BIOLOGICAL CONTROL OF ARMILLARIA ROOT ROT OF SELECTED INDIGENOUS TREES OF MAU FOREST COMPLEX USING ARBUSCULAR MYCORRHIZAE FUNGI AND *Trichoderma* species

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A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Award of the Master of Science degree in Plant Pathology of Egerton University

EGERTON UNIVERSITY

JANUARY, 2015

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted wholly or in part for any award in any institution.

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SM15/2538/09

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RECOMMENDATION

This research thesis was prepared under our supervision and has been submitted to the Graduate School for examination with our approval as supervisors as per the Egerton University regulations.

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DEDICATION

This thesis is dedicated to both my parents. My father, the late Paul Chumo and my mother Christine Chumo, with gratitude for daily examples of a graceful and collaborative approach to life, who encouraged, emboldened and exhorted me always to do the best and believe I will.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisors Prof. Isabel N. Wagara and Prof. Samuel T. Kariuki of Egerton University and Dr. Joyce Jefwa of Tropical Soil Biology and Fertility (TSBF) who provided useful insights and suggestions during the entire research work. Through their continuous efforts they greatly contributed to the success of my research. I would like to express my appreciation to Dr. Mugambi, Head of Mycology Section at National Museum of Kenya and Mary Mureithi for permitting me to work and use the facilities in their laboratory during characterization of mycorrhizae. Thanks to Susan Kabacia, Emma Kibiro and Virginiah Mwirigi for providing valuable technical assistance concerning spore extraction and characterization of mycorrhizae in addition to clearing, staining and assessment of mycorrhizal roots. I would also like to express my gratitude to David Talaam and Samwel Tanui for their technical support concerning dual culture interactions between *Trichoderma* species and *Armillaria* species.

I thank Tea Research Foundation of Kenya and Homegrown (K) Limited-Naivasha for kindly providing the *Armillaria* species and Mycorrhizae spores respectively. This work was funded by the Mushroom initiative Ltd of Hongkong and National Commision for Science, Technology and Innovation to whom I am grateful. I would also like to extend my gratitude towards my family, friends and colleagues for their everlasting support in career and life goals.Special thanks to Kamams family who are the answers to my prayers and for their encouragement, hospitality and support. I am also indebted to a lot of people who at one time or another have contributed to the knowledge and techniques that enabled me to carry out this work. The contribution of all and many more not mentioned here worked together for success of this work. I love you all, with all my heart and soul.

ABSTRACT

Mau Forest Complex is of critical importance for sustaining current and future ecological, social and economic development in Kenya. Mau Forest Complex is highly degraded and tree planting has been highly advocated. However, there is a threat to establishment of the seedlings by *Armillaria* species. The aim of this study was to evaluate efficacy of biological control agents for integrated control of Armillaria root rot of selected indigenous trees of Mau Forest Complex. Two fungal biological control agents, *Trichoderma* species and mycorrhizae, were applied individually and in combination in laboratory-based research and green house conditions to test their efficacy in controlling Armillaria root rot. Microscopic analysis of the mycorrhizal status of the selected indigenous trees from Mau forest complex showed that all the 10 plant species were colonized by arbuscular mycorrhizae fungi. AMF spores were obtained from all rhizosphere soil samples, where low density of AMF spores was generally observed. Visual observation of dual cultured plates showed antagonistic activity of *Trichoderma* species towards *Armillaria* species and an inhibition zone was observed at the margin between the antagonist and the pathogen. These initial results indicated that the strain of *Trichoderma* species can be used as a biocontrol agent against the tested *Armillaria* species. Dual inoculation of *T. asperellum* and arbuscular mycorrhizal fungi significantly reduced the level of root colonisation by arbuscular mycorrhizal fungi while co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* produced a higher percentage of colonization than any other treatment. Ecological measures of diversity used to describe the structure of Arbuscular mycorrhizae fungi (AMF) communities included spore density, species richness, relative abundance, isolation frequency, Shannon–Wiener index of diversity, evenness, Simpson's index of dominance, and Sorenson's coefficient. Differences in spore density and species richness between sites with plant species were tested using one-way ANOVA. The Pearson correlation coefficient was employed to determine the relationships between spore density and species richness, relative abundance and isolation frequency. Correlation analyses with Pearson's correlation coefficients were used to determine if a relationship existed between soil microbial parameters and soil chemical properties. All data was subjected to analysis of variances (ANOVA) using Gen stat and treatment means were compared by Fisher's least significant difference (LSD) test. The selected indigenous trees roots are colonized by AMF. The abundance of arbuscules and vesicle within the roots of these plants suggests that AMF have important influence on plant communities.

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LIST OF ABBREVIATIONS

AMF	Arbuscular mycorrhizae fungi
ASD	Average AMF spore density
ASM	Armillaria selective medium
BCAs	Biological control agents
CFUs	Colony forming units
CWDEs	Cell wall-degrading enzymes
HM	Heavy metal
KEFRI	Kenya Forest Research Institute
MEA	Malt Extract Agar
PDA	Potato Dextrose Agar
PVLG	Polyvinyl Lactophenol Glycerol
RCBD	Randomized Complete Block Design
SE	Standard error
TSBF	Tropical Soil Biology and Fertility
TSM	Trichoderma selected medium

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Mau forest complex is the largest closed-canopy forest ecosystem in Kenya and the largest indigenous Montane forest in East Africa. Tropical forests have the potential to satisfy multiple demands for timber and non-timber forest products, marketed and non-marketed ecosystem services (Guariguata *et al.*, 2010). In addition they have social, cultural and economic significance as sources of important renewable and non-renewable resources (Huete *et al.*, 2008). Despite their importance, they are increasingly under threat from encroachment by the growing population and a subsequent increase in land use and resource extraction (Bonnell *et al.*, 2011). Over the last two decades significant degradation of indigenous trees has occurred in Mau Forest Complex due to illegal extraction of resources and the change of land use from forest to unsustainable agriculture, affecting regional ecosystem services. For instance, levels of pest control and pollination are highly dependent on the abundance and diversity of insects inhabiting indigenous forests (Kremen *et al.*, 2007), while tropical seed dispersal is highly dependent on avian assemblages (Pellikka *et al.*, 2009). For these reasons reforestation has been highly advocated. However, there is a threat to establishment of the tree seedlings by *Armillaria* species that cause Armillaria root rot, which is an important contributor to tree mortality in the forests and has resulted in significant economic losses.

Armillaria species are saproparasitic basidiomycetes that can survive in the soil, on wood and root debris in the absence of any living host and can cause significant damage to forest trees and several crops (Pertot *et al.*, 2008; Savazzini *et al.*, 2009). The *Armillaria* fungi, which are feared by foresters, belong to the most important and cosmopolitan pathogens inside and outside the forest. They can attack almost all species of hardwoods and conifers of all ages (Gibbs *et al.*, 2002). These pathogens induce root rot disease and can be highly aggressive, rapidly advancing through the inner bark to the collar, where they girdle and kill the tree (Kwasna, 2001). Promising systems for the biological protection of growing trees have been studied against *Armillaria* species (Bruce 1998; Palli and Retnakaran, 1998).

Biological control of plant diseases is any means of controlling disease or reducing the amount or effect of pathogens that relies on biological mechanisms or organisms other than man (Hofte and Altier, 2010). Biological control of plant diseases has received more consideration in the recent decades because of its efficacy against fungicide-resistant pathogens in addition to

reduced possibility of resistance development (Brimner and Boland, 2003; John *et al.*, 2010). Moreover many research studies have shown that biological control offers an environmentally friendly alternative to protect plants from soil borne pathogens (Yangui *et al.*, 2008). There is significant interest in finding alternatives to fungicides for suppression of soil borne pathogens due to several negative effects, like development of pathogen resistance, hazards to humans, damage to non-target beneficial organisms, and environmental pollution (Brimner and Boland, 2003; Roberts *et al.*, 2007; Begum *et al.*, 2010). In addition, application of fungicides and fumigants are expensive and can be harmful to plants and their efficacy has been reduced by the appearance of microbial resistance (Sang *et al.*, 2008; Quagliotto *et al.*, 2009).

Antagonists are biological agents that reduce the population density or disease-producing activities of the pathogen and therefore use of antagonistic micro-organism for disease control is being investigated (Jung *et al.*, 2003). Emerging strategies for plant disease control involve application of antagonistic micro-organisms alone or in combination (De Curtis *et al.*, 2010). Unfortunately, biological control agents (BCAs) applied alone are not likely to perform consistently against all pathogens or under different rhizosphere and soil environmental conditions (Roberts *et al.*, 2005). A combination of different antagonists is more likely to have a greater diversity of traits responsible for suppression of one or more pathogens resulting in improved control of pathogens (Postma *et al.*, 2009). Biological control using microbial inoculants has proved to be a reliable component of integrated management of fungal diseases (Tchameni *et al.*, 2011). Among the microbial inoculants, Arbuscular mycorrhizal fungi (AMF) and members of the genus *Trichoderma* have emerged as promising groups of fungi that improve plant nutrition and health (Martínez-Medina *et al.* 2011$_a$).

Fungi of the genus *Trichoderma* are used extensively in the biocontrol of plant diseases (Wijesinghe *et al.*, 2011). They are common components of rhizosphere soil and have been reported to suppress a great number of plant diseases (Martínez-Medina *et al.* 2011$_b$). Species of *Trichoderma* have shown antagonistic activity towards a range of agriculturally devastating phytopathogenic microorganisms, including those within the genus *Fusarium*, *Phytophthora*, *Sclerotinia*, *Rhizoctonia* and *Pythium* (Beaulieu *et al.*, 2011). Arbuscular mycorrhizal fungi belong to the phylum *Glomeromycota* and are key components of soil microbiota where they establish mutualistic symbioses with the majority of plants (Tian *et al.*, 2011; Veresoglou *et al.*, 2012). Alleviation of damage caused by soil-borne pathogens, such as *Phytophthora*, *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotium* and *Verticillium* has been reported widely in mycorrhizal plants (Martínez-Medina *et al.*, 2011$_b$). Therefore, the aim of this study was to

evaluate the efficacy of application of different biocontrol agents: *Trichoderma* species and mycorrhizae, singly or in combination, for the control of Armillaria root rot so as to contribute to a more suitable and environmentally acceptable alternative for sustainable agriculture and forestry.

1.2 Statement of the problem

Mau Forest Complex is highly degraded and tree planting has been highly advocated. However, there is likelihood of a threat to establishment of the seedlings by *Armillaria* species. Armillaria root rot occurs worldwide and causes extensive tree mortality especially during re-planting. There are no effective fungicides for the control of Armillaria root rot. Some studies have indicated that certain fungicides e.g. phenolic fungicides are not only ineffective in reducing the incidence of infection, but also lead to increases in the amount of root colonization by *Armillaria* species. Additionally, many antagonistic soil-inhabiting micro-organisms may be destroyed by the phenolic fungicides, giving a strong, competitive advantage to the surviving *Armillaria* species. Therefore, the aim of the present study was to evaluate the efficacy of arbuscular mycorrhizae fungi and *Trichoderma* species combination in control of Armillaria root rot of some selected indigenous trees of Mau Forest Complex.

1.3 Objectives

1.3.1 General objective

To determine the efficacy of arbuscular mycorrhizae and *Trichoderma* species combination in integrated control of Armillaria root rot of some selected indigenous trees of Mau Forest Complex.

1.3.2 Specific objectives

1. To characterise arbuscular mycorrhizae fungi (AMF) associated with selected indigenous trees of Mau Forest Complex.
2. To determine the antagonistic activity of *Trichoderma* species against Armillaria root rot.
3. To evaluate the efficacy of combined isolates of AMF and *Trichoderma* species in controlling Armillaria root rot.

1.4 Hypotheses

1. AMF associated with selected indigenous trees of Mau Forest Complex have not been characterised.
2. There is no antagonistic activity of *Trichoderma* species against Armillaria root rot.
3. Combined isolates of AMF and *Trichoderma* species do not improve the control of Armillaria root rot.

1.5 Justification

Increasing levels of carbon dioxide and other greenhouse gases in the atmosphere are of growing concern globally and locally, prompting the search for potential ways of storing carbon in trees of terrestrial ecosystems. Trees reduce atmospheric levels of these greenhouse gases by transforming the gases into living matter and play a significant role in addressing global warming. The Mau Complex has a large potential to sequester carbon primarily through reforestation, and conservation of existing forests. Moreover the Mua Complex is of critical importance for sustaining current and future ecological, social and economic development in Kenya and the region. Mau Forest Complex is highly degraded and tree planting has been highly advocated. However, there is a threat to establishment of the seedlings by *Armillaria* species. This is of great concern and warrants investigation. Current management options for Armillaria root rot are extremely limited. Chemical control is most effective, especially when multiple treatments are applied. However, this is limited by the lack of suitable fungicides against the *Armillaria* pathogens and particularly the ecological hazards such as environmental pollution, detrimental health effects for farmers and consumers, and the risk of emergence of resistant pathogen strains. In view of these serious drawbacks, the development of more environmentally friendly control methods, such as biological control using antagonistic microorganisms, can help to complement current strategies for integrated management of Armillaria root rot. The presence of mycorrhizal fungi in the root system of plants is well known to improve plant health, enhance plant growth and in alleviation of stress caused by both biotic and abiotic factors. Studies have shown that this intimate association is accompanied by an increased resistance to root pathogens and in protection against some diseases. In this context, the importance of mycorrhizae fungi colonization for growth and survival of forest seedlings has been widely acknowledged. The filamentous fungi *Trichoderma* species have long been recognized as agents for the control of plant pathogenic fungi. Control has been obtained with *Trichoderma* isolates applied singly and in combination with other microorganisms. The use of combinations of multiple antagonistic organisms may provide improved disease control over the use of single organisms. This is because multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action.

CHAPTER TWO

LITERATURE REVIEW

2.1 Ecological and economical value of Mau forest

Mau Forest complex is the largest closed-canopy forest ecosystem in Kenya, largest water tower and indigenous montane forest in East Africa. It lies between 2,000 m and 2,600 m above the sea level, on the Western slope of the Mau. The Complex forms part of the upper catchments of all but one of the main rivers on the west side of the Rift Valley.Tropical forests comprise a major and critical component of the global earth system through their role in climate hydrologic, biogeochemical cycling, and as a principal reservoir of the planet's biological diversity (Huete *et al.*, 2008). Thus, tropical forest loss, degradation and fragmentation is regarded as a major cause of the current biodiversity crisis (Pellikka *et al.*, 2009).Trees are significant and important components of the environment and provide ecosystem services that are essential ecological processes that support all life on earth (Tzoulas and James, 2010) as well as environmental benefits which cannot be measured on monetary values (Notaro and De Salvo, 2010).

Carbon sequestration benefit is estimated as the physical quantity that includes the growth of the forest biomass, the carbon accumulated in residues and forestry products and their decay and the carbon dioxide emissions saved by replacing fossil fuels with fire wood (Ovando *et al.*, 2010). Tropical forests have a large potential to sequester carbon primarily through reforestation, agroforestry and conservation of existing forests (Lu *et al.*, 2010). Within the carbon cycle, vegetation plays an important role by absorbing carbon dioxide from the atmosphere through photosynthesis and storing the carbon in organic material (Tupek *et al.*, 2010). The ability to remove carbon dioxide from the atmosphere is important in mitigating climate change (Schwaiger and Bird, 2010). Nitrogen fixing trees and shrubs are important features of many of the world's ecosystems where they improve the soil, provide fuel wood to the local population, feed for livestock and provide lumber for high quality furniture (Ewens and Felker, 2010). Proper management of trees and forests is therefore necessary to make resources sustainable, prevent loses from extreme weather events and to reduce global warming.

2.2 Armillaria root rot

Armillaria is a genus of parasitic fungi that live on trees and woody shrubs. *Armillaria* belongs to Family Tricholomataceae, Order Agaricales, class Basidiomycetes, phylum Basidiomycota, and Kingdom Fungi (Agrios, 2005). The genus *Armillaria* comprises about 40 species

worldwide. The rather similar fungi form rhizomorphs in the soil and beneath the treebark, the mycelium shines in the dark, the secondary mycelium is diploid and normally clampless. There are ex-annulate and annulate species (Schmidt, 2006). Most species are heterothallic and have a tetrapolar mating system. *Armillaria mellea* has both homothallic and heterothallic populations (Baumgartner *et al.*, 2011). Populations of *A. mellea* in Europe and North America are heterothallic and are primarily out-crossing (Baumgartner*et al.*, 2010). Populations in Africa (Ethiopia, Kenya, Tanzania, and Sao Tome) and Japan are homothallic and are recognized as subspecies *A. mellea* ssp. *africana* and *A. mellea* ssp. *nipponica* respectively (Baumgartner *et al.*, 2012). China is the only location where both heterothallic and homothallic *A. mellea* are known to occur (Qin *et al.*, 2007).

2.2.1 Aetiology, host range and occurrence

The *Armillaria* species differ in host preference, pathogenicity, geographical distribution, type and frequency of rhizomorphs, and in cultural characteristics such as mat morphology and optimum temperature (Schmidt, 2006). Members of the fungal genus *Armillaria* occur in boreal, temperate, and tropical forests worldwide, and Armillaria root disease is known to occur on all continents except Antarctica (Westwood *et al.*, 2012). In North America, the pathogen is considered a significant agent of biological disturbance (Ayres and Lombardero, 2000). Armillaria root disease is present in all forest regions of Canada, affecting over 200 million hectares (Canadian Forest Service, 2010). *Armillaria ostoyae* is the primary cause of Armillaria root disease in conifers in Canada (Mallett and Mayard, 1998), causes significant mortality among susceptible conifers in British Columbia (Baleshta *et al.*, 2005) and Western North America (Cruickshank *et al.*, 2009). *Armillaria* species are important root rot pathogens of tea in Kenya (Otieno *et al.* 2003_a). In Kenya, some indigenous trees such as camphor (*Ocotea usumbarensis*) and *Cassipourea* spp. have been attacked by *Armillaria* species. This fungus usually attacks trees that have been stressed or weakened by some other factor such as drought or insect attack (FAO, 2007).

Individual *Armillaria* species have such a broad host range that strong associations with particular hosts or landscape characteristics can be difficult to identify (Brazee and Wick, 2009). *Armillaria* occurs worldwide on a wide variety of hardwood and softwood plants including many stone fruit species such as peach (Cox and Scherm, 2006). Armillaria root rot damages a range of plant species including tea, coffee, avocado, banana, pine, eucalyptus, and cypress in highland areas of Kenya (Otieno *et al.*, 2003_b).

2.2.2 Disease development and transmission

Armillaria species can survive in the soil for long periods of time on wood and root debris in the absence of any living host. They are harboured by stumps which act as a source of nutrients and a prolonged reservoir of inoculum (Worrall *et al.*, 2004). Infected roots serve as a source of inoculum (Pertot *et al.*, 2008) and rhizomorphs produced by pathogenic species allow fungi to travel between host plants (Yafetto *et al.*, 2009). These root-like aggregations are a means for Armillaria to spread underground from one tree root system to another (Webster and Webe, 2007). Armillaria does not have an asexual spore stage, but an individual genotype can spread from one infected tree to the root system of a neighbouring tree, by sub-terranean mycelial growth (Figure 1) (Baumgartner *et al.*, 2012).

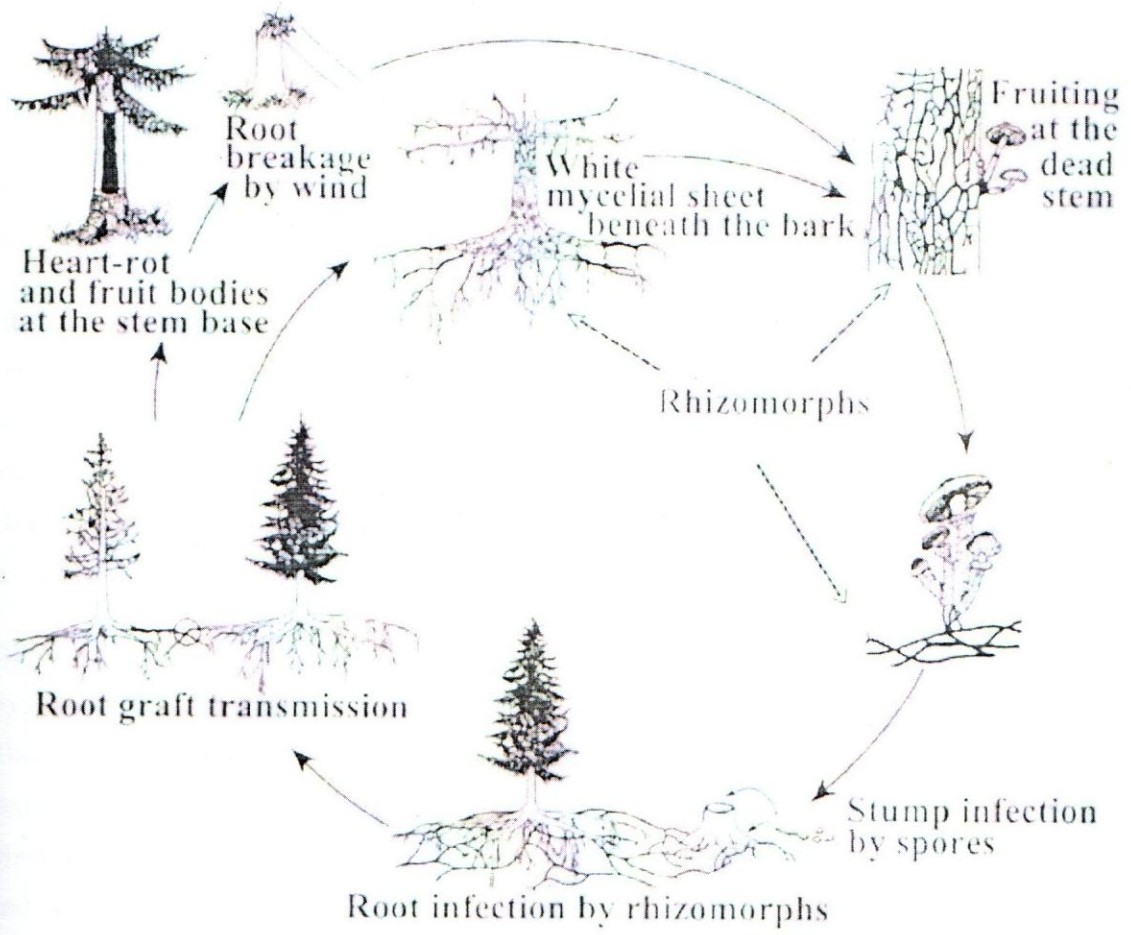


Figure 1: Development and transmission of Armillaria root disease

Source: Schmidt, 2006

2.2.3 Economic importance

Armillaria is a Basidiomycete genus of economic significance, namely with respect to the species that cause Armillaria root disease of horticultural crops, timber trees, and ornamentals (Baumgartner *et al.*, 2011). The genus *Armillaria* includes economically important fungal pathogens inducing root rot in forest trees and cause extensive economical losses (Kwasna, 2001; Lochman *et al.*, 2004). It is an important contributor to tree mortality in the forests and has resulted in significant economic losses (Bendel and Rigling, 2008). The disease assumes importance in one or more of the following ways: (a) the roots are killed and hence the trees die in one or more seasons; (b) the base of trunk may be injured to such an extent that the tree may be blown over under the strain of a heavy crop of fruit; (c) affected trees in bearing usually fail to mature their fruit, particularly in cases of severe infection; or the fruit matures poorly, is stunted and is of an inferior quality; (d) diseased trees often lack the normal amount of foliage; (e) affected plants make little or no growth (Skovsgaard *et al.*, 2010). Armillaria root rot is a lethal disease of many trees and shrubs, some more seriously than others worldwide. A great number of widely related plants, such as forest trees, deciduous trees, vineyard, kiwifruit, are likely to be affected by this disease (Brazee and Wick, 2009). Futhermore, forest die back' has been reported on trees associated with *Armillaria* species (Jurskis, 2005).

