

**Phenotypic characteristics of root nodule Bacteria and
Arbuscular Mycorrhizal Fungi infecting
Acacia polyacantha growing in Ghibe wooded grasslands**

A Thesis

**Presented to the school of graduate studies
Addis Ababa University**

**In partial fulfillment of the requirements of the degree of Master's of
science in Biology**

**By
YONAS YOHANNES**

February 2005

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

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Approved by Examining board

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Declaration

I the under signed declare that this thesis is my original work. It has never been submitted in any institution and that all sources of material used for thesis have been dully acknowledge.

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Acknowledgments

I would like to sincerely thank my advisor Dr Fassil Assefa for his guidance throughout the research work, the useful comments, suggestion and critical feedback of my writing style.

I gratefully acknowledge financial support of Addis Ababa University (AAU) and many thanks are due to the staff of Biology Department who taught me the different courses and Mr Girmay Medhin for his assistance in the statistical analysis.

I am indebted to the staff of Forestry Research Center (FRC) and National Soil Research Center (NSRC) as a whole with special thanks to soil biology and microbiology section for providing me access to the lab, computer facilities, guidance and for the good atmosphere and relaxing during working and mealtime.

Finally, my family and friends deserve all my thanks for their love and support.

Above all, I would deeply thanks to God who keeps me for doing this work according to his plan and will.

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List of symbols and abbreviations

AMF	Arbuscular mycorrhizal fungi
BTB	Bromohymol blue
CEC	Cation Exchange Capacity
CR	Congo red
EARO	Ethiopian agricultural research organization
EC	Exchangeable Cation
FG	Fast growing
FRC	Forestry research center
LM	Large mucoid
LW	Large watery
INVAM	International culture collection of Arbuscular and Vesicular Arbuscular mycorrhizal Fungi
M	Molar
m.a.s.l	Meter above sea level
Meq	milliequivalent
MPN	Most probable number
NSRC	National Soil Research Center
ppm	Parts per million
SD	Small dry
SG	Slowly growing
TY	Tryptone yeast
μM	Micro molar
VAM	Vesicular Arbuscular Mycorrhiza
v/v	Volume/Volume
w/v	Weight/Volume
YEMA	Yeast Extract Mannitol Agar

Abstract

Tree legume (*Acacia polyacantha*) was tested for their capacity to nodulate with indigenous root nodule bacteria in soil collected from Ghibe Valley. The five isolates were characterized by some biochemical and physiological tests. Based on specific growth rate and acid production, the isolates were assigned tentatively to *Bradyrhizobium* and *Rhizobium*. The infective propagules of Arbuscular mycorrhizal fungi (AMF) occurring on the study site was determined by spore count and most probable number (MPN). Average spore count of soil found out 58 per 100g and a hundred gram of field soil contains 189.42 infective propagules. The dominant spore count encountered was *Glomus* like AMF (59.1%) and 40.9% of the rest spores were *Gigaspora-Scutelospora* type AMF. Response to inoculation with *Bradyrhizobium*, AMF, dual inoculation and fertilizer treatments of *Acacia polyacantha* studied in glasshouse condition submitted to 7 treatments. After 70 days, the plants were harvested and nodule number and dry mass, shoot height, dry weight, nitrogen and phosphorus content were determined. Results showed that *A. polyacantha* inoculated with both organisms recorded the highest nodule number, shoot dry weight, nitrogen and phosphorus content ($p < 0.001$). Thus, the extent of rhizobium and mycorrhizal symbiosis in this tree species under green house condition can affect growth.

1. Introduction

The 1991 data obtained from Ministry of Agriculture revealed that the distribution of forest and woodland areas covers 2.8% of the land surface of Ethiopia (MOA, 1991). Out of this *Acacia-Boswellia*, *Commiphora* including wooded grassland cover 16%. It is also estimated that open savannah types of woodlands dominated by acacia species cover more than 20 million hectares of the country.

Ghibe Valley is part of the wooded grassland located in the southwestern part of the country. Different tree and shrubby species are widely distributed in this region. The wooded grassland dominated principally by grasses and scattered *Acacia* species, predominantly *A. polyacantha*, and fig trees (61%, Reid *et al.*, 2000). Eventhough the riparian woodland that is dominated by *Syzigium guineense*, *Acacia polyacantha* and *Ficus sycomrus* cover 3% of the vegetation, they are ecologically very important in maintaining the ecosystem (Reid *et al.*, 2000).

Population increase and their activities have led to the continuous clearing of the above-mentioned trees (particularly *Acacia polyacantha*) for construction, fuel wood and agricultural expansion. The uncontrolled clearing and utilization of these trees has exacerbated the destruction of the riparian vegetation (Reid *et al.*, 1997) that support plant, large mammal and bird species found in the valley. Further and more importantly, the removal of these trees has also threatened the diversity and functioning of other woodland inhabitants, including beneficial microorganisms such as mycorrhizal fungi

and rhizobium which are critical factor in restoring degraded ecosystems (Requena *et al.*, 2001).

In order to meet the ever-increasing demands of forage, wood, fuel and to enhance the fertility of the soil, conservation practice with new plantation programs are very crucial. There has been considerable interest in the use of trees as part of biological approach to manage soil fertility. Revegetation with indigenous tree legumes can therefore be a valuable rehabilitation tool. So plantation forestry (Evan, 1992) and agroforestry (Bene *et al.*, 1977) offer an important opportunity to meet local demands while restoring degraded soil.

Acacia polyacantha is one of the important agroforestry indigenous tree legumes. It has many local uses: first it is an important source of firewood (with high calorific value) and charcoal. The wood is generally hard and therefore used for house construction and manufacturing of furniture and farm implements. Leaves of *A. polyacantha* are an important source of fodder for wild and domestic animals. In addition, it produce gum which is edible and has been used as adhesive and in confectionary (Chikamai, 1996) so generate income for the local people as well as a hard currency for the country. The roots are used medicinally against snakebite and diseases such as gonorrhoea, leprosy and pneumonia (Gupta, 1993).

The establishment of adequate rhizobium and mycorrhizal fungi infection are among the most important means of improving seedling quality and establishment (Jaenicke, 1999). Soil containing various types of rhizobium and there is also high degree of specificity in nodulation and effectiveness (Postgate, 1998). The occurrence of both rapidly (Rhizobium) as well as slowly growing (Bradyrhizobium) infecting some of African *Acacia* species has been reported by Dreyfus and Dommergues, (1981). There are some reports of non-nodulating African acacias trees and shrubs (Faria *et al.*, 1990; Odee *et al.*, 1995). Host Rhizobium interaction also influenced by the provenance of the plant (Dart, 1994), because of these nitrogen-fixing trees (NFTs) shows great variation in nodulation and nitrogen fixation (Zaharan, 2001).

It has been demonstrated that, survival of seedling enhanced by effective rhizobial inoculation (Brad *et al.*, 2001). This is especially important when the seedling planted in degraded site where nutrient deficiencies limit their survival. There is also considerable evidence that inoculation of Arbuscular mycorrhizal fungi (AMF) can increase growth of trees (Diederichs, 1990), basically AMF help the plant in the uptake of less mobile nutrients, particularly phosphorus (Abbott and Robson, 1982). These peculiar characteristics make the AMF very crucial in the tropics where phosphorus deficiencies appear to be a major factor limiting the establishment of most NFTs (Sieverding, 1991).

Different studies have also shown that dual inoculation of AMF and rhizobium improves nodule formation, N₂-fixation, growth and phosphorus nutrition of host plant (Jasper *et al.*, 1989; Dart, 1994; Munro *et al.*, 1998). The two symbiont typically act synergistically

resulting in great nitrogen and phosphorus content in combination than when each is inoculated onto the legume alone.

The knowledge of symbiotic association with tree legumes is much less understood than with legume crops and little information is available about nodulation of woody legumes grown in Ethiopia. There have been studies on the occurrence of root nodule bacterium on some of indigenous tree legumes (Fassil and Kleiner, 1998; Endalkachew *et al.*, 2004). Similarly, few studies have been undertaken on the symbiotic effectiveness of tree legumes with native rhizobia and AMF (Million, 2002; Shasho, 2002). Information on nodulation of *A. polyacantha* from Kenya and elsewhere is available (Odee *et al.*, 1995; Njiti and Galiana, 1996). However, there is no information in local studies about ecophysiological characteristics of root nodule bacteria and response of *A. polyacantha* to dual inoculation with native AMF.

Inoculation with rhizobium and AMF is not only enhancing the establishment of plants, but also improve the fertility of the soil (Requena *et al.*, 2001). Hence prior to inoculation it is important to know how fertile and infective a soil is concerning density of the mycosymbiont, which may be crucial for further investigation in the establishment of seedlings. This study, therefore initiated with the aim to assess the nodulation status *A. polyacantha* in soil sample collected from Ghibe Valley. To isolate associated root nodule bacteria from nodules and characterize them with some phenotypic features. To determine the density of Arbuscular mycorrhizal fungi occurring in the study site beneath

A. polyacantha. Finally, to assess the symbiotic effectiveness of *A. polyacantha* with a standard root nodule bacteria and using mixed AM fungi inoculants.

2. Literature Review

2.1 Leguminous Tree

The leguminosae is a family of flowering plants represented by more than 18,000 species with the sub-families: Caesalpinoideae, Mimosoideae and Papilonoideae. Most of them nodulate and fix nitrogen in an endosymbiotic association with root nodule bacteria. The hitherto studies showed that, about 3003 species including woody and non-woody ones have been identified as N₂ fixers. Of these, 97% of tree species in the Papilonoideae, 90% of the Mimosoidae and 23% of the Caesalpinoidae species examined nodulated (Faria *et al.*, 1990). Sprent (2000) summarized the reports of nodulation in the leguminosae, the author reported that there are still more than 39% of genera, which have never been examined for nodulation, and further there are conflict reports for nodulation of about 3% of the legumes.

2.1.1 Genus Acacia

The genus Acacia is one of the largest genera of leguminous tree and shrubs consisting of three subgenera, Acacia, Aculeiferum and Phyllodineae. It has a wide distribution world wide with 1200 species. Many members of the genus are fast growing multipurpose. Both mycorrhizal infection and nodulation in majority of the group can occur naturally. This ability to enter into a variety of symbioses enables them to be successful and aggressive

colonizers. This has been put to good use in reclamation of degraded soils with higher acidity, high salinity, high aluminum saturation, and low soil fertility (Craig *et al.*, 1991; Ngulube *et al.*, 1993; Cole *et al.*, 1996). As a result of this, they are often recommended to establish plantation in fast disappearing natural vegetation.

In Ethiopia, they are widely distributed in the different agroecological zones and represented by 58 species of which 30% are trees and shrubs (Thulin, 1989). *Acacia polyacantha* an indigenous to Ethiopia is classified in the tribe Acacia, which is a large deciduous tree which can reach 25m tall, but usually up to 15m tall and with a diameter of 70cm (Thulin, 1989; Gupta, 1993). It has open to flat topped crown, feathery leaves with small, narrow leaflets. The flowers are large, creamy-white spikes, up to 12cm with pointer tips. A noticeable feature of the tree is yellowish bark, which peel off to give the stem a whitish appearance. It can be raised from seed and it is reported to be one of the fast growing acacia species and suited for coppicing and pollarding. It is a wide spread in tropical Africa from Gambia in the north to South Africa. It occurs mainly on alluvial or loam soils along river in riverine wooded lands.

2.2 Microbial symbiosis of Tree legumes

Plant-microbial interactions in the rhizosphere may involve a variety of bacteria and fungi with specific functional capabilities that may influence plant growth. The most important of these microorganisms are rhizobium and Arbuscular mycorrhizal fungi (AMF) (Patreze and Cordeiro, 2004). In the context of agroforestry and afforestation of

marginal lands, nitrogen-fixing trees of the family leguminosae have special advantage over other tree species. There is now considerable evidence on the need for inoculating nursery seedling with these beneficial microorganisms (Michelsen, 1993).

2.2.1 *Rhizobium*

Rhizobium is a general term given to a group of symbiotic bacteria capable of infecting and induces N₂-fixing nodules on leguminous plants. Member of the genus *Rhizobium* commonly inhabit the soil. All Rhizobia are root nodulating bacterium and are gram negative, aerobic with out endospore, and normally rod shaped cell. They are motile by one polar or sub polar or two to six peritrichous flagella (Jordan, 1984).

