DIVERSITY AND EXTENT OF COLONIZATION OF ARBUSCULAR MYCORRHIZAL FUNGI IN ASSOCIATION WITH *MELIA VOLKENSII* LOCATED IN KENYA'S ARID AND SEMI-ARID LANDS

Anton Vanhauwere 01711953

Promotor: Prof. dr. ir. Stefaan Werbrouck;

Master's Dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Bioscience Engineering Technology: Agriculture and Horticulture -main subject Horticulture Academic year: 2023 – 2024

Academic year: 2023 - 2024

UNIVERSITEIT GENT

ACKNOWLEDGMENTS

I would like to extend my heartfelt gratitude to Joyce Jefwa for introducing us to the fascinating world of mycorrhiza and guiding us to a deeper understanding of this field. Special thanks to Better Globe Forestry, particularly Jan Vandenabeele, for his invaluable guidance during the first ten days of our stay in Kenya and for his unwavering support throughout our thesis. I am also grateful to Mercy Korir for her assistance with the sampling procedure and to the many local farmers who helped us collect the samples.

A sincere thank you to Prof. Dr. Ir. Stefaan Werbrouck for providing me with the opportunity to undertake this research and for his support. My appreciation extends to Pwani University for supplying the necessary equipment.

I would also like to express my deepest gratitude to my parents and friends for their encouragement and support throughout this journey. A special thanks to Jana, for reviewing and providing feedback on this work. I am also grateful to the lab team in Belgium, especially Amal, for their assistance and support.

Thank you all for your invaluable contributions and support.

TABLE OF CONTENTS

Acknowledgments 2
Table of contents 3
Abstract 5
Introduction 6
1 Literature review 8
1.1 *Melia volkensii* 8
1.1.1 Overview 8
1.1.2 Distribution 8
1.1.3 Botany 9
1.1.4 Usage 10
1.2 Mycorrhiza 11
1.2.1 Ecto mycorrhiza 11
1.2.2 Ericoid mycorrhiza 12
1.2.3 Orchid mycorrhiza 12
1.3 Arbuscular mycorrhizal fungi (AMF) 12
1.3.1 Overview 12
1.3.2 Classification 13
1.3.3 Structures 14
1.3.4 Symbiosis 17
1.4 Molecular analysis 18
2 general methods and materials 20
2.1 Sampling 20
2.2 Writing 20
3 Experiments 23
3.1 Morphological assessment 23
3.1.1 Aim 23
3.1.2 Specific Materials and Methods 23
3.1.3 Results and discussion 26
3.2 Optimizing PCR reaction 30

3.2.1 Aim 30
3.2.2 Specific Materials and Methods 30
3.2.3 Results and discussion 32
3.3 Identification 38
3.3.1 Aim 38
3.3.2 Specific Materials and Methods 38
3.3.3 Results and discussion 38
4 Conclusion 41
5 References 42

ABSTRACT

This thesis explores the diversity and colonization of arbuscular mycorrhizal fungi (AMF) associated with *Melia volkensii*, focusing on different locations with varying land use, tree types, vegetation, and age. A nested PCR protocol was used to study AMF, though challenges with DNA quality and sequencing highlighted the need for next-generation sequencing for more accurate results. Findings reveal that while mycorrhizal colonization was widespread, Site 9, outside the tree's natural range, showed significantly lower colonization, potentially due to environmental and adaptation factors. Statistical analysis identified significant differences in colonization intensity among sites, with agroforestry models supporting better colonization compared to plantations. This research aligns with Kenya's tree planting initiative aimed at mitigating climate change impacts and enhancing agroforestry systems. The thesis includes background information, methodology, experimental results, and recommendations for future research to improve understanding and application of mycorrhizal fungi in *Melia volkensii* agroforestry.

INTRODUCTION

Since the 1950s, extreme weather events like droughts and heavy rainfall have become more frequent, significantly affecting food and water security. Sub-Saharan Africa (SSA) is identified as one of the region's most susceptible to the impacts of climate change (IPCC, 2023). Smallholder and subsistence farmers are particularly at risk, facing locally specific and unpredictable challenges. The diversity of their crops and livestock, along with reliance on non-market relations, adds complexity to both the impacts and required adaptations compared to commercial farms. Despite their vulnerability due to small farm sizes and low technology, resilience factors such as family labor, diversification, and indigenous knowledge provide crucial adaptive capacity (IPCC, 2023; Morton, 2007). Climate change has adversely affected agriculture, slowing productivity growth and reducing crop yields, particularly in mid- and low-latitude regions. Kalele et al. (2021) identified drought and crop pests as the most common climate change impacts in arid and semi-arid lands of Kenya, threatening food security and household socioeconomic welfare, with farmers adopting on-farm adaptation strategies at low rates. Agroecological practices, including agroforestry, support food security, nutrition, and sustainability by working with natural processes and reducing climate risks (IPCC, 2023). Immediate and effective action is crucial, as failing to reduce greenhouse gas emissions and limit global warming to 1.5°C will increasingly threaten health, livelihoods, ecosystems, and biodiversity, with dire consequences for current and future generations(IPCC, 2023).

The Kenyan government wants to counter these negative impacts with an ambitious plan to plant 15 billion trees by 2032, a move aimed at: reducing greenhouse emissions, stopping and reversing deforestation and, restoring 5.1 million hectares of deforested and degraded landscapes through the African Landscape Restoration Initiative which was launched on 22nd December 2022 (Omega & Kiptoo, 2024; Vandenabeele, 2023). There is still a long way to go, but focusing on the arid and semi-arid lands, which cover up to 89% of the country's land area, could be a step in the right direction.

This thesis, in collaboration with Better Globe Forestry (BGF), aims to investigate the diversity and extent of colonization of arbuscular mycorrhizal fungi in association with *Melia volkensii*. This tree, identified by BGF as a valuable native species with significant potential, could benefit from mycorrhizal research to enhance its growth and distribution, particularly within an agroforestry model (Roe & Brooke, 2023). Mycorrhizae, through their symbiotic relationship with plants, can positively impact the survival rates of these trees. This study examines the variation in colonisation intensity of root systems across different locations, which vary in land use (agroforestry, plantation), tree type (planted, wild), vegetation, and age. Additionally, the thesis aims to map the genetic diversity of

these mycorrhizal fungi to explore the advantages of using local inoculum, potentially leading to the production of inoculum to improve soil fertility.

This research serves as a starting point to deepen our understanding of mycorrhiza and their association with *Melia volkensii*, and to encourage and guide further studies in this area. By investigating these relationships, we aim to identify key areas for future focus and exploration.

This research is organized as follows: chapter 1 provides an overview of on *Melia volkensii*, followed by the potential of mycorrhiza. Chapter 2 provides the general methods and materials used in this study. Chapter 3 presents every experiment with the specific methods and materials and gives the results and discussion. And finally chapter 4 summarizes the main conclusions from the research, highlighting key insights. It also proposes future research directions, suggesting areas for further investigation.

1 LITERATURE REVIEW

1.1 *Melia volkensii*

1.1.1 Overview

Melia volkensii, a tree species belonging to the Meliaceae family, is known for its fast growth and versatility. This tree can reach a height of up to 20 m and a diameter of 40 cm within 10–18 years, depending on the growth conditions. Its ability to tolerate drought and produce high-quality termite-resistant timber makes it a popular choice for drylands. Additionally, it provides a range of products, such as poles, posts, fodder, medicine, firewood, and bee forage (Kamondo et al., 2016; Orwa et al., 2009; Stewart & Blomley, 1994). However, the overexploitation of Melia populations, woodland conversion to farmland, and habitat fragmentation have led to a decline in the availability of these products (Kamondo et al., 2016). To address this issue, on-farm conservation strategies for Melia volkensii need to be emphasized, as much of the genetic variation is found in farm populations (Odee, 2004).

1.1.2 Distribution

Melia volkensii, commonly known as 'Mukau', is endemic to the drylands of Eastern Africa, with a natural distribution across Ethiopia, Kenya, Somalia, and Tanzania see figure 1 (Kamondo et al., 2016; Orwa et al., 2009; Stewart & Blomley, 1994). This species thrives in sandy-clay and shallow stony soils but prefers well-drained sandy soils. However, it has also been reported to grow in imperfectly drained soils, as observed in Tharaka-Nithi and Isiolo in Kenya (Orwa et al., 2009). The species occupies a biophysical range at elevations of 350 to 1700 meters above sea level, in areas with mean annual rainfall between 300 and 800 mm, and temperatures ranging from 26 to 38°C. *Melia volkensii* naturally occurs in deciduous bushlands, frequently associated with *Acacia-Commiphora* vegetation (Kamondo et al., 2016). Its adaptability to arid environments makes it a valuable species for ecological restoration and agroforestry projects, contributing to both biodiversity conservation and local livelihoods within its native range.

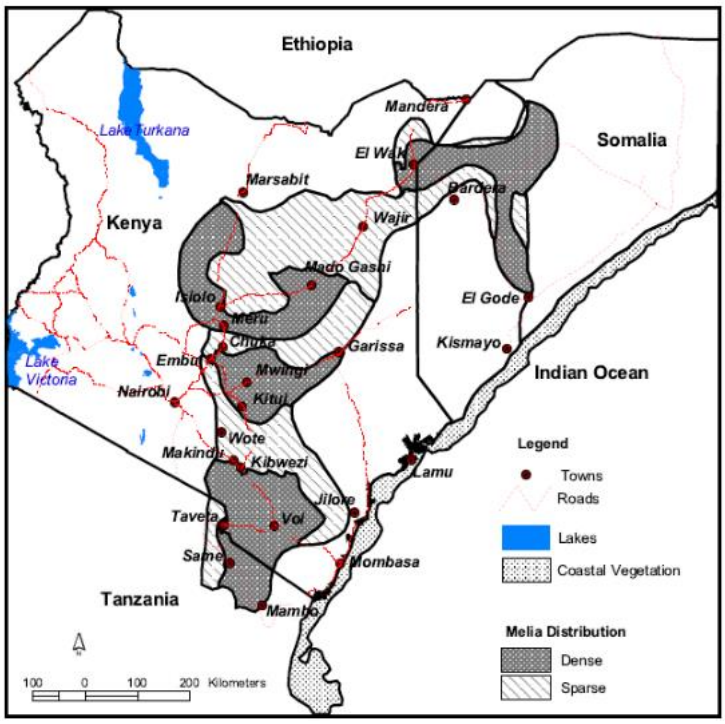


Figure 1 Natural distribution of Melia volkensii(Kamondo et al., 2016)

1.1.3 Botany

Melia volkensii is deciduous, open crowned and laxly branched. Mature trees range between 6 and 20 m tall. Trees with 25 cm diameter are common. The bark is grey, fairly smooth, furrowing with age. Leaves are a light, bright green, bipinnate with (sub)opposite leaflets. Flowers are small, white and fragrant, in loose sprays. Male and female flowers are on the same tree (andromonoecious). The fruit is drupe-like and oval; colour changes from green to pale grey as the fruit matures. Fruit size is normally 4 cm long with a very thick, bony endocarp. *M. volkensii* has been reported to start flowering as early as 2-3 years(Orwa et al., 2009). It sheds its leaves twice a year, flushing new leaves towards the end of the dry season. Flowers and fruits are also produced twice a year, with fruits becoming ripe at the end of the dry season as the leaves emerge(Orwa et al., 2009; Stewart & Blomley, 1994).