Armillaria mellea is an edible, medicinal mushroom also known as honey fungus. It has been reportedly used in treating geriatric patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in limbs and infantile convulsion, while also reportedly exerting neuroprotective effects (Kim *et al.*, 2010; Vaz *et al.*, 2011). Of ecological significance is the fact that *Armillaria* species are white-rot fungi, which degrade lignin, hemicelluloses, and thus have an important role in carbon cycling (Baumgartner *et al.*, 2012).

2.2.4 Symptoms

Symptoms of Armillaria root rot on infected trees include above and below ground symptoms. The most noticeable external symptoms are premature autumn coloration and leaf drop, stunting of growth, yellowing or browning of the foliage, a general decline in the vigour of the plant twig and branch, a thin, reduced, or dead crown, or the presence of fungal fruiting bodies and main stem dieback. White or creamy white, paper-thick, fan-shaped sheets of *Armillaria* mycelium grow over the water-soaked sapwood when exposed on the large roots of trees (Mallett and Mayard, 1998; De Long *et al.*, 2005). *Armillaria* species can be identified by the typical hyphal brushes, which appear on the surface of the wood. Infection by

*Armillaria*species is also recognised by the presence of mycelia fans under the bark of the roots and the stem base (Dobbertin *et al.*, 2001).

2.2.5 Control

Current management options for Armillaria root rot are extremely limited. The root and stump remnants are essential food bases for ensuring longevity of Armillaria inoculum. Their removal is considered to be the single most effective way for minimizing the risk of the disease in tea plantations (Otieno *et al.*, 2003_a). Another possibility is to improve resistance to *Armillaria* species by reducing stress caused by overcrowding (Robinson, 2003). Cultural methods such as exposing of infected crown and upper root area of a tree, removing soil from around the base of the tree to a depth of 9-12 inches are mainly used to control this disease (Thomidis and Exadaktylou, 2012). In addition, fumigation of infested soil is another method used as preventive method to control this disease before establishing new planting (Adaskaveg *et al.*, 1999). The sterol biosynthesis inhibitors cyproconazole, hexaconazole, propiconazole and tetraconazole have been reported as promising fungicides to control Armillaria root rot in vineyards and peach orchards (Aguin *et al.*, 2006; Amiri *et al.*, 2008). Several factors hamper the control of this soilborne fungus. The disease remains undetected until symptomatic plants are observed, by which time *A. mellea* may be widely spread both in soil and within the plant. *Armillaria mellea* forms rhizomorphs that can go deep into the soil, and mycelium that remains protected, beneath the plant bark or inside dead wood, from the action of any control agent (Pronos and Patton, 2007). This fact complicates the matter of control, and is a factor in the economic importance of the disease (Thomidis, and Exadaktylou, 2012).

The group of organisms known as biocontrol agents (BCAs) are the microbial components of soil involved in biological control of pathogens. They are the active ingredients in several biofungicides. The success of biocontrol is highly dependent on the nature of the antagonistic properties and on the action mechanisms of the microorganism. BCAs employ various mechanisms to directly control pathogens: antibiosis, competition for space and nutrients, and mycoparasitism (Whipps, 2001). They can also indirectly induce systemic resistance in the plant to control diseases. Antibiosis is the process whereby metabolites are produced which inhibits the development of a plant pathogen and ultimately cause its death (Pellegrini *et al.*, 2013). Some mycoparasitic microorganisms are used to control soil-borne plant pathogens, such as *A. mellea, Fusarium solani, Microdochium nivale, Myriosclerotinia borealis, Phytophthora* species, *Pythium* species, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahlia* (Daami-Remadi *et al.*, 2006). *Armillaria* species are saproparasitic

basidiomycetes that can survive for a long time in the soil, on wood and root debris even in the absence of any living host (Pellegrini *et al.*, 2013). Because of the limited effectiveness and/or feasibility of conventional management tactics, biological control of Armillaria root rot has been pursued for a number of years. Species of the mycoparasite *Trichoderma*, in particular, have been studied closely. *Trichoderma* can replace *Armillaria* from buried woody inoculum and can suppress root rot caused by the pathogen in citrus orchards when used in combination with sub lethal fumigation (Cox, and Scherm 2006). Furthermore the study of Otieno *et al.* (2003_a), demonstrates the potential of solarization to control Armillaria root rot and confirms that efficacy of antagonistic strains of *T. harzianum* against the pathogen is greater when the antagonist is applied in combination with soil heating, and possibly organic amendments, in an integrated disease management strategy.

2.3 Mycorrhizae

In 1885 Albert Bernard Frank in his study of soil microbial-plant relationships, introduced the Greek term 'mycorrhizae', which literally means 'fungus roots (Siddiqui and Pichtel, 2008). Arbuscular mycorrhizal fungi are among the most abundant soil developing obligate symbiotic associations with the majority of plant families and occur in most terrestrial ecosystems (Gai *et al.*, 2012). In natural ecosystems, the majority of plants form mutualistic associations with arbuscular mycorrhizal fungi, soil inhabiting fungi of the phylum *Glomeromycota*. On a global basis, mycorrhizae occur in about 83% of dicotyledonous and monocotyledonous plants, and all Gymnosperms are mycorrhizal (Smith and Read, 2008). In the symbiosis, the fungus colonises the roots, and forms differentiated arbuscules within cortical cells (Cicatelli *et al.*, 2012). These symbiosis is often mutualistic based largely on exchange of carbon from the plant and Phosphorous delivered by the fungi (Smith and Smith, 2011).

Generally, the fungus is strongly or wholly dependent on the higher plant, and obtain all their organic carbon requirements from their plant partners (Veresoglou *et al.*, 2012). For plants, mycorrhizal associations are either mutualistic or parasitic depending on a range of factors. For example, the availability of N together with C and P may determine which role (mutualistic or parasitic) the mycorrhizae will play in the symbiosis with its host plant (Johnson, 2010). In a P-rich environment, N enrichment can cause a shift to parasitism in the AMF symbiosis since the plant is not limited by below ground resources and will allocate its resources into above ground production instead. N enrichment of P-poor soils on the other hand, can induce a mutualistic AMF symbiosis since the addition of N will force the plants to seek more P supply in order to produce more biomass (Aleklett and Wallander, 2012). Arbuscular mycorrhizal

fungi are key components of soil microbiota and form symbiotic relationships with the roots of most terrestrial plants, improving the nutritional status of their host and protecting it against several soil-borne plant pathogens (Martínez-Medina *et al.*, 2011a). Furthermore, many plant species naturally establish mycorrhizal associations to resist abiotic and biotic stresses such as drought, salinity, cold and nutrient deficiency (Slama *et al.*, 2012).

2.3.1 Basics of arbuscular mycorrhizal fungal biology and taxonomy

Arbuscular mycorrhizal fungi establish a symbiotic relationship with roots of plants called mycorrhizae that are widely recognized as mediators between soil processes and plant community, by enhancing pathogen resistance of their hosts (Barrico *et al.*, 2012). Glomeromycetes (phylum Glomeromycota) are symbionts that form intracellular associations within the roots of most trees and herbaceous plants. Glomeromycetes were previously considered zygomycetes, but in 2002 taxonomists concluded that they form a separate monophyletic group. This phylogeny was based on comparison of ssRNA genes (Solomon, *et al.*, 2008). They belong mainly to four genera, *Acaulospora*, *Gigaspora*, *Glomus* and *Sclerocystis*. Arbuscular mycorrhizal fungi get their name from their characteristic formation of branching structures called arbuscules within the cortical cells of roots (Figure 2).

Arbuscules increase the contact area between plant and fungus and are thought to be the primary sites of exchange of the plant's carbon for the fungus's phosphorus (Bever *et al.*, 2001). The AMF is characterized by the formation of (i) intracellular structures (arbuscules or hyphal coils) within the cortex cells, (ii) intercellular hyphae in the cortex, and (iii) a mycelium that extends well into the surrounding soil (Smith and Read, 2008). It is now recognized that there are two types of AMF with respect to the structures formed in the cortex cells: Arum type mycorrhiza characterized by arbuscules and Paris type that form hyphal coils (Smith and Read, 2008). Arum-type arbuscules are highly dichotomized structures that are produced via the trunk hyphae in the lumen of a cell. Conversely, Paris-type arbuscules are coiled structures that grow in the root cortex intercellularly (Harper *et al.*, 2013). Interestingly, a given AMF can form either arbuscules or hyphal coils depending on the host plant (Dickson, 2004). The arbuscules and coils are the sites of solute exchange with the host, but they are short lived, being active for about 7 days. However, arbuscules are considered to be the part of the AMF symbiosis where most of nutrient exchange between plant and fungus occurs (Reidinger *et al.*, 2012).The hyphae of arbuscular mycorrhizal fungi are coenocytic. These fungi use glucose from their plant partners as their primary energy source, converting the glucose to other, fungus-specific

sugars that cannot return to the plant. Arbuscular mycorrhizal fungi reproduce asexually; there is not yet any direct evidence that they reproduce sexually (Sadava, *et al.*, 2011).

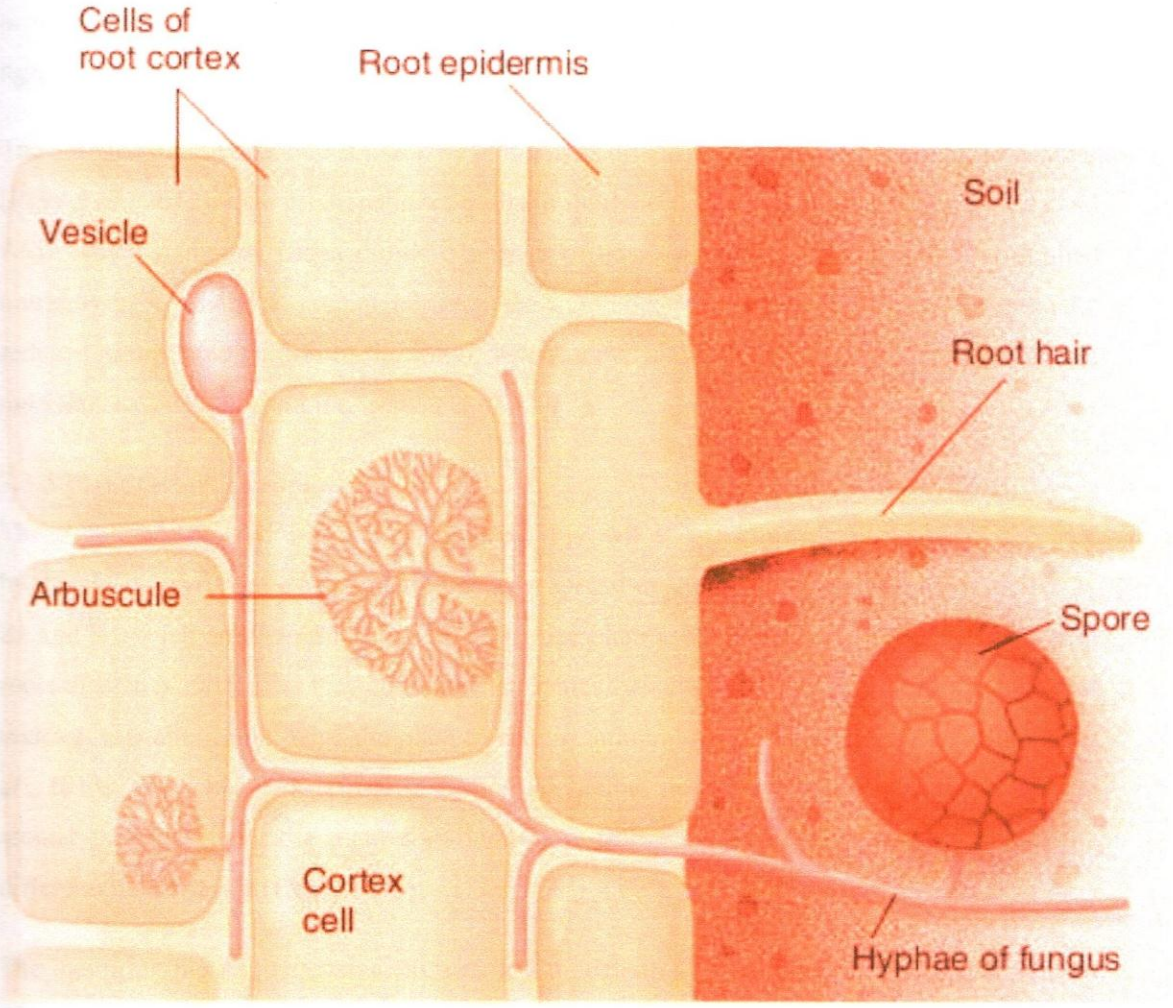


Figure 2: The structure of Arbuscular mycorrhizae

Source: Solomon *et al.*, 2008

2.3.2 Root Colonization

Arbuscular mycorrhizal fungi are obligate biotrophic fungi colonizing the roots of approximately 80% of all terrestrial plants (Vos *et al.*, 2012). Root colonization by mycorrhizal fungi can arise from three sources of inoculum: spores, infected root fragments and hyphae collectively termed propagules or from neighbouring roots of the same or different plants and plant species (Smith and Read, 1997). Colonization is enhanced by a pre-existing network in the soil. Therefore, soil disturbance is one factor which, through disrupting the extraradical mycelium and mixing surface residues into the soil profile selectively interferes with different

AMF, depending on their life and colonising strategies, promoting or impairing specific groups (Brito *et al.*, 2012). This suggests that AMF communities can differ in their response to agricultural disturbance and that the nature and intensity of the agricultural management system needs to be considered when drawing conclusions about AMF diversity and function in agroecosystems (Tian *et al.*, 2013).

The colonization of living host tissue by mutualistic organisms is a delicately balanced process (Plett *et al.*, 2012). One relationship of global importance is the interaction between plant and AMF which is mainly based on the exchange of host supplied carbon and fungus supplied nutrients in a feedback system between the symbionts: the plant supplies photosynthetic reduced assimilated carbon to the fungi, whereas the fungi increase the plant uptake of soil nutrients, mainly of N and P (Asensio *et al.*, 2012).

2.3.3 Plant Growth promotion

The importance of AMF in enhancing host plant growth is well known, and has been explained by the ability of fungal extraradical hyphae to spread in soil and take up nutrients, such as P, Zn and N, which are then translocated to the host plant roots (Piao *et al.*, 2012). AMF live in roots of most plant species they colonize biotrophically in the root cortex, and develop an extra-matrical mycelium that helps the plant to acquire mineral nutrients from the soil (Oztekin *et al.*, 2013). In addition to its role in carbon allocation, the establishment of a mycelial web around the roots from the plant community constitutes a diverse inoculum source for the different plant species (Read, 1998).

The intermingling and extensive extra-radical mycelium allows a more efficient exploitation of soil nutrients and water, thus benefiting the nutrient flow through the soil-fungus-plant system particularly relevant in arid ecosystems (Allen, 2007). In addition, arbuscular mycorrhizal fungi can benefit plants by, production of growth promoting substances, tolerance to drought, salinity and transplant shock and synergistic interaction with other beneficial soil microorganisms such as N-fixers and P-solubilizer (Eftekhari *et al.*, 2012). Moreover both laboratory and field studies provide evidence that mycorrhizal fungi are able to solubilize silicate and carbonate minerals to promote plant growth and precipitate oxalate crystals in the hyphae (Sanz-Montero and Rodriguez-Aranda, 2012).

The most distinct effect of AMF on plant growth is the improved supply of nutrients of low mobility in the soil solution, particularly phosphorus. External hyphae can absorb and transfer phosphorus to the host from soil beyond the rhizosphere depletion zone (Smith and Read,

2008). Due to an increased nutrient uptake, plants forming mycorrhizal associations are often bigger than non-mycorrhizal plants (Smith and Read, 1997). However, plants do not always benefit from AMF and negative plant growth responses to AMF colonization can occur when the net costs of the symbiosis exceed the net benefits (Gange and Ayres, 1999). Individuals of the same plant species frequently vary in AMF colonization levels and in the composition of AMF communities, and the relationship between AMF colonization levels and plant growth responses can range from positive to negative (Reidinger *et al.*, 2012). Moreover, Garrido *et al.* (2010) pointed out that plant biomass allocation can be affected by AMF colonization levels, resulting in increased or decreased biomass allocation to different plant parts. The benefit plants derive from the association with AMF can further depend on the abiotic soil environment (Zaller *et al.*, 2011).

Symbiotic mycorrhizal fungi are crucial agents for plant growth and survival (Fukasawa, 2012). Root-associated fungal communities influence plant succession by facilitating nutrient uptake or otherwise altering plant growth and survival traits (Fujimura and Egger, 2012). AMF have a role in maintaining plant diversity in natural communities, contribute to organic matter cycling and have a strong capacity to mobilize both N and P absorbed by host plants (Piao *et al.*, 2012). For plants in arid environments, association with AMF usually help improve nutrient and water uptake and enhance growth rates (Martínez-García *et al.*, 2012). However, plants do not always benefit from AMF and negative plant growth responses to AMF colonization can occur when the net costs of the symbiosis exceed the net benefits Further, AMF species differ in their effects on plant growth, depending on the identity of both the fungus and the plant (Reidinger *et al.*, 2012)

2.3.4 Suppression of Root Pathogens

Plant diseases can be controlled by manipulation of indigenous microbes or by introducing antagonists to reduce the disease-producing propagules. Futhermore AMF provide defense against root pathogens (George *et al.*, 2012), tolerance of pathogens (Veresoglou *et al.*, 2012) and protection against various abiotic stresses, pests and diseases (Gaidashova *et al.*, 2012). With the increasing environmental and public health hazards associated with pesticides and pathogens resistant to chemical pesticides, AMF may provide a more suitable and environmentally acceptable alternative for sustainable agriculture and forestry (Siddiqui and Pichtel, 2008).

Several mechanisms may operate simultaneously in the enhanced resistance of mycorrhizal plants to soil pathogens. In addition to a possible competition for photosynthates between the AMF and the pathogen, competition for colonization sites has been demonstrated (Pozo *et al.*, 2010). There are many examples of suppression of soil-borne fungal and bacterial root pathogens by inoculation with mycorrhizae. For example, inoculation with AMF provide biological protection against certain soil-borne pathogens of tomato (*Solanum lycopersicum* L), onion (*Allium cepa*), and watermelon (*Citrullus lanatus*) (Ortas, 2012), and protection from other microbial pathogens (Gai *et al.*, 2012). This suppressing effect of AMF is also evident in banana where mycorrhizae fungi reduce the severity of diseases caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *cubense* and *Cylindrocladium spathiphylli*. AMF were also reported to suppress population build-up of the plant-parasitic burrowing nematode *Radopholus similis* in the roots of various banana genotypes (Vos *et al.*, 2012). Plant parasitic nematodes parasitize roots and/or stems of various plants inhibiting plant absorption of nutrients and moisture (Fujimoto *et al.*, 2010). They infect more than 2000 plant species, including almost all cultivated plants, and reduce world crop production by about 5%. Losses in individual fields may become much higher (Moosavi *et al.*, 2010). Biological control using microbial antagonists is one potential alternative to chemical nematicides. Among the biological control agents that have been assessed are egg-parasitic fungi, nematode-trapping fungi, bacteria, and polyphagous predatory nematodes (Burkett-Cadena *et al.*, 2008). More recently, the antagonistic action of mycorrhizal fungi has been demonstrated (Mateille *et al.*, 2010). Asensio *et al.* (2012) reported that AMF enhance plants defences against nematodes.