Previously, the classification of Rhizobia subdivided broadly into fast growing and slow growing types placed in one genus *Rhizobium* (Postgate, 1998). Another attempt of early classification that were grouped strains into what is so-called cross-nodulation were the main taxonomic criteria, with organisms grouped according to the hosts they nodulated. The advent of molecular biology and changes in the method used in bacterial taxonomy provided a new and more powerful approach in shaping previous classification. Currently the number of rhizobia species has increased and new species have been proposed. Root nodule bacteria infecting legumes are currently divided into 44 species in 12 genera, 10 of which belong to the sub class of α -proteobacteria and the rest belong to β -proteobacteria (Young *et al.*, 2001).

2.2.1.1 Nodulation Process

The mode of infection of leguminous plants by the root nodule bacteria has been recorded by Bhuvaneswari *et al.*, (1981). Details of colonization process differ in character from species to species. In general there is a simple exchange of chemical signals between the root and the bacterium (Brewin, 1991). Rhizobium migrates toward plant root by chemotactic response. The bacteria in the soil near by are stimulated to multiply by a secretion from the root of nutrients and growth factors. Wide range of attractant molecules are secreted by the plant root to invite rhizobium to enter into symbioses. It was found out that flavonoids attract rhizobium more specifically (De Trouch and Vanderleyden, 1996).

Entry of Rhizobia or infection occurs through the root hair via plant-derived tunnels, by crack entry or between cells of intact epidermises (Faria *et al.*, 1998). Later, the root hair deformed by bacterial secretions of exopolysacharides (Ervin and Hubell, 1985). Once the root hair is deformed, it serves as the means of invasion of the bacterial cells into the central portion of what becomes the nodule.

2.2.1.2 Symbiotic Nitrogen fixation

Symbiotic nitrogen fixation is strongly related to the nutritional and physiological state of the host plant. The symbiosis between the legumes and the bacteria is mainly under the control of the host plant (Tate, 2000). Nitrogen is the most abundant element in the universe. Although the atmosphere contains about 4×10^{15} tones of N_2 gas (Postgate,

1998), it is unavailable to plants until it is fixed. Interestingly, the majority nitrogen input derived from leguminous plants and some of the vast numbers of under appreciated legumes are tree species (Tate, 2000).

Biological nitrogen fixation requires a complex set of enzymes and a considerable expenditure of ATP. The ability to fix N_2 is restricted to the microsymbiont. The bacteria have the capacity to reduce the nitrogen atom from its most oxidized state (N_2) to its most reduced form (NH_3) by a complex set of enzyme called nitrogenase. The nitrogenase consists of two components, dinitrogenase (FeMo protein) and dinitrogenase reductase (Fe) protein. Under an ideal condition for each atoms of dinitrogen cleaves and reduced a minimum 16 molecule of ATP are utilized (Marschner, 1995).

2.2.1.3 Significance of tree legumes-Rhizobium symbiosis

Plant productivity in many dry land ecosystems is often limited by low soil fertility and moisture stress. Symbiotic nitrogen fixation is an efficient source of nitrogen (Peoples and Ladha, 1995) and has the greatest impact on the return of nitrogen to the cycle. In the context of sustainable agriculture, the need to exploit biological nitrogen fixation (BNF) should be considered as an appropriate alternative strategy directed at sustainability.

BNF is the most important single property of root nodule bacteria. It is estimated that approximately $200\text{kg N ha}^{-1} \text{ yr}^{-1}$ is added to the terrestrial ecosystems, of which 70% is contributed by legume-rhizobia symbioses (Burns and Harley, 1975). The use of nitrogen

fixing trees in agroforestry systems, for example, has shown considerable promise for sustained productivity of soil and increased crop yield (Danso *et al.*, 1992). Therefore in the developing countries where farmers cannot afford nitrogen fertilizer application, BNF can make agriculture more economically viable and competitive (Dakora and Keya, 1997).

There are different methods to estimate the nitrogen fixed, however the measured amounts may differ according to the methods used (Marshner, 1995). Whatever the true figure and regardless of the method used, nitrogen-fixing trees can transform or 'fix' atmospheric nitrogen appreciably. For example, it is estimated that annual nitrogen fixation rates for tree legumes ranges from 43 to 581Kg/ha/yr (Dakora and Keya, 1997) compared to about 15 to 210Kg of N/ha/yr for grain legumes. Nitrogen fixing trees (NFTs) could therefore be an important component of production systems in agroforestry that may be seen in terms of benefit to crop, livestock production and soil fertility.

In general NFTs are rapid growers, propagate easily and grow excellently in dry areas at different conditions. Apart from nitrogen contribution to the soil, these trees have extensive root systems that extract nitrogen and other nutrients from deeper soil horizon and eventually from the water table. And then, they return it to the soil surface with leaf litter and accumulate windblown organic residue near the trunk under their canopy. The fixed nitrogen and other minerals trapped from the deep soil subsequently transferred to

the soil and possibly to the associated plants by microbial degradation or mycorrhizal link.

2.2.1.4 Factor affecting symbiosis

A favorable rhizosphere environment is vital to legume-Rhizobium interactions. Despite the high potential productivity of tree legumes, several environmental factors affect tree growth and symbiotic nitrogen fixation. Among the factors that affect plant growth and symbioses are: soil moisture, soil temperature, soil pH, soil nutrients and soil salinity. In the field these factors operate synergistically and some of them are interdependent one another (Bordeleau and Prevost, 1994).

Water stress in general affects the viability of rhizobia, infection and nodule function (Reddell, 1993). Rhizobia are mesophile and most tropical legumes have few nodules and tolerate temperature up to 40⁰C. Above 40⁰C, soil temperature strongly affects nodulation process and functioning (Arayangkoon *et al.*, 1990).

Soil acidity causes problems in inherent adverse concentration of elements and nutrition of both rhizobium and the legumes. This effect is due to H⁺ concentration, toxic levels of aluminium (Al) and manganese (Mg) and deficiencies of calcium (Ca), phosphorus (P) and Molybdenum (Mo). Soil acidity *per se* can limit rhizobial growth and persistence in soil. Increasing salt concentration may have a detrimental effect on plant growth and symbiotic N-fixation as result of direct toxicity caused by specific ions e.g. Na, Cl, H

CO₂ and a general salt effects such as osmotic effect on water uptake (Tate, 2000). Alkalinity can effect on symbiotic N-fixation via nutritional disorder such as deficiencies in Fe, Mn, Cu and Zn (Bordeleau and Prevost, 1994).

An adequate supply of mineral nutrient is essential for growth of macrosymbiont as well as the root nodule bacteria. Well nourished plants generally nodulated and fix nitrogen better than malnourished ones. Because of the nature of symbiotic association, nitrogen-fixing plants require complex nutrients such as molybdenum and cobalt than non-nitrogen fixing plants in large amount.

2.2.2 Arbuscular Mycorrhizal Fungi

Mycorrhiza is a name for a fungi and plant root association. Mycorrhizal fungi are a major component of the soil micro flora in many ecosystems. Many woody legumes can develop a double symbiosis with both nitrogen-fixing bacteria and mycorrhizal fungi (Barea and Azcon-Aguilar, 1983). In many tropical woody legumes AMF type is the predominate partner (Dart, 1994). Traditionally two classes of mycorrhiza have been recognized based on morphological differences; ecto- and endo-mycorrhiza. However, over the years seven types of mycorrhizal associations have been recognized (Harley and Smith, 1983).

The classification involving different groups of fungi and host plants with distinct morphological patterns:

1. Ectomycorrhiza- these mycorrhizas develop a mantle or sheath outside of the fine root tips;
2. Endomycorrhiza-in this type of association the hyphae penetrate between the cortex and often enter them but they don't form a mantle around the roots;
3. Ericoid mycorrhizas- forming a simple intraradical phase (within roots), consisting of dense coils of hyphae in the outer most layer of root cells (Hartig net), but the hyphae don't form a mantle and don't extend very far into the surrounding soil;
4. Ectendomycorrhiza- might have with or without a mantle, with Harting net and cellular penetrating by hyphal coils;
5. Arbutoid mycorrhiza- with mantle, Harting net and cellular penetration by hyphal coils;
6. Monotropoid mycorrhizal- with mantle, Harting net and cellular penetration;
7. Orchid mycorrhizal- don't form mantle and with cellular penetration by hyphal coils.

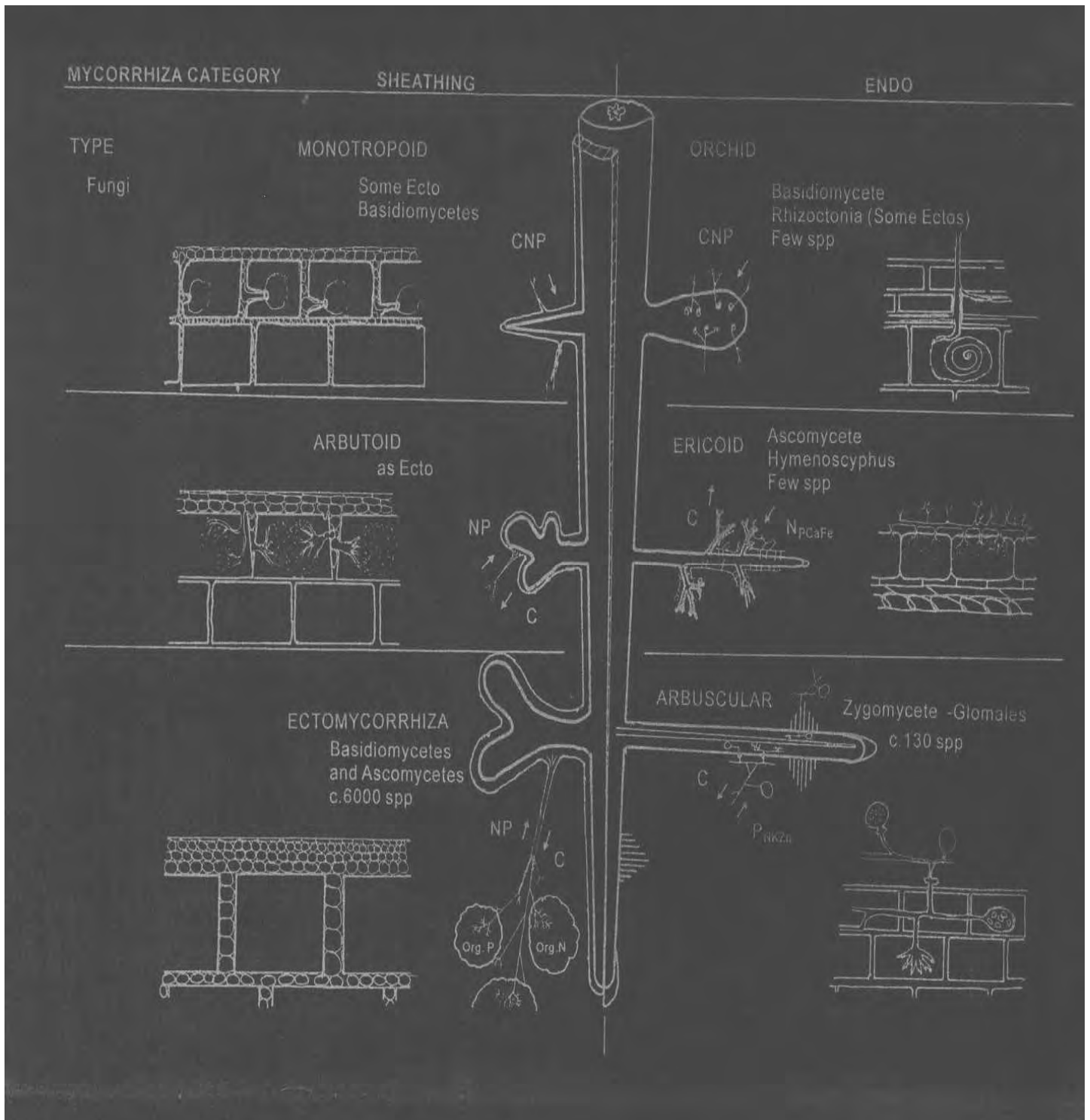


Figure 1. The structural and major nutrient pathways of the six recognized types of mycorrhiza. Extracted from Read, D. J. (1999).

