and availability of the P source. Readily soluble and phytoavailable forms of P will rapidly increase P supply to the plant and decrease the mycorrhizal association but less-soluble forms of P such as rock phosphate have less effect on P supply to the plant and mycorrhizal association. The slow release of P may prevent the tissue P concentration from reaching levels that tend to inhibit mycorrhizal associations (Grant *et al.,* 2004). This effect has been shown for various

slowly available P sources such as rock phosphate (Toro *et al.,* 1997), plant residues (Joner and Jakobsen, 1995) and manure (Joner, 2000).

The effect of P fertilization may also vary depending on the balance of other nutrients present. Mycorrhizal association tends to be highest when low P is combined with an ample supply of other nutrients (Valentine *et al.*, 2001).

2.6 Effect of plant characteristics on mycorrhizal association

Plant species differ in their mycorrhizal dependency. They also differ in degree of association at varying levels of available P (Kahiluoto *et al.*, 2000). For a given available P level, mycorrhizal dependency is related in part to the morphology of plant root system, with plants having extensive fibrous roots often less dependent on mycorrhizae than plants with less extensive root systems (Plenchette *et al,.* 1983). For example flax, which has a small, fibrous root system, is more dependent and responsive to mycorrhizae than barley, a plant with an extensive, fibrous root system (Kahiluoto *et al,.* 2000; Grant *et al.,* 2004).

CHAPTER 3: MATERIALS AND METHODS

3.1 Description of the study area

The study was carried out in two districts namely Bugesera and Rubavu districts representing semi-arid and humid to agroececologies. These two districts were selected in the bigger ICRAF led project funded by the Australian Centre for International Agriculture Research (ACIAR) under which this research falls. More information on the project can be accessed at http://www.worldagroforestry.org/aciar. The study was carried out in one of the three sectors in each district where the project is operating (Nyundo and Rweru in Rubavu and Bugesera, respectively) due to limited time available by the student research.

Bugesera district is located in Eastern province of Rwanda. Its altitude varies between 1300 m and 1667 m with soft slopes. Its relief is mainly constituted of a succession of low plateaux, dry valleys and swamps. The annual precipitation ranges between700-900 mm with the mean atmospheric temperature between 21º C and 29º C. Soils in the region are sandy-loam of moderate fertility (JICA, 2006; MINITERE, 2003). Dominant crops and trees observed are relatively homogenous across Bugesera district - crops: banana, maize, beans and cassava, and trees: Acacia, *Senna spectabilis*, *Grevillea robusta*, *Eucalyptus* (Kiptot *et al.*, 2013; CRA, 2005; http://www.bugesera.gov.rw/). This district is representing the semi-arid regions of Rwanda.

Rubavu is a district in the Western province of Rwanda. The region is characterized by an elevation ranging between 2000 m and 3000 m and higher slopes (the mean slope is 35%). Temperatures are generally cool with an average of 10^0C. Its mean annual rainfall is 1800 mm (Nyandwi and Mukashema, 2011). Major crops in the region include maize, climbing beans, Irish potatoes, wheat and vegetables such as carrots and cabbages along with tea plantations on valley bottoms. The dominant trees observed are *Alnus acuminata* along the contours, *Markhamia lutea* on farm, *Eucalyptus* woodlots, *Grevillea robusta*, bamboo, avocado and some indigenous trees

such as ficus (Kiptot *et al.*, 2013). Rubavu district is a good representative of sub-humid regions of Rwanda.

3.2 Sampling

The rhizosphere soil was sampled from Nyundo and Rweru sectors of Rubavu and Bugesera districts, respectively (Figure 6).

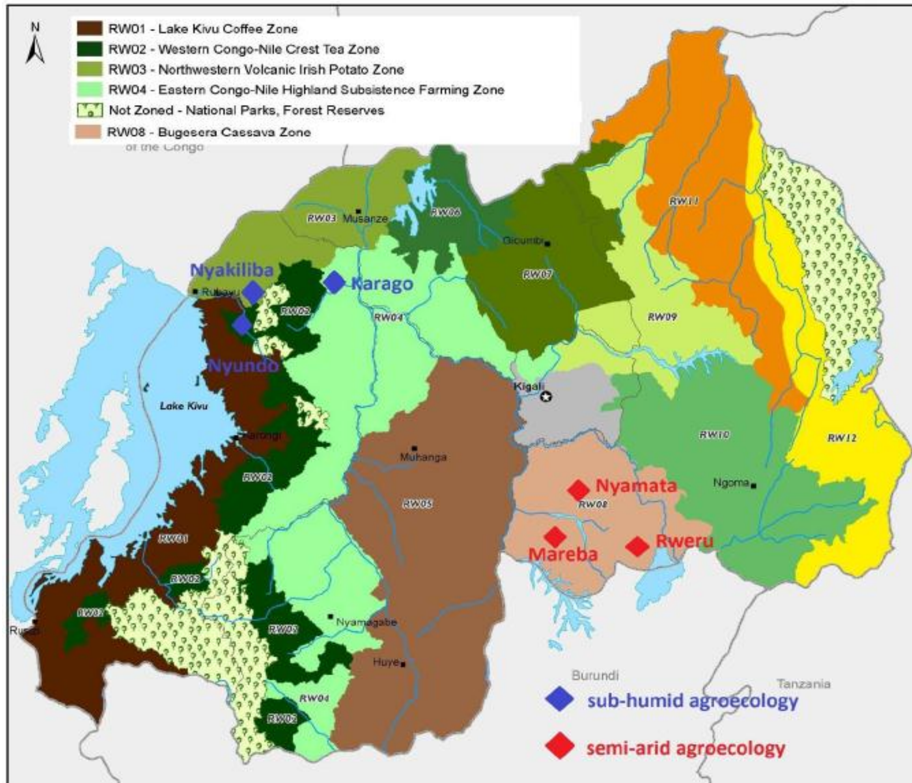

Figure 6: Agroecological Map of Rwanda with selected sites (sectors)

Soil samples were collected using a soil auger to a depth of 0-10 cm. This was done around each tree of the four most common tree species found in the agroforestry systems: *Alnus acuminata*, *Markhamia lutea, Grevillea robusta* and *Eucalyptus* sp. (in Rubavu) and *Acacia polyacantha, Senna spectabilis, Grevillea robusta* and *Eucalyptus* sp. (in Bugesera) in farms where socio economic and tree diversity data had been collected.

Three replications for each tree species were sampled on the same day. Soil samples were collected at three distances from the tree trunk, i.e. 1) 0.5 m from the tree trunk, 2) the edge of the tree crown and, 3) 3 m from the edge of the tree crown. At each distance, the soil was sampled in the eastern and western sides of the tree and the two samples were pooled.

A total of 72 soil samples were collected and stored in sealed plastic bags kept at 4 ºC until AMF spores were extracted, counted and analyzed. Additional information about sampled tree such as replication, sizes of the trees, geographic coordinates of the sites and some soil chemical characteristics were collected (Tables 2 and 3).