2.4 *Trichoderma* species

The genus *Trichoderma* (Ascomycetes, Hypocreales) was first described by Persoon more than 200 years ago, and consists of anamorphic fungi that mainly inhabit soil, organic matter, and decaying trees (Lopes *et al.*, 2012). The genus *Trichoderma* is known to thrive under diverse environmental conditions among billions of aggressive competitors (Nitta *et al.*, 2012). Their ability to survive in different regions can be attributed to diversified metabolic capabilities and natural competitive aggression (Lopes *et al.*, 2012). The beneficial effect of *Trichoderma* is due to a complex of different mechanisms such as: direct mycoparasitism, antibiotic production, nutrient and space competition, enhancement of plant resistance to pathogens and systemic induced or acquired plant resistance (López-Mondéjar *et al.*, 2012).

2.4.1 *Trichoderma*–pathogen interaction

Trichoderma species are free-living fungi that are common in soil and root systems and are well known to solubilize phosphates and micronutrients (Saravanakumar *et al.*, 2013). *Trichoderma* is a secondary opportunistic invader, a fast growing fungus, a strong spore producer, a source of cell wall degrading enzymes and an important antibiotic producer (Vinale *et al.*, 2008). Their ability to synthesize several antagonistic compounds (proteins, enzymes and antibiotics) and micro-nutrients (vitamins, hormones and minerals) improve the biocontrol activity (Wijesinghe *et al.*, 2011). The mechanisms involved in reducing the severity of plant diseases include mycoparasitism, nutrient competition, rhizosphere competence, cell-wall degrading enzymes production, as well as induced defense responses in plants (Munoz-Celaya *et al.*, 2012). Their biological control activity is also attributable to various antimicrobial compounds and their aggressive mode of growth and physiology (Silva Aires *et al.*, 2012). *Trichoderma* species are also able to suppress enzymes secreted by pathogens as pectinases, glucanases and chitinases, through the action of protease secreted on plant surfaces. Thus, it is possible that a combination of several modes of action may be responsible for plant protection afforded by distinct *Trichoderma* strains against different diseases (Fontenelle *et al.*, 2011).

2.4.1.1 Mycoparasitism and lytic enzymes

The complex process of mycoparasitism consist of several events, such as recognition of the host, attack and subsequent penetration and killing. During this process *Trichoderma* secretes hydrolytic enzymes that hydrolyze the cell wall of the host fungus, subsequently releasing oligomers from the pathogen cell wall (López-Mondéjar *et al.*, 2011). Biological control is a complex process that includes recognition of the host by *Trichoderma*, followed by hydrolytic enzymes and antibiotics production triggered by the direct attachment of the mycoparasite to the host fungi (Steindorff *et al.*, 2012). After recognizing the presence of a potential host fungus, *Trichoderma* inhibits or kills the plant pathogen by parasitizing its hyphae, thereby employing hydrolytic enzymes like chitinases and glucanases to degrade the host's cell wall (Reithner *et al.*, 2007). It is believed that *Trichoderma* have the ability to produce hydrolytic enzymes at a constitutive level and detects the presence of another fungus by sensing the molecules released from the host by enzymatic degradation (Figure 3) (Vinale *et al.*, 2008). Moreover, some *Trichoderma* strains secrete hydrolytic enzymes that directly antagonize plant-pathogenic fungi (Morán-Diez *et al.*, 2012). Because of their aggressive lytic capacity, *Trichoderma* strains are involved in the degradation of complex organic compounds in soil. Furthermore, many *Trichoderma* isolates are known for their ability to suppress different

fungal plant pathogens, e.g. *Botrytis cinerea*, *Fusarium* spp., *Phytophthora cactorum*, *Pythium* spp., *Rhizoctonia solani*, and *Verticillium dahlia* (Meincke *et al.*, 2010). In addition to these properties, the ability of certain *Trichoderma* species to induce plant resistance against some plant pathogens, promote plant growth and improve photosynthetic activity of plants greatly boost these microorganisms' biological arsenal (Mbarga *et al.*, 2012). The antagonistic mechanisms of *Trichoderma* species involve the release of lytic enzymes that degrade fungal cell walls and competition with other microbes for space and nutrients (Savazzini *et al.*, 2009).

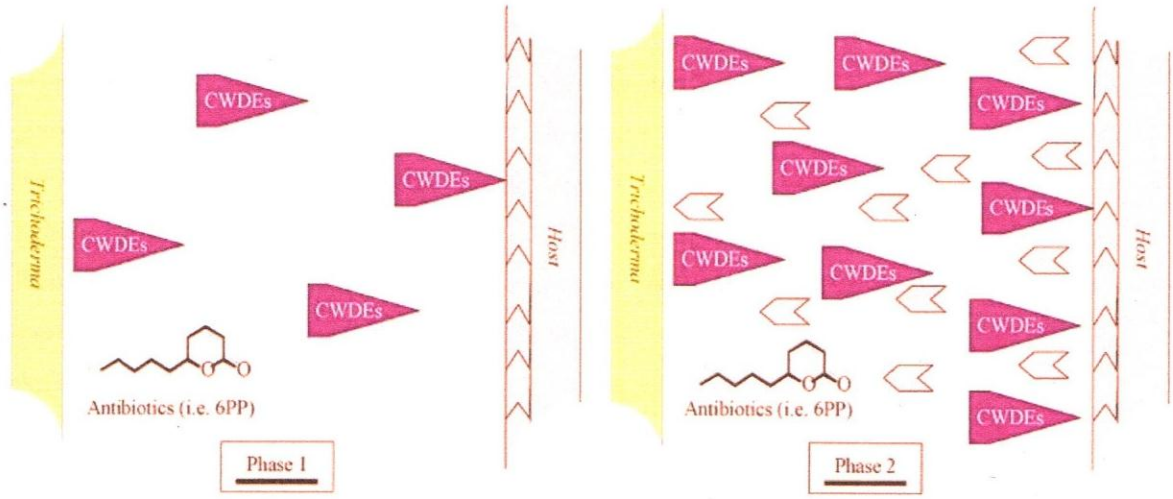


Figure 3: The pre-contact events of the mycoparasitic interaction of *Trichoderma*-host fungus.

Phase 1: The mycoparasite produces high molecular weight compounds that reach the host. **Phase 2:** low molecular weight-degradation products that are released from the host cell walls reach the mycoparasite and activate the mycoparasitic gene expression cascade.
Source: Vinale *et al.*, 2008.

The antifungal arsenal of *Trichoderma* species includes a great variety of lytic enzymes most of which play a great role in biocontrol. Many cell wall-degrading enzymes (CWDEs) from different *Trichoderma* strains have been purified and characterized. Interestingly, when tested alone or in combinations, the purified proteins showed antifungal activity towards a broad spectrum of fungal pathogens (Vinale *et al.*, 2008).

2.4.1.2 Antibiosis and secondary metabolites

Trichoderma species have been studied extensively as potential sources of biocontrol agents and reported to produce a plethora of secondary metabolites showing anti-microbial activity (Anees *et al.*, 2010). The term ''secondary metabolite'' includes a heterogeneous group of

chemically different natural compounds possibly related to survival functions for the producing organism, such as competition against other micro and macro organisms (Vinale *et al.*, 2008). There is increasing circumstantial evidence implicating the existence of secondary metabolites as antibiotics that contribute to their biocontrol activities. The genus *Trichoderma* has afforded many types of bioactive substances including trichothecenes, gliotoxin, and other metabolite groups such as peptides, pyrones, lactones, isonitriles, and peptaibol (Li *et al.*, 2011). Their capability to synthesize different antagonistic compounds such as antibiotics enhance the biocontrol activity (Wijesinghe *et al.*, 2011).

2.4.1.3 Competition with pathogens and soil microbial community

The competitive interactions between *Trichoderma* and other microbes are complex, encompassing competition for nutrients, action of lytic enzymes as well as antibiosis/symbiosis and fungistasis (Klein and Eveleigh, 1998). Some strains are strongly rhizosphere competent, which permits them to colonize roots, grow, and persist on roots and to provide long term benefits in terms of plant health and productivity (Harman *et al.*, 2004_a). Competition for carbon, nitrogen and other growth factors, together with competition for space or specific infection sites, may be also used by the BCA to control plant pathogens (Vinale *et al.*, 2008).

2.4.2 *Trichoderma*–plant interaction

Trichoderma species are recognized as cosmopolitan soil fungi, colonizing a wide range of habitats and ecological niches. These soil fungi are efficient and widely used biological control agents which have been shown to enhance nutrient uptake by plants (Schuster and Schmoll, 2010; de Santiago *et al.*, 2013). Positive results of inoculation with *Trichoderma*, showing biological control capacity, have been reported by increases in biomass as well as nutrient absorption in a broad range of plants including cocoa (*Theobroma cacao* L.), corn (*Zea mays* L.), cucumbers (*Cucumis sativus* L.), garlic (*Allium sativum* L.) and tomatoes (*Solanum lycopersicum* L) (de los Santos-Villalobos *et al.*, 2013). The soil application of *Trichoderma* strains has been demonstrated experimentally to increase the number of roots, thereby increasing the plant's ability to resist drought (Kapri and Tewari, 2010).

2.4.2.1 Plant root colonization

Plant growth and health are strongly influenced by microorganisms colonizing roots and inhabiting the rhizosphere (Mansfeld-Giese *et al.*, 2002). Some species of *Trichoderma* can form intimate associations with plant roots, providing an endemic level of biological control or stimulating plant growth by producing soluble forms of mineral nutrients and growth-

promoting metabolites (Hoyos-Carvajal *et al.*, 2009). The physical interaction between *Trichoderma* and the plant was observed by electron microscopy to be limited to the first few cell layers of plant epidermis and root outer cortex (Yedidia *et al.*, 1999). The hyphae of the BCA penetrate the root cortex but the colonization by *Trichoderma* is stopped, probably by the deposition of callose barriers by the surrounding plant tissues (Vinale *et al.*, 2008). The penetration process of *Trichoderma* into the roots of plants is associated with the ability of the fungus to secrete an arsenal of hydrolytic enzymes which degrade the cell wall, including cellulase. These enzymes are capable of inducing defence mechanisms in plants, probably due to the ability to release fragments of cell wall in plants (Fontenelle *et al.*, 2011). It appears that this interaction evolves into a symbiotic rather than a parasitic relationship between the fungus and the plant, whereby the fungus occupies a nutritional niche and the plant is protected from disease (Vinale *et al.*, 2008). According to Harman *et al.* (2004_a), in rhizosphere-competent strains that grow continuously with the plant, long-term systemic resistance can occur. Even though some *Trichoderma* species grow only on roots, the plant defence reactions can become systemic and protect the entire plant from a range of pathogens and diseases. Besides, the root colonization increases the growth of the entire plant and thus results in an increase in plant productivity and the yields (John *et al.*, 2010).

Plants also derive numerous advantages from root colonization by these opportunistic root symbionts. These include the following:

- Protection of plants against diseases by direct action of the *Trichoderma* strains on pathogenic microbes. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, b-1,3 glucanases and proteases (Gajera, and Vakharia, 2010)
- Protection against plant pathogens because of enhancement of plant resistance to pathogens and systemic induced or acquired plant resistance (López-Mondéjar *et al.*, 2012). For example, through induced resistance, *Trichoderma* strains can control foliar pathogens even when it is present only on the roots (Harman *et al.*, 2004_a).
- Enhancement of plant growth and development, especially of roots. The activity of Trichderma spp. added to soil increases plant growth and development. Some species of *Trichoderma* can form intimate associations with plant roots, providing an endemic level of biological control or stimulating plant growth by producing soluble forms of mineral nutrients and growth-promoting metabolites (Hoyos-Carvajal *et al.*, 2009).

2.4.2.2 Plant growth promotion

Many BCAs, such as fungi, bacteria and viruses, are not only able to control the pathogens that cause plant disease, but are also able to promote plant growth and development. *Trichoderma*-based Biocontrol Agents (BCAs) have an ability to promote plant growth (Wijesinghe *et al.*, 2011). Growth stimulation is evidenced by increases in biomass, productivity, stress resistance and increased nutrient absorption. Increased crop productivity associated with the presence of *Trichoderma* has been observed in a broad range of species, such as carnation (*Dianthus caryophyllus* L), chrysanthemum spp., petunia spp., cucumber (*Cucumis sativus* L.), eggplant (*Solanum melongena* L), pea (*Pisum sativum* L), pepper (*Capsicum annuum* L), radish (*Raphanus sativus* L), tobacco (*Nicotiana tabacum* L), tomato (*Solanum lycopersicum* L), lettuce (*Lactuca sativa* L), carrot (*Daucus carota* L), corn (*Zea mays* L.), poppy (*Papaver somniferum* L), cotton (*Gosyppium* spp.), millet (*Eleusine coracana*), bean (*Phaseolus vulgaris* L), cocoa (*Theobroma cacao* L.), and ornamental grasses (Hoyos-Carvajal *et al.*, 2009). *Trichoderma harzianum* has the ability to directly enhance root growth and plant development in the absence of pathogens and it has been suggested that this could be due to the production of some unidentified growth-regulating factors by the fungus (Sofo *et al.*, 2012). Shukla *et al.* (2012) pointed out that root and shoot length was markedly increased in response to treatment with drought tolerant isolates of *Trichoderma*, prior to altering the water cycle. Vinale *et al.* (2008) suggested that the secondary metabolites such as auxin like compounds or auxin inducing substances by *Trichoderma* -plant interaction might be a reason for the improved growth

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The study was carried out in two sites in Sururu forest located in Eastern Mau, one of the five main Forest Reserves of Mau Forest Complex (Figure 4). Mau Forest Complex is the largest closed-canopy forest ecosystem in Kenya and the largest indigenous montane forest in East Africa, covering an area of more than 400,000 hectares. The forest area has some of the highest rainfall rates in Kenya. Eastern Mau is located about 205 km North West of Nairobi. Site 1 was located in Sururu forest, Gatimu area (2716 m above sea level, 00°38.862 S and 036°01.467 E) where the forest is closed. Site 2 was located in Sururu forest, Mwisho wa Lami area (2814m above sea level, 00°37.089 S and 036°00.012 E) where vegetation is patchily distributed. According to the ground meteorological observation data of a weather station located in Sururu forest, the climatic characteristics in 2011 were as follows: Annual mean temperature was 15°C-24°C, depending on elevation. The average annual rainfall inside the forest was about 1400 mm.

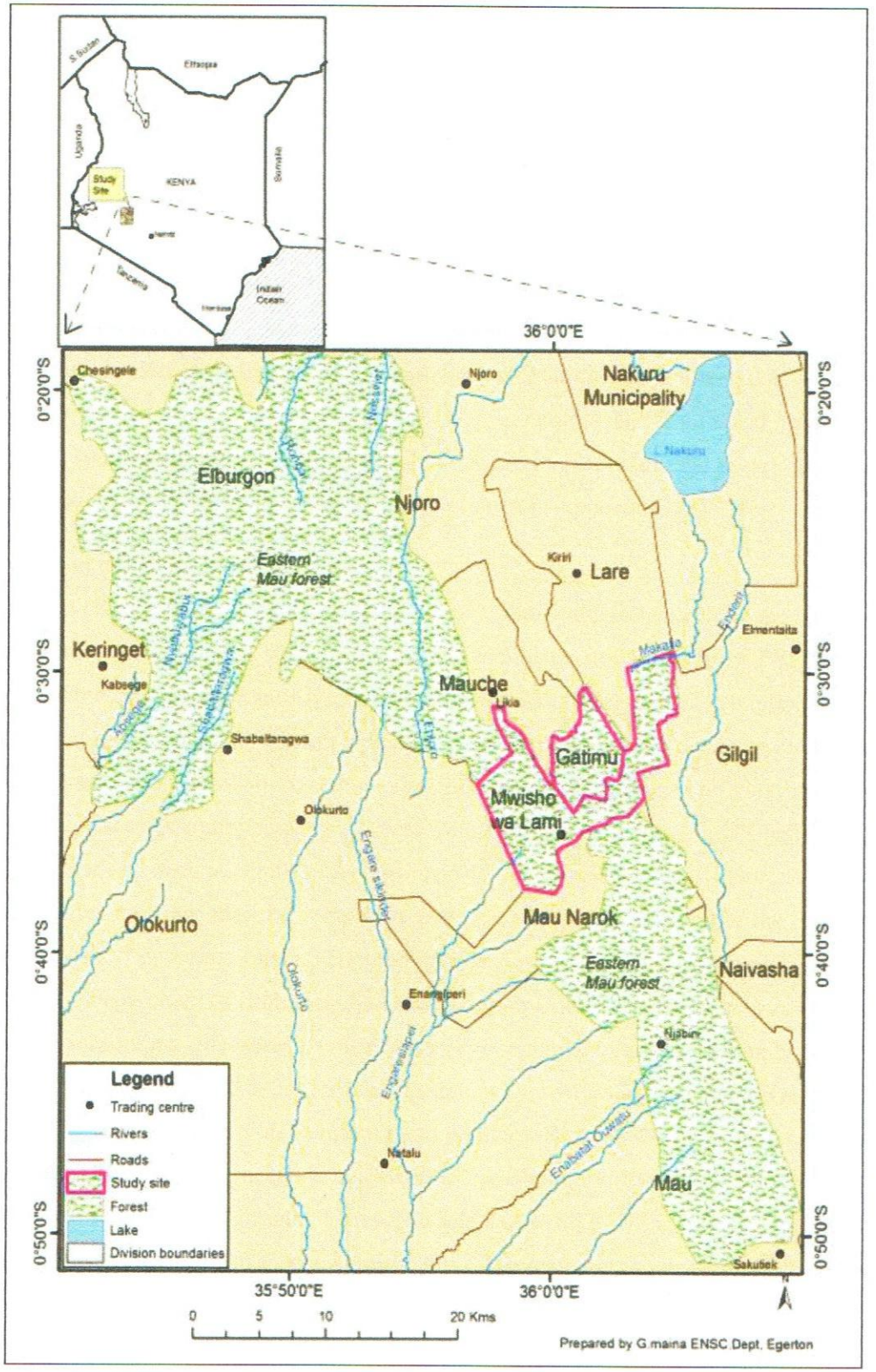


Figure 4: **A section of the Mau Forest Complex showing study site**

3.2 Study plants

Ten woody indigenous tree species were selected among the plants in Sururu forest of the Mau Forest complex ecosystem as the study plants. These plants included *Podocarpus falcatus*, (Thunb.) R.Br. ex Mirb., *Podocarpus latifolius*, (Thunb.) R.Br. ex Mirb., *Olea capensis* (Baker) Friis & P.S. Green, *Olea europaea* subsp. *africana* (Mill.) P. Green, *Prunus africana* (Hook f.) Kalkman, *Hagenia abyssinica* (Bruce) J. F. Gmel, *Juniperus procera* Hochst. ex Endl., *Dombeya torrida* (J.F.Gmel.) Hepper et Friis, *Maytenus senegalensis* (Lam.) Exell and *Rapanea melanophloeos* (L.) Mez. (Thabile Lukhele). All these plants are reported to be multipurpose, threatened and suitable for enrichment planting and reforestation activities. These trees are known to have considerable socio-economic and ecological importance (Michelsen, 1992; Wubet *et al.*, 2003). A list of these species, plant families, growth-habit, size of propagules, habitat or ecology and usage are presented in Appendix 1.

3.3 Collection of soil and root samples

In April 2011 (Rainy season), roots and their rhizosphere soil were collected from the two study sites. Five trees were sampled from each tree species in each study site. A total of 190 root samples and their rhizosphere soils were collected using a stratified random sampling method recommended by Schleuß and Muller (2001). One hundred samples were gathered in Gatimu and ninety samples in Mwisho wa Lami. The roots and their rhizosphere soils of the ten indigenous tree species comprising eight genera were collected in their natural habitats using a 60mm diameter soil corer at depths of 0–15 cm and 15–30 cm (Bertini *et al.*, 2006; Birhane *et al.*, 2010), after ensuring that the roots were connected to plants sampled at the two sites described above. Taxonomic identification of species was made with the assistance of Plant taxonomist in Department of Biological Sciences, Egerton University. Five replicates of roots and their corresponding rhizosphere soils of each representative tree were randomly sampled at standardized distances of 0.5–3m for each sample at the two sites. Equipment was cleaned with water between samples so as to remove soil particles. Rhizosphere soils were collected, put in paper bags to avoid desiccation and taken to the laboratory. Part of the root system of each plant was fixed in 5 ml formalin, 5 ml acetic acid and 90 ml of 70% alcohol, diluted twice (1/2 FAA), and stored at 4°C (Tao and Zhiwei, 2005). The remaining roots were air-dried with their rhizosphere soil for 2 weeks, and then stored in sealed plastic bags until samples could be further processed.

Sampling was done during the long rains and therefore moisture was observed in almost all the soil samples collected. Earthworm species and other soil fauna were found on soil samples in

association with partially decomposed litter, manure and other organic matter (Plate 1). During soil sampling an excessive accumulation of litter was observed under the canopy of *D. torrida* and *H. abyssinica* at the two study sites (Plate 2).