Fruit development takes 12 to 13 months from flowering to maturity. In cultivated orchards, *Melia volkensii* flowers and sets seed throughout the year, with peak flowering and seed collection periods in April to May and July to August. Seeds should be collected when the fruit is ripe, though different stages of fruit development can be found on the same branch, making it challenging to distinguish mature from immature fruits by appearance alone. Although yellow fruits are mature, they are not ready for collection until they develop brown patches (Kamondo et al., 2016; Orwa et al., 2009).

Figure 2 Melia volkensii fruits at different stages of maturity (a) Mature but not ripe, (b) Ripe and ready for collection (c) Over ripe (photo of KEFRI/JICA)

1.1.4 Usage

Melia volkensii is a highly versatile and valuable tree species native to the drylands of Eastern Africa, widely cultivated for its high-quality timber and other beneficial properties. The tree's coarse-textured heartwood, with a density of approximately 0.62, is known for its exceptional durability and resistance to termites and decay, making it a prized resource for constructing door and window frames, shutters, rafters, poles, and furniture (Orwa et al., 2009; Stewart & Blomley, 1994). Beyond its timber value, *Melia volkensii* is one of the most preferred tree species for fodder in arid regions, providing high-quality nutrition for cattle and goats (Roothaert & Franzel, 2001). Unlike other trees in bushland, Melia retains its leaves well into the dry season, offering a critical food source when fodder is scarce (Stewart & Blomley, 1994). Its capacity to thrive on cultivated land, maintaining a lush appearance, makes it particularly useful for livestock feed (Orwa et al., 2009). Additionally, the tree is utilized for producing beehives, medicinal products, and fuelwood, showcasing its multifaceted uses.

In agroforestry systems, *Melia volkensii* is valued for its ability to enhance nutrient cycling, conserve soil, and improve microclimate conditions. Its deep roots draw moisture and nutrients from the water table, making them available to surrounding crops, and they do not interfere with plowing or crop growth. Despite being considered to be a deep rooting tree, (Mulatya et al., 2002) found out that the propagation method has an important role in the root architecture, thus it is advisable to use seedlings. This compatibility with various crops, along with its short rotation period of 10 to 15 years, has made Melia an ideal cash crop for dryland agroforestry in Kenya. Although initial efforts to promote its cultivation faced challenges due to seed dormancy and propagation difficulties, breakthroughs in seedling development and tree establishment have led to its increasing

adoption as a plantation species in semi-arid regions (Kamondo et al., 2016). Farmers regard Melia as a "golden tree" due to its economic benefits and ability to provide diverse products, supporting both agricultural and environmental sustainability in the region (Kamondo et al., 2016; Profi Table Agroforestry Innovations for Eastern Africa, n.d.; Roothaert & Franzel, 2001). As such, *Melia volkensii* continues to play a crucial role in enhancing rural livelihoods and promoting sustainable land management practices.

1.2 Mycorrhiza

Symbiosis between a fungus and a plant occurs when the mycelium of the fungus makes contact with the plant's roots. This association is typically mutualistic, meaning both partners benefit. The fungus derives most or all of its organic carbon from the plant, while the plant gains essential mineral nutrients from the soil through the fungus. With its network of long, fine hyphae, the fungus can explore the soil far more effectively than the plant's coarser roots ever could. This process is invaluable for life on Earth due to its widespread occurrence, yet it often goes unnoticed because it happens almost invisibly underground. Before focusing on arbuscular mycorrhiza (AM), the primary subject of this master's thesis, I will briefly discuss the four important classes of mycorrhiza.

1.2.1 Ecto mycorrhiza

Ectomycorrhizal (ECM) roots are distinguished by a mantle of fungal tissue encasing the root, a Hartig net of hyphae that permeates the spaces between root cells, and an external mycelium that extends into the surrounding soil. Unlike arbuscular mycorrhizal (AM) fungi, ECM fungi can be successfully cultured in laboratory settings, facilitating detailed investigations of their growth and physiology (Smith & Read, 2008). ECM colonization is particularly beneficial for plant growth in nutrient-poor environments, where it enhances nutrient acquisition and lowers growth-limiting thresholds, especially for phosphorus (P) and nitrogen (N). However, the effectiveness of ECM fungi in these roles varies significantly both among and within species, a factor that is critical for predicting their potential applications in agriculture and forestry.

In ecosystems dominated by ECM-associated plants, such as boreal and temperate forests, nitrogen availability is often the primary constraint on productivity. ECM fungi play a crucial role in these environments by accessing nitrogen forms that are otherwise unavailable to plants, contributing to a more nuanced understanding of nitrogen dynamics in such ecosystems (Smith & Read, 2008). While the role of ECM fungi in P and N acquisition is well-documented, their involvement in the uptake of other essential nutrients, such as potassium (K), magnesium (Mg), and calcium (Ca), is less understood (Smith & Read, 2008). Additionally, ECM fungi contribute to plant tolerance against heavy metals and drought, further underscoring their ecological significance. However, their influence on the supply and balance of cations within plants remains underexplored, with

substantial variation observed among different fungal species. This highlights the need for further research to fully understand the diverse functions and potential applications of ECM fungi in both natural and managed ecosystems(Smith & Read, 2008).

1.2.2 Ericoid mycorrhiza

A third group of mycorrhiza is the ericoid mycorrhiza (ERM). These mycorrhiza colonise members of the family *Ericaceae.* They are able to attack a number of simple and complex substrates, providing access to, and mobilization of, N and P. The fungi facilitate transfer of the nutrients originally contained in the polymers to the plant. Secondary the ability of the fungal partner to sequester and, in some cases, to metabolize metal ions that are otherwise toxic to the plant appears to be off ecological significance. This combination of nutritional and non-nutritional attributes of ERM (ericoid mycorrhiza) associations of members of the Ericaceae contributes significantly to the ability of these plants to grow in contaminated mine spoils as well as in their natural heathland habitats (Smith & Read, 2008).

1.2.3 Orchid mycorrhiza

All orchids have a relatively prolonged heterotrophic stage during germination and early growth. The seeds of almost all orchid species, often referred to as 'dust seeds', are extremely small (0.3-14~g) that, in the absence of an endosperm, have few reserves. The lack of reserves to support early seedling development makes all orchids in nature dependent upon the provision of nutrients by mycorrhizal fungi.

During the heterotrophic phase, green orchids (as well as the full mycoheterotrophs) are dependent on carbohydrate translocated to them via the mycelium of a mycorrhizal fungus. In green orchids, the source of the carbohydrate is most frequently dead organic matter supplied by the saprotrophs. The fungus-driven mode of nutrition is retained until the appearance above ground of green leaves, at which stage they are thought to be fully autotrophic (Smith & Read, 2008).

1.3 Arbuscular mycorrhizal fungi (AMF)

1.3.1 Overview

Arbuscular mycorrhizas (AM) represent the most widespread form of mycorrhizal symbiosis, formed by obligately symbiotic fungi across a vast array of host plants, including angiosperms, gymnosperms, and the sporophytes of pteridophytes—all of which possess roots—as well as the gametophytes of some hepatics and pteridophytes, which lack roots. The term "arbuscular" refers to the characteristic arbuscules found within the cortical cells of many plant roots (Smith & Read, 2008). It is highly probable

that fungi originated over 1,000 million years ago, predating current estimates of land colonization, with AM symbioses being similarly ancient. Given their essential role in nutrient acquisition, AM fungi likely played a pivotal role in enabling the colonization of terrestrial environments by plants(Heckman et al., 2001; Redecker et al., 2000).

1.3.2 Classification

Before the introduction of molecular biological techniques, species within the *Glomeromycota* were classified solely based on morphological characteristics. However, the introduction of nuclear ribosomal gene sequencing for AMF spores and environmental samples brought about a significant shift. The rise of molecular methods has greatly expanded the possibilities for classification, making these techniques the preferred approach for studying AMF diversity.

(Redecker et al., 2013) proposes a taxonomic revision based on the consensus of leading experts, offering a robust and stable framework for the systematics of *Glomeromycota*. This framework provides a solid foundation for future research into the phylogenetic relationships and evolutionary history of these vital symbiotic fungi. By addressing the confusion caused by recent publications, this consensus aims to establish a clear and enduring basis for further scientific progress.

Early molecular surveys of *Glomeromycota* revealed phylogroups, some of which correlated with morphospecies, while others did not, leading to other approaches (Helgason et al., 1998; Van Der Heijden et al., 2008). The lack of a clear species concept Therefore, molecular studies of AMF communities primarily identify sequence groups, not species. These groups may eventually be found to correspond to morphospecies or groups of them (Redecker et al., 2003).

A new concept was created to provide a simple way of detecting, identifying, and describing AMF in environmental samples using sequence data. A user-centric approach to *Glomeromycota* taxonomy is emphasized, wherein environmental AMF sequences must be identified to analyze AMF diversity and understand their distribution in ecosystems. This lead to the concept of *Glomeromycota* "virtual taxonomy". A virtual taxon is defined as a phylogenetically distinct group of closely related SSU rRNA gene sequences with a sequence identity of 97% or higher, though this approach can be applied to any marker (Öpik et al., 2014).

It has been implemented in the MaarjAM database (http://maarjam.botany.ut.ee/ Öpik et al., 2010). This virtual taxonomy, primarily based on small-subunit (SSU) rRNA gene sequences, includes increasing numbers of large-subunit (LSU) rRNA gene and internal transcribed spacer (ITS) sequences, along with mitochondrial and protein-encoding gene sequences used for Glomeromycota identification.