2.2.2.1 Taxonomic Description

Taxonomically Arbuscular mycorrhizal fungi (AMF) belong to the fungal class of the Zygomycetes containing a single order Glomales (Morton and Benny, 1990). The bulk of known species belongs to the family Glomaceae that includes the genera *Glomus* and *Sclerocystis*.

The greater difficulty in the classification of Arbuscular Mycorrhizal Fungi emanate from the fact that it is based largely on the structure of their soil borne-resting spore, and they cannot be cultured axenically that necessitates the presence of actively growing plants during their reproduction. This makes them difficult to observe under an *in vitro* condition, as a result of which accurate identification of AMF often requires growing of the host plant with the fungus (Morton, 1993).

Different morphological characters that are used to identify AMF to genus level mainly focused on spores. Because spore contain most of the structural diversity retained in glomalean fungi. The features are: mode of formation of spores, spore sub cellular structure and mode of spore germination (INVAM, 2004). Detail identification requires extra information such as spore wall structure (i.e., composition of layers, thickness, color, ornamentation, etc.) and germination structure (Morton and Bentivenga, 1994).

2.2.2.2 Development of AMF infection

NFTs are known to form association with Arbuscular mycorrhiza with members of the genera *Glomus*, *Gigaspora* and *Acaulospora*. AMF exist in the soil as spore or as vegetative propagules in root fragments. Species of AMF have been reported to lack host specificity as a consequence of this a given fungal propagules obtained from an annual plant can also readily establish on a perennial plants. Similarly a given plant root system can be infected by different group of AMF species (Wilson, 1984). However, some modern literature seems to acknowledge previous works on specificity (Patreze and Cordeiro, 2004).

Resting spore of the fungus, germinating or extra radical hyphae in the soil or hyphae associated with root fragments are infective propagules where the fungal development can start (Brundrett *et al.*, 1996). Different experiment tried to show that plant exudates of volatile compounds, especially (iso) flavonoids, stimulate spore and hyphal growth (Nair *et al.*, 1991). Usually association start when the soil hyphae contact a root of potential host. Penetration takes place between the epidermal cell and often forms an appressorium (Brundrett *et al.*, 1996). Arbuscule and some times vesicles are formed after the aseptate hyphae penetrate the cortex in both directions from the entry point. The successful establishment of mycorrhiza on host root depends on the interaction between the symbiont and environmental factors such as soil moisture, pH and soil fertility (Linderman, 1988).

2.2.2.3 Function and importance of AMF in plant growth

It has been demonstrated that colonization of the roots by AMF improves the productivity of plant in soils of low fertility (Sieverding, 1991). In so doing the hyphae of AMF have the potential to greatly increase the absorbing surface area beyond the root into the surrounding soil to improve the uptake of poorly mobile ions such as P, Zn and Cu where by their uptake depends on the root density per volume of soil. Furthermore the finer hyphae (narrow diameter relative to roots) can also penetrate soil pores that were otherwise inaccessible to root hairs and enhance effective absorption of nutrients.

Studies have also shown that colonization by AMF might also improve the drought tolerance of the host (Auge, 2001). In dry soil phosphorus is limiting even in soil that test high in available phosphorus. But water uptake by mycorrhizal plants increased as a result of direct involvement of AMF on the rate of transpiration and photosynthesis (Allen and Bousalis, 1983). These two peculiar characteristics broadening the ecological niches of plants through changes in the availability of nutrients and water. Therefore, mycorrhizal symbioses are important for survival and establishment of vegetation that might be used to achieve a variety of rehabilitation objectives and agroforestry purpose.

In addition to increase plant productivity, mycorrhizal infection can also alleviate stress due toxicity in soils of high pH and activate plant defense mechanisms (Cordier *et al.*, 1996). In an ecosystem, the extrametrical hyphae produce hydrolytic enzymes that can have an important impact on organic matter mineralization, nutrient availability and also

bind soil particles together and thereby improve soil aggregation (Sieverding, 1991; Brundrett *et al.*, 1996).

2.2.3 Dual symbiosis

Many nitrogen-fixing trees are able to form a tripartite association (Diop *et al.*, 1994). There is abundant literature to support the contention that dual inoculation of rhizobia and mycorrhizal fungi improves the growth of the host plant better than either of the inoculation (Manjunath *et al.*, 1984). When selected rhizobium has been used for a dual inoculation with AMF, significant increase in growth and nodulation has been realized. For example Manjunath and Bagyaraj (1983) found out that, dual inoculation of pigeon pea and cowpea resulted in an increased in shoot, root and nodule dry weight and N and P content of shoot compared with plant inoculated with either organism alone.

In another experiment on the symbiosis of *Acacia saligna* with *Glomus mosseae* and rhizobium in fumigated and unfumigated soil, Benbrahim and Ismaili (2002) found out that dual inoculation significantly increased total dry weight by 56.9%, N and P content of plants by 70.9% and 76.3%. Similar experiments on different hosts carried out and strengthen previous findings, such as Michelson (1992), Michelson and Sprent (1994) and Das *et al.* (1997), all suggested their possible synergistic effect of rhizobium and AMF on growth, nodulation, N-fixation and phosphorus uptake.

However, it is clear from the finding obtained so far, effective rhizobium strains and AMF can increased plant performance in areas of marginal fertility. The mycorrhizal associations circumvents the problem of additional nutrient requirements and assist the plant with increased uptake of nutrients and water through external hyphae particularly in soil of low nutrients. In general the most prominent interaction of Rhizobium with AMF is largely plant mediated and by phosphorus supply via AMF for higher needs in N₂ fixing legumes for successful nodulation (Marschner, 1995).

3. Materials and Methods

3.1 Study site

Ghibe Valley is geographically located in the southwestern part of the country just 180Km to the South West of Addis Ababa on the way to Jimma. It is located at E037⁰15'-037⁰40' and N08⁰00'-08⁰30' and with the elevation 1050m.a.s.l. at river Ghibe bridge. The mean annual temperature of the region is within the range of 18.5⁰C - 25⁰C and the annual rainfall is 1000mm/year (MOA, 1998) where by the highest rainfall is obtained in July. According to MOA (1998), Ghibe valley is categorized under hot to warm sub-humid agro ecological zone. Frequent fire and charcoal production are very common. The vegetative physiognomy is dominated by wooded grassland (61% cover, Reid *et al.*, 1997), because of hot temperature conditions low land plants are found mainly *Acacia* species.

3.2 Soil Sampling

Prior to soil sampling, four sampling units were selected, taking river Ghibe bridge as a reference point (1050 m.a.s.l.). From both sites, two square plots of 100mX100m in opposite directions (on the way to Jimma and Wolkite town), a total of 4 plots were selected as sampling units. From each sampling unit 5 trees of age 15-20 years were randomly selected and approximately 5Kg of soil were taken from the depth 0-30 cm beneath the tree canopy of *A. Polyacantha*. The representative soil samples were then transported to the National Soil Research Center, Ethiopian Agricultural Research organization (EARO), Addis Ababa for analysis, nodulation test and pot experiment. The result of soil physical and chemical analysis is depicted in table 1.

Table 1. Chemical characteristics of the surface soil (0-30cm)

Parameters	Unit	Value
PH (H ₂ O)		6.8
EC	ds/m	0.464
Na	meq/100g soil	0.420
Ca	meq/100g soil	39.77
K	meq/100g soil	1.88
Mg	meq/100g soil	8.97
CEC	meq/100g soil	55.60
Basal saturation	%	92
Total nitrogen	%	0.265
Organic carbon	%	3.788
C/N	-	14.29
Available P	ppm	12.40
Sand	%	24
Silt	%	28
Clay	%	48
Soil class		Clay

3.3 Seed Sample

The seeds of *Acacia polyacantha* were procured from Forestry Research Center, Addis Ababa. Seed testing data are shown in appendix I.

3.4 Nodule induction

Nodule induction was followed the methods described in Vincent, (1970). One kilogram of field soil from each soil sub-samples that were collected from the selected trees were thoroughly mixed and prepared for potting after sieving through 5-mm mesh size.

Plastic pots with 1Kg volume capacity were surface sterilized using 70% alcohol and were used for nodule induction. Seeds were surface sterilized with acidified 2% HgCl₂ (5ml concentrated HCl with 1L water) and thoroughly washed with distilled sterilized water. To aid germination, the seeds were immersed in boiling water for 1 minute, which were then allowed to cool for 24 hour and transferred to circular sterilized petri plates containing filter paper and incubated at room temperature. Eight germinating seeds were then transplanted in each pot, which were thinned to three after a week. The pots were watered with distilled water every day and fertilized with Jensen's nitrogen-free medium twice a week for one month.

Jensen's nitrogen-free medium

CaHPO ₄	1g/l
K ₂ HPO ₄	2g/l
MgSO ₄ .7H ₂ O	0.2g/l
NaCl	0.2g/l
FeCl ₃	0.1g/l
Trace element	1ml

Trace elements:

(H₃BO₃, 2.86g/l ; MnSO₄.4H₂O, 2.03g/l; ZnSO₄.7H₂O, 0.22g/l; CuSO₄.7H₂O 0.08g/l and Na₂MoO₄.2H₂O, 0.14g/l).

3.5 Rhizobium Isolation

The seedlings were excavated two months after planting. The pots were massaged from the edge to loosen contents and pooled the shoots gently to take out from the pots. And then the contents were immersed in a container containing water and washed several times to remove soil particles. For positive seedlings the nodules were detached from the roots and surface sterilized in a solution containing 95% ethanol for 8 seconds and with 5% acidified HgCl₂ for 3 minutes. The nodules were thoroughly (8 times) rinsed with sterile distilled water (Vincent 1970; Somasegaran and Hoben, 1994). The sterilized nodules were transferred into a beaker containing a few drops of sterilized 0.85% NaCl solution and crushed with a sterile glass rod. The sap was streaked on to Yeast Extract

Mannitol Agar (YEMA) containing Congo red (Vincent, 1970) plates and incubated at 28⁰C.

Yeast extract mannitol agar (YEMA)

Mannitol	10 g
K ₂ HPO ₄	5g
MgSO ₄ .7H ₂ O	0.55g
Nacl	0.2g
Yeast extract	0.1g
Agar	15g
Distilled water	1 L
Congo red	10 ml

Plates were periodically examined for growth and single bacterial colonies were picked and re-streaked onto new YEMA plates for purity. Pure isolates were then preserved on YEMA slants containing 0.3% (w/v) CaCO₃ and stored at 4⁰C (Vincent, 1970).

Bacterial designation of isolates

Apo refers to isolate from *Acacia polyacantha*, taking the first letter from the generic name (**A**) and the first two letters from species name (**po**).

3.6 Authentication and effectivity of isolates

Authentication and effectivity study under taken in parallel in three-replicated pot. Surface sterilized seeds as before were sown in sterilized petri dishes. After the emergence of the radicle and hyla five germinating seedlings were transferred into surface sterilized plastic pots (1Kg capacity) containing sterilized sand (121⁰C, 15lb/in²). The seedlings were then inoculated with each of the five homologous isolates grown in YEMA broth for 2 days on a rotary shaker (120 rev/min) at 28⁰C±2⁰C. The seedlings were thinned to two after a week.

After 45 days of growth, the whole plants were removed from the pot. The nodulating capabilities of the isolates were assessed by visual inspection. If only those that showed nodules were considered authentic rhizobial isolate. Effectivity of the five homologous isolates assessed on the basis of nodule most inner color, nodule number and nodule mass and whole plant dry weights after drying at 70⁰C for 72 hours.

3.7 Characterization of the isolates

From authentication and effectivity result, three distinct groups were obtained, (Apo3, Apo4 and Apo5) which showed no difference but differ from Apo1 and Apo2. From these groups isolates Apo2 and Apo3 were selected for pot experiment. And all isolates characterized by morphological and physiological features. They were categorized as wet and dry according to Ahmed *et al.*, (1984). All inoculation were standardized by growing the isolates in shaker incubator with an inoculum size of approximately 10⁸/ml unless otherwise specified. All the physiological and biochemical tests except nitrate reduction

test (adopted from Lindstrom and Lehtomaki, (1988)), were carried out in triplicate on YEMA plates following the methods described in Mohamed *et al.*, (2000); Zerhari *et al.*, (2000). Each plate was spot-inoculated 10 times with 10 μ l of the isolate culture using standardized isolate. Except cultures used to determine minimum-maximum growth temperature, all inoculated plates were incubated at 28⁰C \pm 2⁰C. And all colonies were monitored manually after 10 days growth.