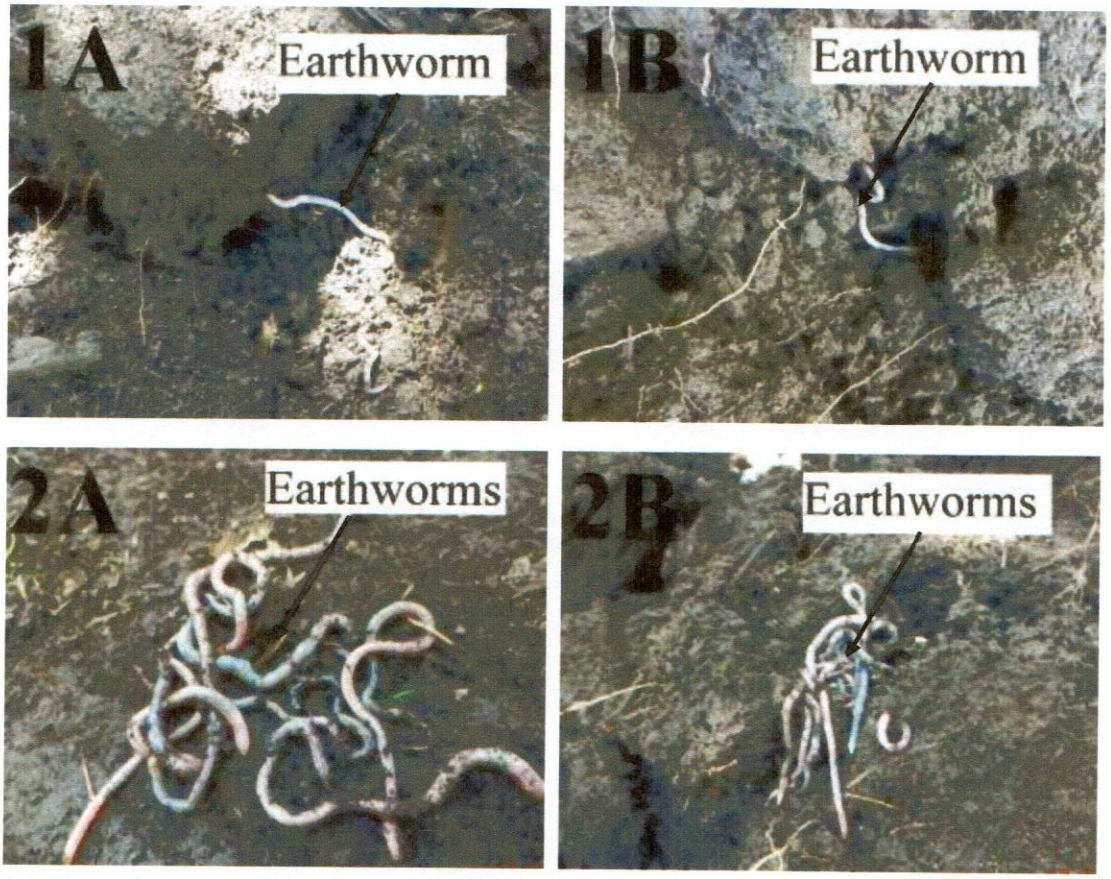


Plate 1: Earthworm species found on soil samples collected in Sururu forest in April 2011 1A-1B Earthworm in Gatimu and Mwisho wa Lami respectively. 2A-2B collected earthworm species in Gatimu and Mwisho wa Lami respectively

Plate 2: Accumulation of leaf litter under the canopy of *Dombeya torrida* and *Hagenia abyssinica* trees in Sururu forest.

1A-1B: under the canopy of *H. abyssinica* in Gatimu and Mwisho wa Lami respectively.
2A-2B: under the canopy of *D. torrida* in Gatimu and Mwisho wa Lami respectively.

3.4 Physical and chemical analysis of soil samples

Soil physico-chemical parameters like soil pH, electrolytic conductivity, available potassium, total nitrogen, total available P and soil organic carbon were analysed at Soil Science Laboratory, Kenya Forestry Research Institute (KEFRI), Nairobi, Kenya following standard procedures. Soil pH was measured in deionized water with a soil water ratio of 1:2 and in calcium chloride with 20g air-dry soil sample mixed with 50ml of a dilute concentration (0.01M) of calcium chloride ($CaCl_2$), shaken for 1 hour and the pH was measured using an electrode. Organic matter was determined using Walkley–Black and total nitrogen was determined using Kjeldahl (Bremmer and Mulvaney, 1982). Electrical conductivity (EC) was

measured in a 1:5 (w/v) aqueous solution (Faithfull, 2002). Atomic absorption spectrophotometry was used to quantify Ca^{2+}, Mg^{2+}, and K^{+} in the water extracts. Magnesium was extracted in a 1M ammonium acetate solution according to NF X31-108 and magnesium concentrations were measured using atomic absorption flame. Available P was extracted with 0.5 N $NaHCO_3$ and P concentration was evaluated colorimetrically according to Olsen and Sommers (1982).

3.5 Mycorrhizal root colonisation assessment

Roots were washed carefully with tap water and cut into 1cm-long segments. About 0.5g root segments were cleared in 10% (w/v) KOH at 90°C in a water bath for 2–3h, the time depending on the size of the roots and their pigmentation. After cooling, the root samples were washed and stained with 0.05% (w/v) Trypan blue (McGonigle *et al.*, 1990). Thirty 1cm-long root segments were mounted on slides in a polyvinyl alcohol–lactic acid–glycerol solution (Koske and Gemma, 1989) and examined at 100× magnification under a compound microscope. Frequency of mycorrhizae in the root system (F%) and intensity (M%) of infection and arbuscule abundance in the root system (A%) were estimated in stained root samples stained after washing with tap water according to Phillips and Hayman (1970). This gave values for total fungal infection and arbuscular development.

3.6 Spore assessment

Spores from the rhizosphere soil samples were isolated through the wet-sieving method described by An *et al.* (1990). Twenty grams of soil sample was weighed and mixed in water in a small plastic container having the capacity of about 1500 ml by stirring thoroughly before decanting through 710µm and 45-µm sieves. Later, the sediments collected on the 45µm sieve were washed into 50 ml centrifuge tubes and centrifuged for 5 min at 1,750 rpm. Water from the tubes was decanted to discard floating debris. Sucrose (48%, w:v) was added to the tubes, mixed thoroughly before centrifuging for 15s at 1,750 rpm. Immediately after centrifugation, sucrose solution was decanted through 45µm sieve. The spores retained on the sieve were rinsed thoroughly with water to wash out the sucrose and later transferred into a Petri dish. Healthy spores were counted under microscope at ×100 magnification. Each spore type was mounted sequentially in Polyvinyl Lactophenol Glycerol (PVLG) (Morton, 1988) and PVLG mixed 1:1 (v/v) with Melzer's reagent for identification (Uhlmann *et al.*, 2006). The spores were examined microscopically and identified down to the genus level or species. The identification was based on spore size, color, surface ornamentation, wall structure as well as

presence and absence of subtending hyphae with reference to the descriptions provided by INVAM (2010) and following descriptions given by Brundrett *et al.* (1996).

3.7 Numbers and distribution of AMF spores

Ecological measures of diversity used to describe the structure of AMF communities included the following indices: spore density, species richness, relative abundance, frequency of occurrence, Shannon–Wiener index of diversity, evenness, Simpson's index of dominance, and Sorenson's coefficient (Zhang *et al.*, 2004; Dandan, and Zhiwei, 2007; Shi *et al.*, 2007; Tian *et al.*, 2011; Gai, *et al.*, 2012) (Table 1). Spore density reflected the total number of spores occurring in 20g soil, species richness (SR) was defined as the number of identified AMF species per 20g soil sample, relative abundance (RA) = (number of spores of a species or genus/total spores) × 100%; isolation frequency (IF) = (number of samples in which the species or genus was observed/total samples) × 100%. Diversity within AMF community and evenness were reflected by Shannon–Wiener index of diversity. Sorenson's coefficient was used to compare similarity existing in the general structure of AMF communities between the two sites. Since only a few spores of one species were isolated, or the collected spores lacked distinguishable fine taxonomic characters, these spores could not be identified to species level and were not considered in the statistical analyses, except as part of total spore density

Table 1: Diversity measures used to describe AMF communities

Diversity measure	Description
Spore density (SD)	The number of spores in 20 g soil sample
Species richness (SR)	The number of identified AMF species per 20g soil sample
Relative abundance (RA)	$RA = \frac{\text{Spore numbers of a species (genus)}}{\text{the total number of identified spore}} \text{X } 100\%$
Isolation frequency (IF)	$IF = \frac{\text{The number of soil samples where a species occurred}}{\text{The total number of soil samples}} \text{X}100\%$
Shannon–Wiener index of diversity (H')	$H' = -\sum P_i \ln P_i$
Evenness (E)	$E = \frac{H'}{H'_{\max}}$
Simpson's index of dominance (D)	$D = \sum [n_i(n_i - 1)/N(N-1)]$
Sorenson's coefficient (C_s)	$C_s = 2j/(a+b)$

P_i is the relative abundance of each identified species per sampling site and calculated by the following formula: $P_i = n_i/N$ and $n_i =$ number of individuals in species I; N is the total number of individuals in all species. $H'_{\max}$ is the maximal H and calculated by the following formula: $H' = \ln S$, where S is the total number of identified species per sampling site. a or b was the total number of identified species per sampling site and j was the number of identified species common to both sites.

(Zhang *et al.*, 2004; Dandan, and Zhiwei, 2007; Shi *et al.*, 2007; Tian *et al.*, 2011; Gai, *et al.*, 2012)

3.8 Source of fungal inoculants

3.8 1 Source of *Armillaria* species

Armillaria species initially isolated from a severely infected *Dombeya torrida* plant (plate 3). The species was isolated using Armillaria selective medium (ASM) and maintained on malt extract agar medium at room temperature.

Plate 3: *Armillaria* species fruiting at the base of tree

A- Basidocarps (mushrooms) produced by *Armillaria* from severely infected tree

B- Stumps colonized by *Armillaria* species

3.8.2. Source of biocontrol agents

The inoculum of AMF and *Trichoderma* isolates were provided by Homegrown (K) Limited-Naivasha. In order to obtain fresh active cultures of *Trichoderma* isolates for *in-vitro* test, isolates were sub-cultured on MEA plates and incubated at 25°C for 7 days.

3.9 Source of potting medium and host plants

The dark brown loam soil collected from Sururu forest of Eastern Mau (2716 m above sea level, S 00°38.862 and E 036°01.467), was sieved (4 mm), mixed with river sand and compost manure (1:1:1) and sterilized by autoclaving for 1 h at 121°C twice, on two consecutive days (Toshihiro *et al.*, 2004). *Dombeya torrida* was used as the host plant. The seeds of these plants were collected from Mau forest complex.

3.10 Experimental design and biological treatments

Two experiments were conducted to test the interaction between AMF and *Trichoderma* species. One experiment (Exp. 1) investigated interaction effects of *T. harzianum* and AMF on

fungal colonization, plant growth promotion and *Armillaria* species suppression. The other experiment (Exp. 2) evaluated effects of *T. asperellum* and AMF on fungal colonization, plant growth promotion and *Armillaria* species suppression. Both experiments had the same treatments as follows. (1) Control (c); (2) *Armillaria* species (A); (3) *Trichoderma* species (T); (4) Arbuscular mycorrhizae fungi (M); (5) *Trichoderma* species and Arbuscular mycorrhizae fungi (T + M); (6) *Trichoderma* species and *Armillaria* species (T+A); (7) Arbuscular mycorrhizae fungi and *Armillaria* species (M+A); (8) *Trichoderma* species and Arbuscular mycorrhizae fungi and *Armillaria* species (T + M +A). Each experiments was conducted in trial one and trial two. Each treatment had four replications and the experiment was laid out in a completely randomized design.

3.11 *In-vitro* study by dual culture interaction

Dual culture interactions between the *Trichoderma* species and the *Armillaria* species were conducted *in-vitro* using a slightly modified protocol used by Latha *et al.* (2011). Discs (5 mm diameter) of the pathogen culture were placed on side of Petri dishes containing MEA, 1 cm away from the edge and Petri dishes were incubated for 14 days at room temperature in the dark to allow the pathogen to grow to approximately 10mm in diameter. Subsequently, 5 mm diameter disc of *Trichoderma* species were cut from the edge of 3-day-old culture then positioned diametrically opposite to that of the *Armillaria* species equidistantly at 70 mm from each other. Four replications were maintained. Monoculture plates of the *Armillaria* species and *Trichoderma* species served as controls. All the plates were incubated at room temperature, and fungal growth was monitored daily for approximately 42 consecutive days. For the determination of ability of *Trichoderma* species to inhibit the mycelial growth of the pathogen, *Armillaria* species was sub cultured from dual culture interactions and placed on Petri dishes containing MEA. All the plates were incubated at room temperature, and fungal growth was monitored daily for approximate 27 consecutive days.

3.12 Greenhouse trials

The experiment was conducted in glasshouse at the department of Biological Sciences, Egerton University.

3.12.1 Inoculation of plants with fungal species

Inocula of mycorrhizal comprised 15 g of mycorrhizal inoculum (spores, hyphae and infected root systems) or 15 g of autoclaved inoculum plus 10 ml of 15g inoculum filtrate through a 25µm filter to correct the potential differences in microbial communities between mycorrhizal

and non-mycorrhizal pots (Wu and Zou, 2010). Inoculum of *Trichoderma* species comprising of powder formulation was prepared according to manufactures instruction (Dudu tech). Sterile water (100 mL) was added to the 10g of the formulation and mixed with a sterile stirring rod. The resulting suspension was introduced to potted *D. torrida* seedlings. The inoculations of *Trichoderma* species were done at 0, 30, 60, 90, 120 and 150 days from the beginning of the experiment in order to maintain sufficient populations of the antagonist in the soils and hence to favor biological control, as suggested by Knudsen *et al.* (1991). *Armilllaria* species inoculum was prepared by growing the culture on malt extract agar and incubating at 25°C for 14 days. Each plant was inoculated after every 1 month of growth by placing four agar plugs from the 14-day *Armilllaria* species (Baumgartner *et al.*, 2010). The inoculum was introduced to the root zone of potted plants. The three fungal inocula, *Armilllaria* species, *T. harzianum* and *T. asperellum* were applied singly or in combination according to the treatment.

3.12.2 Plant growth

Selected tree seeds were surface sterilized by immersing them in a 1% solution of sodium hypochlorite for 5 min, rinsing in sterile distilled water and air drying on sterile filter paper (Coskuntuna and Ozer, 2008). The seeds were then incubated in water agar plates at 28°C in the dark for two days. Five pre-germinated seeds of *D. torrida* seedlings were planted in pot filled with potting medium mixed with fungal inocula according to the treatment combination. After three weeks the seedlings were thinned to one seedling per pot. The experiment was laid out in a complete randomized design with eight treatments and four replicates. Final harvest was done after 28 weeks and the roots washed in sterile water.

3.12.3 Determination of colony forming units of *Trichoderma* species on *Dombeya torrida* rhizoplane

The estimation of colony forming units (CFUs) of *Trichoderma* species in the rhizoplane were determined using a slightly modified protocol used by Rosa and Herrera (2009). One gram fresh root tissue, previously disinfected in sodium hypochlorite solution for 6 min, was cut into pieces and transferred to test tubes containing 100 ml sterile distilled water. This serially diluted, and 0.1 ml of each dilution was finally plated on fresh *Trichoderma* selective medium (TSM) (Elad *et al.*, 1981). The plates were incubated for 14 to 18 hours at 25°C in the dark. There were five replicates for each plate. The population counts of *Trichoderma* colonies on each Petri dish were recorded, and data expressed as colony forming units (CFUs) per gram fresh root.

3.12.4 *Armillaria* species assay

At the end of the experiments plants were assayed for viability of *Armillaria* species using a slightly modified protocol used by Otieno *et al.* (2003a). One gram fresh root tissue, previously disinfected in sodium hypochlorite solution for 6 min, was cut into pieces and transferred to Armillaria semi-selective medium. Inoculated Petri dishes were incubated at room temperature in the dark for at least 21 days and observed for the growth of *Armillaria* species.

3.12.5 Measurement of plant parameters

At the end of the experiment plant height, shoot and root fresh and dry weights were measured. Roots were separated from the soil by washing. Shoot and root were dried using an oven at 105°C for 4 days and then dry weights were measured.

3.13 Data analysis

For all data (soil, plant growth parameters, mycorrhizal parameters), treatments were compared using one-way analysis of variances (ANOVA) ($p<0.05$) using Gen stat. The Pearson correlation coefficient was employed to determine the relationships between spore density and species richness, relative abundance and isolation frequency. Both experiments were conducted two times and data from the repeated trials were pooled. Data was subjected to ANOVA and treatment means were compared by Fisher's least significant difference (LSD) test. Correlation analyses with Pearson's correlation coefficients were used to determine if a relationship existed between soil microbial parameters and soil chemical properties.

CHAPTER FOUR

RESULTS

4.1 Soil Characteristics of the two study sites

Tables 2 and 3 summarize the main physical and chemical soil characteristics of the two sites. Soil units in both Gatimu and Mwisho wa Lami were moderately acidic. In Gatimu, pH (H_2O) ranged from 5.2 to 6.7 and pH ($CaCl_2$) ranged from 4.6 to 6.1, while in Mwisho wa Lami pH (H_2O) ranged from 5.8 to 6.8 and pH ($CaCl_2$) ranged from 4.8 to 6.3. The amounts of organic matter levels at the two sites were very high ranging from 3% to 6.6% and 3.7% to 8.6% in Gatimu and Mwisho wa Lami respectively. The amounts of potasium levels at the two sites were also very high averaging 5.06 me/100g and 3.034me/100g in Gatimu and Mwisho wa Lami respectively. All soils showed low electrical conductivity. The two sites exhibited low available phosphorus content (available P), ranging from 0.4ppm to 5.8 ppm in Gatimu and 0.6 ppm to 5.3 ppm in Mwisho wa Lami repectively. The two sites showed medium Magnesium and Calcium content. The amounts of Calcium levels averaged 6.553 me/100g and 9.361 me/100g in Gatimu and Mwisho wa Lami respectively. Magnesium levels averaged 1.606 me/100g and 1.9167 me/100g in Gatimu and Mwisho wa Lami respectively. Correlation analysis demonstrated that Organic C was positively significantly correlated with total N in both Gatimu and Mwisho wa Lami sites (Pearson product-moment correlation coefficient $r= 0.596$, and $r= 0.721$, $p<0.05$, respectively).

Table 2: Physical and chemical properties of the top 0–15 cm soil used in the study in Gatimu (G) and Mwisho wa Lami (ML)

Characteristics	Gatimu (G)		Mwisho wa Lami (ML)	
	Mean	SE	Mean	SE
pH (H_2O)	5.90	0.13	6.35	0.09
pH $CaCl_2$	5.31	0.12	5.81	0.14
Electrical conductivity (ms/cm)	0.24	0.02	0.25	0.01
Nitrogen (%)	1.36	0.12	1.32	0.13
Total organic carbon (%)	5.88	0.60	4.82	0.30
Available P (ppm)	4.03	0.65	2.80	0.53
Extractable K (ppm)	1258.01	74.21	1143.04	44.46
Calcium (ppm)	2332.88	342.17	3846.79	258.60
Magnesium (ppm)	369.56	29.45	434.87	26.92

The reported values were from composite samples of five sub-samples

Table 3: Physical and chemical properties of the top 15–30 cm soil used in the study in Gatimu (G) and Mwisho wa Lami

Characteristics	Gatimu (G)		Mwisho wa Lami (ML)	
	Mean	SE	Mean	SE
pH (H_2O)	6.10	0.15	6.15	0.11
pH $CaCl_2$	5.36	0.15	5.50	0.15
Electrical conductivity	0.27	0.03	0.28	0.02
Nitrogen (%)	1.10	0.16	1.10	0.16
Total organic carbon (%)	4.34	0.30	4.39	0.42
Available P	2.25	0.56	3.26	0.54
Extractable K	1365.70	85.49	1229.20	55.11
Calcium (ppm)	2920.13	314.07	3678.21	321.23
Magnesium (ppm)	411.42	41.04	452.79	34.94

The reported values were from composite samples of five sub-samples

Soil chemical analytic data on nitrogen, organic carbon and and available phosphorous are presented in Tables 4, 5, 6, and 7. The highest value of total nitrogen, organic carbon and and available phosphorous were detected in soil samples collected from the root zone of *H. abyssinica* and *D. torrida*, respectively, while the lowest nitrogen organic carbon and total available phosphorus was measured under *Maytanus senegalensis* and *Rapanea melanophloease.*

Table 4: Some chemical characteristics of the top 0–15 cm soil of the tree species/site of sampling used in the study from Gatimu (G)

Tree species	Organic C			Available P	
	Total N (%)	C%	C/N ratio	mg kg^{-1}	P/N ratio
P. falcatus	1.00	6.60	6.60	5.50	5.50
P. latifolius	1.30	6.70	5.15	5.80	4.46
O. capensis	1.70	5.50	3.24	4.20	2.47
O. europaea	0.80	3.90	4.88	3.90	4.88
P. africana	1.30	6.90	5.31	3.00	2.31
H. abyssinica	1.97	7.80	3.96	6.40	3.25
J. procera	1.12	6.90	6.16	2.10	1.88
D. torrida	1.89	8.30	4.39	6.73	3.56
M. senegalensis	1.30	2.70	2.08	1.90	1.46
R. melanophloease	1.20	3.50	2.92	0.80	0.67

The reported values were from composite samples of five sub-samples

Table 5: Some chemical characteristics of the top 0–15 cm soil of the tree species/site of sampling used in the study from Mwisho wa Lami (ML)

Tree species	Organic C			Available P	
	Total N (%)	C%	C/N ratio	mg kg^{-1}	P/N ratio
P. falcatus	1.20	4.50	3.75	4.30	3.58
P. latifolius	1.33	4.70	3.53	1.10	0.83
O. capensis	1.27	5.00	3.94	1.30	1.02
O. europaea	0.87	4.50	5.17	2.10	2.41
P. africana	1.63	5.20	3.19	0.60	0.37
H. abyssinica	1.86	6.50	3.49	5.30	2.85
J. procera	1.11	6.10	5.50	3.40	3.06
D. torrida	1.86	5.80	3.12	4.80	2.58
M. senegalensi	0.70	3.40	4.86	2.70	3.86
R. melanophloease	1.20	3.80	3.17	3.00	2.50

The reported values were from composite samples of five sub-samples

Table 6: Some chemical characteristics of the top 15–30 cm soil of the tree species/site of sampling used in the study from Gatimu (G)

Tree species	Organic C			Available P	
	Total N (%)	C%	C/N ratio	mg kg^{-1}	P/N ratio
P. falcatus	0.97	4.30	4.43	1.00	1.03
P. latifolius	0.80	4.00	5.00	0.60	0.75
O. capensis	0.79	3.94	4.99	0.40	0.51
O. europaea	0.70	3.90	5.57	0.70	1.00
P. africana	1.67	3.60	2.16	1.10	0.66
H. abyssinica	1.98	5.60	2.83	4.70	2.37
J. procera	0.77	4.50	5.84	4.50	5.84
D. torrida	1.88	6.60	3.51	5.20	2.77
M. senegalensis	0.72	3.70	5.14	1.60	2.22
R. melanophloease	0.67	3.30	4.93	2.70	4.03

The reported values were from composite samples of five sub-samples

Table 7: Some chemical characteristics of the top 15–30 cm soil of the tree species of sampling used in the study from Mwisho wa Lami (ML)

Tree species	Organic C			Available P	
	Total N (%)	C%	C/N ratio	mg kg^{-1}	P/N ratio
P. falcatus	0.90	4.30	4.78	1.70	1.89
P. latifolius	0.70	5.40	7.71	4.50	6.43
O. capensis	0.90	4.40	4.89	4.20	4.67
O. europaea	0.80	3.70	4.63	0.60	0.75
P. africana	1.57	3.80	2.42	3.70	2.36
H. abyssinica	1.79	6.30	3.52	4.70	2.63
J. procera	0.67	3.70	5.52	1.30	1.94
D. torrida	1.75	5.90	3.37	5.40	3.09
M. senegalensis	0.52	3.10	5.96	2.40	4.62
R. melanophloease	0.97	2.60	2.68	2.10	2.16

The reported values were from composite samples of five sub-samples

4.2 Mycorrhizal status of the selected indigenous trees

Microscopic analysis of the mycorrhizal status of the selected indigenous trees from Mau forest complex showed that all the 10 plant species were colonized by arbuscular mycorrhizae fungi. Frequency of colonization (F%) and intensity of colonization (M%) were high in *D. torrida* and *H. abyssinica* and much lower in *R. melanophloease* and *M. Senegalensi* plant species (Tables 8). Intensity of mycorrhizal colonization of all replicates of the ten plants species ranged from 7 to 29% and the frequency ranged from 18 to 82%.