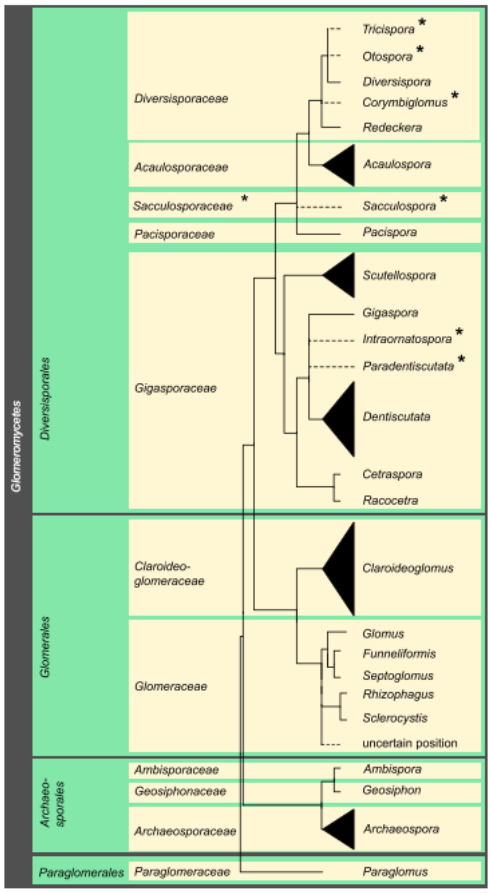


Figure 3: Consensus classification of the Glomeromycota (Redecker et al., 2013)

1.3.3 Structures

Root colonization by arbuscular mycorrhizal (AM) fungi can be initiated from three main sources of inoculum present in the soil: spores, infected root fragments, and hyphae, collectively known as propagules. An AM root system consists of three critical components: the root itself and two associated mycelial networks—one in the soil and the other within the root apoplast. The presence of intraradical hyphae, arbuscules, and extraradical mycelium, along with spores produced in the extraradical mycelium, are characteristic features of AM associations (Smith & Read, 2008).

The extraradical mycelium of arbuscular mycorrhizal (AM) fungi, composed of hyphae and spores, plays a crucial role in the formation, function, and persistence of mycorrhizal associations. The primary function of the hyphae is the uptake and translocation of mineral nutrients from the soil to the plant(Smith & Read, 2008). Spores serve as reserve and propagation organs, ensuring the continuity of the fungal lifecycle. The intraradical mycelium, found within the root, consists of hyphae, arbuscules, and sometimes vesicles. In the Arum-type AM, a single hyphal branch penetrates the wall of a cortical cell, forming a trunk hypha that repeatedly branches to create an arbuscule. In contrast, the Paris-type

AM develops complex coils and fine branches that constitute the arbuscules. These two AM types represent the extremes of a developmental continuum that also includes mycorrhizas characterized by straight hyphae spreading longitudinally within, rather than between, cortical cells, as well as instances where both intercellular and intracellular hyphal development occurs (Dickson, 2004). The morphology of AM fungi is influenced by both the specific plant species and the fungal species involved in the colonization. Vesicles are thick-walled, lipid-rich structures that can vary in shape—from ovoid and irregularly lobed to box-like—depending on the fungal species and their formation site within the root. They likely serve as crucial storage organs and play a significant role as propagules within root fragments. However, much remains unknown about their biology, particularly regarding their germination and the mobilization of stored reserves. Environmental conditions significantly affect vesicle development in arbuscular mycorrhizal (AM) fungi(Smith & Read, 2008).

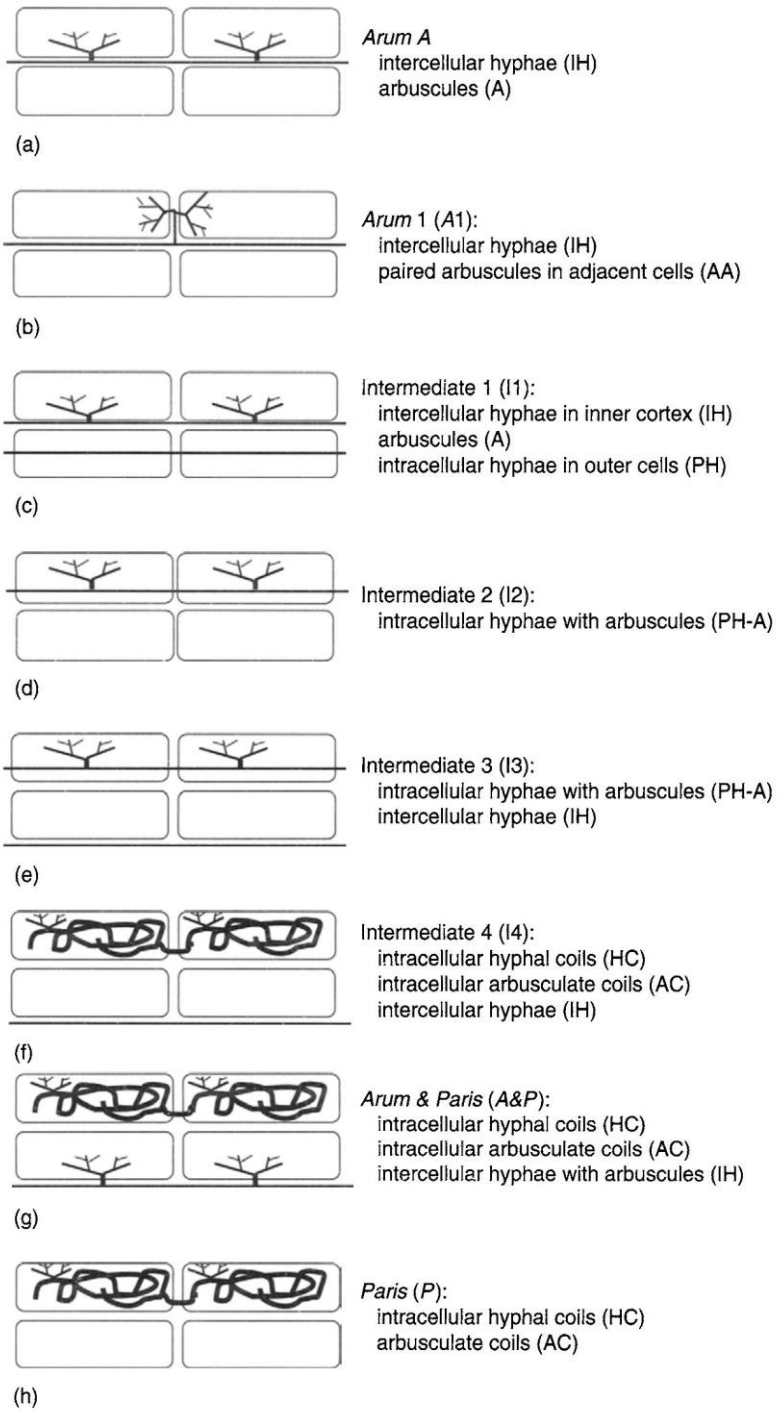


Figure 4 Diagrams of colonization types observed in roots of different plant species, colonized by different AM fungi

1.3.4 Symbiosis

Arbuscular mycorrhizal (AM) fungi form essential symbiotic associations with the roots of most terrestrial plants, playing a critical role in nutrient acquisition and overall plant health. These fungi rely on organic carbon from their plant partners, receiving between 4% to 20% of the net photosynthate produced by the plant. This carbon is vital for the growth, maintenance, and nutrient uptake of the fungi, allowing them to support their host plants in various ways, particularly by improving the absorption of immobile nutrients such as phosphorus (P), zinc (Zn), and copper (Cu) from the soil. This mutualistic relationship not only enhances the plant's nutrient uptake but also contributes to better plant water relations, resulting in higher stomatal conductance and improved drought resistance compared to non-mycorrhizal plants (Smith & Read, 2008).

The importance of AM fungi extends beyond nutrient acquisition and water management. Their role in reducing nutrient loss from soils is increasingly recognized as vital for sustainable agriculture. Nutrient losses through leaching and gaseous emissions can be both environmentally damaging and economically costly. AM fungi help mitigate these losses by expanding the nutrient interception zone around plant roots, thus reducing the impact of rain-induced leaching events(Cavagnaro et al., 2015). This function is particularly significant in the context of ecological intensification, where reliance on ecosystem services rather than synthetic inputs is seen as key to achieving food security in an environmentally sustainable manner.

Enhancing the symbiotic potential of AM fungi in agricultural systems is a promising strategy for improving resource use efficiency. Research indicates that practices such as reduced tillage and the use of cover crops can significantly enhance AM fungal colonization and diversity, even in conventional, intensively managed systems (Bowles et al., 2017). Cover cropping, in particular, has been shown to sustain AM formation regardless of tillage practices, highlighting the importance of maintaining continuous root associations for AM fungal development. This finding is particularly relevant for organic or low-input farming systems, where tillage may be necessary for weed control and organic matter incorporation (Bowles et al., 2017).

Beyond their direct benefits to crops, AM fungi also play a crucial role in maintaining ecosystem health and biodiversity. The diversity of AM fungi below ground is a major factor contributing to the maintenance of plant biodiversity and overall ecosystem functioning (van der Heijden et al., 1998). AM fungi influence key ecosystem processes such as soil aggregation, which is vital for soil structure and ecosystem resilience (Rillig & Mummey, 2006). Protecting and promoting AM fungal diversity is therefore essential for sustainable land management practices that aim to preserve ecosystem functions.

In the context of environmental stress and contaminated soils, the role of AM fungi becomes even more critical. Indigenous and stress-adapted AM fungal isolates have potential as biotechnological tools for the restoration of degraded ecosystems. Screening and utilizing pollutant-tolerant AM fungi can enhance the effectiveness of phytoremediation efforts, although our understanding of the molecular mechanisms involved in AM fungal responses to abiotic stress remains limited (Lenoir et al., 2016).

Overall, the symbiosis between AM fungi and plants is a cornerstone of sustainable crop production and ecosystem management. By leveraging this relationship through conservation practices, site-specific crop selection, and reduced reliance on chemical fertilizers, we can address the current challenges of sustainable agriculture. The integration of AM fungi into plant nutrition management strategies offers a promising pathway to enhancing crop resilience, improving nutrient uptake, and mitigating the impacts of abiotic stress (Singh & Singh, 2019).

1.4 Molecular analysis

Traditionally, microscopic analysis has been used to observe fungal structures and measure their abundance and identity. However, this approach is limited by the extensive genetic variability of fungi contrasted with their limited morphological diversity (Gorzelak et al., 2012). Almost all identification systems for arbuscular mycorrhizal fungi (AMF) rely on ribosomal DNA (rDNA), as this genome region contains highly conserved as well as variable sectors, enabling taxonomic distinction at various levels (Redecker et al., 2003).

Designing a single primer for all glomalean fungi, while excluding plants and other fungi, has proven challenging, resulting in the development of multiple primers, each with its own advantages and disadvantages (see table 1). The majority of primers used in AMF research target rDNA, specifically regions such as the small subunit (SSU—18S), large subunit (LSU—25S), internal transcribed spacer (ITS), or combinations thereof (Gorzelak et al., 2012). Various DNA-based molecular identification techniques have been designed for several target regions, incorporating numerous approaches (Gollotte et al., 2004; Krüger et al., 2009; Lee et al., 2008; Redecker & Redecker, 2000; Schüßler et al., 2001; Simon,' et al., 1992; Victorino et al., 2020).