Morphological and cultural characteristics

The test isolates (\sim 10⁸cell/ml) were inoculated into YEMA medium and incubated at 28⁰C \pm 2⁰C. They were periodically checked for growth about 10 days. Colony size and appearance were recorded as SD (small dry), LM (large mucoid), or LW (large watery) according to Ahmed *et al.*, (1984).

Urea hydrolysis

Urea hydrolysis was performed following the method described in Lindstrom and Lehtomaki (1988). Hydrolysis of urea by the test isolates was investigated on YEMA agar medium containing 2% (w/v) urea and 0.012% phenol red. A red color appearance in plates containing the urea designated as positive result.

Nitrate reduction

Nitrate reduction test was carried out in liquid TY medium containing 0.1% (w/v) KNO₃ following the method described in Lindstrom and Lehtomaki (1988). **Tryptone yeast (TY) Medium (g/L)** (Tryptone, 5.0; Yeast extract, 3.0; CaCl₂.H₂O, 0.87; Distilled water,

1L). Each isolates ($\sim 10^8$ cell/ml) were inoculated onto the TY broth and were grown for 4 days at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Sulfanilic acid and α -naphthylamine were used as indicators and result designated as + when red color appeared after a few drops of the indicator added into the medium.

Growth rate determination

Growth study carried out following the methods describe in Somesagaran and Hoben (1994). First, each isolate were streak on YEMA plates and single colony is transferred into a test tube containing 10 ml YEM broth which is then incubated overnight on a rotary shaker (120 rev/min) at $28 \pm 2^{\circ}\text{C}$. Then one ml of cell suspension (*ca* 10^8 cell) was added to a 250 ml Erlenmeyer flask containing 100 ml of YEM broth that is then incubated as before. Growth was monitored turbidometrically by taking samples at interval of 4 hours and determined during logarithmic growth. Absorbance was recorded with a spectrophotometer at a wavelength 600 nm.

Acid and Base production

Acid or alkaline production of the five homologous isolates were detected on YEMA media containing 0.5% Bromothymol blue (BTB). The test isolates were grown in YEM broth for 2 days and streak onto BTB-YEMA and then incubated for seven days. Change in the color of the medium into yellow is an indication of acid production that is a characteristic of fast growers according to Jordan (1984).

Carbon source utilization

The test carbohydrates were prepared 10%(w/v) solution in water. The different carbohydrates as a carbon source were tested by replacing mannitol but yeast extract was reduced to 0.05g/L according to Somasegeran and Hoben (1994). Arabinose was filter sterilized using Millipore size 0.2 μ m and added to the medium after sterilization but heat stable carbohydrates were autoclaved together with the medium. YEMA plates without an added carbon source and with mannitol were used as a negative and positive control respectively. Data recording were done qualitatively following the method described in Somasegeran and Hoben (1994) as follows: G= good, W=week, and 0=no growth comparing with the control.

pH-tolerance

The ability of the isolates to grow at acid or alkaline pH was tested on YEMA plates. Prior to autoclaving, the media were adjusted to low pH range 4 and high pH 9 using acetic acid and sodium hydroxide respectively. Additional plates adjusted at pH 7 were used as a positive control. Growth of the test isolates were evaluated qualitatively following the method described in Lupwayi and Haque (1994) as follows : negative,- =no growth, +=minimal growth, and ++=maximal growth as it appeared in the control.

Salinity tolerance

The ability of the five homologous isolates tolerance to sodium chloride (NaCl) and calcium chloride (CaCl₂) were assessed through determining the growth on YEMA plate

containing 1, 2, 3% (w/v) at pH 7.0. Standard YEMA plates inoculated with the respective isolates were included for comparison purpose. Data presentation and interpretation was similar with pH tolerance test.

Growth temperature

Growth temperature for the test isolates was determined on YEMA plates. The plates were incubated at the following temperatures: 4, 10, 15, 20, 25, 30, 35, and 40°C, and growth were monitored for 10 days. Data presentation and interpretation was similar with pH tolerance test.

Antibiotic resistance

The resistances to antibiotics were tested on YEMA plates to which the following antibiotics were added: Streptomycin (120µg/ml), Chloramphenicol (200µg/ml), Nalidixic acid (100µg/ml), Rifampicin (100 µg/ml) and Kanamycin (100µg/ml). The antibiotics were filter sterilized using Millipore size 0.2µm and added to the medium after sterilization. Plates without an added antibiotic was employed as a positive control. Rhizobial growth on antibiotic contain YEMA were determined qualitatively and recorded as negative (-) and + for no growth and positive growth respectively. If growth is not clear it was recorded as +/- according to Lupwayi and Haque (1994).

Heavy metal resistance

The resistances to heavy metals were tested on YEMA plates to which the following heavy metals were added: $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 500 $\mu\text{g/ml}$, $\text{HgCl}_2 \cdot \text{H}_2\text{O}$, 5 $\mu\text{g/ml}$ and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 500 $\mu\text{g/ml}$. The heavy metals were filter sterilized using Millipore size 0.2 μm and added to the medium after sterilization. Inoculation, incubation and data presentation were similar with antibiotic test.

3.8 Characterization of Arbuscular mycorrhizal fungi

3.8.1 Spore extraction

Spore extraction were adopted from Sieverding (1991); Brundrett *et al.*, (1996). Soil and root fragment samples were taken around the tree trunk of the 20 randomly selected trees of *A. polyacantha*. From each tree 1Kg samples from the top 30 cm of the soil profile were collected and each sample from the respective sampling units pooled together separately. From these samples, sub-samples 50g of soil and root materials were suspended in 1-liter of water for 20 sec., the soil suspension was then decanted over a series of sieves with 425, 219, 125 and 50 μm mesh size. The washing and decantation process was repeated till the water was clear. The contents of 219, 125, and 50 μm mesh size sieves were transferred with some water into 100 ml tubes. Each suspension was then centrifuged for 5 min at 2000 revolution per minutes (RPM).

After having decanted the supernatant, each soil-spore mixture of the pellet was homogenized in 50% sucrose solution and then centrifuged for 1 minute at 2000 RPM.

Finally, the supernatant contained the spores were washed quickly onto a 50 µm mesh sieves and filtered. After repeated washing, the spores were collected manually with micropipette and then transferred into a sterile petridish and stored at 4⁰C until counted.

3.8.2 Identification of AMF

The spore samples were separated by typifying morphological features such as shape, color and hyphal connection and spore surface using a dissecting microscope at 30 to 50X magnification and further mounted on microscopic slides. Details of spore ornamentation and wall structure examined after making squashed spore over a microscopic slide and observed under a compound microscope to determine fungal isolates tentatively to genus level (INVAM, 2004).

3.8.3 Quantification of AM fungal propagules

Two methods (direct spore count and most probable number) were used to assess the density of propagules of mycorrhizal fungi in the sampled soils. From the homogenized soil, four 100g-sub samples from each sampling unit were taken separately for estimation of spore density. The plates containing the spores were taken out from the refrigerator and sterile water was added to the plate. Then, the plates were agitated and transferred the contents into a grid-lined plastic petridish and counted at 30 to 50X magnification.

Secondly, the most probable number (MPN) was used following the methods described in Sieverding (1991). Four fold dilutions series with 5 replications as a standard

technique were used. Fifty plastic cups of about 300ml volume were used as a potting vessel. The cups were divided into ten sets each containing 5 cups. The 10 sets represent dilution starting 4^0 , 4^{-1} , 4^{-2} ...and 4^{-9} . Each cup was initially filled with 150g of autoclaved soil, which was later layered with 50g (containing slightly air dried field soil diluted with an autoclaved soil), and finally covered with 50g of autoclaved soil. Eight surface sterilized sorghum seeds were seeded in each cup and after a week thinned to five following germination. The seedlings were watered each day with distilled water and maintained in a greenhouse and harvested three month after emergence of the plant.

Fifty test tubes were prepared in the same enumeration as given to the plastic cups. Roots within the diluted portion were taken out and washed from the adhering soils using tap water. From each pot a sub-sample of 2g fresh roots was removed and cut into 2-3cm long pieces approximately. They were then transferred into a test tube containing 10% KOH and autoclaved (121°C , 15 lb/in^2) for 15 min. Cleared roots captured on a fine sieve ($50\mu\text{m}$ mesh size), rinsed thoroughly with water and transferred into a staining solution Trypan blue (0.05% w/v) in lactoglycerol (1:1:1 lactic acid, glycerol and water), autoclaved (121°C , 15 lb/in^2) for 15 minutes. Stained roots were examined for infection using dissecting microscope.

The number of infective propagule calculated by $d=10^{\log(x.\log a-k)}$

Where x= sum of infected pot per replication

a= the factor of dilution

k= a constant from fisher table

d= # of infective propagules

3.8.4 Inoculum Production (Trap culture)

Inoculum production was conducted following the method described in Brundrett *et al.*, (1996) with slight modification recommended in INVAM (2004). Sterilized sand and field soil were mixed in a ratio of 1:3 (v/v) and filled in a pot of 3Kg capacity. From the most frequent occurred spore type i.e. Glomus like AMF, two hundred spores of uniform appearance were selected and picked up using a micropipette and transferred into sterile petriplates containing filter paper. The collected spores were then washed into a wide 3cm deep hole in pots that was filled with sterilized sand and soil. Before sorghum seeds were planted, the spores were covered with sand and watered with modified Hogland's solution II following the methods described in Mitiku and Osrio, (2001). The seedlings were maintained in a greenhouse and watered every day with deionized water and once a week with nutrient solution. Before three weeks of harvesting, the seedlings watered every three days for two weeks and withdraw completely for subsequent week. The seedlings were allowed to grow 4 months and then harvested.

Modified Hoagland's solution II

Stock solution	working solution (ml/L stock solution)
MNH ₃ NO ₃	1
MKNO ₃	6
MCa (NO ₃) ₂	4
MMgSO ₄ . 7H ₂ O	2
Micronutrient solution	1

Micronutrient solution: (H₃ BO₃ , 2.86 g; MnCl₂.4H₂O, 1.81g; ZnSO₄.7H₂O , 0.22g; CuSO₄. 5H₂O, 0.08g and Na₂MoO₄. H₂O, 0.02g)/L.

3.9 Pot Experiment

A pot experiment was conducted in a greenhouse at the National Soil Research Center (NSRC) in Addis Ababa, Ethiopia (2394 m.a.s.l, maximum mean temperature 25-31⁰C, and minimum mean temperature 19-25⁰C). Plastic pots each of 3 kg capacities were surface sterilized as before and filled with sterilized field soils (121⁰C, 15 lb/in²) for one hour.

The mycorrhizal fungus applied was crude inoculum which had been grown in trap culture, consisted of colonized sorghum roots and adhering soil at a rate of 100g/pot homogenized with the sterilized soil. The root of the host crop was cut into small pieces and mixed with the rhizosphere soil on the surface of plastic sheet swabbed with 90% alcohol.

Seeds of *A. polyacantha* were surface sterilized as before and allowed to germinate on petridish until both hyla and radicals were properly seen. Eight germinating seeds of uniform appearance were transplanted in each pot. Selected rhizobium isolates (Apo2 and Apo3) were cultured overnight on YEM broth; 1ml of sample ($\sim 10^8$ cell) was then inoculated into the base of each seedling. After fifteen days, the number of plants per pot was reduced to three. All pot received basal nutrients at the following rates per pot: 0.5g, K_2SO_4 ; 62mg, $ZnSO_4 \cdot 7H_2O$ and 2.4mg, $NaMoO_4 \cdot 2H_2O$ and watered daily with distilled water according to Somasegaran and Hoben (1994).

The plants were cut at the soil level after 70 days of transplanting. The dry matter yield of shoot was determined after drying at $70^{\circ}C$ for 72 hours. The phosphorus concentration of above ground dry matter was determined calorimetrically using vanadomolybdo phosphoric acid yellow color method (Jackson, 1958). Total nitrogen concentration of the above ground plant dry matter was determined by the Kjeldahl digestion. After the root system is thoroughly washed free of soil, nodules were collected, counted and weighed after drying at $70^{\circ}C$ for 72 hours (Jackson, 1958).