Table 2: Summarized information of the sampled tree replicates (Rubavu District)

Tree species	Sampled tree replicate	Geographic coordinates of the sites			Important chemical characteristics of the site	
		Latitude S	Longitude E	Elevation (m)	Mean pH	Mean P (ppm)
Alnus acuminata	1	01°44’33”	029°20’54”	2025	4.9	10.32
	2	01°44’28”	029°20’59”	1998	4.9	10.32
	3	01°44’27”	029°21’01”	2006	4.9	10.32
Markhamia lutea	1	01°44’50”	029°21’00”	2114	4.9	10.32
	2	01°44’04”	029°21’14”	1967	4.9	10.32
	3	01°44’01”	029°21’16”	1959	4.9	10.32
Grevillea robusta	1	01°44’28”	029°20’57”	2007	4.9	10.32
	2	01°44’51”	029°21’01”	2126	5.1	10.07
	3	01°44’50”	029°21’00”	2119	5.8	19.73
Eucalyptus sp.	1	01°44’50”	029°21’00”	2116	5.8	19.73
	2	01°44’43”	029°20’47”	2089	5.1	10.07
	3	01°44’52”	029°21’01”	2144	5.1	10.07

Table 3: Summarized information of the sampled tree replicates (Bugesera District)

Tree species	Sampled tree replicate	Geographic coordinates of the sites			Important chemical characteristics of the site	
		Latitude S	Longitude E	Elevation (m)	Mean pH	Mean P (ppm)
Senna spectabilis	Tree N° 1	02°17'30"	030°15'23"	1336	5	24.98
	Tree N° 2	02°17'39"	030°15'14"	1352	5	24.98
	Tree N° 3	02°17'40"	030°15'13"	1349	5.1	8.8
Acacia polyacantha	Tree N° 1	02°17'28"	030°15'05"	1329	6.1	58.4
	Tree N° 2	02°17'30"	030°15'04"	1324	5.1	8.8
	Tree N° 3	02°17'28"	030°15'02"	1329	6.1	58.4
Grevillea robusta	Tree N° 1	02°17'32"	030°15'22"	1342	5	24.98
	Tree N° 2	02°17'33"	030°15'22"	1339	5	24.98
	Tree N° 3	02°17'28"	030°15'24"	1334	5	24.98
Eucalyptus sp.	Tree N° 1	02°17'38"	030°15'14"	1353	5	24.98
	Tree N° 2	02°17'38"	030°15'14"	1344	5.1	8.8
	Tree N° 3	02°17'37"	030°15'12"	1342	5	24.98

3.3 Extraction of AMF spores

Soil samples were air-dried before extraction and counting of AM fungal spores. AMF spores extraction was done using the method adapted from Ingleby (2007). A 50 g sample of air-dried soil was mixed with water to obtain a 1L suspension, and the suspension was strongly agitated (The purpose of this step is to disperse the soil aggregates and release AMF spores). The liquid was then poured onto a nest of sieves (200 µ pore size on top to allow passage of spores but retain large soil and organic matter particles, and 45µ on the bottom to retain AMF spores yet allow passage of the

finest soil particles). The collected residue in the smallest sieve was washed and transferred into 50ml centrifuge tubes and centrifuged with water for 5 minutes at 1,800 rpm. The supernatant was then discarded and the pellet re-suspended in 48% (w/v) sucrose and centrifuged again for 1 min at 1,800rpm. The supernatant (with spores) was poured onto 45µ sieve and rinsed with water to remove the sugar. The remaining debris on the sieve were transferred to a petri dish for initial observation and collection of AMF spores under dissecting microscope with 40X magnification. Spore abundance was expressed as the number of AMF spores per gram of soil.

3.4 Morphological identification of AMF Spores

This was done using the method adapted from Ingleby (2007). Spores were grouped into different genera according to their morphological characteristics. Microscopy slides were prepared for each different spore morphotype with polyvinyl-alcohol and polyvinyl-alcohol plus Melzer's solution with 1:1 ratio. After the uniformity of the morphological groups was confirmed under a dissecting microscope, the different morphotypes were examined under a stereomicroscope at 400X and tentatively identified to the genus level. This morphological spore identification was based mainly on spore size, shape, color, wall structure, hyphal attachment, ornamentation and Melzer's solution reaction (INVAM, 2004; Ingleby, 2007).

3.5 Genetic characterization

3.5.1 DNA extraction from AMF spores

This was done as per SOP-BM12/V01. A single AMF spore was transferred with the forceps and placed on the wall of the Eppendorf tube. It was then crushed under the microscope with the white tip while adding 2 µl of NaOH 0.25 N. The tubes were then heated at 95°C (water bath) for 2 min. 1 µl of Tris-HCl 0.5 M (pH = 8.0) and 2 µl of HCl 0.25 N were added to the tubes heated again for 2 min at 95°C (water bath). The result was directly used as template for PCR amplification.

3.5.2 Internal Transcribed Spacer (ITS) - PCR amplification, sequencing and molecular characterization

The isolated DNA was amplified using nested PCR following the method adapted from Dhami (2005). During the first amplification step, the ITS regions along with 5.8S regions of different spores was amplified by the PCR using the primers NS5 (5'-AAC TTA AAG GAA TTG ACG GAA G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The 25µl reaction volume for first PCR amplification contained the following:

Template DNA	1µl
PCR buffer	2.5µl
$MgCl_2$ (50 mM)	0.75µl
dNTPs (2 mM)	2.0µl
Forward primer	1.0µl
Reverse primer	1.0µl
Taq polymerase (5U/µl)	0.15µl

The volume was made up to 25µl with sdd water.

The PCR amplifications were performed as described by Dhami (2005):

An initial denaturation step of 94^0C for 3 min. followed by 35 cycles of 94^0C for 2 min. (Denaturation), 50^0C for 1 min. (Annealing), and 72^0C for 2 min. (Extension) with a final extension of 72^0C for 8 min. Controls containing no DNA template were included in PCR amplification to test for the presence of contamination of reagents and reaction buffer. Aliquots (5 µl) of amplification products were run through 1% agarose gel at 150 V during 60 min and stained with Ethidium bromide and visualized in a UV-Transilluminator.

1-2 µl of aliquot of I^{st} PCR product was used as template for the 2nd step of PCR amplification with specific primers ARCH1311 (5' – TGC TAA ATA GCC AGG CTG Y – 3'), GLOM1310 (5'- AGC TAG GYC TAA CAT TGT TA- 3'), LETC1670 (5' – GAT CGG CGA TCG GTG AGT – 3') along with ITS4. Table 4 from Redecker

(2000) shows AMF species targeted by used specific primers in nested PCR. The reaction volumes and concentrations of various reagents were the same as in case of the Ist amplification step. PCR conditions were as follows: An initial denaturation step of 94^0C for 3 min. followed by 35 cycles of 94^0C for 2 min., 60^0C for 1 min., and 72^0C for 2 min., with the final extension of 72^0C for 8 min. The aliquots (5µl) of amplified products were run through 1% agarose gel at 150 V during 60 min, stained with Ethidium bromide and visualized in a UV-Transilluminator.