For researchers focusing on AMF using Illumina MiSeq, the 18S-based approach is recommended, as it provides broad coverage of the subphylum Glomeromycotina with minimal amplification of non-AMF sequences (Berruti et al., 2017).

Table 1 General AMF primers for use in amplifying community DNA (Gollotte et al., 2004; Gorzelak et al., 2012; Helgason et al., 1998; Krüger et al., 2009; Lee et al., 2008; Simon,' et al., 1992; Trouvelot et al., 1999; Van Tuinen et al., 1998)

Reference	Direction	Primer pairs	Sequence	Fragment size
van Tuinen et al. (1998)	Forward	LR1	GCA TAT CAA TAA GCG GAG GA	750 bp
	Reverse	NDL22	TGG TCC GTG TTT CAA GAC G	
Trouvelot et al. (1999)	Forward	LR1		~300 bp
	Reverse	FLR2	GTC GTT TAA AGC CAT TAC GTC	
Simon (1992)	Forward	VANS1	GTC TAG TAT AAT CGT TAT ACA GG	550 bp
	Reverse (universal)	NS21	AAT ATA CGC TAT TGG AGC TGG	
Gollotte (2004)	Forward	FLR3	TTG AAA GGG AAA CGA TGG AAG T	400 bp
	Reverse	FLR4	TAC GTC AAC ATC CTT AAC GAA	
Lee (2008)	Forward	AML1	ATC AAC TTT CGA TGG TAG GAT AGA	800 bp
	Reverse	AML2	GAA CCC AAA CAC TTT GGT TTC	
Simon (1992)	Forward	NS31	TTG GAG GGC AAG TCT GGT GCC	550 bp
Helgason (1998)	Reverse	AM1	GTT TCC CGT AAG GCG CCG AA	
Sato et al. (2005)	Forward	AMV4.5NF	AAG CTC GTA GTT GAA TTT CG	300 bp
	Reverse	AMDGR	CCC AAC TAT CCC TAT TAA TCA T	
Kruger (2009)	Forward	SSUmCf	see reference for details	1500 bp
	Reverse	LSUmBr		
Kruger (2009)	Forward	SSUmAf	see reference for details	1800 bp
	Reverse	LSUmAr		

2 GENERAL METHODS AND MATERIALS

2.1 Sampling

Sampling was conducted across 9 sites, as outlined in Table 2, with locations proposed by Better Globe Forestry (BGF) due to their familiarity with the diverse growing environments of *Melia volkensii*. Each site was characterized by tree age, type (wild, spontaneously growing, or planted), and land use (plantation or intercropped). For the first 9 sites, the vegetation type was consistent—northern *Commiphora-Acacia* buschland—but the last site, located in the Arabuko Sokoke forest, falls outside the natural distribution of *Melia volkensii*. Details of these characteristics are provided in Table 3.

At each site, three samples were collected, with the initial sampling point chosen arbitrarily and the subsequent points located 10 meters apart to form a triangle when feasible. Fine roots were collected from each tree for mycorrhizal colonization assessment, with samples taken from three trees per site, resulting in a total of 25 root samples. The collection involved tracing the fine roots back to the trunk, starting from the base of the tree and moving outward. The roots were carefully excavated, preserved in 70% ethanol, and sealed in plastic.

To obtain the small roots necessary for analysis, we used a Djembe and machete, beginning near the trunk and excavating large roots until we reached the desired size. This process was labor-intensive, and in some cases, it was not possible to collect three samples. For wild trees, where only one tree was available, samples were collected from that single tree.

2.2 Writing

I used ChatGPT, an AI tool from OpenAI, to help with editing and refining some parts of this thesis.

Table 2 Root collection summary

Location id	Location description	Coordinates	Date collect
1	Farm Simon Muli	-0.589744, 37.935092	11/07
2	Kiambere plantation BGF	-0.682656, 37.919872	11/07
3	Farm Fred K	-0.765306, 38.478358	12/07
4	Farm Mwema Mwilu	-0.800806, 38.517781	12/07
5	Farm Sarah	-0.771742, 38.351028	12/07
6	Farm Julius	-0.769336, 38.408364	12/07
7	Farm Joyce	-0.751178, 38.482389	12/07
8	Kibwezi Mukuyu Farm	-2.375972, 38.078833	14/07
9	KEFRI Gede	-3.300542, 39.990425	15/07

Farm Mwema Mwilu

Farm Joyce

Figure 5 photos of sampling locations

Kiambere plantation BGF

KEFRI Gede

Figure 6 photos of sampling locations

Table 3 Location characteristics

Location id	Type of tree	Age	Vegetation	land use
1	Planted	5-6y	Northern *Acacia–Commiphora* bushland	Agroforestry
2	Planted	8 month	Northern *Acacia–Commiphora* bushland	Plantation
3	Wild tree	25y; 6-7y	Northern *Acacia–Commiphora* bushland	Agroforestry
4	Planted	2-3y	Northern *Acacia–Commiphora* bushland	Agroforestry
5	Planted	6y	Northern *Acacia–Commiphora* bushland	Agroforestry
6	Wild tree	10y	Northern *Acacia–Commiphora* bushland	Agroforestry
7	Wild tree	60-70y	Northern *Acacia–Commiphora* bushland	Agroforestry
8	Planted	13y	Northern *Acacia–Commiphora* bushland	Agroforestry
9	Planted	8y	Myombo forest	Plantation

3 EXPERIMENTS

3.1 Morphological assessment

3.1.1 Aim

The aim of this study is to identify potential differences in mycorrhizal colonization across various locations, vegetation types, tree types, and land uses. Understanding these differences will help us determine the key factors that promote or hinder the presence of mycorrhizae in the soil, guiding future efforts to maintain or manage these beneficial fungi. This broad comparison also lays the groundwork for more extensive research in the future. Specifically, we aim to determine whether factors such as tree age or different land uses influence the AM fungal community associated with *Melia volkensii*.

3.1.2 Specific Materials and Methods

3.1.2.1 Root staining and assessment

Roots were chopped into 1–2 cm long segments before clearing. Fresh roots were gently cleansed using tap water and were fixed in 70% ethanol in tightly sealed plastic bags and stored at room temperature until they were transported to the laboratory. The preserved roots were stained using a modified procedure (Grace & Stribley, 1991a; Koske & Gemma, 1989). Roots were treated with 2.5% KOH for 15–20 min. at 121 °C in an autoclave. Clear pieces of roots were rinsed with tap water to remove KOH. The roots were further bleached with H2O2/ NH4 for 20 min at room temperature. Then the roots were acidified with 1% HCl (v/v) for 1 night at room temperature. Finally were stained for 3min at 121°C in an autoclave with 0.5% Aniline blue in acidic glycerol (Grace & Stribley, 1991b). After the staining stage, the roots were de-stained in acidic glycerol. They were stored in acidic glycerol for microscopic analysis. Three slides with 20 randomly selected stained roots (0.5 – 1cm long root) were prepared from every site. PVLG (polyvinyl lactate glycerol) was used as mounting agent. Due to the thickness of some roots, some had to be cut in half to mount them properly.

A total of 60 root samples per site were examined under x20 and x40 magnification using an Olympus light microscope for both qualitative and quantitative analysis. The presence of mycorrhizal structures, including extraradical and intraradical hyphae, vesicles, arbuscules, and coils, was assessed within the roots. The degree of colonization was scored using the Trouvelot method, allowing us to calculate the intensity of mycorrhization and the abundance of arbuscules (see Figure 7)(Ho-Plágaro et al., 2020). Trouvelot proved to be a more accurate method compared to McGonigle although both

methods are prone to misestimations (Kokkoris et al., 2019). Three parameters were calculated (Ho-Plágaro et al., 2020)

a) Frequency of mycorrhiza in the root system

Frequency of colonization (F%) = (Number of mycorrhized fragments/Number of total fragments) * 100

b) Intensity of the mycorrhizal colonization in the entire root system

Relative mycorrhizal root length, relative mycorrhizal intensity (M%) = (95*n5+70*n4+30*n3+5*n2+ 0.5*n1)/ (number of total fragments)

where n5 = number of fragments rated 5 according to mycorrhizal colonization; n4 = number of fragments rated 4; etc.

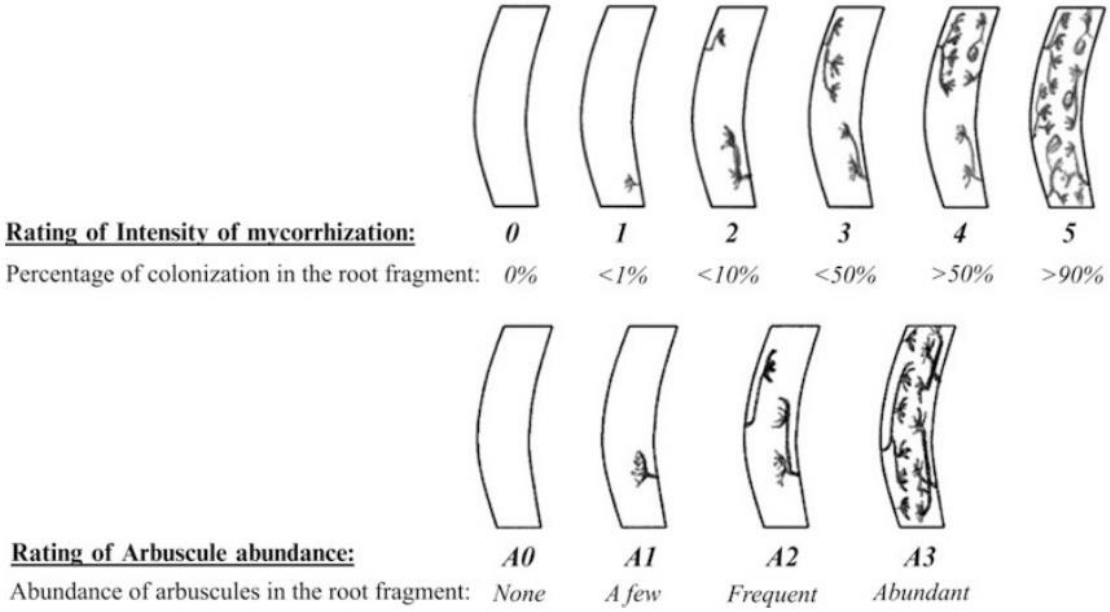
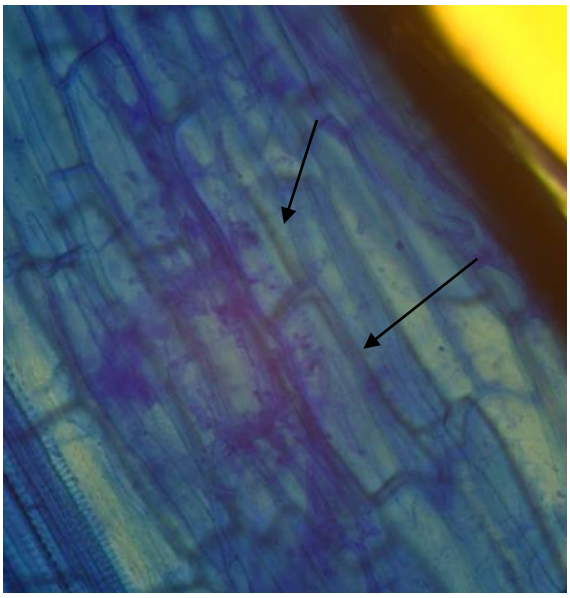