From each pot inoculated with AMF a sub sample of 2g fresh roots was removed and cut into small segment of 2-3 cm approximately. They were then transferred into staining tubes, cleared and stained in similar procedures as described before in MPN test. Estimation of the proportion of roots in a sample that contains mycorrhizal structures were quantified using the grid line intersect methods (Giovannetti and Mosse, 1980).

Pieces of stained roots were spread out with random orientation on 9 cm diameter round plastic petridish with a grid lined. Percentage of root colonization was calculated by:

$$\% \text{ of root length colonized} = \frac{\sum (\text{intersects with mycorrhizal})}{\sum (\text{intersects with non mycorrhizal})} \times 100$$

Experimental design and data analysis

Completely randomized designs of 7 treatments with three times replication were used.

The treatments were:

1. Plant inoculated with isolate Apo3 (T1),
2. Plant inoculated with isolate Apo2 (T2),
3. Un-inoculated plants, negative control (T3),
4. Un-inoculated plants with N fertilizer (10mM KNO₃), positive control (T4),
5. Plant inoculated with Apo3 and AMF (T5),
6. Plant inoculated with Apo2 and AMF (T6),
7. Plant fertilized with 10mM KNO₃ and inoculated with AMF (T7).

* All plants were grown in sterilized soil.

Comparisons between treatments were carried out using ANOVA. Tukey's test was applied after ANOVA. All statistical analyses were performed with the program SPSS v 12.0 (SPSS Inc., Chicago, IL., USA).

4. Result

4.1 Authentication and relative effectiveness

Authentication and relative effectiveness of the five isolates were undertaken by inoculating them in sand pond cultures. All isolates were authenticated as root nodule bacteria by forming nodules on the test host plant. The five isolates, however, showed differences in symbiotic effectiveness based on nodule number and mass, and plant dry weight. Out of the five isolates Apo3, Apo4 and Apo5 were found to be more effective than the Apo2 and Apo1. The later displayed the lowest value in terms of plant dry weight and nodule number and mass.

Table2. Effectivity of test isolates on sand culture

<i>Isolates</i>	<i>Nodule number Per pot</i>	<i>Nodule fresh Weight (mg/pot)</i>	<i>Plant dry Weight (g/pot)</i>
<i>Apo1</i>	10.40 ±2.50 ^c	8.23 ±1.24 ^c	0.73 ±0.20 ^c
<i>Apo2</i>	13.60 ±1.40 ^b	31.33 ±1.98 ^b	1.13 ±0.25 ^b
<i>Apo3</i>	21.30 ±4.52 ^a	46.50 ±2.05 ^a	1.86 ±0.43 ^a
<i>Apo4</i>	18.00 ±3.60 ^a	48.80 ±1.90 ^a	1.80 ±0.30 ^a
<i>Apo5</i>	19.00 ±2.00 ^a	45.43 ±2.54 ^a	1.75 ±0.61 ^a

Means and S.D followed by the same letter within a column are not significantly different at 5% level by Tukey's test

Numbers are means and S.D of three replicates (2 plants in each pot)

4.2 Characterization of Rhizobium and AMF

4.2.1 Characterization of Rhizobium

Staining and microscopic examination revealed that all isolates were gram-negative, short to medium rods. Morphologically all nodules were indeterminate type and reddish-brown in color. Colonies of root nodule bacteria showed differences in appearance and diameter, Apo1 displayed large colonies (2-3mm) and mucoid appearance with excessive production of polysaccharide where as the rest isolates showed small dry (SD) with less than 1mm in size after 10 days of growth on YEMA. Furthermore, Apo1 showed a yellow color change on the BTB-YEMA medium where no color change was recorded for the rest of the isolates. Isolate displayed different doubling times ranging from 1.7h to 7.6h.

All isolates were able to hydrolyse urea indicated by the red color on the test plates containing 2% urea. Similarly, addition of a few drops of Sulfanilic acid and α -naphthylamine to TY broth showed a red color, which is a positive reaction for nitrate reduction.

Table 3. Cultural characteristics of isolates from *A. polyacantha* after 10 days of Growth

Characteristics	Isolates				
	Apo1	Apo2	Apo3	Apo4	Apo5
Morphology	Short rod	Medium rod	Short rod	Short rod	Short rod
Staining	Negative	Negative	Negative	Negative	Negative
Mean generation time (h)	1.7	7.6	6.4	6.1	6.7
Colony appearance	LW	SD	SD	SD	SD
Colony diameter (mm)	2-3	≤1	≤1	≤1	≤1
Change of pH on YEMA+BTB	Yellow	Blue	Blue	Blue	Blue
Pattern of nodulation	FG	SG	SG	SG	SG

LW, large watery; SD, small dry; FG, fast grower; SG, slow grower

All isolates tested were able to grow at pH between 4 and 9, at temperatures between 4⁰C and 40⁰C and tolerated NaCl concentration from 1 to 3%. But isolates unable to grow in the presence of calcium chloride. All isolates grew well on monosacharides (D-glucose, D-fructose, Xylose and Arabinose). However, unlike the fast grower isolate Apo1, the slow growers showed weak colony morphology on the disaccharides lactose and sucrose (Table4).

Table 4. Physiological characteristics of isolates isolated from *A. polyacantha*

Characteristics	Isolates				
	Apo1	Apo2	Apo3	Apo4	Apo5
Urea Hydrolysis	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Carbon source utilization					
Glucose	G	G	G	G	G
Fructose	G	G	G	G	G
Arabinose	G	G	G	G	G
Ribose	G	G	G	G	G
Sucrose	G	W	W	W	W
Lactose	G	W	W	W	W
pH tolerance at 4	++	++	++	++	++
pH tolerance at 9	++	++	++	++	++
Growth on 3% NaCl	++	++	++	++	++
Growth on 1% CaCl ₂	-	-	-	-	-
Growth on YEMA at 4 ⁰ C	++	++	++	++	++
Growth on YEMA at 40 ⁰ C	++	++	++	++	++
Susceptibility to antibiotics					
Streptomycin	-	+/-	+	+/-	+/-
Chloramphenicol	+/-	+/-	+/-	+/-	+/-
Nalidixic acid	+	+	+	+	+
Rifampicin	+	+	+	+	+
Kanamycin	-	-	-	-	-
Heavy metal resistance					
Cu	-	-	-	-	-
Hg	+	+	+	+	+
Al	-	-	-	-	-

+, Positive result; ++, Maximal growth; +/- Growth not clear; -, No growth
G, Good; W, Weak

The levels of intrinsic antibiotic resistance were found to be slightly different between Apo1 and the others Apo2, Apo3, Apo4 and Apo5 (Table 4). isolate Apo1 was inhibited by Kanamycin and Streptomycin; and generally resistant to Nalidixic acid and Rifampicin. The others were also fully inhibited by Kanamycin and uniform pattern of response towards Streptomycin, Chloramphenicol, Nalidixic acid and Rifampicin except Apo3, which showed difference pattern of resistance towards Streptomycin. Pattern of

heavy metal resistance was uniform in all isolates tested; they showed resistance against Hg but sensitive by Al and Cu.

4.2.2 Characterization of Arbuscular Mycorrhizal Fungi

In the present study based on major differences in spore appearance, two classes of spore were detected. Morphologically, the most dominant spore type encountered and placed in the first tentative class was globose shaped with yellow to light brown colors, that was borne terminally from a flaring subtending hyphae (Table 5). The spore surface of this class appeared as shiny with smooth ornamentation. Apart from this, another group was characterized by brown color spore and by swollen/bulbous subtending hyphae. In this group the spores appeared as spiny with rough surface ornamentation. The spore wall layer of the first group characterized by thicker with simple irregular structure; whereas, the second group showed complex wall structure consisting of a thicker outer wall.

Table5. AM Fungal spore type and relative abundance in soil sample collected from the depth 0-30 cm

Sampling Site	Relative Abundance (%)	Spore character					Identification
		Shape	Color	Outer wall layer	Subtending Hyphae	Ornamentation	
1	75* (33.5)	Globose	Light brown	Thick	Bulbous	Dull/pits/spines	Gigaspora-Scutellospora
	149*(66.5)	Globose	Yellow to brown	Irregular/ Thick	Flaring	Shiny/smooth	Glomus
2	100*(47.2)	Globose	Light brown	Thick	Bulbous	Dull/pits/spines	Gigaspora-Scutellospora
	112*(52.8)	Globose	Yellow to brown	Irregular/ Thick	Flaring	Shiny/smooth	Glomus
3	97*(42.2)	Globose	Light brown	Thick	Bulbous	Dull/pits/spines	Gigaspora-Scutellospora
	133*(57.8)	Globose	Yellow to brown	Irregular/ Thick	Flaring	Shiny/smooth	Glomus
4	86*(34.6)	Globose	Light brown	Thick	Bulbous	Dull/pits/spines	Gigaspora-Scutellospora
	162*(65.4).	Globose	Yellow to brown	Irregular/ Thick	Flaring	Shiny/smooth	Glomus

Numbers followed by * are spore count per 400g soil and numbers within the brackets are relative value in %

The number of AMF spore counts from the samples was between 45 to 79 spore/100g. Whereas AMF assay on trap culture showed that a 100g of field soil samples contained 189.42 infective propagules (Table 6).

Table 6. Density of infective propagules AMF based on spore count and MPN

Method of quantification	Density 100g ⁻¹
Spore count	57.86*
MPN	189.42 (88.71 – 403.64) ^a

*Entries given are mean number of fungal spore
(Numbers)^a is lower and upper limit confidence at 95%

4.3 Pot experiment

The pot experiment showed different responses to the effect of single, double inoculation of endosymbionts to the host plant. This manifested by the data shown in table 7. The lowest value of the different parameter; nodule number and weight, shoot weight, height, total nitrogen and total phosphorus on the host plant was recorded on T3. Whereas, the highest values in most parameter recorded were clearly shown on T5 and T7. Similarly, T1 and T4 showed the next highest values of yield related character with T6, T2 and T3 with decreasing order.

The single inoculation of the two rhizobial isolates Apo3 and Apo2 showed significant differences in nodule number and dry weight where by Apo3 showed a significant nodule number and mass than Apo2, which also showed significant different in other parameter

investigated. The average number of nodules on plants inoculated with Apo2 isolates (18.7 per pot) was significantly less ($p < 0.001$) than for plants inoculated with Apo3 isolate (38.3 per pot). Considering the dual inoculated plant, T5 (Apo3+AMF) resulted the best nodule number and dry weight, which was 50/pot and 176.5 mg/pot respectively, whereas in T6 (Apo2+AMF) it was found 28/pot and 75.4 mg/pot on average respectively (Table7). In general, the highest nodule number was observed on plant inoculated with Apo3 and AMF in T5 (50 per pot). Whereas, the smallest nodule number (18.7 per pot) was observed in treatment 2 inoculated with isolate Apo2. Similarly, among all other treatments the highest nodule dry weight was recorded on dual inoculated pot in treatment 5, which was 176.5 mg per pot and the smallest recorded in T2 which was 53.1mg per pot (Table 7).