4.3 Spore density, species richness and the distribution of AMF

AMF spores were obtained from all rhizosphere soil samples, where low density of AMF spores was generally observed. A total of 3,966 spores of AMF were wet-sieved from the 190 rhizosphere soil samples collected in Sururu Forest from which 32 morphotypes were identified (Plate 3). Samples from Gatimu and Mwisho wa Lami yielded relatively low densities, ranging from 2 to 63 and 2 to 70 spores per 20g, respectively. Samples from the two sites were dominated by large spores followed by medium spores then small spores (Figure 5). Within the AMF spores obtained, 11% (419 spores) were small, 35% (1407 spores) were medium and 54% (2140 spores) were large.

Table 8: Frequency and intensity of mycorrhizal colonization of plants collected from Gatimu and Mwisho wa Lami

		Colonization			
		Gatimu		Mwisho wa Lami	
Plant species	Family	Frequency (F%)	Intensity (M%)	Frequency (F%)	Intensity (M%)
P. falcatus	Podocarpaceae	42.67±7.92	10.93±2.05	36.67±5.48	10.60±1.85
P. latifolius	Podocarpaceae	50.00±6.41	13.53±2.77	42.00±6.80	12.33±2.67
O. capensis	Oleaceae	50.67±7.41	17.07±2.80	55.33±5.54	17.00±2.85
O. europaea	Oleaceae	53.33±3.80	13.13±0.89	53.33±3.50	12.53±1.11
P. africana	Rosaceae	44.00±1.94	13.73±1.66	34.67±4.30	10.53±2.15
H. abyssinica	Rosaceae	74.00±3.86	25.13±2.93	66.00±5.62	24.27±2.26
J. procera	Cupressaceae	56.67± 4.08	18.87± 1.45	-	-
D .torrida	Sterculiaceae	81.33±4.90	29.20±3.79	82.00±3.43	26.40±2.97
M. senegalensi	Celastraceae	18.66±2.26	7.13±1.59	18.00±2.26	5.53±0.77
R. melanophloease	Myrsinaceae	20.00±4.08	5.86±0.97	22.67±5.31	7.2±1.89

The reported values were from composite samples of five sub-samples Mean ±S: E:

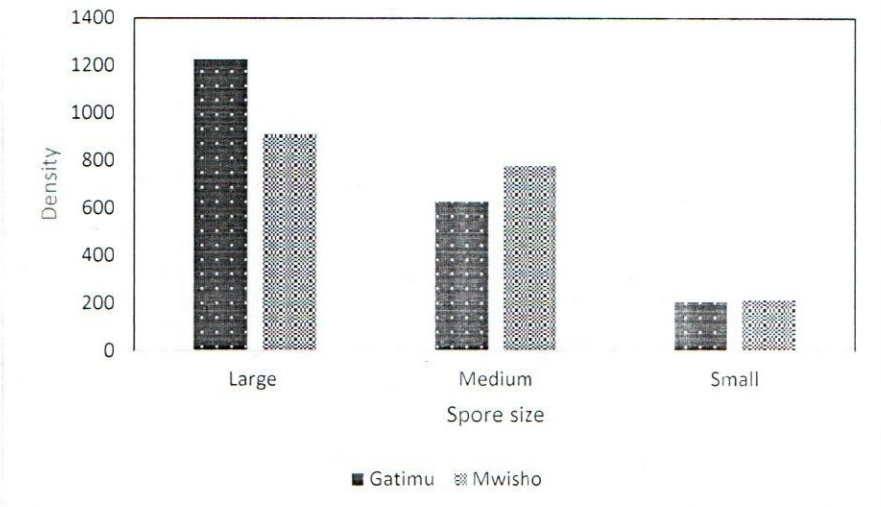


Figure 5: Spore size distribution in Gatimu and Mwisho wa Lami

Spore density, species richness and the distribution of AMF species in the rhizosphere soil of the 10 plant species are presented in Table 9. The average spore density of AMF showed similar trends in Gatimu (G) and Mwisho wa Lami (ML). In Gatimu (G) the mean density was 20.63 spores per 20g dry soil while the mean density in Mwisho wa Lami (ML) was 21.20 spores per 20g dry soil. The average species richness showed the same tendency: mean of 3.22 species (range of 1–5) in Gatimu and mean of 3.14 species (range of 1–6) in Mwisho wa Lami. Further, correlation analysis demonstrated that spore density of AMF was positively significantly correlated with species richness in both sites (Pearson product-moment correlation coefficient $r= 0.854$ and $r= 0.809$, $p< 0.050$, respectively).

Table 9: Average spore densities and species richness (SR) of AMF in soil samples of tree species collected in Mau forest

		Gatimu (G)		Mwisho wa Lami (ML)	
Plant species	Family	SR	ASD	SR	ASD
P. falcatus	Podocarpaceae	3.5± 0.23	19.10±2.16	3.9±0.31	21.90±1.30
P. latifolius	Podocarpaceae	2.3± 0.15	11.40± 1.07	2.8±0.13	15.3±1.78
O. capensis	Oleaceae	2.6± 0.16	12.80± 1.58	2.9±0.10	16.60±1.51
O. europaea	Oleaceae	2.7± 0.15	12.60± 1.55	3.2±0.133	15.90±2.19
P. africana	Rosaceae	3.8±0.13	19.90±2.02	2.6±0.31	10.4±1.63
H. abyssinica	Rosaceae	3.7±0.21	41.20±3.97	3.8±0.33	41.60±3.70
J. procera	Cupressaceae	4.1± 0.10	27.60±3.563	–	–
D. torrida	Sterculiaceae	4.5±0.17	42.30±3.63	3.8±3.37	50.40±3.37
M. senegalensi	Celastraceae	2.9±0.23	11.70±2.02	2.8±0.20	7.60±1.64
R. melanophloease	Myrsinaceae	2.1±0.23	7.70±1.16	2.5±0.31	11.10±1.72

Notes: ASD: Average AMF spore density (number of AMF spores in 20g soil) and species richness (SR) from the corresponding plant rhizosphere

One-way ANOVA showed that both spore density and species richness differed significantly between different plant species at both sites (Tables 10, 11, 12, and 13). There were also greater diversity and evenness indices of AMF in Gatimu than in Mwisho wa Lami. However Simpson's index of dominance was greater in Mwisho wa Lami than in Gatimu (Table 14). The community diversity was higher and the distribution of AMF species was more uniform in Gatimu (E= 0.97) than in Mwisho wa Lami (E= 0.94). In comparing the general AMF community structure in the two sites, Sorenson's coefficient of AMF community was 0.92. Furthermore the results of the current work showed that there was a significant positive correlation between relative abundance and isolation frequency of AMF species in Gatimu than in Mwisho wa Lami (Pearson product-moment correlation coefficient r= 0.914and r= 0.935, $p<0.050$, respectively),

Table 10: Mean spore densities of AMF in soil samples collected from Gatimu

Tree species	Family	Spore densities	Standard error
R. melanophloease	Myrsinaceae	7.70a	1.16
P. latifolius	Podocarpaceae	11.40a	1.07
M. senegalensi	Celastraceae	11.70a	2.02
O. europaea	Oleaceae	12.60ab	1.55
O. capensis	Oleaceae	12.80ab	1.58
P. falcatus	Podocarpaceae	19.10bc	2.16
P. africana	Rosaceae	19.90c	2.03
J. procera	Cupressaceae	27.60d	3.56
H. abyssinica	Rosaceae	41.20e	3.97
D .torrida	Sterculiaceae	42.30e	3.64

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test.

Table 11: Mean species richness of AMF in soil samples collected from Gatimu

Tree species	Family	Species richness	Standard error
R. melanophloease	Myrsinaceae	2.100a	0.23
P. latifolius	Podocarpaceae	2.300ab	0.15
O. capensis	Oleaceae	2.600abc	0.16
O. europaea	Oleaceae	2.700bc	0.15
M. senegalensi	Celastraceae	2.900c	0.23
P. falcatus	Podocarpaceae	3.500d	0.22
H. abyssinica	Rosaceae	3.700de	0.21
P. africana	Rosaceae	3.800de	0.13
J. procera	Cupressaceae	4.100ef	0.10
D .torrida	Sterculiaceae	4.500f	0.17

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Table 12: Mean spore densities of AMF in soil samples collected from Mwisho wa Lami

Tree species		Spore densities	Standard error
M. senegalensi	Celastraceae	7.60a	1.64
P. africana	Rosaceae	10.40ab	1.63
R. melanophloease	Myrsinaceae	11.10ab	1.72
P. latifolius	Podocarpaceae	15.30b	1.78
O. europaea	Oleaceae	15.90bc	2.18
O. capensis	Oleaceae	16.60bc	1.51
P. falcatus	Podocarpaceae	21.90c	1.30
H. abyssinica	Rosaceae	41.60d	3.70
D .torrida	Sterculiaceae	50.40e	3.37

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Table 13: Mean species richness of AMF in soil samples collected from Mwisho wa Lami

Tree species		Species richness	Standard error
R. melanophloease	Myrsinaceae	2.500a	0.31
P. Africana	Rosaceae	2.600ab	0.31
M. senegalensi	Celastraceae	2.800ab	0.20
P. latifolius	Podocarpaceae	2.800ab	0.13
O. capensis	Oleaceae	2.900ab	0.10
O. europaea	Oleaceae	3.200bc	0.13
H. abyssinica	Rosaceae	3.800cd	0.33
D .torrida	Sterculiaceae	3.800cd	3.37
P. falcatus	Podocarpaceae	3.900d	0.31

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Table 14: Diversity measurements of AMF communities in the Gatimu (G) and Mwisho wa Lami (ML)

Ecological parameters	Gatimu	Mwisho wa Lami
Shannon–Wiener index of diversity (H')	5.58	5.39
Evenness (E)	0.97	0.94
Simpson's index of dominance (D)	0.043	0.058
Sorenson's coefficient of AMF community (Cs)	0.92	

4.4 AMF community composition

The AMF communities both in Gatimu and Mwisho wa Lami soil samples were dominated by families such as Acaulosporacae, Dentiscutataceae, Glomaceae, Racocetraceae, Scutellosporaceae. Among these, Gigasporaceae and Dentiscutataceae were dominant at the two sites with Relative abundance (RA) of 47.86% and 20.03% respectively in Gatimu and 39.78% and 21.05% respectively in Mwisho wa Lami (Table 15, Figure 6).

Table 15: Relative abundance (RA) and spore number of AMF families in Gatimu (G) and Mwisho wa Lami (ML)

Family	Gatimu		Mwisho wa Lami	
	Spore number	RA%	Spore number	RA%
Acaulosporacae	165	8.48	240	14.03
Dentiscutataceae	395	20.30	360	21.05
Gigasporaceae	931	47.87	680	39.77
Glomaceae	227	11.67	110	6.43
Racocetraceae	227	11.67	320	18.71

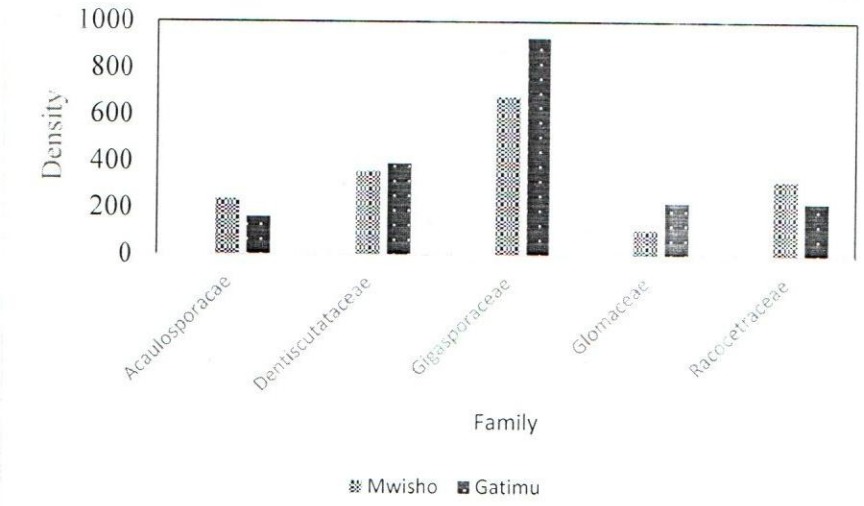


Figure 6: Density of some AMF fungal families in Gatimu and Mwisho wa Lami

A total of thirteen AMF species belonging to five genera were identified according to morphological characteristics of spores extracted from soil samples collected from Sururu Forest (Table 16 and Plate 3). Six species of these identified species belonged to the genus *Scutellospora*, three to *Glomus*, two to *Acaulospora*, and one each to *Dentiscutata* and *Racocetra* (Figures 7). Among the identified AMF species the majority of species detected from spores were common to both sites. Eleven AMF species were isolated on both sites (*A. denticulate*, *Acaulospora* sp. 1, *D. nigra*, *Gigaspora* sp. 1, *Glomus* sp. 1, *Glomus* sp. 2, *Glomus* spp., *Racocetra* sp. 1, *S. heterogama*, *S. nigra*, *Scutellospora* sp. 1, *Scutellospora* sp. 2, S. *spinosissima* sp. nov.). Two of the species (*Scutellospora* sp. 2, *Glomus* sp. 2) were found only in Gatimu.

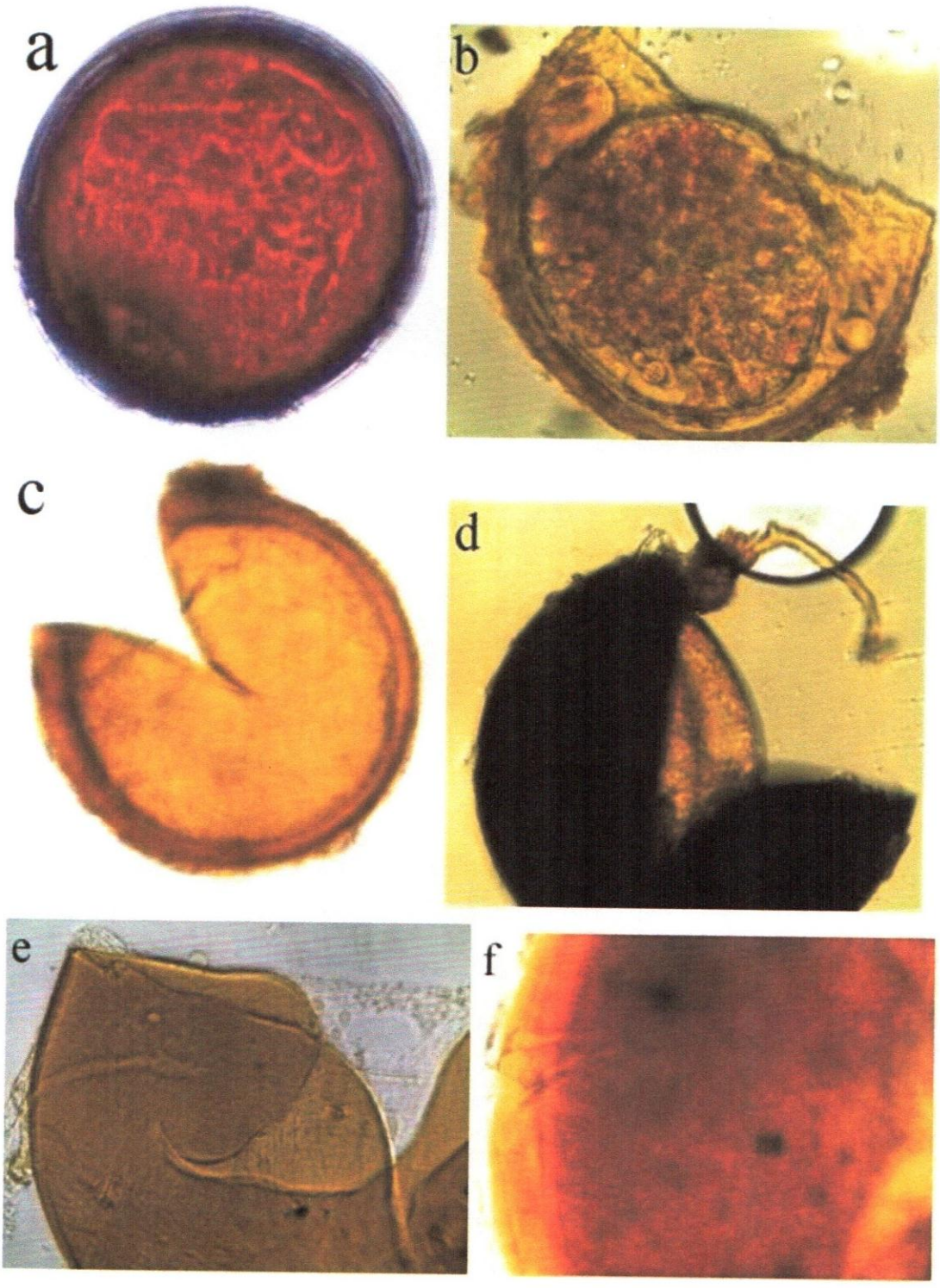
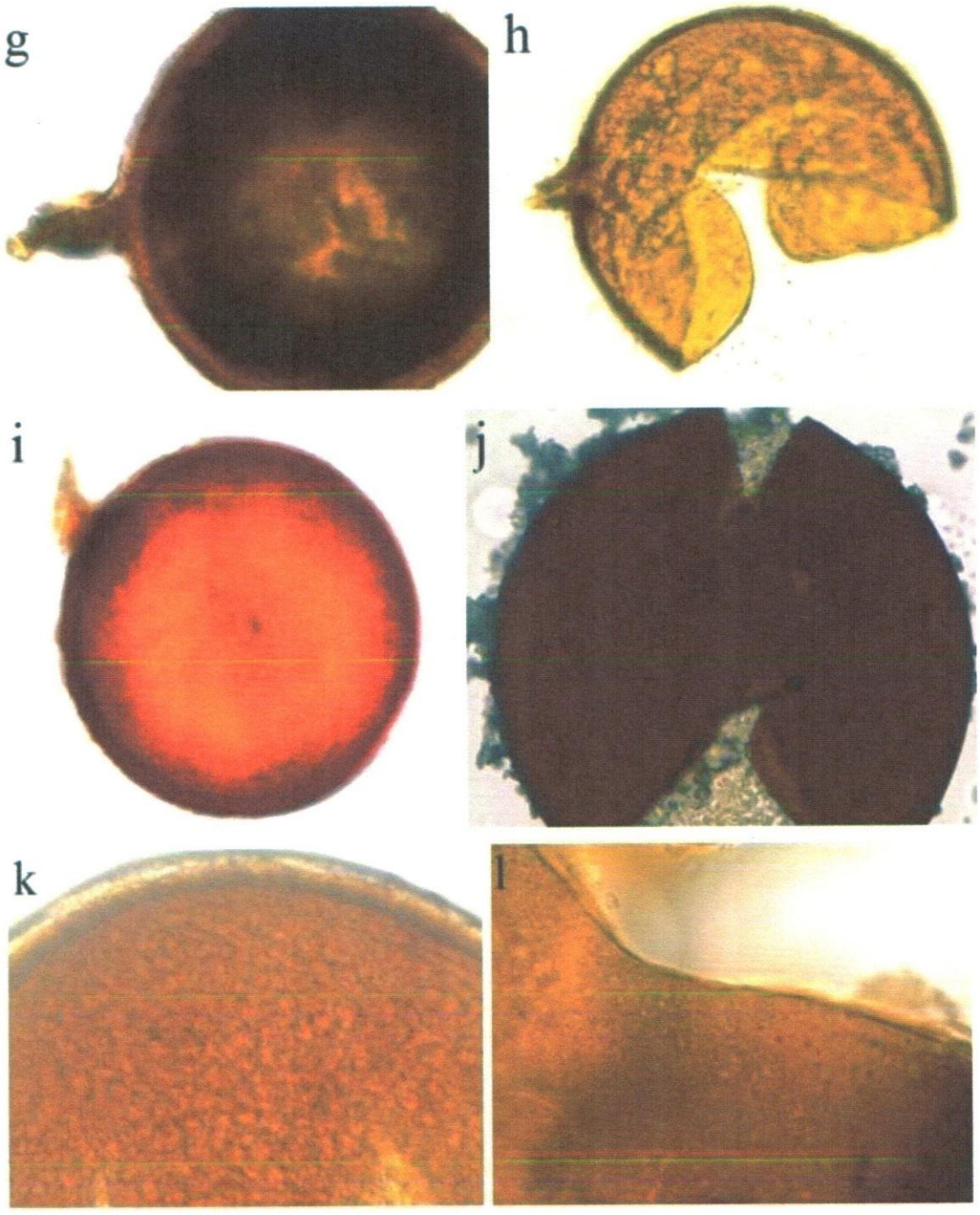


Plate 4: Photographs of mycorrhizal spores (Leica DM 500 Magnification ×40).