Table 4: Used specific primers in nested PCR and targeted AMF species

ARCH1311	**GLOM1310**	**LETC1670**
Glomus occultum HA771	*Glomus intraradices*	*Glomus versiforme*
Glomus occultum CL700	*Glomus sinuosum*	*Glomus etunicatum*
G. leptotichum/A. gerdemannii NC176	*Glomus versiforme*	*Glomus claroideum*
Acaulospora trapei NB112	*Acaulospora rugosa*	*Glomus sp. S329*
Acaulospora trapei AU219	*Acaulospora spinosa*	*Glomus intraradices*
Glomus etunicatum	*Entrophospora colombiana*	*Glomus sinuosum*
	Gigaspora albida	*Glomus mosseae*
	Gigaspora gigantean	
	Scutellospora dipapillosa	
	Scutellospora pellucida	

The amplified PCR products were purified using Thermo Scientific GenJET PCR purification kit. The purified DNA was further transferred to sequence analysis program. Using bioinformatics tools, AMF species were identified.

3.6 Greenhouse experiments

3.6.1 AMF Spores Propagation: Trap Cultures Development

The aim of the cultures is to maintain a living collection of the organisms in study and obtain fresh spores for further inoculation. The trap cultures were set in pots, using the soil sampled from Bugesera agroforestry system. Three pots with sterilized small stones to facilitate water drainage were filled with a mixture of sterilized soil and sand in a ratio 1:1 at ¾. Sorghum seeds were sowed in each pot as symbiotic partner plant to AMF spores. Three different morphotypes of AMF spores were sowed in the three pots, respectively. They were maintained in green house for 8 weeks with a regular watering.

3.6.2 AMF spores propagation: Trap Cultures Development

The experiment was conducted in a greenhouse with natural lighting and temperature. The experiment was arranged in a design with three replicates for each treatment. Treatments were factorial combinations of two factors; including AMF inoculation (AMF inoculum vs non-mycorrhizal control) and P addition (0; 0.9, 1.9 and 2.9 g P per pot) (See Table 5).

Table 5: Summary of maize treatments with AMF

Amount of added P (g per pot)	Inoculation with indigenous AMF of Bugesera and Rubavu districts							Maize without any inoculums
	AMF mono-inoculation			Combination of AMF morphotypes				
	Morphotype 1	Morphotype 2	Morphotype 3	Morphotypes 1 & 2	Morphotypes 1 & 3	Morphotypes 2 & 3	Morphotypes 1, 2 & 3	
0	3	3	3	3	3	3	3	3
0.9	3	3	3	3	3	3	3	3
1.9	3	3	3	3	3	3	3	3
2.9	3	3	3	3	3	3	3	3

Fertilizers N and K were added as 1.9 g of N per pot in the form of urea and 1.9 g per pot in the form of KCl. All the amount of used fertilizers were calculated using the fertilization application rate for maize in Rwanda of 17:17:17 at 300Kg per ha. The maize variety used in our experiment is ZM607

Mycorrhizal inoculum consisted of soil, spores, mycelium and infected root fragments picked from the trap cultures. Each pot was inoculated with 100 g inoculum for the AMF treatment. Each pot was filled with 5 kg of autoclaved soil. The inoculum was placed 20 mm below the seeds prior to sowing. Maize seeds were surface sterilized in a 70% alcohol solution for 5 min then washed several times with sterilized distillated water. Five seeds of maize were sowed in each pot and thinned to three after seedling emergence. Watering was done daily and plants were harvested 60 days after germination.

3.7 Evaluation of maize roots colonization

This was adapted from from Ingleby (2007) and Habte and Osorio (2001).

3.7.1 Collection of Maize Roots Samples

Entire roots of maize were picked from the soil, washed free of soil and tertiary roots were collected to obtain a representative sample. Roots samples were stored in plastic vials within 70% ethanol before staining for AMF assessment.

3.7.2 Roots Staining

Before AMF infection could be observed, root samples were taken through a series of stages during which roots were cleared in potassium hydroxide, bleached in alkaline hydrogen peroxide, acidified in hydrochloric acid, stained in trypan blue and destained using a method adapted from Ingleby (2007) and Habte and Osorio (2001).

Maize roots previously stored in 70% ethanol were stained for AMF assessment. Ethanol was poured and 2.5% KOH was added for clearing root samples. The roots

were heated in an oven at 70^0C for 1 hour; KOH was poured and roots were rinsed with tap water. Alkaline Hydrogen peroxide (comprised of 60ml of 20-30% NH_4OH and 90ml of 30% H_2O_2 and 840ml distilled water) was added to remove the phenolic substances. The roots were placed in the oven at 70^0C for 20 minutes. The roots were then rinsed with tap water, 1% HCl was added and the root samples were left for 24 hours. HCl was poured and ,without rinsing the roots, 0.05% trypan blue (500ml glycerol, 450ml water, 50ml of 1% HCl and 0.5g trypan blue) staining reagent was added and placed in the oven for 1 hour at 70^0C. The stain was then poured and de-staining solution, acidic glycerol (500ml glycerol, 450ml water, 50ml of 1% HCl) added.

3.7.3 Slide Preparation and Roots Analysis for AMF Colonization

Roots were removed from the de-staining solution, and placed in a Petri dish. A small amount of water was added into the Petri dish, and with forceps and a surgical blade on a holder, roots were cut into approximately 1cm pieces. 30 pieces of roots were mounted on a glass microscope slide and a drop of lactic acid added as a mounting reagent. The cover slip was gently lowered from the edge and roots gently squashed. Slides were examined under the compound microscope at 100X magnification and the frequency of AMF colonization (arbuscules, coils, and vesicles, internal and external hyphae) recorded for each sample. The frequency of AMF was recorded as the number of root fragments infected with AMF and was expressed in percentage.

CHAPTER 4: RESULTS

4.1 Abundance of AMF Species

Spore abundance in the rhizosphere of different tree species from the agroforestry systems of sub-humid Rubavu and semi-arid Bugesera districts in Rwanda are shown in Tables 6 and 7. The distribution of AMF on the basis of spore density showed difference among the experimental tree species. In Bugesera, the highest spore counts were observed under *Acacia polyacantha* (Mean of 5.4 to 9.9 spores/ g soil) and the least spore counts were encountered under *Grevillea robusta* (3.1 to 3.4 spores/g of soil). In Rubavu, the highest spores counts were found under *Alnus acuminata* (2.9 to 4.4 spores/g of soil) while the least count were detected under *Eucalyptus sp.* (1.6 to 2.4 spores/g of soil).

Around individual trees, AMF spores abundance varied with the distance from the tree trunk but not consistently. Some tree species rhizospheres (3/8 cases) showed greater mean spore density within rhizosphere in close proximity to trees, 2/8 cases showed the highest spore density at the end of the tree canopy and 3/8 cases were with higher spore density away from the canopy.

Table 6: Abundance of AMF spores in the agroforestry system of sub-humid Rubavu district.