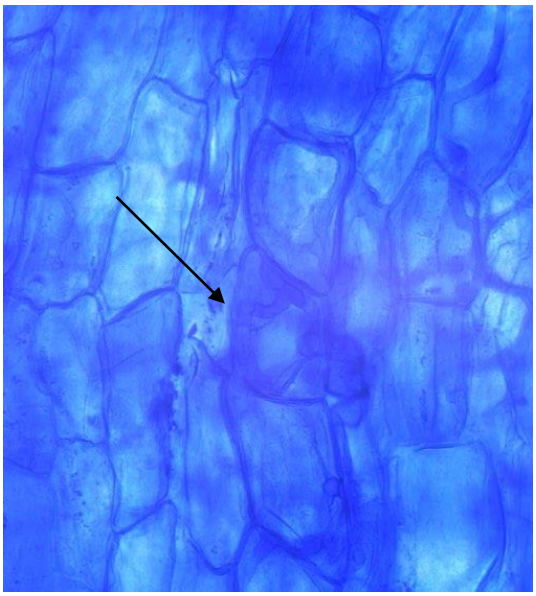
Figure 7 Scoring scale to evaluate the level of mycorrhizal colonization and the arbuscule abundance in root fragments (Ho-Plágaro et al., 2020)

3.1.2.2 Data analysis

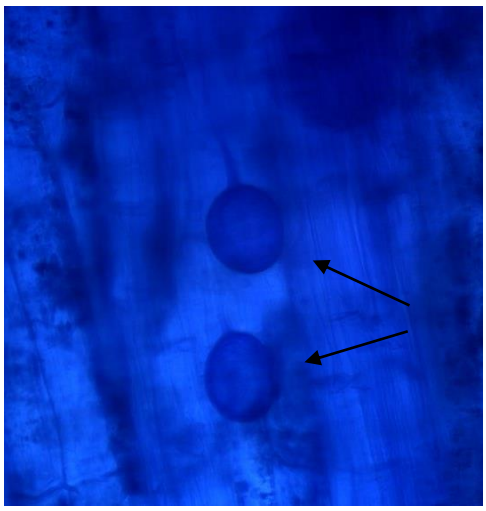
Data analysis was performed in R. Due to the non-normal distribution of the data, the Kruskal-Wallis test was used to assess differences across multiple groups, followed by Dunn's test for post-hoc comparisons to identify specific group differences. Significance was determined at a 0.05 threshold.



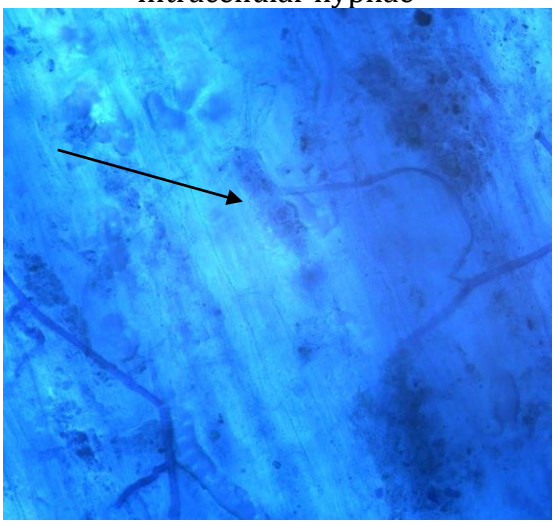
Intracellular hyphae forming arbuscules



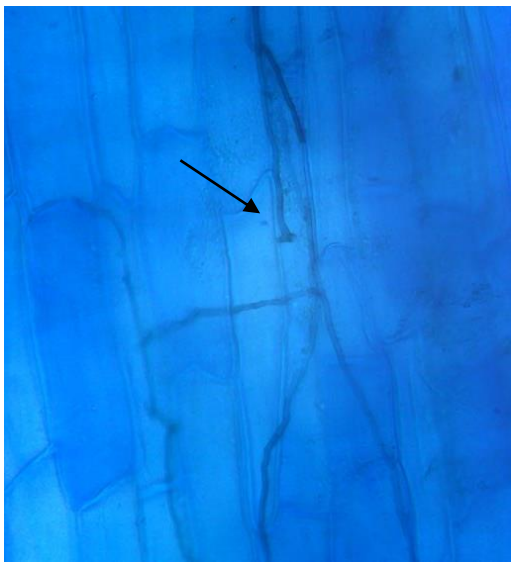
Intracellular hyphae



Vesicles



Intracellular hyphae with arbuscule



Hyphae with entry point

Figure 8 Presence of stained mycorrhizal feature

3.1.3 Results and discussion

An initial observation is the frequency of mycorrhiza within the root systems across the different sites (see figure). Notably, mycorrhiza was present at every location, colonizing the trees and the majority of root samples. However, the frequency at Site 9 was significantly lower. This could be attributed to the fact that Site 9 is outside the natural distribution range of *Melia volkensii*, meaning the mycorrhiza there might be less adapted to colonizing this species (Kamondo et al., 2016). Additionally, this site is coastal, and the sampling period was quite wet, possibly leading the trees to invest less energy in symbiosis with mycorrhiza. This factor will be further discussed in the analysis of colonization intensity.

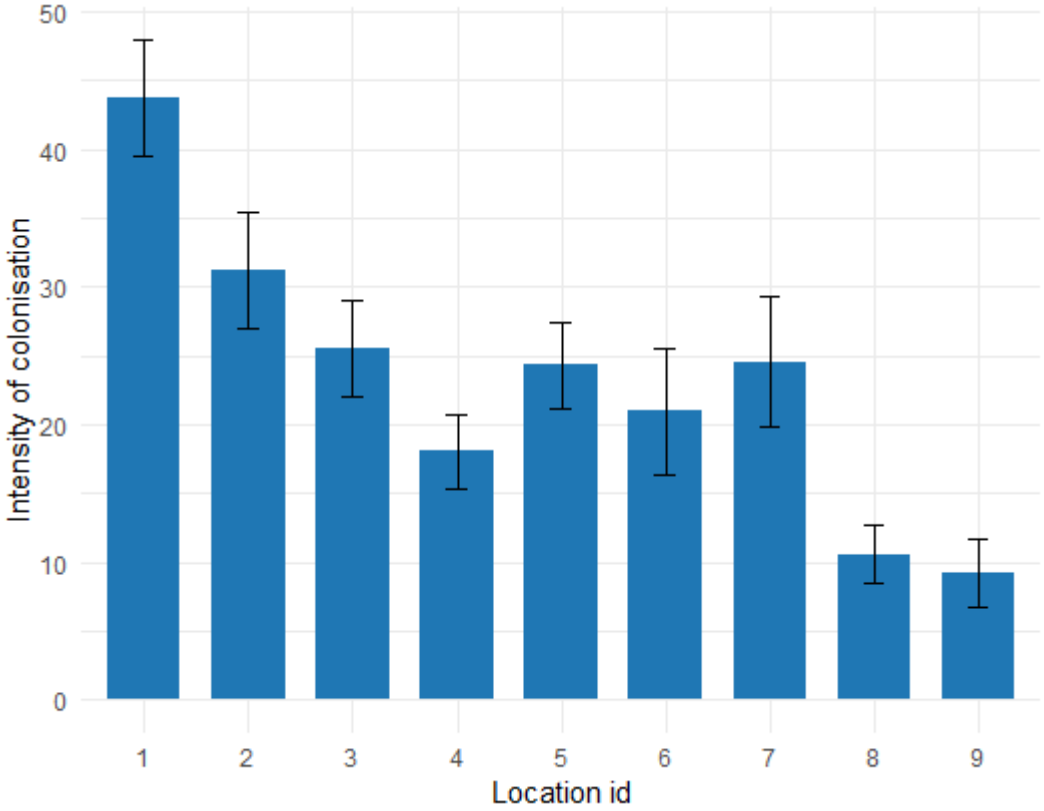
Next, we examine the intensity of mycorrhizal colonization across the entire root system, as depicted in the first bar plot (see figure). At first glance, there appear to be noticeable differences between locations. Statistical analysis confirms that these differences are significant. The Kruskal-Wallis test was rejected at the 5% significance level ($P = 2.353e-13$). While this test indicates that there are differences, it does not specify which distributions differ from each other. For this, a post-hoc analysis was performed using Dunn's test.

The Dunn's test revealed that Site 1 differs significantly from Site 4 ($P = 0.0014$), Site 6 ($P = 0.0467$), Site 8 ($P < 0.0001$), and Site 9 ($P < 0.0001$). Site 2 differs significantly from Site 8 ($P = 0.0016$) and Site 9 ($P < 0.0001$). Site 3 shows significant differences from Site 8 ($P = 0.0074$) and Site 9 ($P < 0.0001$). Site 4 differs significantly from Site 9 ($P = 0.0476$), while Site 5 shows significant differences from Site 8 ($P = 0.0205$) and Site 9 ($P < 0.0001$). These results suggest that locations 8 and 9 differ significantly from the other sites, which are clustered together in one area. This may provide a possible explanation. As noted earlier, location 9 is outside the natural range of the species. Age does not appear to play a significant role in the intensity of colonization.