The spores extracted from rhizosphere soils of ten selected indigenous trees of Mau Forest Complex. (a) *Acaulospora denticulata* (b) *Acaulospora* sp. 1 (c) *Glomus* sp. 1, (d) *Scutellospora nigra*, (e) *Scutellospora* sp. 1, (f) *Scutellospora* sp. 2,



The spores extracted from rhizosphere soils of ten selected indigenous trees of Mau Forest Complex. (g) *Scutellospora spinosissima* sp. nov, (h) *Glomus* sp. 2, (i) *Scutellospora heterogama*, (j) *Dentiscutata nigra*, (k) *Glomus* sp. 3, (l) *Gigaspora* sp. 1

Table 16: Families and species of AMF identified from Mau forest

Family	Fungal species
Acaulosporacae	*Acaulospora denticulata*
	Acaulospora sp. 1 (Mau forest)
Dentiscutataceae	*Dentiscutata nigra* (J.F. Redhead) Sieverd. F.A. Souza & Oehl
Gigasporaceae	*Scutellospora heterogama*
	Scutellospora nigra (J.F. Redhead) C. Walker & F.E. Sanders, 1986)
	Scutellospora sp. 1 (Mau forest)
	Scutellospora sp. 2 (Mau forest)
	Scutellospora spinosissima sp. nov
	Gigaspora sp; 1 (Mau forest)
Glomaceae	*Glomus* sp. 1 (Mau forest)
	Glomus sp. 2 (Mau forest)
	Glomus sp. 3 (Mau forest
Racocetraceae	*Racocetra* sp. 1 (Mau forest)

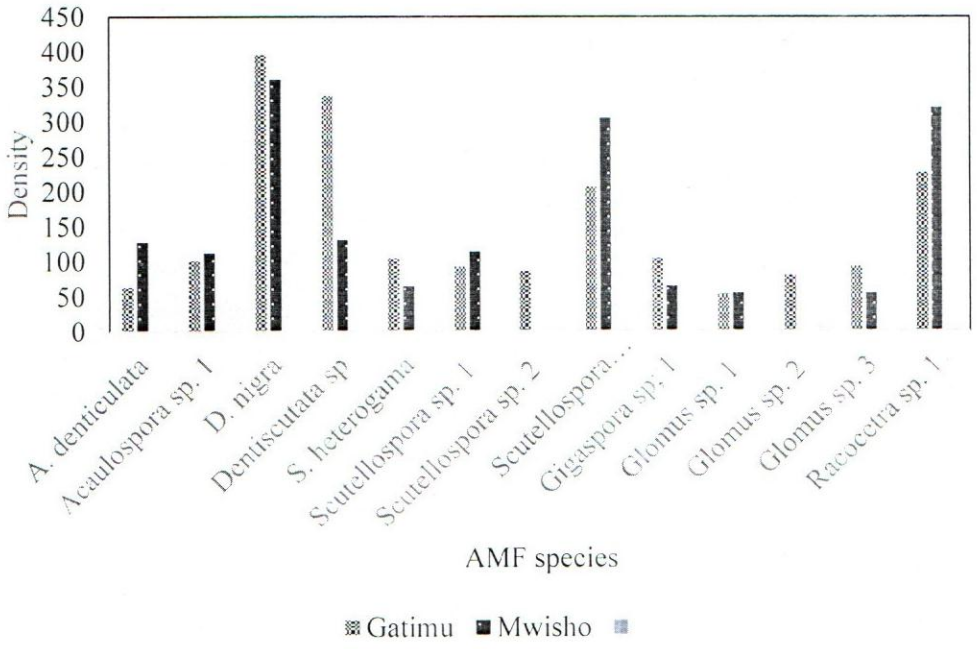


Figure 7: Composition of AMF community in Gatimu and Mwisho wa Lami soil samples

Based on relative abundance and isolation frequency, the three dominant species in Gatimu were *Dentiscutata nigra*, *Scutellospora nigra* and *Scutellospora spinosissima* sp. nov, whereas there were only two dominant species (*Dentiscutata nigra* and *Scutellospora spinosissima* sp. Nov) in Mwisho wa Lami (Table 17). Furthermore the results of the current work showed that there was a significant positive correlation between relative abundance and isolation frequency of AMF species in both Gatimu and Mwisho wa Lami (Pearson product-momentcorrelation coefficient r= 0.9044 and r= 0.9787, $p<0.050$, respectively), and it appeared that species producing more spores usually had a wide distribution, while species that produced fewer spores usually had small geographic ranges. However, a few AMF species, such as *Acaulospora* sp. 1 (5.19% of RA, 16% of IF), *Scutellospora* sp. 1(4.78% of RA, 14% of IF), *Gigaspora* sp; 1 (5.35% of RA, 17% of IF), *Scutellospora heterogama* (5.35% of RA, 17% of IF), in Gatimu and *Acaulospora* sp. 1 (6.55% of RA, 23.33% of IF), *Glomus* sp. 1 (3.22% of RA, 14.44% of IF), in Mwisho wa Lami, had low relative abundances but were widely distributed (Table 17).

Table 17: The Relative abundances (RA) and Isolation frequency (IF) of AMF species

	Gatimu (G)		Mwisho wa Lami (ML)	
Fungal species	RA	IF	RA	IF
Acaulospora denticulata	3.29	23	7.485	21.11
Acaulospora sp. 1	5.19	16	6.550	23.33
Dentiscutata nigra	20.31	62	21.053	62.22
Scutellospora heterogama	5.35	17	3.801	8.89
Scutellospora nigra	17.33	42	7.661	21.11
Scutellospora sp. 1	4.78	14	6.667	14.44
Scutellospora sp. 2	4.42	40	_	_
Scutellospora spinosissima sp. nov	10.64	17	17.836	45.56
Gigaspora sp; 1	5.35	17	3.801	6.67
Glomus sp. 1	2.78	7	3.216	14.44
Glomus sp. 2	4.11	2	_	_
Glomus sp. 3	4.78	8	3.216	7.78
Racocetra sp. 1	11.67	39	18.713	46.67

4.5 *In-vitro* study by Dual culture interaction

4.5.1 Effect of *Trichoderma harzianum* on the growth of *Armillaria* species *in-vitro*

The diameter of *Armillaria* species was only 10mm at 14 days after inoculation (Plate 4, 2). *Armillaria* species had slower growth than *T. harzianum*. *Trichoderma harzianum* grew on all possible sides of the pathogenic fungus (*Armillaria* species) in the plate (Plate 4, 1C). *T. harzianum* came into contact with all sides of the *Armillaria* species on the fifth day after inoculation to suppress further growth of the pathogen (Plate 4, 1E) and later *T. harzianum* started to overgrow on the pathogen. Visual observation of dual cultured plates showed antagonistic activity of *T. harzianum* towards *Armillaria* species and an inhibition zone was observed at the margin between the antagonist and the pathogen (Plate 4, 1E). Growth of *Armillaria* species was inhibited by encroachment of *T. harzianum* and close-up of this zone was evident (Plate 4, 1F). No further growth of the pathogen was observed in *Trichoderma* controlled plates. Later, the plates became covered exclusively by *T. harzianum* and the pathogen was destroyed completely after about 24 days of incubation. *Armillaria* species were grown fully on the control plates (Plate 4, 4). These initial results indicated that the strain of *T. harzianum* can be used as a biocontrol agent against the tested *Armillaria* species.

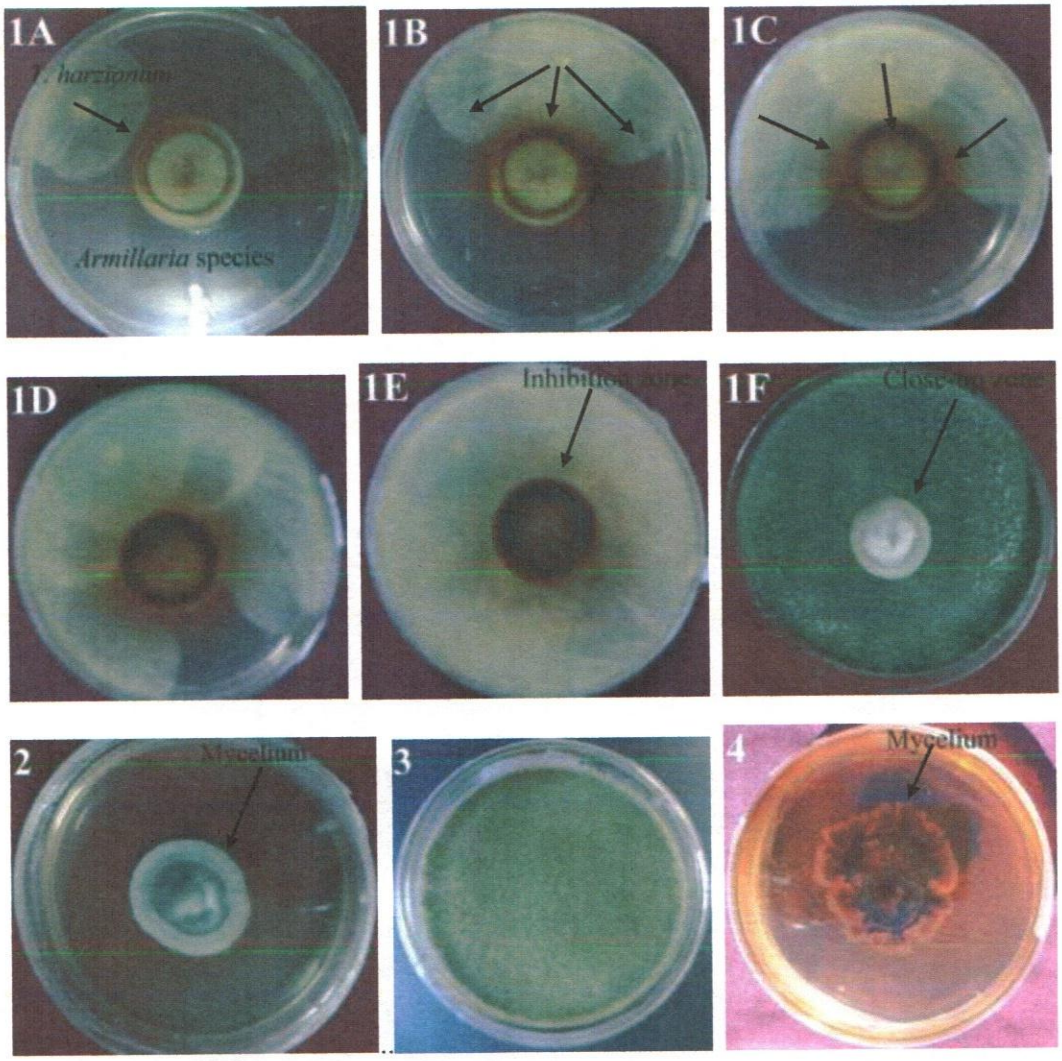


Plate 5: Antagonism of *Trichoderma harzianum* against *Armillaria* species following a direct confrontation test

1A-1F: *T. harzianum* growth and inhibition of *Armillaria* species growth after 1, 2, 3, 4, 5 and 6 days respectively (arrow marks show growth of Trichoderma). 2: *Armillaria* species 4: *Armillaria* species (after prolonged incubation) 3: *T. Harzianum*.

4.5.2 Effect of *Trichoderma asperellum* on the growth of *Armillaria* species *in-vitro*

The diameter of *Armillaria* species was between 15 - 17 mm 14 days after inoculation (Plate 5, 2). *Trichoderma asperellum* grew on all possible sides of the pathogenic fungus (*Armillaria* species) in plate (Plate 5, 1C). *Trichoderma asperellum* came into contact with all the sides of the pathogen on the fouth day after inoculation and started to suppress further growth of the pathogen (Plate 5, 1D). In test MEA plates, *T. asperellum* inhibited the growth of *Armillaria* species within five days after inoculation and started to overgrow on the pathogen (Plate 5, 1F).

Trichoderma asperellum showed antagonistic activity against *Armillaria* species in the direct confrontation tests. Antagonism resulted in the cessation of growth of *Armillaria* species when in contact with *T. asperellum* and an inhibition zone was also observed (Plate 5, 1E). A close-up of this zone is shown in (Plate 5, 1F) and a dense sporulation followed by an overgrowth of *T. asperellum* on the strains of *Armillaria* species was observed on the sixth day (Plate 5, 1F). No further growth of the pathogen was observed in *Trichoderma* controlled plates. Total destruction of *Armillaria* species by the antagonistic action of *T. asperellum* was confirmed by the absence of growth of two strains of *Armillaria* species in the selective culture medium. *Armillaria* species were grown fully on control plates after about 21 days of incubation (Plate 5, 4).

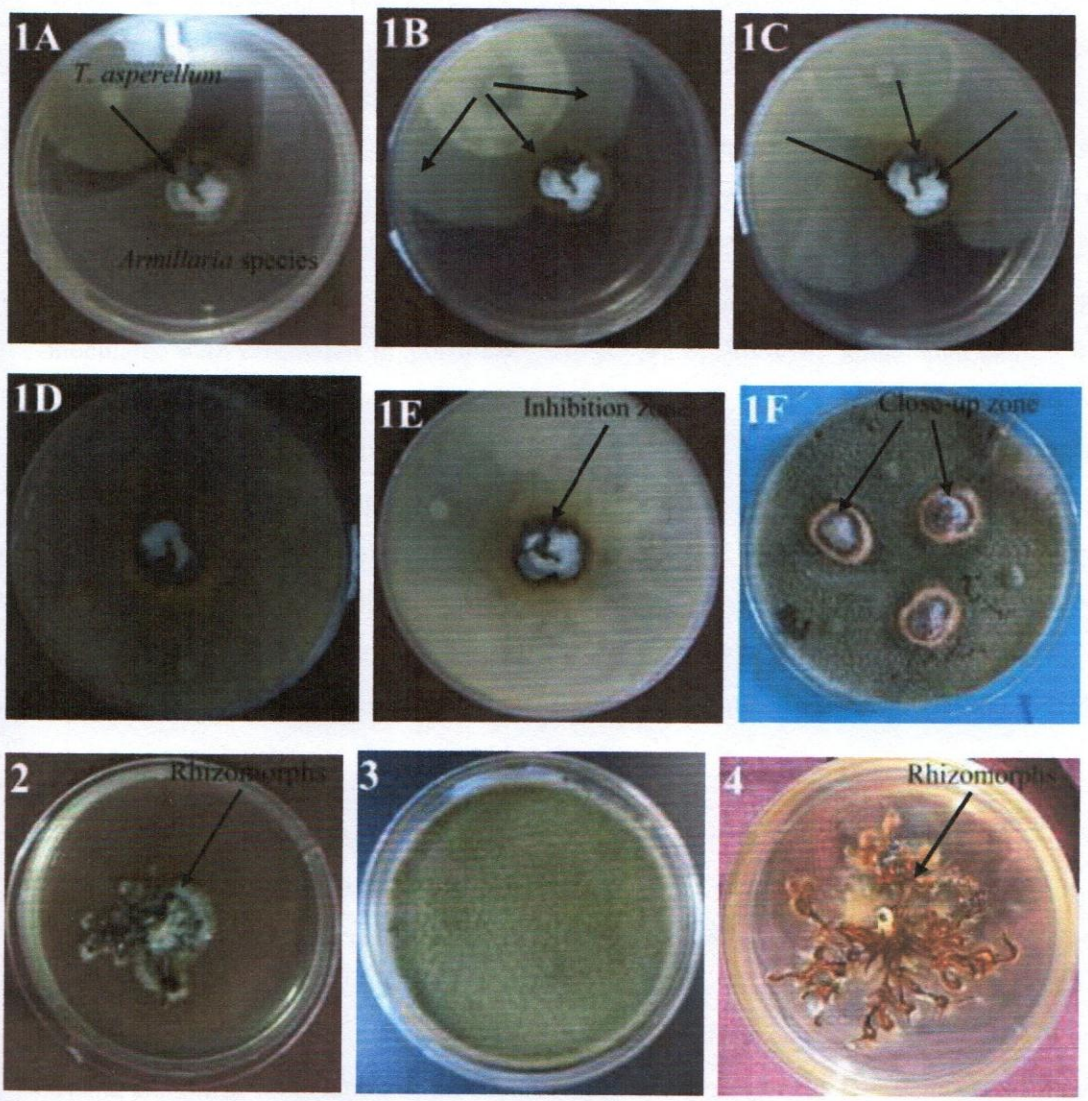


Plate 6: Antagonism of *Trichoderma asperellum* against *Armillaria* species following a direct confrontation test

1A-1F: *T. asperellum* growth and inhibition of *Armillaria* species growth after 1, 2, 3, 4, 5 and 6 days respectively (arrow marks show growth of Trichoderma). 2: *Armillaria* species. 4: *Armillaria* species (after prolonged incubation) 3: *T. asperellum*

4.6 Interaction between AMF and *Trichoderma harzianum* under greenhouse conditions

4.6.1 Plant shoot and root fresh weight

Growth parameters of *D. torrida* seedlings grown in soils inoculated with arbuscular mycorrhizal fungi and *T. harzianum* were variably affected when examined after 28 weeks. Single inoculation of arbuscular mycorrhizal fungi did not significantly change shoot fresh weight compared with control plants, while inoculation with *T. harzianum* alone significantly increased shoot fresh weight compared with control plants. Plants inoculated with *T. harzianum* alone had significantly increased shoot fresh weight compared to the plants inoculation with arbuscular mycorrhizal fungi. Plants co-inoculated with *T. harzianum* and arbuscular mycorrhizal fungi showed the highest fresh weights. Co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* significantly increased root and shoot fresh weights compared to plants inoculated with arbuscular mycorrhizal fungi alone or *T. harzianum* alone (Table 18). Inoculations with *T. harzianum* only resulted in better root weight compared to inoculation with arbuscular mycorrhizal fungi alone. On the other hand, fresh shoots of plants inoculated with arbuscular mycorrhizal fungi alone only were weightier than control plants. Although there were no significant changes in the shoot fresh weight due to inoculation of arbuscular mycorrhizal fungi, an increased shoot/root ratio was observed in arbuscular mycorrhizal fungi inoculated plants compared to control plants (Table 18). A significant interaction between the arbuscular mycorrhizal fungi and *T. harzianum* was observed regarding the shoot/root ratio. Combination of *T. harzianum* and mycorrhizal fungi resulted in higher increases of root weight than shoot weight as illustrated by the decrease in the shoot/root ratio. Moreover, smaller fresh shoot/root ratios were recorded in plants inoculated with *T. harzianum.*

4.6.2 Plant height

The other important parameters reflecting the seedling vigour of plants include the direct measurements of plant growth such as plant height. Although arbuscular mycorrhizal fungi inoculated plants had increased the plant height, the difference was not significant from the control. *Trichoderma harzianum* inoculants significantly increased the plant height compared to the arbuscular mycorrhizal fungi inoculated plants. Plant height was found to be enhanced by the combined inoculation of arbuscular mycorrhizal fungi and *T. harzianum* compared to

individual inoculations. The highest plant height of 83.50cm was recorded in treatment which received combined inoculation of two groups of microorganisms (Table 18).

Table 18: The shoot and root fresh weight (g) and the shoot/root ratio of *Dombeya torrida* plants inoculated with *Trichoderma harzianum* and/or AMF 28 weeks after planting

Treatment	Plant height (cm)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Shoot/root ratio
C	53.38ab	12.25a	3.59a	3.38b
A	49.25a	12.38a	3.62a	3.39b
A+M	62.62bc	13.1a	4.05a	3.21b
M	69.25c	13.59a	3.75a	3.70b
A+T	71.75 cd	19.05b	8.43b	2.80ab
T	69.25 c	19.43b	6.62ab	2.96b
A+T+M	75.88cd	24.04bc	15.57c	1.71a
M+T	83.50d	26.42c	17.14c	1.72a

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test.

Treatments

A-*Armillaria* species; C-control; M-mycorrhizae; T- *Trichoderma harzianum*

4.6.3 Root colonisation

A significant interaction between the biocontrol agents, arbuscular mycorrhizal fungi and *T. harzianum* was observed regarding arbuscular mycorrhizal fungi root colonization. The presence of *T. harzianum* increased arbuscular mycorrhizal fungi root colonization compared to plants inoculated with the arbuscular mycorrhizal fungi alone, co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* producing a higher percentage of colonization than any other treatment (Table 19). The number of CFUs in the assays performed ranged between 27200 and 48400. In non-inoculated treatments, there were no detectable levels of native *T. harzianum*. Significant differences in the number of *T. harzianum* colony forming units (CFUs) recovered from the roots were observed for the treatments involving co-inoculation with arbuscular mycorrhizal fungi, with respect to inoculation with *T. harzianum* alone (Table 19). Among all the treatments challenged with *Armillaria* species it was observed that there was no disease incidence 28 weeks after inoculation. When the roots were cultured

in-vitro using Armillaria selective medium, there was no growth of *Armillaria* species in the medium confirming the absence of the pathogen (Table 19).