Tree species	Sampled tree replicate	Tree size		AMF spores abundance (Number of spores/g of soil)		
		Height (m)	Diameter at breast height (m)	0.5m from tree	End of tree crown	3m from tree crown
Alnus acuminata	1	15.5	0.22	6.6	5.8	4.2
	2	14.0	0.20	3.5	5.1	3
	3	11.5	0.18	3.1	2	1.6
	Mean and sd*			4.4(±1.9)	4.3(±2.0)	2.9(±1.3)
Markhamia lutea	1	10.5	0.14	1.1	2.2	1.7
	2	13	0.17	1.8	2.3	1.6
	3	14.5	0.18	2.9	2.8	2.5
	Mean and sd			1.9(±0.9)	2.4(±0.3)	1.9(±0.5)
Grevillea robusta	1	22	0.27	1.7	2.8	3
	2	21.5	0.26	1.6	3	2.8
	3	19.5	0.24	2.5	1.5	1.8
	Mean and sd			2.0(±0.5)	2.4(±0.8)	2.5(±0.6)
Eucalyptus sp.	1	17.5	0.25	1.8	2.1	1
	2	19	0.27	1.3	3	2.2
	3	16.8	0.22	2	2.2	1.6
	Mean and sd			1.7(±0.3)	2.4(±0.5)	1.6(±0.6)

*Mean values are the mean of n; sd = standard deviation

Table 7: Abundance of AMF spores in the agroforestry system of semi-arid Bugesera district

Tree species	Sampled tree replicate	Tree size		AMF spores abundance (Number of spores/g of soil)		
		Height (m)	Diameter at breast height (m)	0.5m from tree	End of tree crown	3m from tree crown
Senna spectabilis	1	7.5	0.14	9.9	8.2	7.5
	2	6.0	0.13	6.4	5	3.6
	3	5.5	0.11	3.4	4.4	6.1
	Mean and sd*			6.5(±3.2)	5.8(±2.0)	5.7(±1.9)
Acacia polyacantha	1	7.0	0.17	2.9	5	5.4
	2	7.6	0.19	4.8	4.9	5.9
	3	8.2	0.22	8.7	9.2	18.5
	Mean and sd			5.4(±2.9)	6.3(±2.4)	9.9(±7.4)
Grevillea robusta	1	12.0	0.17	3.2	3.4	4.9
	2	11.5	0.16	3.4	2.6	1.8
	3	10.5	0.14	3.6	3.4	3
	Mean and sd			3.4(±0.2)	3.1(±0.4)	3.2(±1.5)
Eucalyptus sp.	1	11.0	0.14	2.9	4.1	4.6
	2	13.5	0.21	2	5.8	6.5
	3	11.5	0.16	5.3	4.9	4.3
	Mean and sd			3.4(±1.7)	4.9(±0.8)	5.1(±1.2)

*Mean values are the mean of n; sd = standard deviation

These results showed significant differences among some experimental tree species. In Rubavu, *Alnus acuminata* gave a significantly greater density of AMF spores compared to *Markhamia lutea* (p=0.001), to *Grevillea robusta* (p=0.018) and to *Eucalyptus sp.* (p=0.011) which on their side do not show any statistical difference among their rhizosphere AMF spores density. In Bugesera agroforestry system, *Senna spectabilis* with no significant difference to *Acacia polyacanta* showed a significantly greater density of AMF spores than *Grevillea robusta* and *Eucalyptus sp.* (p=0.0002 and 0.030 respectively). Also, spores densities around *Acacia polyacanta* and *Eucalyptus sp.* were significantly greater than the density around *Grevillea robusta* (p=0.022 and 0.042, respectively).

The difference in spore density recorded between the two agroforestry systems (Figure 7) was statistically significant with p=0.036. The spore count varied from 3.1 to 9.9 spores per gram of soil in Bugesera and 1.6 to 4.4 spores per gram of soil in Rubavu (Tables 6 and 7).

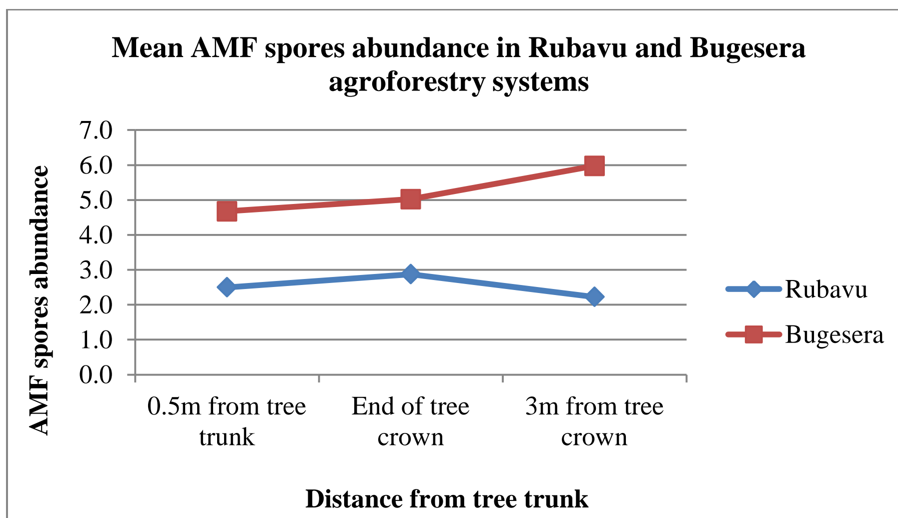

Figure 7: Mean AMF spore abundance around most common tree species by distance from tree trunk

4.2 AMF Spores Characterization

4.2.1 Morphological Identification of AMF Spores

Based on major differences in spore morphological appearance, four different types (genera) of spores were detected from the agroforestry systems in the semi-arid and humid agroecologies of Rwanda. These morphotypes AMF1, AMF2, AMF3 and AMF4 were identified into four genera i.e. *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora,* respectively (Figure 8) based on their morphological features. All spore types were found in all rhizosphere soil samples. In all rhizosphere soil samples, *Glomus* was the predominant taxonomic, the second dominant AMF genus encountered was *Gigaspora*. The third dominant was genus *Scutellospora* and the last was the genus *Acaulospora*. The characteristics shown by the different types of spores are indicated (Table 8).