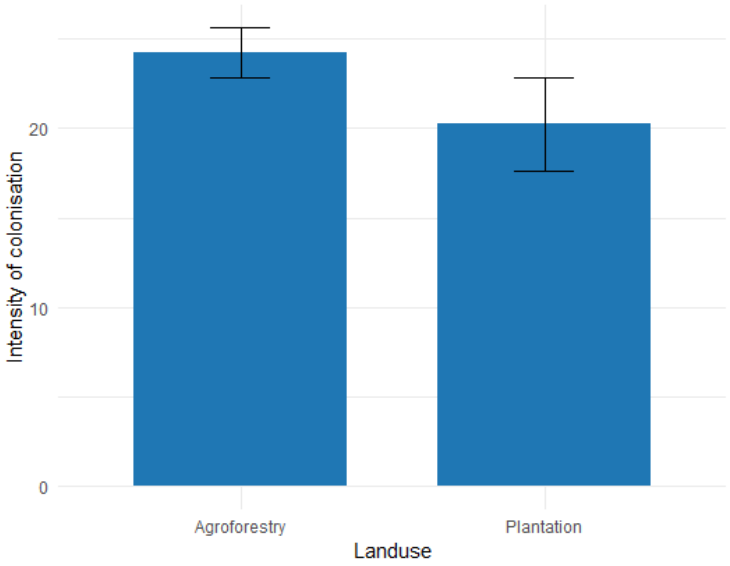
When we shift focus from location to differences between tree types, no significant differences were found (P = 0.1563). However, significant differences were observed in land use (P = 0.00679) and vegetation (P = 9.327e-09). The significant difference in vegetation supports the hypothesis that Melia trees are less colonized outside their

Location id	Mycorrhiza	No mycorriza	Frequency of mycorrhyza
1	59	1	98.33
2	56	4	93.33
3	58	1	98.31
4	58	2	96.67
5	56	4	93.33
6	28	2	93.33
7	28	2	93.33
8	51	8	86.44
9	45	15	75.00

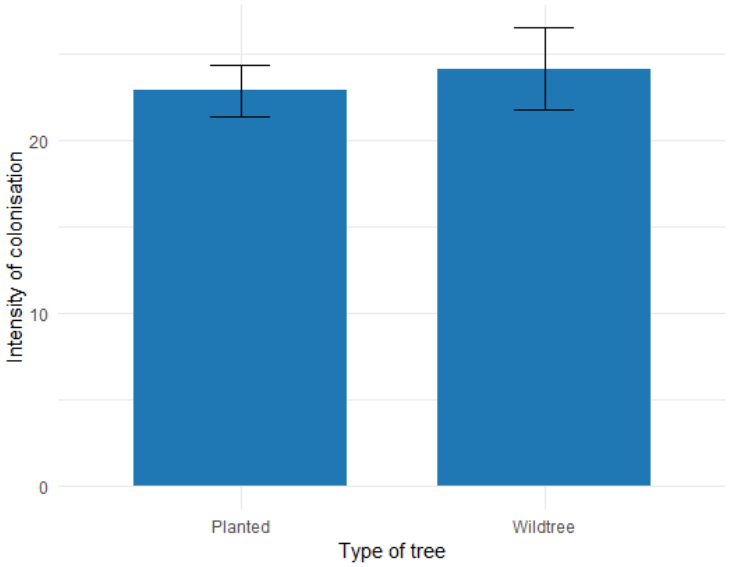
natural range. Additionally, the notable difference in land use suggests that an agroforestry model is more conducive to colonization than a plantation. This finding is unexpected, as plantations typically experience less soil disturbance, which might be anticipated to result in higher colonization intensity. However, the plantation group includes a site outside the natural range, which could skew the results.



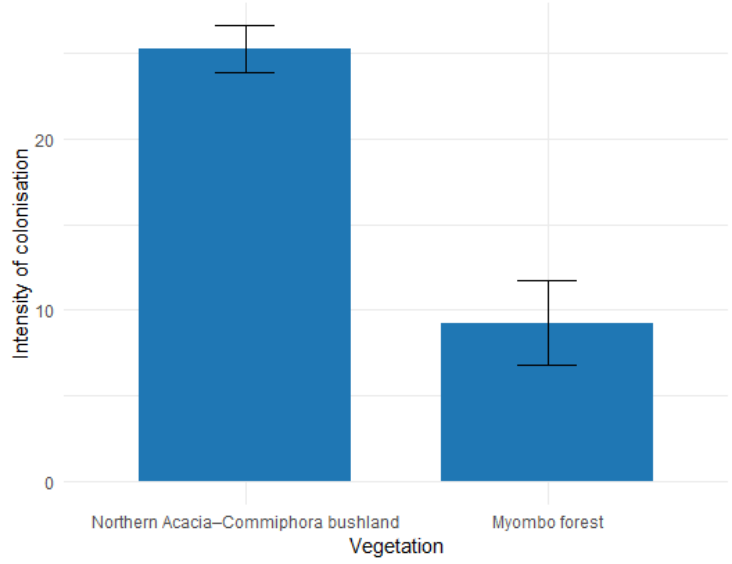
Site	Intensity	n
1	43.73333	60
2	31.21667	60
3	25.57627	59
4	18.06667	60
5	24.30833	60
6	20.96667	30
7	24.56667	30
8	10.58475	59
9	9.25000	60



Land use	Intensity	n
Agroforestry	24.21	358
Plantation	24.16	120



Type	Intensity	n
Planted	22.89	359
Wild tree	24.16	119



Vegetation	Intensity	n
Northern *Acacia-Commiphora* bushland	25.21	418
Myombo forest	9.25	60

3.2 Optimizing PCR reaction

3.2.1 Aim

This thesis aims to explore the genetic diversity of mycorrhizal fungi associated with *Melia volkensii*, with the potential to harness local inoculum for improving soil fertility. Despite encountering challenges with PCR amplification during the experiment, this section outlines the steps taken to optimize the PCR process, paving the way for smoother procedures in future studies.

3.2.2 Specific Materials and Methods

Seven samples were analysed, as shown in the table below. For budgetary reasons, samples 5 and 8, as well as samples 6 and 7, were combined due to their similar characteristics.

Location id	PCR sample id
1	a
2	b
3&6	c
4&5	d
7	e
8	f
9	g

3.2.2.1 DNA-extraction

Usage of Qiagen DNeasy® Plant Pro kit

1. Add 5–100 mg of fresh or frozen plant tissue and 500 µl of Solution CD1 to a 2 ml tissue disruption tube. Vortex briefly to mix.
2. Homogenize using one of these methods: 2a. Vortex: Secure tissue disruption tubes to a Vortex Adapter (cat. no. 13000-V1-24) and vortex at maximum speed for 10 min.
3. Centrifuge the tissue disruption tubes at 12,000 x g for 2 min.
4. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided). Note: Expect 350–450 µl. The supernatant may still contain some plant particles.
5. Add 200 µl Solution CD2 and vortex for 5 s. Note: For problematic samples, add 250 µl Solution CD2.
6. Centrifuge at 12,000 x g for 1 min at room temperature. Avoiding the pellet, transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided). Note: Expect 400–500 µl.
7. Add 500 µl of Buffer APP and vortex for 5 s.
8. Load 600 µl lysate onto an MB Spin Column. Centrifuge at 12,000 x g for 1 min.
9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB spin column.
10. Place the MB spin column into a clean 2 ml collection tube (provided).
11. Add 650 µl Buffer AW1 to the MB spin column. Centrifuge at 12,000 x g for 1 min. Discard the flow-through and place the MB spin column back into the same 2 ml collection tube.
12. Add 650 µl of Buffer AW2 to the MB spin column. Centrifuge at 12,000 x g for 1 min. Discard the flow-through and place the MB spin column into the same 2 ml collection tube.
13. Centrifuge at up to 16,000 x g for 2 min. Place the MB spin column into a new 1.5 ml elution tube (provided).
14. Add 50–100 µl of Buffer EB to the center of the white filter membrane.
15. Centrifuge at 12,000 x g for 1 min. Discard the MB spin column. The DNA is measured by means of Nanodrop and is now ready for downstream applications. (Qiagen DNeasy Plant pro Kit, n.d.)

3.2.2.2 PCR-protocol

A nested PCR protocol was employed, involving an initial PCR with less specific primers followed by a second PCR using more specific primers. This approach aims to enhance the binding efficiency of the second primer pair with the correct DNA sequences. The first primer pair is: Forward AML1: 5’ ATCAACTTTCGATGGTAGGATAGA 3’; Reverse AML2: 5’GAACCCAAACACTTTGGTTTCC 3’; Amplicon size ~800bp

, and the second primer pair is: Forward AMADF: 5‘ GGGAGGTAGTGACAATAAATAAC 3’; Reverse AMDGR: 5’ CCCAACTATCCCTATTAATCAT 3’; Amplicon size ~420(Victorino et al., 2020). The binding site is localized on the SSU ribosomal DNA (see figure). Following each PCR, gel electrophoresis was performed to verify the results.

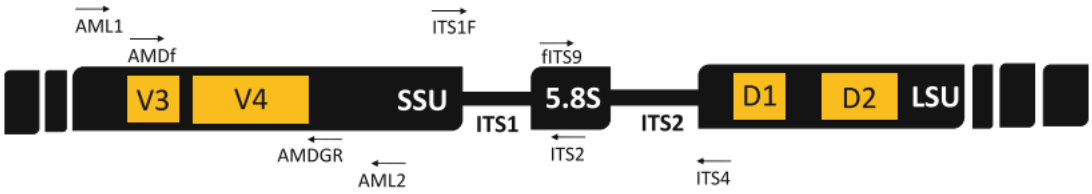


Fig. 1 Ribosomal RNA primer map. Major regions targeted by most used primers for fungal identification: ITS1F, ITS2, and ITS4 [16, 17], fITS9 [18], AMADF and AMDGR [19], and AML1 and AML2 [20]

Ingredients

- 5x Green GoTaq Reaction buffer
    - Contain 7.5mM MgCl2 for a final concentration of 1.5mM in a 1X reaction
- dNTP Mix (10mM)
- GoTaq® G2 DNA polymerase (5u/µl)(Promega, n.d.)
- Forward AML1: 5’ ATCAACTTTCGATGGTAGGATAGA 3’
  Reverse AML2: 5’ GAACCCAAACACTTTGGTTTCC 3’
  Amplicon size ~800bp
  Forward AMADF: 5‘ GGGAGGTAGTGACAATAAATAAC 3’
  Reverse AMDGR: 5’ CCCAACTATCCCTATTAATCAT 3’
  Amplicon size ~420

3.2.3 Results and discussion

3.2.3.1 Extraction + nanodrop

After extracting the DNA using the Qiagen DNeasy® Plant Pro Kit, the concentration and purity were assessed with a Nanodrop. The Nanodrop measurements revealed low values and indicated poor purity, which likely complicated the PCR process.

PCR sample id	DNA concentration (ng/µl)
a	10.1
b	6.0
c	14.5
d	8.4

e	6.3
f	5.5
g	9.6

3.2.3.2 Optimization PCR protocol

Below, the ingredients and protocol for the different attempts are outlined. In the first three attempts, only two samples were used to test the protocol. It is likely that there was an issue with the extraction of these samples, as testing with another sample was successful.