As it is shown in table 7, shoot dry weight and height were significantly different among and between the rhizobial isolates and dual inoculation with AMF ($p < 0.001$). Taking the mean into consideration, isolate inoculated with Apo3 in T1 alone significantly increase the dry shoot weight by 43% over T2; 67% over the negative control (T3) and 3% over N fertilized pot (T4). However, there is no statistical difference between the fertilized pots in T4 and T1 (Table7). Comparing the dual inoculated plant, T5 (Apo3+AMF) showed significant improvement on shoot dry weight than T6 (Apo2+AMF), which were 3.5 g/pot and 2.1 g/pot respectively (Table 7). Although T5 showed the highest magnitude in shoot dry weight, it is not statistical different from T7 which was recorded 3.4 g shoot dry

weight/pot (Table 7). In general the highest shoot dry weight recorded in T5 and the next in T7, T1, T4, T6, T2 and T3 with decreasing order

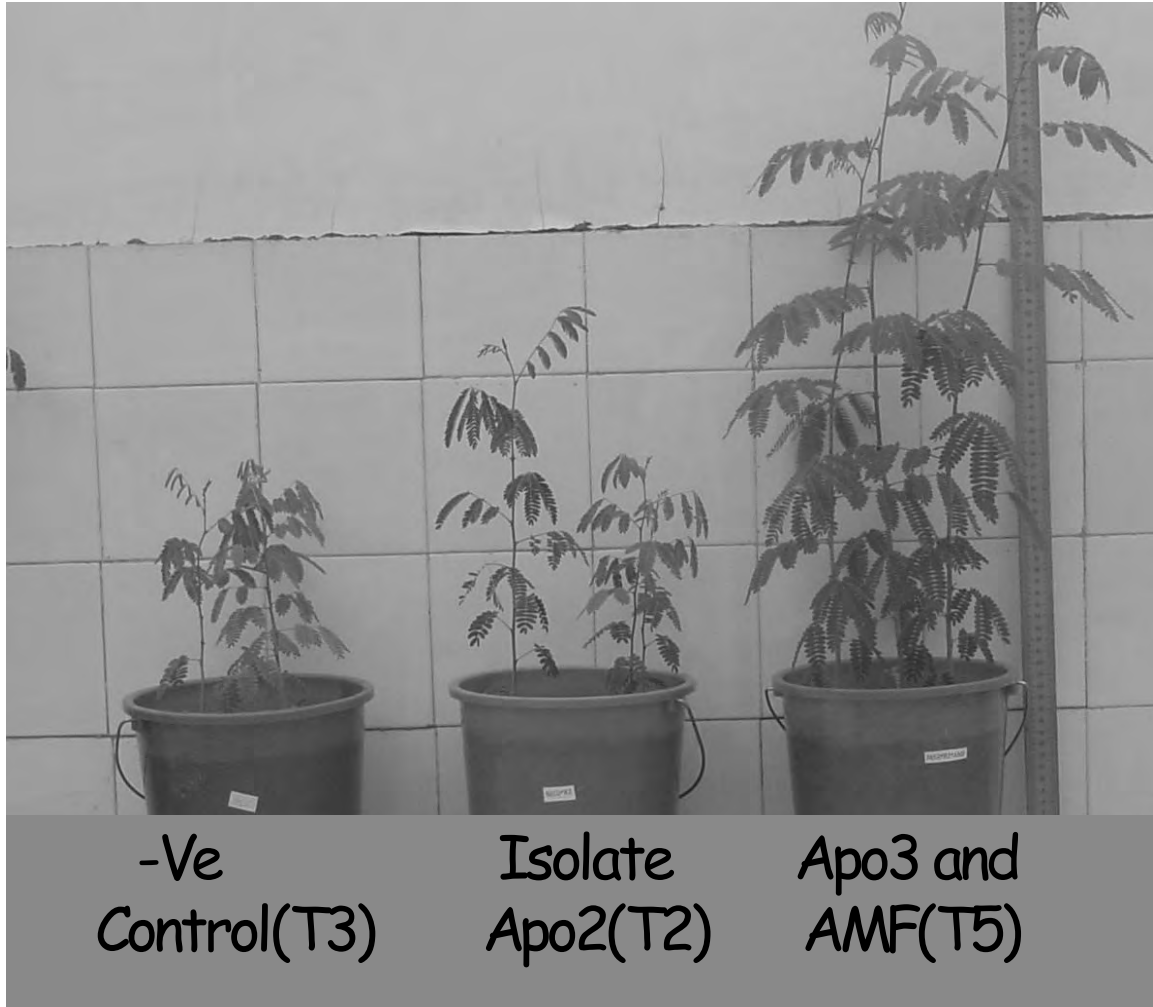


Figure 2. Greenhouse experiment showing difference between T5, T2 and T3.

Considering height and taking the maximum value into consideration at 70 days after transplanting *A. polyacantha* inoculated with rhizobium (Apo3) in treatment 1 was 1.5 times taller than plant inoculated with Apo2 (T2), 2.1 taller than T3 and almost similar with fertilized pot in T4 (Table 7). Result on height measurement on dual inoculated

plant showed significant difference between T5 and T6 ($p < 0.001$). Comparing dual inoculated plant with T7, no statistical difference with T5 but significantly differ from T6 (Table7). In general, treatment effect on height was showed that, T5 was the highest figure recorded and the next T7, T1, T6, T2 and T3 according to decreasing order (Table7).

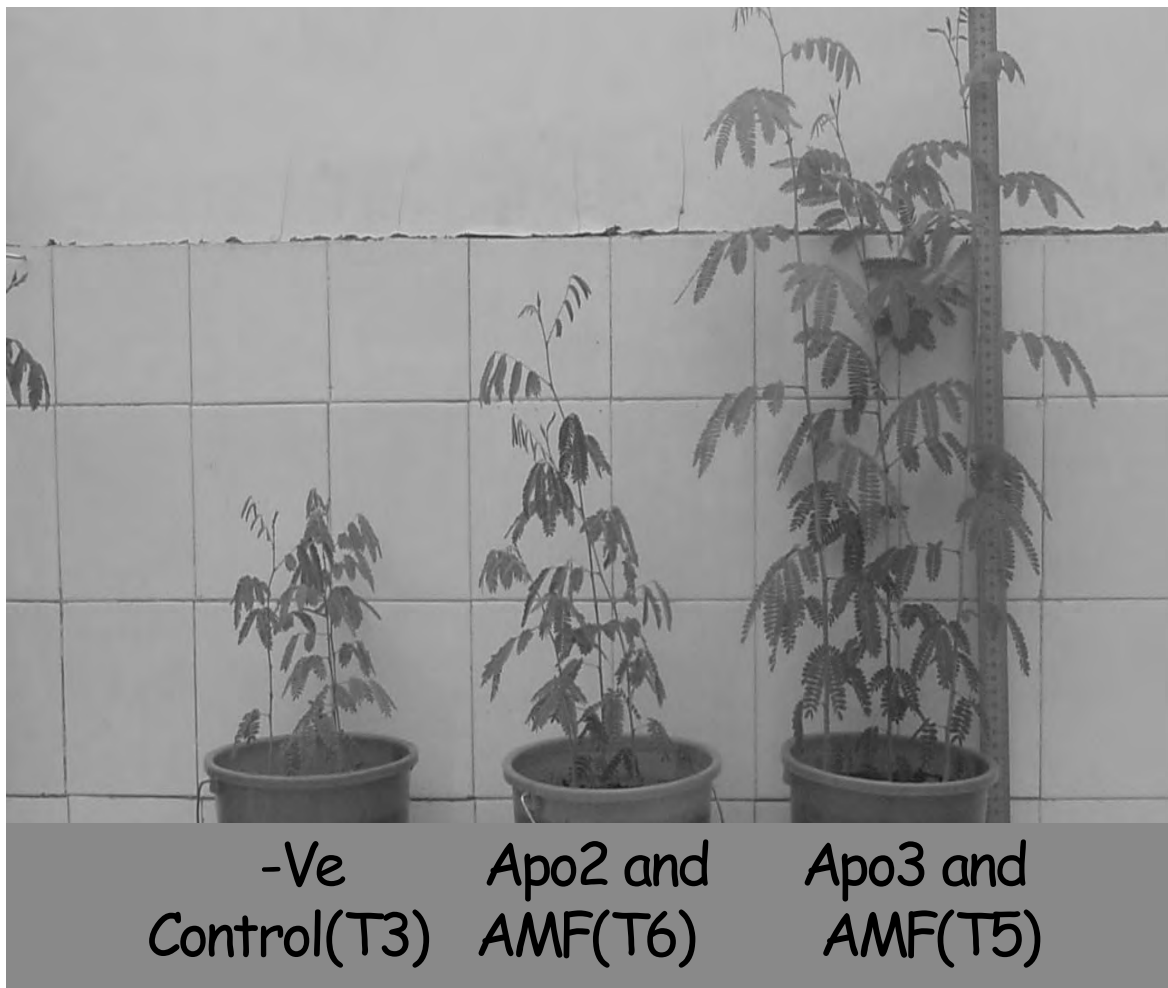


Figure 3. Greenhouse experiment showing difference between T5, T6 and T3.

Similarly total shoot N content followed the trend observed in shoot dry weight and height ($p < 0.001$). Comparing the two rhizobial isolates, Apo3 inoculation in treatment 1

showed 27.9mg/g and T2 showed 17.9mg/g total shoot N that is 1.5 less than that of Apo3 (Table 7). No statistical difference between T1 and T4, which was recorded 27.8 mg/g of shoot total N (Table 7).



Figure 4. Greenhouse experiment showing difference between T4, T1 and T3.

The result of this experiment revealed that plant inoculated with Apo3 and AMF in T5 contain higher amount of total shoot N (32.2mg/g) followed by plant inoculated with AMF and fertilized with nitrogen (T7), which was 32mg/g but not significantly different from T5 and the lowest was recorded in T3 which was 16mg/g (Table 7).

The total shoot phosphorus concentration of the seven treatment showed significant different between them (Table 7). It was found out that Apo3 inoculation in treatment 1 showed highest shoot P content than Apo2 in treatment 2 ($p < 0.001$), which was 1.6mg/g and 1.2mg/g respectively (table 7).

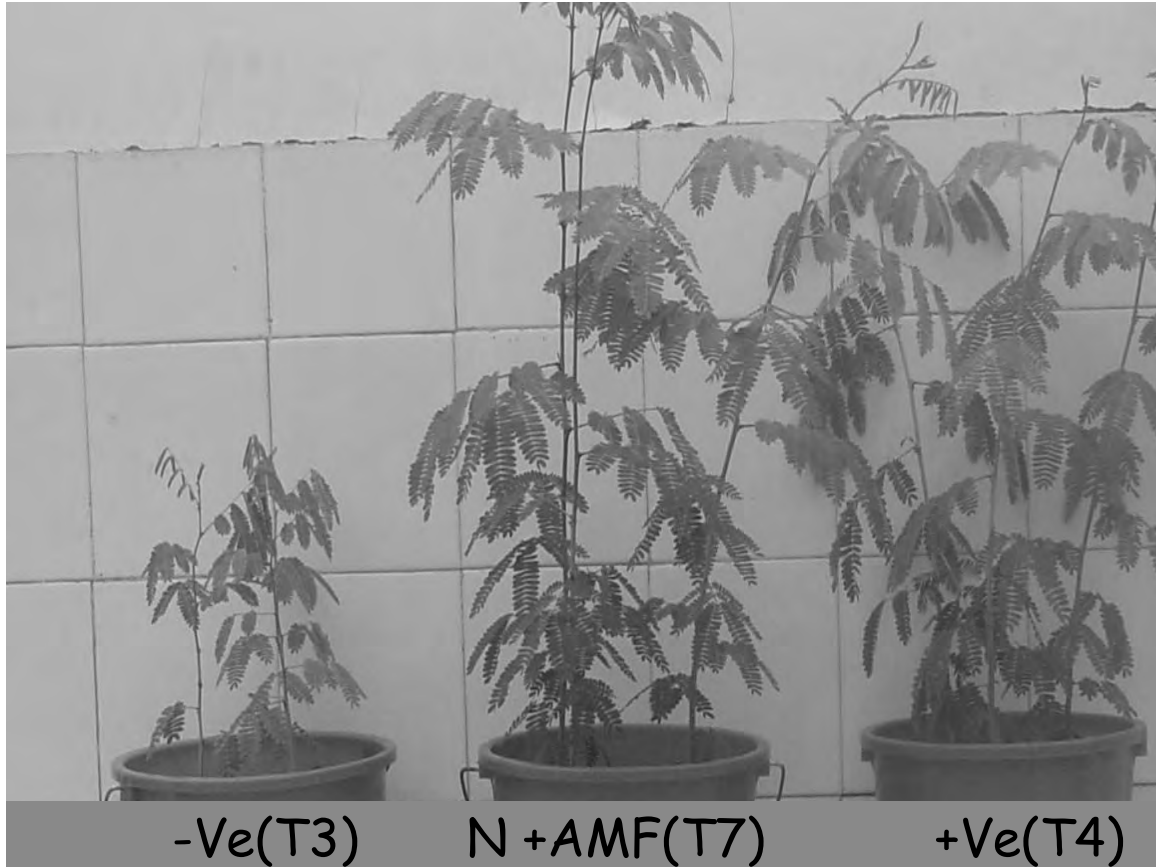


Figure 5. Greenhouse experiment showing difference between T7, T4 and T3.