Table 8: AMF Spores Morphological Characteristics

Morphotype	Color	Shape	Ornamentation	Melzer's solution reaction	Subtending hypha	Size	Spore wall layers	Tentative genus
AMF1	Light yellow to Brown	Spherical	No	Yes	Present	Small	1 or laminated layers	***Glomus***
AMF2	White to Gray	Globose	No	No	Present	Big	3 laminated layers	***Gigaspora***
AMF3	Brown	Globose	Yes	Yes	Present	Small	2 layers	***Scutellospora***
AMF4	Brown to Black	Round	Yes	No	Absent	Big	2 or 3 layers	***Acaulospora***

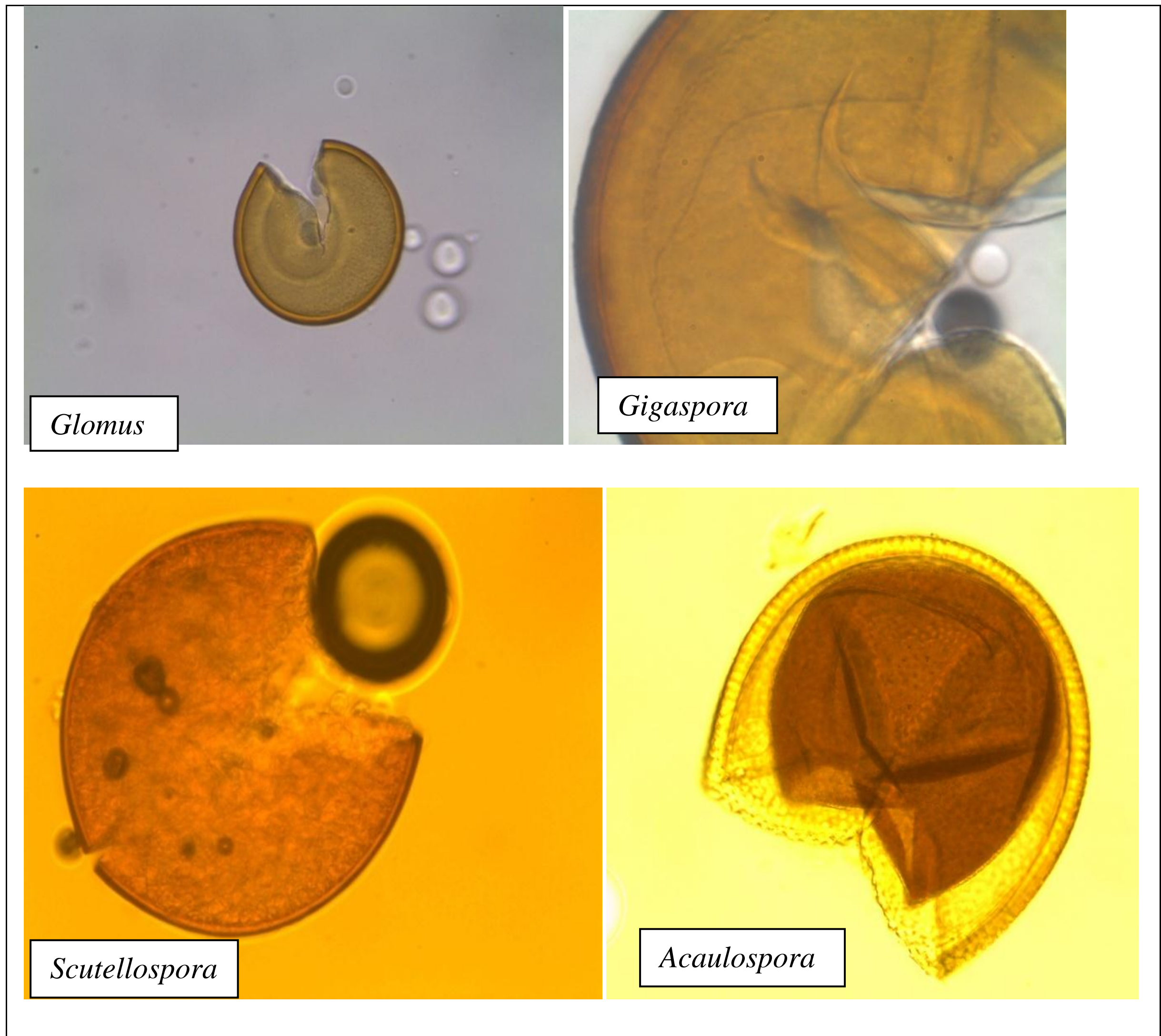

Figure 8: Photomicrographs of arbuscular mycorrhizal fungi spores (400X magnification)

4.2.2 Genetic Characterization of AMF Spores

The universal primer pair NS5 and ITS4 was used to amplify fungal rDNA (partial 18S, ITS1, 5.8S, ITS2 and partial 28S rDNA) extracted from different spores. Between 500 and 1200 bp fragments were obtained from numbers 1, 9, 10, 11, 12, and 16 of spore samples. Figure 9 shows first PCR results from the first 17 treated AMF spore samples.

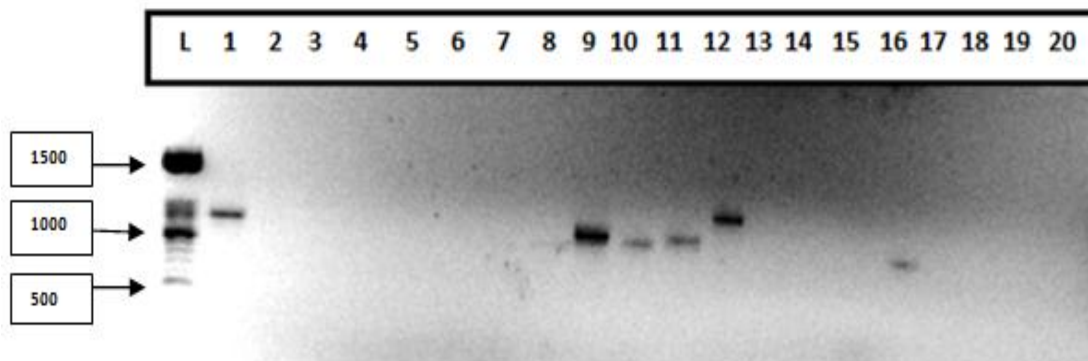

Figure 9: NS5 and ITS4 PCR amplification products of DNA extracted from some spore samples

Key:
No. 1 -17 : Samples JD1- JD17
No. 18- 20: Negative Controls
L : 100bp molecular markers/ladders

After universal primer pair amplification, the amplified product was taken as a template for nested PCR in which species specific primers were used and between 500 and 1200 bp fragments were obtained from numbers 1, 6, 8, 9, 10, 11, 12, and 16 of spore samples (Figure 10).

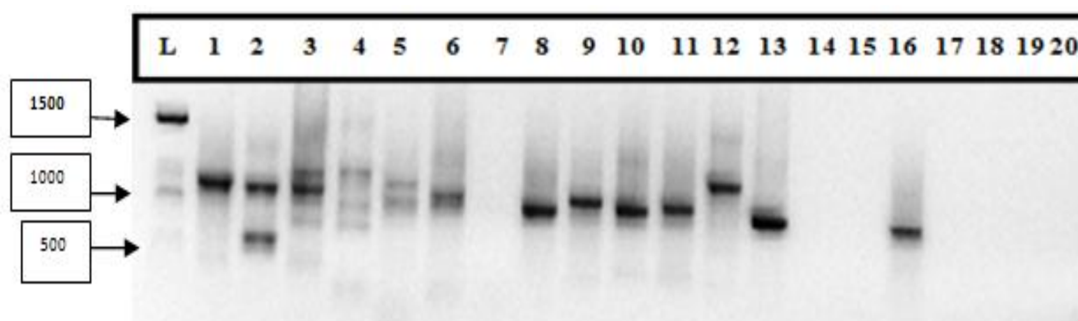

Figure 10: Nested PCR amplification products from some AMF spores samples with specific primer pairs

Key:

No. 1 – 17: Samples JD1- JD17
No. 18- 20: Negative controls
L: 100bp molecular marker/ladder

DNA was extracted from a total of 102 spore samples belonging to different morphotypes. Due to the little amount of DNA per single spore, only 33 DNA samples (ie 32.35%) showed clear bands on agarose gel and were subjected to sequencing; three of them were sequenced successfully. Using bioinformatics tools, only one species AMF *Gigaspora margarita* was identified. This supplements the results from morphological AMF spores identification which had shown *Gigaspora* as one of the morphologically identified genera. However, from results of nested PCR with AMF species targeted by the specific primers ARCH1311, GLOM1310 and LETC1670 (Table 4); and making reference to results from morphological identification, the genera *Glomus*, *Gigaspora*, *Acaulospora* and *Scutellospora* were detected as being present in the treated AMF spores samples.