Table 19: The AMF root colonization and *Trichoderma harzianum* CFUs 28 weeks after planting and Armillaria root rot incidence

Treatment	*T. harzianum* CFUs	AM root colonization	Armillaria root rot incidence
A	0.00a	0.00 a	0.00
C	0.00a	0.00 a	0.00
M	0.00a	43.20 b	0.00
M+A	0.00a	52.00 b	0.00
T+M+A	27.20b	80.00c	0.00
T+M	30.20b	84.00c	0.00
T+A	45.80c	0.00 a	0.00
T	48.40c	0.00a	0.00

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Treatments

A-*Armillaria* species; C-control; M-mycorrhizae; T-*Trichoderma harzianum*

4.7 Interaction between AMF and *Trichoderma asperellum* under greenhouse conditions

4.7.1 Plants shoot and root fresh weight

Growth parameters of *D. torrida* seedlings grown in soils inoculated with arbuscular mycorrhizal fungi and *T. asperellum* were variably affected when examined after 28 weeks. Plants inoculated with *T. asperellum* alone had significantly increased shoot fresh weight compared to the control plants. Single inoculation of arbuscular mycorrhizal fungi did not significantly change shoot fresh weight compared with control plants. Plants co-inoculated with *T. asperellum* and arbuscular mycorrhizal fungi showed the highest fresh weights. Co-inoculation with arbuscular mycorrhizal fungi and *T. asperellum* significantly increased root and shoot fresh weights compared to *T. asperellum* alone or arbuscular mycorrhizal fungi alone (Table 20). Inoculations with *T. asperellum* only resulted in better root weight compared to inoculation with arbuscular mycorrhizal fungi alone. On the other hand, fresh shoots of plants inoculated with arbuscular mycorrhizal fungi alone were weightier than control plants. Although no significant changes in the shoot fresh weight due to inoculation with arbuscular mycorrhizal fungi, an increased shoot/root ratio was observed in arbuscular mycorrhizal fungi inoculated plants compared to control plants (Table 20). A significant interaction between the

arbuscular mycorrhizal fungi and *T. asperellum* was observed regarding the shoot/root ratio. Combination of *T. asperellum* and arbuscular mycorrhizal fungi resulted in higher increases of root weight than shoot weight as illustrated by the decrease in the shoot/root ratio. Moreover, smaller fresh shoot/root ratios were recorded in plants inoculated with *T. asperellum.*

4.7.2 Plant height

The other important parameters reflecting the seedling vigour of plants include the direct measurements of plant growth such as height. Although arbuscular mycorrhizal fungi inoculated plants had increased plant height, the difference was not significant from the control. *Trichoderma asperellum* inoculants significantly increased the plant height compared to the arbuscular mycorrhizal fungi inoculated plants. Plant height was found to be enhanced by the combined inoculation of arbuscular mycorrhizal fungi and *T. asperellum* compared to individual inoculations. The highest plant height of 82.25 cm was recorded in treatment which received combined inoculation of the two of microorganisms (Table 20).

Table 20: The plant height, shoot and root fresh weight (g), and the shoot/root ratio of *Dombeya torrida* plants inoculated with *Trichoderma asperellum* and/or AMF, 28 weeks after planting

Treatment	Plant height (cm)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Shoot/root ratio
C	61.62ab	13.42a	3.96a	3.54c
A	61.75ab	13.50a	3.98a	3.54c
M	65.38 ab	13.56a	4.01a	3.55c
A+M	52.25a	15.66a	4.58a	3.73c
T	72.00bc	18.26ab	9.050b	2.04ab
A+TA	71.75bc	18.39ab	6.74ab	2.78 bc
A+TA+M	74.62bc	21.54bc	14.66c	1.56a
M+TA	82.25c	23.82c	16.15c	1.56a

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Treatments

A-*Armillaria* species; C-control; M-mycorrhizae; T-*Trichoderma asperellum*

4.7.3 Root colonisation

There were variations in root colonisation of *D. torrida* seedlings grown in soils inoculated with arbuscular mycorrhizal fungi and *T. asperellum* when examined after 28 weeks (Table 21). Plants grown in soil inoculated with arbuscular mycorrhizal fungi alone were successfully colonized whereas non-inoculated plants remained non-mycorrhizal. Dual inoculation of *T. asperellum* and arbuscular mycorrhizal fungi significantly reduced the level of root colonisation by arbuscular mycorrhizal fungi (Table 21). Population density (CFU) of *Trichoderma asperellum* in the rhizosphere soil of combine inoculated plants (*Trichoderma asperellum*-arbuscular mycorrhizal fungi) was almost similar to that of plants inoculated with *Trichoderma asperellum* alone. The number of CFUs in the assays performed ranged between 39400 and 44000. There was no significant difference between the CFU of *Trichoderma asperellum* associated with roots of single and combine-inoculated plants (*Trichoderma asperellum*-arbuscular mycorrhizal fungi) 28 weeks after planting. No mycorrhizal colonization was recorded in control treatment (Table 21).

Table 21: The AMF root colonization and *Trichoderma asperellum* CFUs 28 weeks after planting and Armillaria root rot incidence

Treatment	*T. asperellum* CFUs	AM root colonization	Armillaria root rot incidence
A	0.00 a	0.00a	0.00
C	0.00 a	0.00a	0.00
T	44.00b	0.00a	0.00
T+A	42.60b	0.00a	0.00
T+M+A	39.40b	19.40b	0.00
T+M	40.00b	21.80b	0.00
M	0.00 a	41.40c	0.00

Data in the same column sharing a letter in common do not differ significantly ($P<0.05$) by the Fischer's least significant difference test

Treatments

A-*Armillaria* species; C-control; M-mycorrhizae; T-*Trichoderma asperellum*

CHAPTER FIVE

DISCUSSION

5.1The diversity of arbuscular mycorrhizal fungi in Mau forest

The ecosystem surveyed is characterized by low temperature and high rainfall for most of the year according to data collected from weather station located at Sururu forest of Eastern Mau. All the 10 selected indigenous study plants surveyed in this study showed AMF colonisation and AMF spores in their respective rhizosphere soils, confirming the earlier findings suggesting that the roots of 90 to 95% of vascular plant species are colonized by mycorrhizal fungi in a symbiotic association in natural ecosystem (Arocena *et al.*, 2012; Chmura and Gucwa-Przepiora, 2012). These results are in agreement with previous work of Birhane *et al.* (2010) who observed that all 43 plant species of trees and shrubs in dry deciduous woodlands of Ethiopia that are dominated by the economically important frankincense tree *(Boswellia papyrifera)* are arbusucular mycorrhizal. Work by Onguene and Kuyper (2001) also showed that 79% plants were arbuscular mycorrhizal in the rain forest of south Cameroon. In another study of Tao and Zhiwei (2005), 95% of plants surveyed were arbuscular mycorrhizal in hot and arid ecosystem in southwest China.

AMF colonization was observed by the presence of structures including arbuscules and vesicles in roots of investigated plants. The morphological diversity of the different fungal structures observed within the roots and diversity of spores from the rhizosphere soil samples suggested that the roots were colonized by at least two different fungal morphotypes. The mycorrhizal status of *P. falcatus, P. latifolius, O. capensis, O. europaea, P. africana, H. abyssinica, J. procera, D. torrida, M. senegalensis* and *R. Melanophloeos* are reported for the first time from the Mau forest ecosystem. All 10 plant species belonging to 7 families including Podocarpaceae, Oleaceae, Rosaceae, Cupressaceae, Sterculiaceae, Celastraceae and Myrsinaceae were colonized by AMF. Six of these seven families have been reported in earlier studies on Ethiopian trees to be mycorrhizal (Wubet *et al.*, 2003, Birhane *et al.*, 2010). These observations confirm results from other surveys in Africa that showed most or all of the woody species being mycorrhizal and a majority being arbusucular mycorrhizal. Wubet *et al.* (2003) reported *Albizia gummifera, Albizia schimperiana, Aningeria adolfi-friedericii, Croton machrostachyus, Ekebergia capensis, H. abyssinica, J. procera, P. falcatus, P. africana, Olea europaea*.ssp.*cuspidata*, and *Syzygium guineense*, in the dry Afromontane forests of Ethiopia as typically being arbusucular mycorrhizal. Similarly Michelsen (1992), reported the presence of AMF in *C. macrostachyus, J. procera, O. europaea* ssp.*cuspidata* and *P. falcatus* based on his study on nursery grown seedlings in Ethiopia.

Arbuscular mycorrhizal colonization and spore population in the present study varied significantly in different tree species. The variation in the percentage colonisation in the roots and AMF spore population in the rhizosphere soils of different trees recorded showed that the plant species had a low to high range of mycorrhizal colonisation. Although the mycorrhizal colonization was observed in all plants from the two sites, the percentage of the colonization was variable among tree species. Differences in colonization may be attributed to variation of the tree species in mycorrhizal dependency. An additional trend was found in the relationship between plant species and colonization level, where the highest values of colonization were noted in *D. torrida* and *H. abyssinica* and least colonization levels were recorded under *M. senegalensi* and *R. melanophloease*. Colonization levels of the same species did not differ much between the two sites. The results of the present study are consistent with the report of Onguene and Kuyper (2001), who reported variation in AMF colonisation in different trees from the rain forest of south Cameroon. Borde *et al.* (2010) pointed out that the variation in percentage root colonisation has been reported to be affected by seasonal sporulation, seasonal variation in the development of host plants and nutrient availability in the soils. Furthermore, variations in spore density and colonization of AMF associated with different host plant species may be generated by a variety of potential mechanisms, including variations in host species phenology, variations in host species, mycorrhizal dependency, host plant-mediated alterations of the soil microenvironment, or other unknown host plant traits (Lorgio *et al.*, 1999; Eom *et al.*, 2000).

Presence of a variety of AMF spores in the rhizosphere soils of studied tree species and high AMF community similarity between the two sites suggested that most AMF found in Mau Forest Complex could colonize a variety of plant species, and further supported a lack of host specificity among AMF isolated from this ecosystem. The variation in the present study may be the result of variations in host species and host plant-mediated alterations of the soil microenvironment. The host plant species may have direct effects on the abundance and colonization of AMF through their influence on the soil microenvironment. Organic matter which serves as a nutrient sink for the plants could also regulate the intensity of mycorrhizae (Siddiqui and Pichtel, 2008). Under the canopy of *H. abyssinica* and *D. torrida* values for both total organic C and total N were higher than any other study trees species. During soil sampling an excessivea ccumulation of litter was observed under the canopy of those particular two trees at the two study sites. Accordingly, spores and sporocarps population and level of AMF colonization were higher under *D. torrida* followed by *H. abbysinica* and lower for *M. senegalensi* and *R. melanophloease*. According to He *et al.* (2002), spore density and

colonization of AMF were higher under *Artemisia herbaalba* and *Atriplex halimus* canopies, and lower under *Zycophyllum dumosum* and *Hammada scoparia* canopies. The study of McGrady-Steed *et al.* (1997) showed that biodiversity could regulate ecosystem predictability in terrestrial ecosystems. This suggests that AMF diversity could be used to investigate the function of AMF in maintaining plant biodiversity and ecosystem function during the conservation and restoration of diverse natural ecosystems (Dandan and Zhiwei, 2007).

A total of 32 AMF morphotypes were wet sieved from the 95 rhizosphere soil samples collected from Sururu Forest, from which nine AMF were identified. This number is closer to that from the study of Jefwa *et al.* (2012), that reported a total of 22 AMF morphotypes affiliated to Glomaceae, Acaulosporaceae, Archaeosporaceae and Gigasporaceae were isolated from the banana farming systems of central Kenya. This study confirmed the widespread occurrence of arbuscular mycorrhizal fungi in the respective rhizosphere soils of selected studied tree species of Mau Forest Complex. The diversity of AMF observed in the Sururu forest is however low compared to 40 species recorded in the Cameroon tropical rainforest (Mason *et al.*, 1992) and 43 AMF species recorded in the hot-dry valley of the Jinsha River, southwest China (Dandan and Zhiwei, 2007). However, it is closer to 15 species recorded in the acid soils of western Kenya (Shepherd *et al.*, 1996), and 12 species recorded in agroforestry systems in the miombo ecozone of Malawi (Jefwa, 2004).

AMF spores were obtained from all rhizophere soil samples, but the counts showed considerable variations. Spore population varied significantly in plant rhizosphere of different tree species. The highest spore population were found in the rhizophere soil samples of *D. torrida* and *H. abyssinic* and lowest spore population levels were recorded under *M. senegalensi* and *R. melanophloease* rhizophere soil samples. Differences in spore population could be attributed to variation in the tree species in root turnover. Spore densities are known to vary greatly in different ecosystems. Values range from dozens to 10,000 spores per 100 g soil (Tao and Zhiwei, 2005). In the present study, the density of AMF spores was relatively low ranging from 1 to 30 spores per 20g soil. Spore densities recorded seem low as compared to other studies. For example, higher counts of AMF spores ranging from 85 to 5315 spores per 100g of dry soil, with an average of 476 has been reported from the tropical rain forest of Xishuangbanna, southwest China (Zhao *et al.*, 2001). Similarly, Tao *et al.* (2004) recorded counts ranging from 5–6400 spores per 100g soil in the dry tropics in avalley-type savanna in south west China. However, spore abundance in the present study is comparable with other previous investigations. Muleta *et al.* (2007), recorded counts ranging from 4 to 67 spores per

100g soil in Bonga natural coffee forest, southwestern Ethiopia. In another study of Birhane *et al.* (2010), spore density ranging from 8 to 69 spores per 100g dry soil were recorded in three different dry deciduous woodlands of Northern Ethiopia.

The low number of spores reported in this study could be attributed to the following factors: Sampling was made during the rainy season, which may have suppressed the number of spores due to the excess moisture that could either cause decomposition of spores or initiate spore germination. During rainy season, mycorrhizae is predominantly in vegetative form of hyphae, mycelia and colonization state in roots. It has been reported that intra and extrametrical mycelia increases during the rainy season, because spore germination is favoured. This in turn increases mycorrhizal colonization and decreases spore abundance (Ragupathy and Mahadevan, 1993). Guadarrama and Alvaarez-Sanchez (1999) demonstrated that the highest numbers of species and spores were observed during the dry season, with a marked decrease during the rainy season. Sporulation in the dry season is as a result of root senescence because there is a likelihood of high root turnover especially in annuals or competition. Sporulation is a result of stress and reproduction stage therefore would be observed in the dry season when roots are decomposing, dying or at flowering stage in the tropics. Secondly, earthworms and other soil fauna which may feed on mycorrhizal fungal spores were frequently encountered during soil sampling processes. The negative influence of the soil fauna such as earthworms on AMF populations has been reported (Brown, 1995). The AMF mycelium is important in nutrient transfer in soil, but this process is affected by the activities of earthworms. Earthworms may graze preferentially on soil containing AMF propagules and as a result, concentrate them in the casts (Yu *et al.*, 2005). The study of Mangan and Adler (2000) reported the consumption of arbuscular mycorrhizal fungi by terrestrial and arboreal small mammals. The low number of spores in the natural forest does not necessarily mean that AMF abundance is low since AMF survive in soils as spores, extraradical mycelium, vegetative hyphal fragments and infected root pieces. The existence of these propagules in the soil may explain roots colonization potential of AMF in soils from natural forest (Brito *et al.*, 2012; Zubek *et al.*, 2012).

The presence of different AMF spores in the rhizosphere soils of Mau Forest Complex suggests that this type of ecosystem may be characterized by a high diversity of AMF. Most of the AMF spores (54%) obtained by wet-sieving were large. The dominance of large spores may be a selective adaptation to low temperature and high rainfall that is characteristic feature in this ecosystem. The presence of larger spores may indicate less disturbance of this forest ecosystem. Jefwa *et al.* (2012), reported that larger spores are fewer in agroecosystems with smaller spores

more dominant. This suggested that AMF may take an important role in the developing and sustaining of vegetation in this forest. AMF cannot be ignored in the reestablishment of this ecosystem and large AMF spores might have a potential application in the practice. Picone (2000) reported that small spores were more frequent and had a low seasonal variation than larger spores. In addition, small spore species are adapted to the hot and arid environment.

5.2 Ecological implications of AMF biodiversity

In comparing the general AMF community structure in Gatimu and Mwisho wa Lami, the Shannon–Weaver index revealed low levels of diversity of 5.58 among the sampled soils and Sorenson's coefficient of AMF community was 0.92. Furthermore the results of the current work showed that there is a significant positive correlation between relative abundance and isolation frequency of AMF species in Gatimu than in Mwisho wa Lami (Pearson product-moment correlation coefficient r= 0.914 and r= 0.935, respectively). Similarly spore density of AMF was positively significantly correlated with species richness in Gatimu than in Mwisho wa Lami (Pearson product-moment correlation coefficient r= 0.854 and r= 0.809, respectively). Thus, though vegetation distribution differed slightly in the two sites, there was a high degree of overlap in AMF fungal species composition between the closed forest (Gatimu) and patchily distributed forest (Mwisho wa Lami).

Furthermore, it was indicated that many of the AMF species identified in this study had broad dispersal and that the environmental conditions seemed to be more influential in determining the structure of AMF communities than the vegetation. Similar results were observed by Zhang *et al.* (2004) who found that there was high AMF composition similarity (Cs = 0.71) between the deforested land and natural forest, and that the deforestation did not largely influence the AMF species composition in the subtropical region of Dujangyan, south-west China. The current study also revealed significant non-uniform distributions of the dominant AMF species, and AMF community structure associated with different host plant species varied considerably. Since functional differences in AMF (either inter or intraspecies) could lead to different levels of plant-fungus compatibility, and since the variation in functional diversity within one AMF species can be greater than differences between different AMF species or even genera (Munkvold *et al.*, 2004), there are clearly opportunities for significant host preference to develop among AMF species. Kennedy *et al.* (2002) demonstrated that increasing local biodiversity could act as a barrier to enhance invasion resistance, and the study of McGrady-Steed *et al.* (1997) showed that biodiversity could regulate ecosystem predictability in terrestrial ecosystems. This suggests that AMF diversity could be used to investigate the

function of AMF in maintaining plant biodiversity and ecosystem function during the conservation and restoration of diverse natural ecosystems (Pande and Tarafdar, 2004).

5.3 AMF community composition

In this study, a total of 13 AMF species were identified belonging to six genera, five families and two orders from Sururu forest. AMF species that could not be matched with known taxa including *Gigaspora* sp. 1 (Mau forest), *Glomus* sp. 1 (Mau forest), *Glomus* sp. 2 (Mau forest), *Glomus* spp. (Mau forest), *Racocetra* sp. 1 (Mau forest), *Acaulospora* sp. 1 (Mau forest), *Scutellospora* sp. 1 (Mau forest) and *Scutellospora* sp. 2 (Mau forest) are reported for the first time from this forest ecosystem. These morphotypes could not be assigned species epithets as they did not match with other described species reported elsewhere. There is a possibility that these are new species. However, other AMF species observed in this study could be matched with species observed in surveys of AMF in other ecosystems. These species include *A. denticulate*, *D. nigra*, *S. heterogama*, *S. nigra*, S. *spinosissima* sp. nov. Dandan and Zhiwei (2007) reported 42 morphospecies of AMF. Among them, 28 were in the genus *Glomus*, 7 in *Acaulospora*, 4 in *Scutellospora*, 2 in *Entrophospora* and 2 in *Gigaspora* in the hot-dry valley of the Jinsha River, southwest China. In the present study, *Dentiscutata nigra* was the most frequent species as well as the most abundant species at the two study sites, suggesting that they may be particularly adaptable to this forest ecosystem. *Glomus* species were less frequent in this forest ecosystem despite the fact they are the most common AMF isolated throughout the world (Shi *et al.*, 2007). *Glomus* are said to be more common in agroecosystems and Gigasporaceae are almost absent in these agroecosystems but present in forest ecosystems. This could be attributed to disturbance and there is an indication that the two sites are relatively less disturbed and the degree of disturbance could vary depending on how much of the Gigasporaceae are present (Jefwa *et al.*, 2009). According to Dandan and Zhiwei (2007), the most common and frequent genus was *Glomus*, and several species of *Glomus* and Gigaspora were the most common and frequent among the 43 species present in hot-dry ecosystem of the Jinsha River, southwest China. They may be particularly due to its adaptation to arid conditions because Al-Raddad, (1993) reported that the genus *Glomus* was dominant in arid climates due to its resistance to high soil temperatures. In these forest ecosystem AMF belonging to the genus *Scutellospora* had the highest number of species at the two study sites although they occurred in much lower abundance, suggesting that this particular genus is adaptable to these elevations. The composition of AMF vary at different elevations, both at genus and at the species level. Furthermore, some of the AMF, such as *Scutellospora*, preferred a specific range of elevations (Gai *et al.*, 2012).

5.4 *In-vitro* study by dual culture interaction of *Armillaria* and *Trichoderma* species

The results from the present study have implications for the use of *in-vitro* tests as part of a general screening for efficacy of biological control agents. In the present investigation, inhibition of mycelial growth of *Armillaria* pathogens by *T. harzianum* and *T. asperellum* clearly showed that the antagonistic property of the two *Trichoderma* species was effective against the pathogen. These results clearly demonstrate that *Trichoderma* species inhibits the growth of the target *Armillaria* pathogen through its ability to grow much faster than the pathogens, thus competing efficiently for space and nutrients. Keca (2009) demonstrated that *Trichoderma viride* grow six times faster than *Armillaria* species and immediately after contact between *Trichoderma* and *Armillaria*, hyphae of *Trichoderma* began to grow over *Armillaria* mycelium. Vinale *et al.* (2008) suggested that *Trichoderma* is a fast growing fungus, a strong spore producer, a source of cell wall degrading enzymes and an important antibiotic producer. Several studies have shown that fungi can be used as biological control agents in a number of applications, including antagonism/competition with other fungi and insect control (Fernández-Sandoval *et al.*, 2012). Different researchers have reported the antagonistic activity of different *Trichoderma* isolates against plant pathogenic fungi including *Botrytis cinerea*, *Botryodiploidea theobromae*, *Colletotrichum gloeosporioides*, and soil borne fungi including *Rhizoctonia*, *Sclerotina*, *Pythium* and *Fusarium* (Wijesinghe *et al.*, 2010).