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	5µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.125µl	1.25u
dNTP Mix	0.5µl	0.2mM each dNTP
Forward primer: AML1	0.5µl	
Reverse primer: AML2	0.5µl	
Template DNA	1µl	
Nuclease-free water to	25µl	

PCR attempt 1 AML1-AML2 (Promega Tm Calculator, n.d.; Victorino et al., 2020)

Temperature	Time	Repeat
95 °C	15min	
95 °C	60s	x35
57 °C	60s	x35
72 °C	60s	x35
72 °C	7min	
4 °C	hold	

PCR attempt 2 AML1-AML2 (NEB Tm Calculator, n.d.; Victorino et al., 2020)

Temperature	Time	Repeat
95 °C	15min	
95 °C	60s	x35
49 °C	60s	x35
72 °C	60s	x35
72 °C	7min	
4 °C	hold	

PCR attempt 3 AML1-AML2

Temperature	Time	Repeat
95 °C	15min	
95 °C	60s	x35
53 °C	60s	x35
72 °C	60s	x35
72 °C	7min	
4 °C	hold	

After observing a faint band in Sample a, we aimed to improve the results by increasing the concentration of primers and GoTaq® DNA polymerase to eliminate any potential limitations. Additionally, the concentration of template DNA was raised, and a hot start was tested with some samples for comparison. The hot start had minimal impact on the results, so it was omitted in subsequent steps. The best results were achieved by raising the annealing temperature to 57°C, which allowed the primers to bind more specifically to the DNA target sequence.

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.5µl	1.25u
dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AML1	1µl	onverdund
Reverse primer: AML2	1µl	onverdund
Template DNA	10µl	
Nuclease-free water to	50µl	

PCR attempt 3 AML1-AML2

Temperature	Time	Repeat
95 °C	10min	
80 °C	5min	Add GoTaq
94 °C	45s	x35
53 °C	60s	x35
72 °C	60s	x35
72 °C	7min	
8 °C	hold	

PCR attempt 5 AML1-AML2

Temperature	Time	Repeat
95 °C	10min	
94 °C	60s	x35
57 °C	60s	x35
72 °C	60s	x35
72 °C	7min	
8 °C	hold	

3.2.3.3 Nested PCR

Following the initial PCR, a nested PCR was performed. However, the process encountered issues with the formation of dimers. To address this problem, the annealing temperature was increased to 58°C, and various concentrations of the PCR product from the previous reaction were tested. Ultimately, a concentration of 1.5 µl was found to be optimal for these samples, resulting in a visible band on the gel electrophoresis (see figure).

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.125µl	1.25u
dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AMADF	1µl	onverdund
Reverse primer: AMDGR	1µl	onverdund
Template DNA	10µl	
Nuclease-free water to	50µl	

PCR attempt 1 AMADF-AMDGR

Temperature	Time	Repeat
95 °C	10min	
94 °C	40s	x30
54 °C	40s	x30
72 °C	45s	x30
72 °C	7min	
8 °C	hold	

PCR attempt 2 AMADF-AMDGR

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.125µl	1.25u

dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AMADF	0.5µl	onverdund
Reverse primer: AMDGR	0.5µl	onverdund
Template DNA	1µl	
Nuclease-free water to	50µl	

Temperature	Time	Repeat
95 °C	10min	
94 °C	40s	X25
58 °C	40s	X25
72 °C	45s	X25
72 °C	7min	
8 °C	hold	

PCR attempt 3 AMADF-AMDGR

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.125µl	1.25u
dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AMADF	0.5µl	onverdund
Reverse primer: AMDGR	0.5µl	onverdund
Template DNA	5µl	
Nuclease-free water to	50µl	

PCR attempt 1 AMADF-AMDGR

Temperature	Time	Repeat
95 °C	10min	
94 °C	40s	X30
58 °C	40s	X30
72 °C	45s	X30
72 °C	7min	
8 °C	hold	

PCR attempt 4 AMADF-AMDGR

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]

GoTaq® G2 DNA polymerase	0.125µl	1.25u
dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AMADF	0.5µl	onverdund
Reverse primer: AMDGR	0.5µl	onverdund
Template DNA	1.5-2-3µl	
Nuclease-free water to	50µl	

Temperature	Time	Repeat
95 °C	10min	
94 °C	40s	X25
58 °C	40s	X25
72 °C	45s	X25
72 °C	7min	
8 °C	hold	

PCR attempt final AMADF-AMDGR

Component	Final volume	Final concentration
5X Green GoTaq Reaction buffer	10µl	1X (1.5mM $MgCl_2$)[2]
GoTaq® G2 DNA polymerase	0.125µl	1.25u
dNTP Mix	1µl	0.2mM each dNTP
Forward primer: AMADF	0.5µl	onverdund
Reverse primer: AMDGR	0.5µl	onverdund
Template DNA	1.5µl	
Nuclease-free water to	50µl	

Temperature	Time	Repeat
95 °C	10min	
94 °C	40s	X25
58 °C	40s	X25
72 °C	45s	X25
72 °C	7min	
8 °C	hold	

To conclude this section, a few recommendations are offered. Given the poor DNA quality after extraction, it is crucial to use a sufficiently high DNA concentration in the PCR mixture. Additionally, maintaining a high concentration of primers is important to ensure effective binding. Lastly, the annealing temperature should not be too low, as this can reduce primer specificity and lead to suboptimal results.

3.3 Identification

3.3.1 Aim

This experiment aims to map the genetic diversity of the mycorrhizal fungi to evaluate the benefits of using local inoculum, with the potential to produce such inoculum to enhance soil fertility. Following PCR, we sought to identify the possible fungal species present in the samples. Due to the extensive nature of next-generation sequencing, we opted for a more straightforward Sanger sequencing approach. The results of this analysis are presented below.

3.3.2 Specific Materials and Methods

The DNA was sent to Eurofins using the Mix2Seq kit for sequencing. The results were then emailed back. BioEdit was used to process the sequences, which were subsequently BLASTed using the NCBI database.

3.3.3 Results and discussion

During the alignment process, it quickly became apparent that Sanger sequencing was not an adequate method for obtaining a comprehensive view of species richness. Overlapping base pair detection in the chromatograms complicated the alignment of forward and reverse strands, resulting in very short sequences of 160-200 base pairs. This issue likely arises from the presence of multiple AMF species in a single sample. To address this, the best samples were selected for BLAST analysis. The results, including the top five hits from each BLAST search, are presented below, with query cover, E-value, and percentage identity reported. Query cover measures the proportion of the query sequence aligned with the database sequence, E-value indicates the likelihood of obtaining the alignment by chance, and percentage identity reflects the proportion of identical residues between the aligned sequences.

In every sample, a member of the *Glomeromycota* family was consistently identified. Although the results showed good values for the three parameters, the relatively short sequences limited the precision of species identification. Nonetheless, these findings confirm the presence of AMF in the samples, indicating that the PCR was successful. The results suggest that *Glomus* species are predominant, with possible presence of *Septoglomus*, *Funneliformis*, and *Rhizophagus*. However, these results should be considered illustrative.

To obtain a more accurate representation of AMF diversity, the application of next-generation sequencing techniques, such as Illumina sequencing, is recommended for

future research. This approach offers greater potential for in-depth analysis and understanding of AMF communities.

Sample b

Scientific Name	Query cover	E value	Percentage Identity
uncultured Glomus	100%	3e-52	100%
uncultured Glomus	100%	3e-52	100%
Septoglomus sp.	100%	3e-52	100%
Glomus sp.	100%	3e-52	100%
Funneliformis sp.	100%	3e-52	100%

Sample c

Scientific Name	Query cover	E value	Percentage Identity
uncultured Rhizophagus	100%	0.0	98.45%
uncultured Glomus	100%	0.0	98.45%
uncultured Glomus	100%	0.0	98.45%
Rhizophagus sp. MAY-2012	100%	0.0	98.45%
uncultured Glomus	100%	0.0	98.45%

Sample e

Scientific Name	Query cover	E value	Percentage Identity
Glomeromycotina sp.	100%	1e-61	100%
Fungal endophyte sp.	100%	1e-61	100%
uncultured Glomus	100%	1e-61	100%
uncultured Glomus	100%	1e-61	100%
uncultured Glomus	100%	1e-61	100%

Sample f

Scientific Name	Query cover	E value	Percentage Identity
Glomus sp. MUCL 41833	100%	8e-69	99.33%
Glomus microcarpum	100%	1e-67	99.33%
Glomus microcarpum	100%	1e-67	99.33%
Glomus microcarpum	100%	1e-67	99.33%
Glomus microcarpum	100%	1e-67	99.33%

Sample g

Scientific Name	Query cover	E value	Percentage Identity
Rhizophagus sp.	100%	1e-38	100%
Glomus sp.	100%	1e-38	100%
Glomus sp.	100%	1e-38	100%
Glomus sp.	100%	1e-38	100%
Glomus sp.	100%	1e-38	100%

4 CONCLUSION

This study aimed to evaluate the mycorrhizal colonization of *Melia volkensii* across various locations, focusing on factors such as vegetation, tree type, and land use. The findings reveal that while mycorrhizae were present at every site, Site 9, which lies outside the natural range of *Melia volkensii*, exhibited significantly lower colonization frequency. This is likely due to the mycorrhizae at this site being less adapted to colonize *Melia volkensii*. Additionally, environmental factors such as the site's coastal location and recent wet conditions might have contributed to reduced investment in mycorrhizal symbiosis by the trees.

Statistical analysis of colonization intensity using the Kruskal-Wallis and Dunn's tests indicated significant differences among sites. Locations 8 and 9 stood out with distinct differences compared to other sites, which clustered together, suggesting that geographical and possibly environmental factors significantly impact mycorrhizal colonization. The results also demonstrated that vegetation and land use are important factors influencing colonization. Specifically, an agroforestry model was found to support better colonization compared to a plantation model, contrary to expectations based on soil disturbance levels.

The nested PCR approach used for species identification encountered issues with low DNA quality and short sequence lengths, which impacted the accuracy of species identification. The use of next-generation sequencing methods, such as Illumina sequencing, is recommended for future research to provide a more comprehensive understanding of AMF diversity and distribution.

In summary, the study highlights the importance of considering both environmental conditions and land use practices when evaluating mycorrhizal colonization. Future research should address the limitations encountered in DNA sequencing and explore the application of advanced sequencing technologies to better characterize AMF communities and their interactions with *Melia volkensii*.