No statistical difference between T2 and T3, however, these two treatments were significantly different from T4 which shoed significant improvement in total shoots P content than T2 and T3 (Table 7). Comparing dual inoculated plant in T5 and T6 with

that of T7, no statistical difference with T5 but significantly differ from T6 which was recorded 1.6 mg/g, almost 1.3 times less than T5 and T7 (Table7).

Highest percentage of mycorrhizal colonization was recorded in treatment 5 (39 %), next in treatment (35 %) and last in treatment 6, which was 21 % (Table 7). In general comparing dual inoculated plant in treatment 5 and 6; and fertilized plant and inoculated with AMF in treatment 7 with their respective single inoculation in T1 and T2 and fertilized plant in T4 they showed significant improvement in all parameter investigated (Table 7).

Table 7. Effect of rhizobium and AMF inoculation on Number and dry weight of nodules, shoot dry weight, height, N and P content and % root colonization of *A. polyacantha*

Trt	Nodule Number	Nodule dry weight mg/pot	Dry weight g/plant	Height cm	Total Shoot N mg/g	Total Shoot P mg/g	AMF root colonized (%)
T1	38.3 ±4.5 ^{ab}	162.0 ±4.0 ^b	3.0 ±0.2 ^b	46.8 ±2.6 ^b	27.9 ±0.2 ^b	1.6 ±0.1 ^b	-
T2	18.7 ±4.0 ^c	53.0 ±6.0 ^d	1.7 ±0.2 ^d	32.1 ±2.1 ^d	17.9 ±0.1 ^d	1.2 ±0.1 ^c	-
T3	-	-	1.2 ±0.1 ^e	22.4 ±1.2 ^e	16.0 ±0.1 ^e	1.1 ±0.1 ^c	-
T4	-	-	2.9 ±0.3 ^b	45.7 ±3.5 ^b	27.8 ±0.1 ^b	1.5 ±0.1 ^b	-
T5	49.7 ±7.5 ^a	175.0 ^c ±5.0 ^a	3.5 ±0.2 ^a	54.3 ±2.1 ^a	32.2 ±0.2 ^a	2.0 ±0.2 ^a	38.9
T6	28.0 ^c ±6.0 ^b	75.0 ±7.0 ^c	2.1 ±0.2 ^c	38.2 ±3.1 ^c	20.1 ±0.2 ^c	1.6 ±0.2 ^b	21.3
T7	-	-	3.4 ±0.1 ^a	52.8 ±2.4 ^a	31.9 ±0.3 ^a	2.0 ±0.1 ^a	34.7

* Means and S.D followed by the same letter within a column are not significantly different at 5% level by Tukey's test. Numbers are means and S.D of three replicates (3 plants in each pot)

5. Discussion

In the present study, *Acacia polyacantha* was found to be nodulated by root nodule bacteria with indeterminate nodule type which are the characteristics of tropical woody legumes (Ndoye *et al.*, 1994). The pattern of nodulation showed that it is nodulated by both fast growing Apo1 and slow growing root nodule bacteria Apo2, Apo3, Apo4 and Apo5 (Table 3). Similarly, several researchers showed that *Acacia* species are nodulated by both fast grower (*Rhizobium*) and slow grower (*Bradyrhizobium*) species Dreyfus and Dommergues, (1981); Odee *et al.*, (1995); Njiti and Galiana, (1996); Fassil and Kleiner, (1998).

Although the diversity of tree rhizobia is so various and hence difficult to assign into a particular genus and species without genetic information Sutherland and Sprent (1993), it is possible to use standard phenotypic features as a tool to make a taxonomic comparison of a given isolate with representative established root nodule bacteria. Consequently, phenotypic features such as growth rate and colony morphology as described by Jordan (1984) were applied to make preliminary classification of the five isolates.

Based on growth rate and acid base production (Table 3) the test isolates Apo1 was identified as fast grower (FG) and Apo2, Apo3, Apo4 and Apo5 were grouped into slow

grower (SG). Consequently, they were tentatively classified into fast growing (FG) and slow growing (SG). SG isolates according to Jordan (1984) are those with generation time of greater than 5h and with a cell mass diameter of less than 1mm in 10 days of incubation at 25-30⁰C. FG isolate is the one with a doubling time of less than 2h and with a size of 2- 4mm in 3-5 days of incubation at 25-30⁰C. Generation times of the isolates were found to be within the range reported on fast and slow growing (Zhang *et al.*, 1991; Fassil and Kleiner, 1998; Mohamed *et al.*, 2000; Endalkachew *et al.*, 2004).

All isolates were found to reduced nitrate and hydrolyse urea. However, there are contrast reports on these characters indicating diversity among rhizobial isolates. For Example, Zhang *et al.*, (1991) who reported that, from 62 fast- and slow growing rhizobia isolates from *Acacia* and *Prosopis* species, 55 of them were unable to hydrolyse urea and only 8 of them reduced nitrate. According to Lindstrom and Lehitomaki (1988), out of 82 strains tested only 30% of them reduced nitrate and more than 68% hydrolyse urea.

Result on carbohydrate utilization test showed that the disaccharides, hexose and pentose sugar supported growth of all test isolates, particularly the fast grower, similarly with mannitol that is often used in the laboratory to grow them. Similar observation that support the present result were also found by Fassil and Kleiner, (1998); Marsudi *et al.*, (1999) who reported that the fast growers utilized the disaccharides but the slow grower didn't grow well on the disaccharides. Similarly Zerhari *et al.*, (2000) tested 48 strains of fast and slow growers, all of the isolates showed good growth on the carbohydrates

tested; this findings also supported by other studies such as Zhang *et al* (1991); Mohamed *et al.*, (2000). In an experiment made by Lindstrom and Lehitomaki (1988), the majority of carbon source tested served as good source of growth both for slowly growing and fast growing isolates.

In the present study all isolates were found to grow at 4⁰C and 40⁰C, is in agreement with previous reports (Basak and Goyal, 1980; Odee *et al.*, 1997; Fassil and Kleiner, 1998; Zerhari *et al.*, 2000). A study made by Zhang *et al.*, (1991), out of the 12 slow growers tested, none of them failed to grow at 38⁰C. According to the result obtained by Lindstrom and Lehitomaki (1988), the Tmax value of majority the strains (most of isolates from temperate origin) tested were within the range of 31 to 37⁰C. Out of the 30 isolates tested more than 50% of them even grow at 42⁰C according to Mohamed *et al.*, (2000). However, growth at these temperatures doesn't mean that they can fix nitrogen efficiently but rather they will take an advantage of survival in the period of thermal stress and so recover afterwards (Michiels *et al.*, 1994).

Intolerance to NaCl may be a limiting factor in the establishment of rhizobia in the soil. The experiment here showed that both isolates were similar resistant pattern towards NaCl and were in the range reported by other published works (Zhang *et al.*, 1991; Odee *et al.*, 1997; Marsudi *et al.*, 1999). Similar observations have been seen for isolates with the Morocco's (Zerhari *et al.*, 2000) and Libya's tree rhizobia (Mohamed *et al.*, 2000). Zerhari *et al.*, (2000) found out that more than 50% of his strains grown between 3.5 - 4%

and one strain grew at 5% salt concentration. Solute can inhibit growth of microorganisms in several ways and hence salt tolerant strains can give them advantage to survive and multiply in salt affected regions. And here again their symbiotic nitrogen fixation efficiency also depends on both partners (Craig *et al.*, 1991).

Antibiotic resistance tests were made based on five antibiotics representing different mode of action. In most of antibiotics and heavy metals resistance tested, it is found out that no major difference were observed in this experiment, except Apo3 which showed resistance against streptomycin where as the rest failed to resist (Table 4). It is known that antibiotic sensitivities differ among and within root nodule bacteria species (Jordan, 1984), because of this they are used as a supplementary diagnostic character to show diversity among strains. Similar pattern of antibiotic resistance observed in the test isolate in the present study may be due to the fact that, only *A. polyacantha* was tested as a host plant, so it may invited restricted group of isolates among others. In view of the differences between the geographical isolates, it is difficult to generalize, this diversity also expected to strains isolated from the same site from different hosts. Such diversity occurring in a given site could be explained by soil micro sites having distinct marginal edapho-climatic condition, which induces different strain adaptation (Postgate, 1982).

In general, experiment on tolerance to the different phenotypic trait such as salt, pH and temperature showed that tree legumes isolates display higher tolerance to these factors than the other *Rhizobium* species isolated from temperate legumes reported by Jordan,

(1984) and Lindstrom and Lehitomaki (1988). It is clear from the foregoing discussion that there may be strain-to-strain variation in terms of different environmental factors. Such differences are welcomed in making the recipient strain improvement to compete in soil under adverse natural conditions. Since harsher environmental conditions are the major constraints to plant productivity that are directly and indirectly associated with nitrogen availability (Zaharan, 1991). Furthermore, physiological and biochemical properties alone will have strong ecological implications to select good inoculants together with choosing legume species and their provenance in field experiments.

Based on spore morphological characters observed and comparing the features stated in Morton, (1988); Brundrett *et al.*, (1996) and INVAM (2004), the extracted spores were tentatively assigned within genera *Glomus* and *Gigaspora-Scutellospora* type AMF. The dominant AMF spores encountered in the sampled soils were found to be the genus *Glomus* (59.1%). The presence of conspicuous flaring structure directly below the spore on the spore-bearing hyphae (Brundrett *et al.*, 1996) is a typical feature of the genus *Glomus*. On the other hand, the next dominant spore type encountered were *Gigaspora - Scutellospora* type AMF (41.9%). The presence of conspicuous swelling directly below the spore on the spore-bearing hyphae (Brundrett *et al.*, 1996) also known as a bulbous suspensor-like cell, characterizes *Gigasporineae* taxa that encompass the genera *Gigaspora* and *Scutellospora*.

Similar dominant spore type was also observed by (Million, 2002), who reported that more than 80% spore extracted beneath Acacia trees were *Glomus*. On another study Munro *et al.*, (1998) found out that *Glomus* species were the dominant spore beneath the host tree *Acacia tortilis*. However, the result of the present finding was in marked contrast with Shasho, (2002), who reported that 50.7% of the extracted spores from the rhizosphere of *Erythrina brucei* were Gigaspora and 38.8% were *Glomus*. This contrast might be due to the hosting preference of the host plant.

The distribution of endomycorrhizal on the basis of spore density showed that a hundred grams of soil samples contains 57.9 spores on average. However, the MPN estimate that give a better count of infective AMF propagules showed 189.4 per 100g (Table 6). In fact, count on AMF propagules is said to give more realistic estimate than spore density since it considered the life cycle of AM fungi in the soil and includes non-sporulating AM fungal species (Mitiku and Osrio, 2001).

Although the exact number of propagules are not established the high or low number of propagules for infection to determine, Mitiku and Osrio (2001) considered highly infective when a soil contain at least four infective propagules per g dry soil, similarly Sieverding (1991) defined a high density as in excess of 20 infective propagules per g dry soil, where as Harinikumar and Bagyaraj (1996) suggested 37 infective propagules per g dry soil was a low level compared to 1000-fold value. Armstrong *et al.*, (1992) employed MPN to estimate the number of AMF propagules under scrublands and native pasture.

They came up with low mycorrhizal inoculum density with 1 and 0.2-propagules/g soil for native pasture and scrubland respectively. In the present study the MPN estimate of 1.8 per g soil, is considerably lower than those reported except by Armstrong *et al.*, (1992). This indicate that the prevailing environmental condition and the host have different influence on AMF propagules

The average number of AMF spores extracted 57.9/100g (Table 6), relatively small compared with some of the previous findings such as Shasho, (2002) who reported more than 300 spore/100g soil beneath *E. brucei*. In another studies on undisturbed soils of secondary semi-deciduous moist tropical forest in Cameron, Musoko *et al.*, (1994) found out 200-500 spores per 100g soils.

Several possibilities may explain the lower mycorrhizal propagules number. The highest number of spores obtained in Musoko *et al.*, (1994) study may be due to the fact that, the land is under the natural vegetation where there is was no disturbance on the vegetation cover. Ghibe Valley, according to the hypothesis made by Reid *et al.*, (1997), is a wooded grassland of secondary nature derived from a more densely wooded landscape resulted from human intervention. It is known that grasslands are tending to low in spores density (Sieverding, 1991). So changes of the natural habitat occurred in the present study site may contribute the change in population and low number of spore density.