GenBank search using BLAST resulted in retrieval of four AMF sequences. However, these sequences showed a closer similarity to one AMF species, *Gigaspora margarita*. Phylogenetic position of *Gigaspora margarita* in relation to other AM fungi is shown in Figure 11. Our samples are indicated by JD3R, JD19R, JD3LETC, JD19LETC.

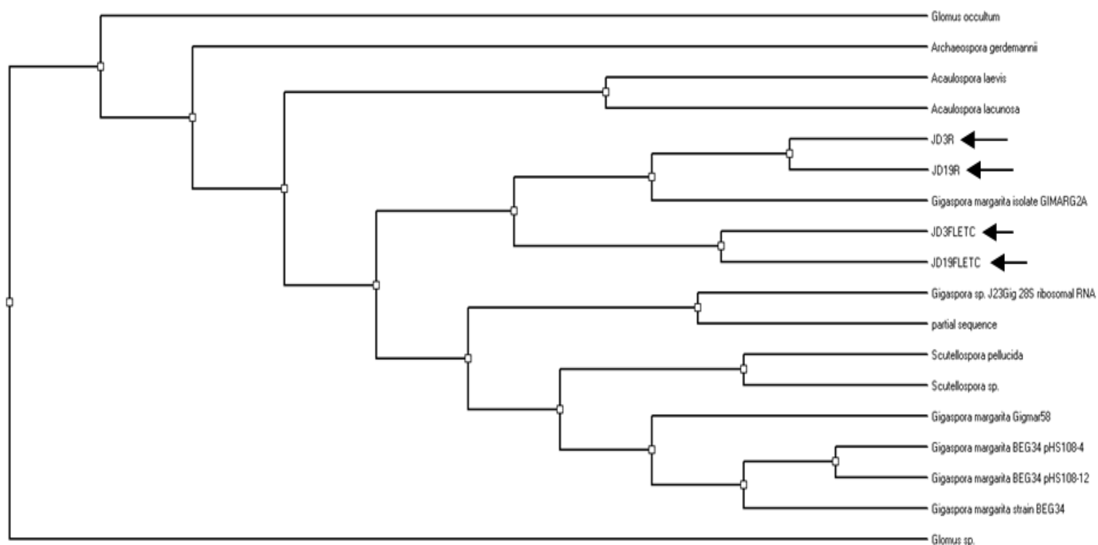

Figure 11: Phylogenetic tree depicting the relationship between *Gigaspora margarita* and other reference taxa

4.3 Maize roots colonization by AMF

A total of 96 samples including 84 pots of maize plants treated with AMF inoculum and 12 controls were organized into 32 treatments and tested for AMF colonization. All the plant roots in the treatments with AMF were colonized by AMF and contained few abuscules and/or vesicles (Figures 12 A and B). No mycorrhizae were found in all treatments without mycorrhizal inoculation (Figure 12 C).

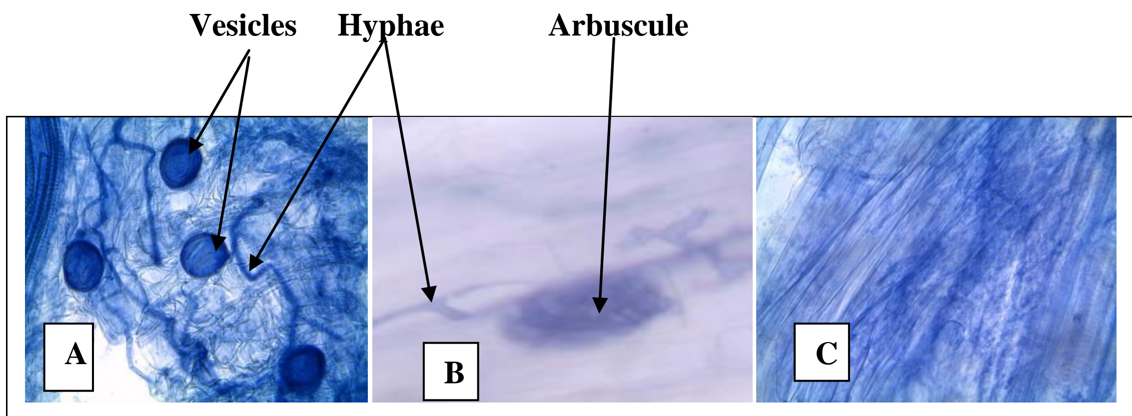

Figure 12: Photomicrographs for AMF structures in maize roots (100X): Vesicles (A), Arbuscule (B), No AMF colonization (C)

The minimum and maximum percentage values of roots colonization detected were 10% and 100%, respectively, but, in general, the mean percentages varied between 40% and 70%. In our study, there was no statistically significant difference brought by different P fertilizer application levels.

The minimum and maximum percentage values of roots colonization detected were 10% and 100%, respectively, but, in general, the mean percentages varied between 40% and 70%. In the current study, there was no significant difference obtained by application of different levels of P fertilizer.
Figure 13 shows mycorrhizal root colonization percentage by phosphorus fertilization application.

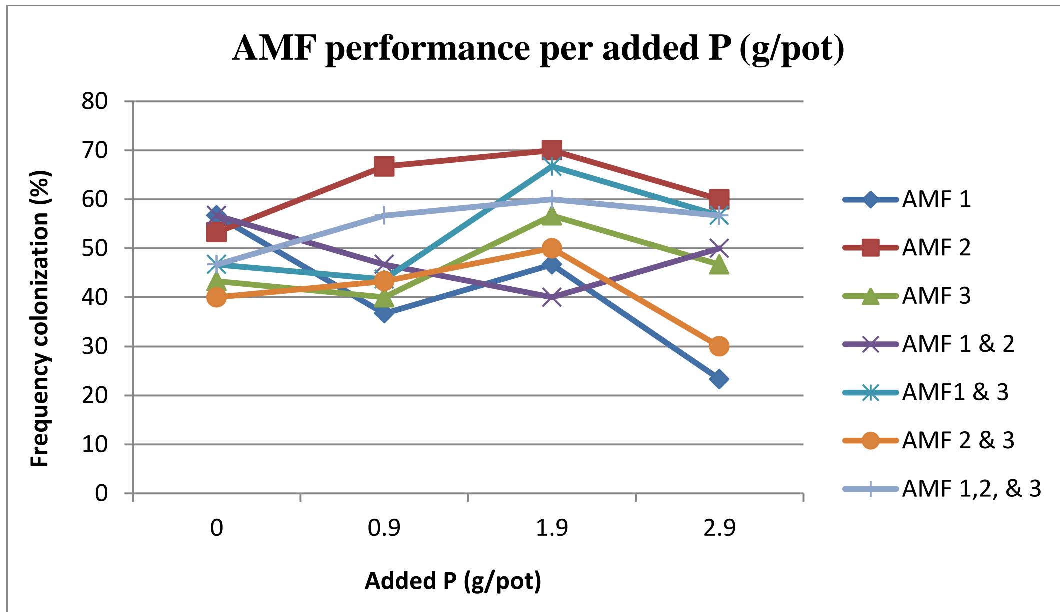

Figure 13: Mycorrhizal root colonization percentage by phosphorus fertilization levels