5 REFERENCES

Berruti, A., Desirò, A., Visentin, S., Zecca, O., & Bonfante, P. (2017). ITS fungal barcoding primers versus 18S AMF-specific primers reveal similar AMF-based diversity patterns in roots and soils of three mountain vineyards. *Environmental Microbiology Reports*, *9*(5), 658–667. https://doi.org/10.1111/1758-2229.12574

Bowles, T. M., Jackson, L. E., Loeher, M., & Cavagnaro, T. R. (2017). Ecological intensification and arbuscular mycorrhizas: a meta-analysis of tillage and cover crop effects. *Journal of Applied Ecology*, *54*(6), 1785–1793. https://doi.org/10.1111/1365-2664.12815

Cavagnaro, T. R., Bender, S. F., Asghari, H. R., & van der Heijden, M. G. A. (2015). The role of arbuscular mycorrhizas in reducing soil nutrient loss. *Trends in Plant Science*, *20*(5), 283–290. https://doi.org/10.1016/J.TPLANTS.2015.03.004

Dickson, S. (2004). The Arum-Paris continuum of mycorrhizal symbioses. *New Phytologist*, *163*(1), 187–200. https://doi.org/10.1111/j.1469-8137.2004.01095.x

Gollotte, A., Van Tuinen, D., & Atkinson, D. (2004). Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species Agrostis capillaris and Lolium perenne in a field experiment. *Mycorrhiza*, *14*(2), 111–117. https://doi.org/10.1007/s00572-003-0244-7

Gorzelak, M. A., Holland, T. C., Xing, X., & Hart, M. M. (2012). Molecular approaches for AM fungal community ecology: A primer. In *Journal of Microbiological Methods* (Vol. 90, Issue 2, pp. 108–114). https://doi.org/10.1016/j.mimet.2012.04.010

Grace, C., & Stribley, D. P. (1991a). A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. *Mycological Research*, *95*(10), 1160–1162. https://doi.org/10.1016/S0953-7562(09)80005-1

Grace, C., & Stribley, D. P. (1991b). A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. *Mycological Research*, *95*(10), 1160–1162. https://doi.org/10.1016/S0953-7562(09)80005-1

Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L., & Hedges, S. B. (2001). Molecular Evidence for the Early Colonization of Land by Fungi and Plants. *Science*, *293*, 1129–1132.

Helgason, T., Daniell, T. J., Husband, R., Fitter, A. H., & Young, J. P. W. (1998). Ploughing up the wood-wide web? *Nature*, *394*, 431.

Ho-Plágaro, T., Tamayo-Navarrete, M. I., & García-Garrido, J. M. (2020). Histochemical Staining and Quantification of Arbuscular Mycorrhizal Fungal Colonization. In *Methods in Molecular Biology* (Vol. 2146, pp. 43–52). Humana Press Inc. https://doi.org/10.1007/978-1-0716-0603-2_4

IPCC. (2023). *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (P. Arias, M. Bustamante, I. Elgizouli, G. Flato, M. Howden, C. Méndez-Vallejo, J. J. Pereira, R. Pichs-Madruga, S. K. Rose, Y. Saheb, R. Sánchez Rodríguez, D. Ürge-Vorsatz, C. Xiao, N. Yassaa, J. Romero, J. Kim, E. F. Haites, Y. Jung, R. Stavins, … C. Péan, Eds.). https://doi.org/10.59327/IPCC/AR6-9789291691647

Kalele, D. N., Ogara, W. O., Oludhe, C., & Onono, J. O. (2021). Climate change impacts and relevance of smallholder farmers' response in arid and semi-arid lands in Kenya. *Scientific African*, *12*. https://doi.org/10.1016/J.SCIAF.2021.E00814

Kamondo, B., Kariuki, J., KEFRI, A. L.-, Published, K., & 2016, undefined. (2016). Guideline on production, distribution and use of improved Melia seed and seedlings in the drylands of Kenya. *Kefri.OrgBM Kamondo, JG Kariuki, AM Luvanda, GM Muturi, D OchiengKEFRI, Kenya Published by, 2016•kefri.Org*. http://www.kefri.org/assets/publications/extension/Guideline%20on%20Production,%20Distribution%20and%20Use%20of%20Improved%20Melia%20Seed%20and%20Seedlings%20in%20the%20Drylands%20of%20Kenya.pdf

Kokkoris, V., Pogiatzis, A., & Hart, M. M. (2019). Contrasting common measures of arbuscular mycorrhizal fungal root colonization. *Journal of Microbiological Methods*, *167*. https://doi.org/10.1016/j.mimet.2019.105727

Koske, R. E., & Gemma, J. N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research*, *92*(4), 486–488. https://doi.org/10.1016/S0953-7562(89)80195-9

Krüger, M., Stockinger, H., Krüger, C., & Schüßler, A. (2009). DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist*, *183*(1), 212–223. https://doi.org/10.1111/J.1469-8137.2009.02835.X

Lee, J., Lee, S., Peter, J., & Young, W. (2008). *Improved PCR primers for the detection and identi¢cation of arbuscular mycorrhizal fungi*. https://doi.org/10.1111/j.1574-6941.2008.00531.x

Lenoir, I., Fontaine, J., & Lounès-Hadj Sahraoui, A. (2016). Arbuscular mycorrhizal fungal responses to abiotic stresses: A review. *Phytochemistry*, *123*, 4–15. https://doi.org/10.1016/J.PHYTOCHEM.2016.01.002

Morton, J. F. (2007). *The impact of climate change on smallholder and subsistence agriculture*. https://www.pnas.org

Mulatya, J. M., Wilson, J., Ong, C. K., Deans, J. D., & Sprent, J. I. (2002). *Root architecture of provenances, seedlings and cuttings of Melia volkensii: implications for crop yield in dryland agroforestry*.

NEB Tm Calculator. (n.d.). Retrieved 24 May 2024, from https://tmcalculator.neb.com

Odee, D. (2004). Analysis of genetic structure in Melia volkensii (Gurke.) populations using random amplified polymorphic DNA. *African Journal of Biotechnology*, *3*(8), 421–425. http://www.academicjournals.org/AJB

Omega, M., & Kiptoo, E. (2024, January 8). *From Tree Planting to Tree Growing: A Paradigm Shift Towards 30% Tree Cover*. https://kippra.or.ke/from-tree-planting-to-tree-growing-a-paradigm-shift-towards-30-tree-cover/

Öpik, M., Davison, J., Moora, M., & Zobel, M. (2014). Dna-based detection and identification of glomeromycota:The virtual taxonomy of environmental sequences1. In *Botany* (Vol. 92, Issue 2, pp. 135–147). https://doi.org/10.1139/cjb-2013-0110

Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J. M., Reier, Ü., & Zobel, M. (2010). The online database Maarj *AM* reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist*, *188*(1), 223–241. https://doi.org/10.1111/j.1469-8137.2010.03334.x

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Melia volkensii*.

Profi table Agroforestry Innovations for Eastern Africa. (n.d.).

Promega. (n.d.). Retrieved 22 May 2024, from https://be.promega.com/products/pcr/taq-polymerase/gotaq-g2-dna-polymerase/?catNum=M7841

Promega Tm Calculator. (n.d.). Retrieved 24 May 2024, from https://be.promega.com/resources/tools/biomath/tm-calculator/

Qiagen DNeasy plant pro kit. (n.d.). Retrieved 22 May 2024, from https://www.qiagen.com/us/products/discovery-and-translational-research/dna-

rna-purification/dna-purification/genomic-dna/dneasy-plant-pro-and-plant-kits?catno=69204

Redecker, D., Hijri, I., & Wiemken, A. (2003). MOLECULAR IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGI IN ROOTS: PERSPECTIVES AND PROBLEMS. In *Folia Geobotanica* (Vol. 38).

Redecker, D., Kodner, R., & Graham, L. E. (2000). Glomalean Fungi from the Ordovician. *Science*, *289*, 1920–1921. https://www.science.org

Redecker, D., & Redecker, D. (2000). Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. In *Mycorrhiza* (Vol. 10). Springer-Verlag.

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S. L., Morton, J. B., & Walker, C. (2013). An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). *Mycorrhiza*, *23*(7), 515–531. https://doi.org/10.1007/S00572-013-0486-Y/FIGURES/4

Rillig, M. C., & Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytologist*, *171*(1), 41–53. https://doi.org/10.1111/J.1469-8137.2006.01750.X

Roe, K., & Brooke, Q. (2023). Potential of Melia volkensii in Kenya's ASAL. *Miti*, *57*, 44–45.

Roothaert, R. L., & Franzel, S. (2001). Farmers' preferences and use of local fodder trees and shrubs in Kenya. In *Agroforestry Systems* (Vol. 52). Kluwer Academic Publishers.

Schüßler, A., Schwarzott, D., & Walker, C. (2001). A new fungal phylum, the Glomeromycota: Phylogeny and evolution. *Mycological Research*, *105*(12), 1413–1421. https://doi.org/10.1017/S0953756201005196

Simon,', L., Lalonde, M., & Bruns2, T. D. (1992). Specific Amplification of 18S Fungal Ribosomal Genes from Vesicular-Arbuscular Endomycorrhizal Fungi Colonizing Roots. In *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*. https://journals.asm.org/journal/aem

Singh, S. P., & Singh, M. K. (2019). Mycorrhiza in sustainable crop production. *Agronomic Crops: Volume 2: Management Practices*, 461–483. https://doi.org/10.1007/978-981-32-9783-8_22

Smith, S. E., & Read, D. (2008). *Mycorrhizal Symbiosis* (3rd ed.). Academic Press. https://doi.org/10.1016/B978-0-12-370526-6.X5001-6

Stewart, M., & Blomley, T. (1994). Use of Melia volkensii in a semi-arid agroforestry system in Kenya. *Commonwealth Forestry Review*, *73*(2), 128–131.

Trouvelot, S., Van Tuinen, D., Hijri, M., Gianinazzi-Pearson, V., Trouvelot, S., Van Tuinen, D., Hijri, M., & Gianinazzi-Pearson, V. (1999). Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization. In *Mycorrhiza* (Vol. 8). Springer-Verlag.

Van Der Heijden, M. G. A., Bardgett, R. D., & Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, *11*(3), 296–310. https://doi.org/10.1111/J.1461-0248.2007.01139.X

van der Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., & Sanders, I. R. (1998). Mycorrhizal fungal diversity determinesplant biodiversity, ecosystemvariability and productivity. *Nature*, *369*, 69–72.

Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., & Gianinazzi-Pearson, V. (1998). Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Molecular Ecology*, *7*(7), 879–887.

Vandenabeele, J. (2023). Kenya sees it big. *Miti*, *57*, 6.

Victorino, Í. M. M., Berruti, A., Orgiazzi, A., Voyron, S., Bianciotto, V., & Lumini, E. (2020). High-Throughput DNA Sequence-Based Analysis of AMF Communities. In *Methods in Molecular Biology* (Vol. 2146, pp. 99–116). Humana Press Inc. https://doi.org/10.1007/978-1-0716-0603-2_9