The results of the present study showed that *T. asperellum* showed a better growth of inhibition of *Armillaria* species compared to *T. harzianum* in the dual cultures interaction. The antagonistic effect of *T. aviride*, *T. harzianum* and *Trichoderma virens* against isolates of *Aspergillus niger* Van *Tieghem* that causes a disease called black mold on certain fruits and vegetables such as grapes (*Vitis* spp.), onions (*Allium cepa* L), and peanut (*Arachis hypogaea* L) was studied by Gajera, and Vakharia, (2010). Among the three antagonists tested, *T. viride* was found to be more effective than *T. harzianum* and *T. virens*. However, Shalini and Kotasthane (2007) screened seventeen *Trichoderma* strains against *Rhizoctonia solani in-vitro* and all strains including *T. harzianum*, *T. viride* and *Trichoderma aureoviride* more or less inhibited the growth of *R. solani*.

In the dual cultures, *Trichoderma* isolates grew on all possible sides of *Armillaria* species in plate to suppress further growth of pathogen within four to five days for *T. asperellum* and *T. harzianum*, respectively. No further growth of the pathogen was observed on dual cultured plates, where there was a dense sporulation followed by an overgrowth of *T. asperellum* on *Armillaria* species on the fifth day. *Trichoderma* species showed antagonistic activity towards

Armillaria species and an inhibition zone was observed. Later, *Trichoderma* overgrew on *Armillaria* species and destroyed it completely after 21 days of incubation. No growth of pathogens were observed when *Armillaria* species was sub-cultured from dual cultured plates. Dumas and Boyonoski (1992) investigated the mycoparasite mechanisms of *Trichoderma* species against rhizomorphs of *Armillaria gallica* using scanning electron microscopy. They observed events such as direct penetration, coiling of the *Trichoderma* hyphae around the *Armillaria* hyphae and disintegration of rhizomorph content.

The first physical contact between *T. asperellum* and *Armillaria* species, and that of *T. harzianum* and *Armillaria* species ocurred within four days and five days respectively, followed by growth inhibition which was similar to that reported by Almeida *et al.* (2007) where *T. harzianum* was found to be antagonistic against *R. solani* within 2-3 days. *Trichoderma* species showed initial faster growth and covered more than half of the plate within 2-3 days. In this study, the presence of an inhibition zone in the dual cultures of *Trichoderma* species and *Armillaria* species suggests the secretion of a diffusible inhibitory substance by *Trichoderma* species into growing media. The pathogens were grown fully on control plates after prolonged incubation leading to formation of rhizomorphs. Benhamou and Chet (1993) illustrated many interactions of *Trichoderma* with pathogens (*Rhizoctonia* and *Pythium*), whereby *Trichoderma* had grown parallel to pathogen, grown along the pathogen and grown around the pathogen. Normal degradation of pathogen mycelia would take place after penetrating with appressorium-like structures and they observed the growth of Trichodermal hyphae within the pathogen (Benhamou and Chet, 1993).

Visual analysis of the development of the confrontation showed the inhibition of *Armillaria* species growth shortly after coming into contact with the *Trichoderma* isolates. This cessation of growth was followed by an all-out invasion of the *Armillaria* species by the *Trichoderma* species. The results are in agreement with the findings of Mbarga *et al.* (2012) which showed the inhibition of *Pythium myriotylum* growth shortly after coming into contact with the *T. asperellum* strains. Present findings indicated that the strains of *T. asperellum* and *T. harzianum* can be used as biocontrol agents against the *Armillaria* species. The idea that mycoparasitism is one of the main ways for *Trichoderma* to combat pathogens is supported by previous studies. For example, John *et al.* (2010) reported the mycoparasitic activity of *Trichoderma viride* on *Pythium arrhenomanes*, a pathogen of soybean. However, they observed that, initially, *P. arrhenomanes* showed a faster growth leading to more than half of the plate being covered within two days. But later, after five days, *T. viride* overgrew *P. arrhenomanes* and destroyed

it completely. In the study of Mbarga *et al.* (2012) where the potential of four *T. asperellum* isolates were assessed as biological agents to control *Pythium myriotylum*, the causative agent of cocoyam root rot disease, the biological principle of their actions was discussed. According to these authors, all *T. asperellum* strains were antagonistic to *P. myriotylum* and could be aggressive mycoparasites. The confrontation tests between *T. asperellum* and *P. myriotylum* showed an inhibition of the mycelial growth of the pathogen. Similarly, a study by de los Santos-Villalobos *et al.* (2013) showed that *T. asperellum* has the ability to control anthracnose infection and development, a positive and ecologically sound role to play in mango plantations.

5.5 Interaction between AMF and *Trichoderma harzianum*

This study clearly demonstrated that inoculation of *T. harzianum* either individually or in combination with AMF enhanced growth in *D. torrida* seedlings under greenhouse conditions. The combination of AMF and *T. harzianum* resulted in the greatest improvement in growth compared to uninoculated controls. These results are in agreement with previous work that has demonstrated improvement of plant growth parameters by arbuscular mycorrhizae and other BCAs. Sukhada *et al.* (2011) showed that BCAs like arbuscular mycorrhizal fungi, *T. harzianum* and *P. fluorescens* improved plant growth parameters in papaya individually and in combination and also had a protective effect against the root pathogen *Phytophthora* under field conditions. Haggag and Abd-El latif (2001) found that the combined inoculation of *Glomus mosseae* and *T. harzianum* enhanced growth of Geranium plants. Similarly, combined inoculation of *T. aureoviride* and *G. mosseae* had a synergistic effect on the growth of marigold plants (Calvet *et al.*, 1993). Erman *et al.* (2011) also point out that whey, AMF and *Rhizobium* inoculation alone or in combination provided significantly increased yield and yield components over the control in chickpea.

The results of the current study may be explained by the different modes of action of *T. harzianum* and AMF fungi and their interaction in dual inoculated *D. torrida* seedlings. Co-inoculations of plant beneficial microorganisms in plant production systems may improve the efficacy of desired features in relation to biocontrol and plant growth promotion (Larsen *et al.*, 2009). Growth improvement of plants could be due to the synergistic activity of *T. harzianum* and AMF on host plant. These beneficial microorganisms might have colonized around the root and increased the root biomass and helped to increase the availability of nutrients. In this regard, Martínez-Medina *et al.* (2011_b) observed a synergistic effect on AMF root colonization due to the interaction between *T. harzianum* and *Glomus constrictum* or *Glomus intraradices*. The synergistic effect produced by the interaction between *T. harzianum* and AMF in the

current study could have been caused by a direct beneficial action of soluble exudates and volatile compounds produced by the saprophytic fungus as reported by Calvet *et al.* (1992). No negative interaction was observed in these results, in contrast to the results of Martinez *et al.* (2004). According to these authors, root and shoot weights of soybean were decreased by co-inoculation with *Trichoderma pseudokoningii* and *Gigaspora rosea.*

The current findings, further demonstrate that the growth of *T. harzianum* around *D. torrida* seedlings roots was suppressed by the presence of AMF. However, the presence of *T. harzianum* improved root colonization of *D. torrida* roots by AMF. McAllister *et al.* (1994) who studied the interactions between *G. mosseae* and several other saprophytic soil fungi found that the population of *Trichoderma koningii* and *Fusarium solani* in the rhizosphere of maize plants was decreased by *G. mosseae* when these saprobes were inoculated two weeks after the AMF, but not when they were inoculated at the same time as *G. mosseae.* Similarly, McAllister *et al.* (1995) also reported similar reduction of the population of a saprophytic fungus *Aspergillus niger* in the rhizosphere of maize and lettuce when *G. mosseae* was established in the plants. Fracchia *et al.* (1998) observed antagonistic, synergistic and neutral interactions between *G. mosseae* and associated saprophytic fungi.

The suppression of *T. harzianum* growth observed in the current investigations could have been directly caused by the AM fungus and/or indirectly by AM symbiosis-mediated factors. The establishment of AMF symbiosis in plants is known to change physiological and biochemical properties of the host plant and these changes may alter the composition of root exudates which play a key role in the modification of the microbial population qualitatively and quantitatively in the mycorrhizosphere (Siddiqui and Pichtel, 2008). Moreover, direct competition between extraradical mycelia of the AMF and *T. harzianum* fungi for the colonization sites and nutrients could also have occurred. Green *et al.* (1999) who found the mutually inhibitory interaction between *T. harzianum* and the external mycelia of an AMF *Glomus intraradices* suggested that the competition for nutrients as the possible cause. Additionally, the nature and extent of arbuscular mycorrhizal associations appears a crucial factor influencing rhizosphere microbial communities (Veresoglou *et al.*, 2012). Although the *T. harzianum* population was lower in the dual inoculated plants, the AMF colonization in roots was enhanced in compensation. Similarly, Calvet *et al.* (1992) observed a significant enhancement of *G. mosseae* growth by the presence of *T. harzianum in-vitro.*

Trichoderma isolates were able to colonize the root surface of *D. torrida* seedlings, characterized by the growth of the colony forming units by all the root surface of *Trichoderma*

inoculated *D. torrida* seedlings suggesting that the isolates used in this study are rhizosphere-competent strains. According to Fontenelle *et al.* (2011) the penetration process of *Trichoderma* species into the roots of plants is associated with the ability of the fungus to secrete an arsenal of hydrolytic enzymes which degrade the cell wall, including cellulase. These enzymes are capable of inducing defense mechanisms in plants, probably due to the ability to release fragments of cell wall in plants. According to Harman *et al.* (2004_b) the activation process of broad spectrum systemic resistance by *Trichoderma* species begins with the colonization of plant roots by this fungus. Shoresh and Harman (2008) also provided evidence that the existence of a relationship between increased growth and induced resistance in plants by root colonizing microorganisms such as *Trichoderma* species and non-pathogenic rhizobacteria. According to these authors, these effects could be mediated by different elicitors. Thus, the possibility of combining plant growth promotion and induction of disease resistance makes the use of *Trichoderma* species in disease control programs even more attractive.

5.6 Interaction between AMF and *Trichoderma asperellum*

This study presents the first assessment of AMF and *T. asperellum* to enhance growth and resistance of *D. torrida* against *Armillaria* species, the causal agent of Armillaria root rot disease under greenhouse conditions. Differences that emerged from measurements of plant growth parameters following the inoculation of plants with *T. asperellum* and AMF provided a number of lines of evidence to show that microbial inoculations could improve growth of *D. torrida* seedlings. In this study, significant increases in plant height, root and shoot weights were observed after inoculation with *T. asperellum* and AMF, although these increases were not similar for all treatments. Microbial inoculants are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Mader *et al.*, 2011). The smallest shoot/root ratios were obtained with the treatment involving the dual inoculation with *T. asperellum* and AMF, showing that the combined inoculation of *T. asperellum* and AMF was not essential for the promotion of plant growth. This may be explained by the different modes of action of *T. asperellum* and AMF and their interaction in dual inoculated *D. torrida* plants. The interaction between AMF and *T. harzianum* and its effect on plant growth may vary depending on the inherent characteristics of the AMF and the *T. harzianum* strain (Martínez-Medina *et al.*, 2011_a). Several reports have demonstrated that the interaction of these two groups of microorganisms may be beneficial for both plant growth and plant disease control (Martínez-Medina *et al.*, 2009). Although saprophytic fungi have been reported to influence AMF colonization and host plant response, the effects of the saprophytic

fungi on AMF formation differ depending on the inherent characteristic of both agents (Martínez-Medina *et al.*, 2011$_a$). AMF colonization levels of *D. torrida* seedlings were significantly reduced when dually inoculated with *T. asperellum*. This could be due to inhibition of AMF by *T. asperellum* through parasitism. As a biocontrol agent, the mechanism of action of *T. asperellum* is known to be based on mycoparasitism (Mbarga *et al.*, 2012). It could, therefore, be possible for *T. asperellum* to have had a deleterious effect on the AMF as reported by Rousseau *et al.* (1996).

In the current investigations, inoculations with AMF and *T. asperellum* resulted in higher increases of root weight than shoot weight as illustrated by the decrease in the shoot/root ratio. The shoot/root ratio of a plant is a good indicator of plant stress, whereby the lower the shoot/root ratio, the more stressed the plant (Naseby *et al.*, 2000). The smallest shoot/root ratios were obtained with the treatment involving *T. asperellum* alone and the dual inoculation with *T. asperellum* and AMF, showing that the combined inoculation of *T. asperellum* and AMF was not essential for the promotion of plant growth. Similarly, root and shoot weights of soybean were decreased by co-inoculation with *T. pseudokoningii* and *Gigaspora rosea* (Martinez *et al.*, 2004). Since the present experiment was conducted under controlled conditions with daily watering, a possible cause of stress could have been nutrient limitations and thus, a decrease in shoot/ root ratio could indicate such stress (Naseby *et al.*, 2000). Under these conditions, a larger increase of root weight is related to the expansion of the root system as a result of their search for more nutrients. While other reports indicate positive interactions following the combined inoculation of saprophytic fungal species and AMF (Srivastava *et al.*, 2010; Erman *et al.*, 2011), the reduced effectiveness of the combined use of the saprophytic fungus *T.asperellum* and the AMF in this study, suggests that the interactions of saprophytic fungi with AMF may differ depending on the AMF, the host plants and the species of the saprophytic fungus.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The Sururu forest presents diversity with respect to both tree species and indigenous AMF populations. The selected indigenous trees' roots were all colonized by AMF and the rhizospheres under *D. torrida* and *H. abyssinica* harbored highest numbers of AMF spores. This project will benefit the community living around Mau forest complex in that they will collect rhizosphere soils under *D. torrida* and *H. abyssinica* to be used in raising tree seedlings for reafforestation of the forest

Trichoderma harzianum and *T.asperellum* reduced the mycelia growth of *Armillaria* root rot pathogen significantly and destroyed it completely after prolonged incubation and therefore, can be incorporated for integrated management of the disease.

Under green house conditions, plant growth was found to be enhanced by the combined inoculation of arbuscular mycorrhizal fungi and *Trichoderma* species compared to individual inoculations. The study clearly demonstrated that inoculation of AMF and *T. harzianum* either individually or in combination enhanced root colonization in *D. torrida* seedlings under greenhouse conditions. However, negative interference between AMF and *T. asperellum* on root colonization has been demonstrated

6.2 Recommendations

1. The rhizospheres soils from under *D. torrida* and *H. abyssinica* indigenous trees that harbor higher abundance and biodiversity of AMF spores could be used for inoculation of tree seedlings to promote establishment and management of the natural environment in the Mau forest.

2. Mycorrhizae dependency test to evaluate individual AMF species against all tree species to be evaluated

3. A study should also be conducted on the impact of earthworm population on spore abundance.

4. The *Trichoderma* species with antagonistic activities against Armillaria should be formulated into products that can be economically used for management of Armillaria root rot in forest ecosystems.

5. The inhibitory effects of the *Trichoderma* species and AMF should be evaluated against *Armilllaria* species under field growth conditions inorder to explore their whole potential as biocontrol agents suppressing Armillaria root rot in the forest ecosystems.

6. For the successful use of both AMF and *Trichoderma* species in a sustainable plant production system, it is highly recommended to first study the functional compatibility between the AMF, the *Trichoderma* species and host plants, not only in terms of plant nutrients and growth responses, but also in terms of biocontrol activity.

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APPENDIX 1

A: List of studied species with information of their habit, size of propagule, habitat or ecology and usage

Species/family	Growth Habit	Size of propagule	Habitat/ecology	Usage
Podocarpus falcatus (Podocarpaceae)	Tree, upto 45 m tall	Fruit, 13 –15 mm	Commonly known as podo or East African yellow wood. Commonly found in the semi-humid lower highland forests of the central and eastern highlands between 1600–2500 m	Firewood, poles, timber, Paneling, boxes, boardsplywood, ornamental, Shade tree and agroforestry, medicine, treatment of breast cancer (bark), liver, meat allergies, appetite and stimulants.
Podocarpus latifolius (Podocarpaceae)	Tree up to 40 m tall		This tree occurs from central to southern Africa 900-3200m it dominates the higher sub humid to humid upland forest of Mt. Kenya. Also found in Tugen Hills and Mau Forest Complex. In Kenya it grows between 1500 and 3500m often forming pure stands above 2600m.	Firewood, poles, timber, boxes, bakery boards, medicine (roots), ornamental. Shade tree, treatment of breast cancer (bark), liver, meat allergies, appetite and stimulants.
Oleacapensis (Oleaceae)	Tree, up to 40 m tall	Drupe, 1.5×1 cm	A tree found from Ethiopia to west and central Africa and south through Kenya, Uganda and the democratic republic of Congo to Angola. In Kenya it is widely distributed	Dried roots are used for swollen joints, Medicine (roots and bark), charcoal, Roundworms (bark). Paneling, flooring, tool

			occurring from coast province (Taita hills) through to western and Nyanza province and also highland parts of northern Kenya. Altitude 1100-2600M. Agro-climatic zone II-III.	handles, bee forage, veneer, ceremonial (Maasai) furniture.
Olea europaea (Oleaceae)	Tree, up to 15 m tall	Seeds, 7×5 mm	Widely distributed in dry forest and forest margins, often with *Podocarpus* and *Juniperus* forests between 1500–2500 m. Hardy and drought resistant once established even in poor soils.	Firewood, charcoal, timber, poles, posts, tooth brushes, walking sticks, flooring, utensils, seasoning (fermenting and milk), bee forage, wind break paneling, carving, clubs, farm implements, edible fruit, olive oil, medicine, skin fungal infections; Stem bark decoction used in South Africa as antipyretic Roundworms, Malaria and associated fever. Symbol of Justice and Knowledge for Jews, but was also the image of prosperity and beauty.
Prunus africana (Rosaceae)	Tree, upto 40 m tall	Drupe, 1×0.7 cm	*Prunus africana* is widespread tree in montane habitats of Africa. Widely distributed in humid and semi-humid lower highland forests, humid and semi-humid upper highland forests and humid mountain wood lands between 1500–2300 m.	Firewood, charcoal, poles, timber, utensils, earrings, bee forage, carving, mulch, green manure, shade, windbreak, medicine (leaves, bark), veterinary medicine. Leaves used as inhalant for fever and stem bark for stomach and malaria, stem bark effectively treats

				prostate gland hypertrophy and benign protatic hyperplasia.
Hagenia abyssinica (Rosaceae)	Tree, up to 20 m tall	Fruit small	*Hagenia abyssinica* is a slender tree up to 20 m tall, with a short trunk and thick branches. Formerly it was the commonest high altitude rain forest tree. Currently found scattered in moist and wet climatic zones between 2300–3500 m	Firewood, poles, timber, fire breaks, furniture, flooring, carving, medicine (female flowers, bark and roots), veterinary medicine, mulch, green manure, bee forage, ornamental, soil conservation
Juniperus procera (Cupressaceae)	Tree, up to 35 m tall	Fruit berry like.	Commercially known as African pencil cedar, the largest Juniper in the world and one of the two important coniferous trees of Ethiopia. Valuable timber tree in highland forests of Ethiopia b /n 1500–3000 m	Firewood, the wood is termite tolerant and mostly used for fence, posts, poles, timber, joinery, beehives, flooring, wind breaks, medicine (twigs bark, leaves and buds) veterinary medicine, ornamental, ceremonial, shade tree
Dombeya torrida (Sterculiaceae)	Tree, upto 20 m tall	Seeds, 3–4 mm	Tree, upto 20 m Montane Aningeria-Albizia-Croton and Juniperus forest, montane scrub 1600–3100m Kenya, Ethiopia, south Sudan, Djibouti, Uganda, West North Tanzania, East Zaire, Rwanda and Burundi	Firewood, charcoal, poles, tool handles, ladders, spoons, bows, timber, , furniture, flooring, carving, yokes, poles, medicine (bark and roots), bee forage, soil improvements, mulch, fibre (from bark for ropes), shade tree.

Maytenus senegalensis (Celastraceae)	A tropical African shrub or tree from north Africa Somalia to Senegal, south to south Africa, in Madagascar and east to Bangladesh with a wide altitude range from sea level to up to 2400M. In Kenya it is found in most parts of the country except the dry north eastern parts in wooded grassland and riverine vegetation in the drier parts.	Firewood, charcoal, poles, timber, tool handle, walking sticks, combs, fodder, bee forage, life fence, dry fencing, shade, ornamental, flowers are put in coconut oil to perfume it for use as body oil, medicine (leaves, bark and roots). Leaves, stems and roots of these species are used in the local medicine by traditional healers to treat dys-entery, snake bites, wounds and respiratory diseases
Rapanea melanophloeos (Myrsinaceae)	Found from the coast to the western parts of the country and most tropical Africa to South Africa. It is more common in highland forests to an altitude as high as 3800M.	Firewood, charcoal, furniture-making and violins.The bark are used medicinally for respiratory problems, stomach, muscular and heart complaints. The berries are dried, ground and made into an extract or infusion like tea which is taken as an antihelminthic. Most parts of the plant are used as medicine (mainly against intestinal worms).Treatment of womb after delivery, veterinary medicine

Sources of information: Getachew *et al.*, (2002); Stewart, (2003); Wubet *et al.*, (2003); Fashing, P. J. (2004); Battinelli *et al.*, (2006); Bianco, and Ramunno, (2006); Muthaura *et al.*, (2007); Lans *et al.*, (2007); Lee and Lee, (2010) Long *et al.*, (2010) and indigenous knowledge from Mau Forest Complex.