Comparing performance among different morphotypes AMF1, AMF2 and AMF3; AMF2 showed a statistically higher performance than AMF1 and AMF2 at p = 0.004 and 0.022, respectively. When separately combined to AMF1 and AMF3, the performance of AMF2 got statistically decreased with p = 0.03 and 0.008, respectively. However, when all the morphotypes AMF1, AMF2 and AMF3 were combined, the performance of AMF2 did not show any significant decrease.

CHAPTER 5: DISCUSSION

5.1 Abundance of AMF spores

In comparison to the findings from other studies in similar or different host plants of the tropical area, the abundance of AMF spores got in the agroforestry systems of Rubavu and Bugesera districts is generally moderate. High abundance of 775 to 1240 spores 100 g^{-1} soil were found in *A. albida* Del. in Senegal (Diop *et al*., 1994); 500 to 1500 spores 100 g^{-1} soil in *A. farnesiana* and *A. planifrons* in moderately fertile alkaline soils in India (Udaiyan *et al*., 1996); 110 to 2600 spores 100 g^{-1} soil in tropical forest and pasture (Picone, 2000) and 5 to 6400 spores 100 g^{-1} soil in a valley savanna of the dry tropics (Tao *et al*., 2004). By contrast, low spore densities of 11 to 32 spores 100 g^{-1} soil were detected in dry deciduous woodlands of Northern Ethiopia associated with different *Acacia* species (Birhane *et al*., 2010). Low AMF spore numbers were also recorded in a survey of *Acacia* tree species (49 to 67 spores 100 g^{-1} soil) in India (Lakshman *et al*., 2001) and in *Acacia* and *Prosopis* tree species (8 to 51 spores 100 g^{-1} soil) in Senegal (Ingleby *et al*., 1997).

This moderate level of AMF spores density in the agroforestry systems of Rubavu and Bugesera districts is in agreement with previous findings on natural and agricultural systems. It agrees with research findings of Picone (2000) who found a total of 110-770 AMF spores 100 g^{-1} in forest and 830-2600 spores 100 g^{-1} in pasture. Conversion from natural habitats to agricultural lands has been identified as one of the leading causes for loss of biodiversity worldwide. As shown by previous researchers, some modern agricultural practices such as continuous monoculture, non host crop in rotation and tillage can impact on the AMF association, both directly, by damaging or killing AMF and indirectly, by creating conditions unfavorable to AMF. These practices especially tillage can also cause soil erosion, reduce soil fertility and disrupt biodiversity in general including the previous crops. In cultivated lands, therefore, AMF population, species composition and diversity are often decreased compared to natural ecosystems (Helagson *et al.,* 1998).

The abundance of AMF is also influenced by many factors including soil P and pH. However, in this study, the influence of P was not recorded. In their study, Zerihun *et al* (2013) showed a significant negative correlation between AMF spore density and available P. These findings were similar to some reports from India and Northern Europe (Udaiyan *et al*., 1996; Kahiluoto *et al*., 2001). In contrast to this, Muleta *et al.* (2007) observed a positive relationship between spore number and available P in soil samples from natural coffee forest in Ethiopia. They suggested that available P level in their study sites was not high enough to inhibit mycorrhizal development. Similarly to this view of Muleta *et al.* (2007), the available P in our sampled sites may not be at a level of inhibiting AMF spores development.

Another important soil factor on AMF development is pH and this is positively correlated with AMF abundance (Don-Rodrgue *et al*., 2013). Low soil pH negatively affects AMF species richness (Toljander *et al.,* 2008). Therefore, the low pH in our sampled sites (Mean pH=4.9-5.8 in Rubavu and 5.0-6.1 in Bugesera) may also be one of the causes of the noticed moderate abundance of AMF spores in the regions.

Results obtained in this study are supported by various findings from previous studies. Various researches showed that rhizosphere in close proximity to trees has a greater spore densities relative to the soil beyond the tree canopies (Mutabaruka *et al*., 2002; Pande and Tarafdar, 2004; Prasad and Mertia, 2005). In addition, studies in agroforestry coffee (*Coffea arabica L.*) systems observed higher spore densities in the rhizosphere of coffee plants under shade trees compared to monocultural coffee systems (Muleta *et al.,* 2007; Muleta *et al.,* 2008). The explanation was given by Tadesse and Fassil (2013) that greater numbers of spores under the tree canopies in agroforestry systems may be due to greater amounts of roots at this specific site. In contrast, other studies have showed no effect or in some cases a negative effect of trees on AM fungi (Boddington and Dodd, 2000; Leal *et al.*, 2009). Spores of AMF may also occur in clumped distributions in the field (St. John and Koske, 1988), not correlated to root distribution (Friese and Koske, 1991) as cited by Douds (1999).

The difference in spore density between the two agroforestry systems could be explained partly by a certain number of reasons. Most of Bugesera parts are made of valleys and a succession of low plateaux. This relief implies soft and middle slopes and smooth flow of rainwater that do not much transport away AMF spores, and then resulting in the accumulation of AMF spores in the soil around the host plants. This is not the case at Rubavu district as the region is characterized by a higher elevation and higher slopes causing strong flows of rainwater and erosion transporting away both soil and AMF spores. This could explain partly the higher density of AMF spores at Bugesera zone than Rubavu. This is in agreement with Chandrasekara *et al*., (2005) who concluded that there is a decreasing trend in spore density and spore diversity with increasing elevation. The researchers concluded that higher density of spores at lower elevations could be explained by the accumulation of spores, which are coming down with rainwater.

Another possible explanation for the higher density of spores at Bugesera zone than Rubavu may be the fact that during the period the soil samples were collected, Bugesera zone was experiencing a dry period and most of possible host crops were out of season. The AMF may have remained as spores without germinating. This was not the case at Rubavu as it was the period of much rain, and the soil samples were collected in agricultural lands with growing maize where very many AMF spores might have germinated to colonize the maize roots. This is supported by the view of Janos (1980) who pointed out that the variation in spore population could be attributed to many factors in a given site. The researcher argues that AMF spores always need live root contacts for germination since they are obligate fungi; they may persists as spores in the absence of suitable hosts.