The pronounced seasonal variation in AMF spore density is thoroughly documented (Hayman, 1982; Brundrett *et al.*, 1996). In this study, soil samples were collected during the end of rainy season when the root systems were actively growing. Spore density tends to increase after the period of maximum root growth during which vegetative growth was minimal. Consequently, carbon and nutrients are reallocated for sporulation.

The number of spores also may depend on the species of AMF present under the host (Zahra and Thomas, 2003). For example, some *Glomus* species may have to build up large amounts of fungal biomass before sporulation can occur. In a pot experiment Brundrett *et al.*, (1999) found out that *Glomus* species sporulated after several generations. Furthermore, lower propagule numbers found in the present study may be attributed to the location of the propagules at different depths of the soil profile (Jakobsen and Nielsen, 1983). These authors reported that 70-85% of spores were found in the top 40-45cm; in the present study only the top 30cm depth was sampled.

Inoculation with rhizobium is known to improve growth and nitrogen fixation of legumes. The ability of rhizobia to form nodules (infectiveness) and their capacity to fix atmospheric nitrogen (effectiveness) are important parameters to assess the ecological and evolutionary relationship between rhizobia and their hosts (Chanwory *et al.*, 1991).

In the present glasshouse experiment the rhizobial isolates showed considerable variation in their ability to form effective symbiotic associations with *A. polyacantha* although they

form nodules. Single inoculation of Apo3 in T1 showed a significant difference in nodule number and dry weight than isolate Apo2 in T2. This was also expressed in increased shoot dry weight, height and total N; indicating that the symbiotic effectiveness of the two isolates were different. Different works also showed that selection and inoculation of effective rhizobium increased nodulation, shoot dry weight, height and total shoot nitrogen content (Burdon *et al.*, 1999; Bala and Giller, 2001; Million, 2002).

Dual inoculation of different isolates Apo3 and Apo2 in T5 and T6 with *Glomus* also showed difference between the effectiveness of individual root nodule bacteria regardless of the mycorrhizal endosymbiont. The dual inoculated *A. polyacantha* in T5 (Apo3+AMF) showed 1.8 times larger in nodule number, increased nodule dry weight by 57.5%, shoot dry weight by 40%, 1.4 times taller in height and increased total shoot N and P content by 37.5% and 20% respectively than T6 (Table 7). Comparing dual inoculated *A. polyacantha* with their respective single inoculated plant in T1 and T2, they displayed significant improvement in yield related character between T2 and T6; T1 and T5 (Table 7). Similarly, earlier observations have shown significant improvement in nodulation, plant growth and nutrient content (N and P) in double inoculated *Acacia saligna* Benbrahim and Ismaili (2002); *Acacia abyssinica* (Million, 2002).

The efficacy with which the assimilation gaseous N₂ in the rhizobial root nodules differ in the presence and absence of mycorrhizal fungi. Colonized plant in AMF inoculated pots (T5, T6 and T7) had a combined root and hyphal density, which permit effective use

of a much greater volume of soil and further from symbiotic nitrogen fixation (T5 and T6). There are reports that infection of plant root by AMF stimulating nodule formation by increasing the volumes of soil exploited by the plant and have more access to the growth limiting nutrients, predominantly phosphorus, as a result of which there may be more fixation of nitrogen and subsequently increases in plant biomass (Prasad, 1998). A further stimulus to plant biomass productivity associated with mycorrhizal inoculation is due to nitrogen (Smith, 1980) and efficient water uptake from the soil (Auge, 2001). The present investigation supporting previous reported that a combination of effective rhizobium and mycorrhizal inoculation resulted in considerably greater plant matter production than with either microbial component taken singly.

In the present study, in all parameter investigated the least value was recorded in the negative control (T3) and the highest was recorded in *A. polyacantha* dual inoculated with Apo3 and AMF in T5 (Table 7). In *A. polyacantha*, host benefit, expressed as the percentage change in host response compared to non-inoculated plant in T3 with the next least value in T2 and the highest record in T5, ranged from 31 to 65% for shoot dry weight, 30 to 59% for shoot height, 11 to 53% for total shoot nitrogen and 8 to 45% for shoot phosphorus content (Table 7).

On the other hand, *A. polyacantha* fertilized with KNO_3 in the positive control (T4) showed significant improvement in yield related character than negative control (T3) and plant inoculated with isolate Apo2 in T2. Similarly, fertilized plant in T7, which was

inoculated with AMF showed better performance than T1, T2, T3, T4, and T6 (Table 7). The higher yield related character in fertilized plant in T4 and T7 was due to the higher nitrogen availability in the rooting medium, which stimulates growth during the early phase. An increase supply of nitrogen enhance stem elongation and increases shoot-root ratio. Such phenomenon takes place in both perennial and annual plant species (Levin *et al.*, 1989).

There is much information on the negative effect of high soil nitrogen on the nodulation induced by rhizobia. According to Desta and Angaw (1986) soil nitrogen content greater than 0.20% classified as very high. Considering this value as a reference, soil analysis data showed that total nitrogen content is reasonably high (Table 1). However, pot experiment result showed that the soil nitrogen content was not limiting for rhizobial inoculation and fertilizer application response, this argument is strongly supported by the plant performance on inoculated and/or fertilized pot (T1, T2, T5, T6 and T7) comparing with the negative control (T3). The fact that a higher concentration of shoot total nitrogen recorded in plant inoculated with Apo3, dual inoculated in treatment 5 (Apo3 + AMF), fertilized and/or inoculated with AMF in T4 and T7 respectively were clearly due to an efficient uptake of nitrogen from the growing medium (T4 and T7) or from effective symbiotic nitrogen fixation (T1 and T5).

On the other hand, according to Ngeborg (1986), soil phosphorus content ranked as medium when available P is within the range of 10-18ppm, the available soil P in the

present study (12.4ppm) fall under this category (Table 1). According to Mitiku and Osrio (2001) for most mycorrhizal root, available soil phosphorus below 40ppm is mainly optimum, so the P content of the study soil was not limiting as far as mycorrhizal association is concerned. Even available soil P at 60ppm, Michelson (1993) under nursery condition found out significant plant improvement from mycorrhizal inoculation. From this study therefore, the highest shoot P content in plant inoculated with AMF is due to the action of extensive mycorrhizal formation, which increases in root length densities and in turn aiding the plant to take up nutrients from the soil.

Percentage of root colonization by AMF observed in this experiment was relatively high compared with Shasho (2002), who found out 25-28% infected roots of *Erythrina brucei* in pot experiment under greenhouse condition. However, percentage of mycorrhizal colonization observed was in marked contrast with that of Munro *et al.*, (1998), who found out 56% for *Acacia tortilis* and 67.2 % for *Prosopis juliflora* in nursery conditions. Apart from the hosting ability of the legumes and differences in experimental set up, high root infection level observed in this experiment compared with Shasho's finding might be due to nutritional factor particularly phosphorus. The available P that observed in Shasho (2002) was 37ppm that is high compared with the present study (12.4ppm). These findings support the generally accepted idea that percentage of colonization decreased as the supply of soil P increased (Siqueira *et al.*, 1998).

On the other hand the result of mycorrhizal colonization of *A. polyacantha* in the present study was almost similar to those reported by Million (2002) who found out AMF colonization in two indigenous tree species (*A. abyssinica* and *A. tortilis*) 22.5-35 % on average, and by Michelson (1993) in root segments of *A. abyssinica* (22-38%) in nursery conditions. In general, the poor performance of plant recorded in sterile soil of uninoculated treatment (T3) indicating that growth of *A. polyacantha* is less successful in the absence of the soil microorganisms such as rhizobia and AMF.

6. Conclusions and Recommendation

In dry parts of the country not only patch of trees but also free standing trees are being cut down by people in their search for various purposes. Such activities together with natural disaster consequences soil erosion, depletion of soil nutrient, decline in soil fertility and aggravate desertification. There are a number of indications that rhizosphere microorganisms colonize roots of plants and improve productivity. This can be caused by a number of organisms, of which rhizobium and mycorrhizal fungi predominately contributes to improve plant growth by influencing nitrogen and phosphorus uptake.

Phenotypic traits such as growth rate, carbohydrate metabolisms, pH changes of growth medium, salinity and maximum temperature of growth etc., are good indicator of taxonomic relationship between the different isolates. However, results obtained from such findings are too often subjective and by no means fail-safe. In order to get a more

complete picture about their taxonomy and evolutionary relationship one should rely on more powerful approach such as genotypic information.

Similarly a description of the fungal isolates that is based on spore count and MPN may not reflect the contribution of each fungal genus or species to the community. But, it can be concluded that the native AMF are capable of infecting *A. polyacantha* and also MPN technique estimate mycorrhizal propagule number better than spore count. The comparison of the yield parameters obtained from the greenhouse experiment revealed that microorganisms are an essential below ground component in establishment and sustainability of plants. From this it can be concluded that inoculation of *A. polyacantha* with effective rhizobium and AMF constituted an advantage over un-inoculated control. However caution should be observed when interpreting the findings about the responsiveness of *A. polyacantha*, because most of enhancement effect of rhizobium and AMF on host plant growth and nutrient uptake were obtained from greenhouse experiment.

And finally, this study didn't test the whole range of root nodule bacteria and fungal species potentially infecting *A. polyacantha*. The number of root nodule bacteria tested from the study site was too small to draw firm conclusion as to the geographical origin of the strains. Similarly Glomus types were originally was selected because they produced large numbers of spores. Therefore other root nodule bacteria and fungal species covering

the whole range of agro-ecological zone of the study site should be explored and assayed with *A. polyacantha* to know whether this is a general phenomena.

7. Reference

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Appendix 1: Seed testing data of *A. polyacantha*

Parameter	value
Seed weight no/Kg	11,000
Purity%	77
Germination %	92
Moisture content%	6.61
Pre sowing treatment	90 ⁰ C for 60min
Testing date	5/4/04

Appendix 2: Number of infected cups set at 4 fold dilution from Ghibe soil

<i>Dilution Level</i>	<i>Rep I</i>	<i>Rep II</i>	<i>Rep III</i>	<i>Rep IV</i>	<i>Rep V</i>	<i>Sum of infected cups</i>
<i>4⁰</i>	+	+	+	+	+	5
<i>4¹</i>	+	-	+	+	+	4
<i>4²</i>	-	+	+	+	+	4
<i>4³</i>	+	+	+	-	-	3
<i>4⁴</i>	+	+	-	+	-	3
<i>4⁵</i>	+	-	-	-	+	2
<i>4⁶</i>	-	-	-	-	-	0
<i>4⁷</i>	-	-	-	-	-	0
<i>4⁸</i>	-	-	-	-	-	0
<i>4⁹</i>	-	-	-	-	-	0
<i>Total number of infected cups</i>						21

Appendix 3: K-values extract from table VIII Fisher and Yates for four dilutions
to determine the MPN

X	K value	Y	K value
0.4	0.707	3.5	0.550
0.6	0.618	3.0	0.548
0.8	0.577	2.5	0.545
1.0	0.559	2.0	0.537
1.5	0.555	1.5	0.522
2.0	0.553	1.0	0.488
2.5	0.552	0.8	0.464
		0.6	0.431
		0.4	0.370

Appendix 4: ANOVA Showing the effect of rhizobium and AMF treatment on variability of nodule dry weight, shoot height and dry weight, shoot total N and P content in *A. polyacantha*

Parameter	Source of variation	d.f	Sum of square	Mean square	F-value	p value
Nodule Number	Between treatment	3	1382.917	460.972	32.927	<0.001
	Within treatment	8	112.000	14.00		
	Total	11				
Nodule Dry Weight	Between treatment	3	40230.234	13410.078	888.249	<0.001
	Within treatment	8	120.775	15.097		
	Total	11	40351.012			
Shoot Height	Between treatment	6	2344.283	390.714	119.624	<0.001
	Within treatment	14	45.727	3.266		
	Total	20	2390.01			
Shoot Dry Weight	Between treatment	6	12.827	2.138	223.142	<0.001
	Within treatment	14	0.134	0.011		
	Total	20	12.962			
Shoot Total N	Between treatment	6	10.369	1.728	455.371	<0.001
	Within treatment	14	0.053	0.004		
	Total	20	10.423			
Shoot Total P	Between treatment	6	46.441	7.740	261.490	<0.001
	Within treatment	14	0.414	0.030		
	Total	20	46.855			