Environmental differences are also important factors in determining spore production by AMF. It is known that high temperature can increase AMF sporulation (Guadarrama and Álvarez-Sánches, 1999). These characteristics could also be used to explain the reason for the higher abundance of AMF spores in Bugesera characterized by a climate quite hot with the annual temperatures averaging between 21º C and 29º

C (http://www.bugesera.gov.rw/) compared to Rubavu with an average temperature of 10ºC (Nyandwi and Mukeshimana, 2011).

5.2 Characterization of AMF spores

Four AMF genera were morphologically recovered from soil samples and *Glomus* was the predominant taxonomic group. The dominance of *Glomus* was also reported in dry afromontane forests of Ethiopia (Tesfaye *et al*., 2003b), in tropical rain forest of Xishuangbanna, China (Zhao *et al., 2001*), in tropical rain forest in Mexico (Guadarrama and Alvarez-Sanchez, 1999), and in arid and semi-arid lands of North Jordan (Mohammad *et al.,* 2003). *Glomus* species were also the most frequently encountered AMF in the fecal samples collected from terrestrial and arboreal small mammals in a Panamian cloud forest with 87% frequency of occurrence in the samples (Mangan and Alder, 2000). Million, (2002) also reported that more than 80% spore extracted beneath *Acacia tortilis* was *Glomus*. Similarly Munro *et al*., (1998) found out that *Glomus* spores were the dominant beneath the host tree *Acacia tortilis*. The acidity of the soil in the regions may have favored this genus as reported by Frioni *et al*., (1999). Gigaspora, the second dominant AMF genus in the sampled agroforestry systems, was also found to be abundant at lower pH (Frioni *et al*., 1999). The least dominant spore type was the genus Acaulospora. This is supported by Tadesse and Fassil (2013) where their explanation on this was based on a probable limited capacity of Acaulospora genus to compete with other AMF genera.

5.3 AMF colonization analysis of Maize

This study confirmed that indigenous AMF species associated with agroforestry systems in the semi-arid and humid agroecologies of Rwanda are capable of infecting maize roots. The study also confirmed the results from various previous researches that maize is highly colonized by AMF. High root colonization percentage observed in maize could be due to higher compatibility between the AMF and the plant. Characteristics of root surface besides the anatomical structure of the roots may also

influence the early stages of the plant-fungus interaction (Brundrett and Kendrick, 1990).

Maize possesses a root surface covered by two kinds of mucilage: a gelatinous material produced by the root cap, and another firmer and uniformly thickened, attached to the epidermal cells. When the roots elongate in maize, the mucilaginous mantle is detached only with epidermal and hypodermic cells contrary to some other plants in which this mucilaginous mantle is detached with the cortical cells. These anatomical root characteristics may influence AMF development and be responsible for the high maize roots infection with AMF since the roots keeps the sites where symbiosis is established (cortex) (Mc Cully, 1987).

Although statistically significant difference brought by different P fertilizer application levels in this study was not observed, the percentage to which AMF colonized plant roots appeared to be normally dependent on soil content in phosphorous; it varied with phosphorus inputs but not consistently (Figure 13). Furthermore, the best conditions for AMF spores proliferation seemed to differ among morphotypes/species. These results are in agreement with previous researches. While classifying AMF species according to their sensibility to various parameters, *G. etunicatum*, *Acaulospora sp*, *G. margarita*, *A. mellea* and *S. pellucid* were grouped as species sensitive to P (Carrenho *et al.,* 2007).But, in general, the benefits of AMF including root colonization are greatest in systems where phosphorous inputs are low. Heavy usage of phosphorus fertilizer can inhibit mycorrhizal colonization and growth. As the soil's phosphorus increases, the amount of phosphorus increases in plant's tissues (Kahiluoto *et al.,* 2000; Liu *et al*., 2000), and this affects membrane phospholipids in plant cells, thus influencing their membrane permeability and the release from the roots of carbohydrates that nourish the fungi (Schwab *et al.*, 1991).

The apparent fluctuations observed may be due to AMF individual differences in their tolerance for phosphorus fertilizer input. In the majority of the treatments (5/7), addition of 1.9 g of P for fertilization seemed to be the optimum for AMF colonization of maize roots and AMF morphoype 2 was the most effective. From this, the colonization frequency decreased as more phosphorus fertilizer was added. The

explanation to this was given by Grant *et al.,* 2004. Phosphorus fertilization does not always reduce mycorrhizal association. If the available P in the soil is very low, mycorrhizal colonization and spore production may be restricted and mycorrhizal associations may be increased by P application (Grant *et al.,* 2004).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This work recognizes the density, diversity and effectiveness of indigenous AMF species associated with agroforestry systems in the semi-arid and humid agroecologies of Rwanda. The investigation was done on most common tree species found within the agroforestry systems. These are *Alnus acuminata*, *Markhamia lutea, Grevillea robusta* and *Eucalyptus* sp. in Sub-humid Rubavu district while those in semi-arid Bugesera district were *Acacia polyacantha, Senna spectabilis, Grevillea robusta* and *Eucalyptus* sp. The results of this work revealed that AMF spores abundance in the soil varied with the distance from the tree trunk but not consistently. A significant difference in spore density was recorded between the tree species and between the regions. The highest spore count was recorded from *Acacia polyacantha* (Mean of 5.4 to 9.9 spores/ g soil) in Bugesera and from *Alnus acuminata* (2.9 to 4.4 spores/g soil) in Rubavu. The tree species can therefore be confirmed to be the right trees within respective regions to be mixed with crop plants as far as AMF are concerned to optimize crop yields.

Under a morphological point of view, four different genera were identified, ie *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora.* All genera were found in all rhizosphere soil samples. AMF are commonly considered to be non-specific with respect to their host and their ability to colonize particular plant species. *Glomus* was the predominant taxonomic group in all soil samples. Genetically, only one species *Gigaspora margarita* was identified at species level.

On the other hand, the results from inoculation of maize with AMF showed their ability to infect the plant. Although AMF root colonization percentage was not statistically affected by phosphorus fertilization levels, 1.9 g of P for fertilization seemed to be more suitable for AMF colonization of maize roots. The minimum and maximum percentage values of roots colonization detected were 10% and 100%, respectively, and, in general, the mean percentages varied between 40% and 70%.

The results obtained should extend the knowledge of native AMF population in the perspective of using them as biofertilizer to reduce the negative effect of phosphorous fertilization on maize.

6.2 Recommendations

Indigenous AMF of agroforestry systems in Rwanda could be considered to be a future tool in agriculture especially as bio-fertilizer. Therefore, it is recommended that:

1. A complete inventory of AMF in all agroforestry systems of Rwanda should be very important;
2. A deep study on ecology of AMF native to Rwanda agroforestry systems and host range of AMF is of enormous importance before application.
3. Their relation with nutrient dynamics and other soil characteristics of Rwanda territory should be evaluated before use.
4. Indigenous AMF organisms of Rwanda agroforestry systems should receive the proper management.

Finally, this research recommends great interest in AMF native to Rwanda as the subject of future research with relation to food security within the country.

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