Diversity and Phytobeneficial properties of indigenous root nodule bacteria and Arbuscular Mycorrhizal Fungi associated with *Erythrina* species and their effect on growth of the host plant under greenhouse conditions

By

Belay Berza Beyene

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This is to certify that the Dissertation prepared by **Belay Berza**, entitled: **Diversity and Phytobeneficial properties of indigenous root nodule bacteria and Arbuscular Mycorrhizal Fungi associated with *Erythrina* species and their effect on growth of the host plant under greenhouse conditions** and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy (Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Supervisor: Fassil Assefa (Associate Prof.)

Signature______________________________

Date April 2021

Dedication

This Dissertation is dedicated to my late father, Berza Beyene, and my mother, Daynate Yanew, who sent me to school and paved my way to education when I was not aware of the values of education.

Declaration

I declare that this Dissertation submitted entitled **"Diversity and Phytobeneficial properties of indigenous root nodule bacteria and Arbuscular Mycorrhizal Fungi associated with *Erythrina* species and their effect on growth of the host plant under greenhouse conditions"** by me for the Degree Doctor of Philosophy (PhD) in the department of Microbial, Cellular and Molecular Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University is my own independent work and has not been previously submitted by me or anybody elsewhere. Any material obtained from other sources is duly acknowledged in the Dissertation.

PhD candidate ______________________________

Belay Berza Beyene

April, 2021

Diversity and Phytobeneficial properties of indigenous root nodule bacteria and Arbuscular Mycorrhizal Fungi associated with *Erythrina* species; and their effect on growth of the host plant under greenhouse conditions

Belay Berza

ABSTRACT

Erythrina is a leguminous tree used in agro-forestry practices in the southern and southwestern Ethiopia. The legume-rhizobium symbiosis provides N to plants, while Legume-AMF symbiosis enhances P availability. Despite the crucial agro-forestry attributes, there were scarcities of information on species diversity of arbuscular mycorrhizal fungi (AMF) associated to *E. brucei*, genetic and functional diversity, and eco-physiological stress tolerance traits of bacteria isolated from the root nodules of *E. abyssinica*. The objectives of this study were to; 1) determine AMF species diversity and richness associated with *Erythina brucei*; 2) the genetic and functional diversity and eco-physiological stress tolerance traits of bacteria isolated from the root nodules of *E.abyssinica ;*3) evaluate inorganic phosphate solubilizing efficiency and multiple phytobeneficial properties of root nodule bacteria associated with *E. abyssinica*; 4) evaluate the symbiotic effectiveness of *Bradyrhizobium* species, and evaluate the effects of multiple inoculation of the consortia of microorganisms on *Erythrina abyssinica* growth, nodulation, and shoot TN and P contents in greenhouse condition. Soil samples were collected from different *E.spp* growing areas involved various land use types and trap cultures were established. AMF spore extraction and species identification was done from soils obtained from the rhizosphere of *E. brucei*. The root nodule bacteria of *E. abyssinica* were obtained by plant infection method. The genetic diversities were studied using Amplified ribosomal DNA restriction analysis (ARDRA). The *nifH* was gene screened and the taxonomic position was determined using 16S rRNA sequence analysis. Plant growth promoting traits were also evaluated and selected microbial inputs were studied for their effects on *E. abyssinica* growth, nodulation, nitrogen fixation and uptake P in the greenhouse condition. Eleven AMF genera and 33 species were recovered. The ARDRA grouped the bacterial isolates in to twelve clusters at 70 % similarity level. *NifH* gene was amplified in 15 (50%) of the root nodule bacteria the 16S rRNA sequence analysis grouped the bacterial in to nine genera (species): *Bradyrhizobium* (n=3), *Paenibacillus* (n=2), *Bacillus* (n=2), *Staphylococcus* (n=2), *Enterobacter* (n=2), *Achromobacter* (n=1), *Acinetobacter* (n=3), *Gluconobacter* (n=4) and *Stenotrophomonas* (n=2). *Achrmobacter, Acinetobacter, Bacillus, Gluconobacter, Paenibacillus, Staphylococcus* and *Stenotrophomonas* are the first reports from E. *brucei* root nodules. In addition, *Bradyrhizobium cajani* and *Bradyrhizobium cytisi* are the first reports from *E. abyssinica* root nodules and from Ethiopia as well. The 61.9%, 76.2%, 47.6%, 33.3% and 19.04% of the isolates were inorganic phosphate solubilizers, IAA, NH_3, HCN and chitinase producers respectively. The maximium shoot length and dry weight improvements (140%) and (267%) were recorded by multiple inoculations involved *B. shewense* (AU27) + *Glomus* sp.1(AMF1) + *Acaulospora* sp.1 (AMF2) + *A. soli* (AU4). Similarily, the maximum shoot total nitrogen improvement (260%) was recorded in treatment received *B. shewense* (AU27) + *A. soli* (AU4) inoculation; whereas the highest shoot P improvement

(1200%) was recorded by *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) inoculation. AMF species richness was affected by land use types and geographic locations. Phylogenetically and functionally diverse bacteria were inhabited in the root nodules of *E. brucei*. Multiple inoculations enhanced *E. abyssinica* growth and development, biomass production, nitrogen fixation and multiple inoculations also improved phosphorus uptake by the host plant. Synergistic interaction was observed among the microsymbionts and macrosymbiont. More studies are required to explore the rhizosphere of *E. abyssinica* and the performance of the microbial inputs which exhibited potential performance in greenhouse needs evaluation in the field condition.

Keywords: *E. brucei*, *E.abyssinica* AMF, Inoculation, *Bradyrhizobium*, ARDRA, genetic diversity, *nifH*

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Acronyms/Abbreviations

AMF-Arbuscular Mycorrhizal Fungi

BNF-Biological Nitrogen fixation

ARDRA-Amplified Ribosomal DNA Restriction Analysis

TN-Total Nitrogen

P-Phosphorous

IAA-Indole-3- Acetic Acid

HCN- Hydrogen Cyanide

NH3-Ammonia

DBT- Department of Biotechnology, India

TWAS- The World Academy of Science

MSSRF- M S Swaminathan Research Foundation

MoE- Ministry of Education, Ethiopia

MoSHE-Ministry of Science and Higher Education, Ethiopia

PGPR-Plant Growth Promoting Rhizobacteria

PGPM-Plant Growth Promoting Microorganisms

IAR-Intrinsic Antibiotic Resistance

ISR-Induced Systemic Resistance

TCP-Tricalcium Phosphate

Al-P-Aluminum Phosphate

Fe-P-Iron phosphate

EPS-Exopolysaccharide

KPS-Capsular polysaccharide

LPS-Lipolysaccharide

PSM-Phosphate Solubilizing Microorganisms

PSB-Phosphate Solubilizing Bacterial

ePGPR-Extracellular PGPR

iPGPR-Intracellular PGPR

SOM- Soil Organic Matter

OM-Organic Matter

ROC-Root Organ Culture

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1. General Introduction

Legumes are one of the most widely distributed families of flowering plants on earth next to the Family Graminiae (Graham and Vance, 2003). Most legumes have unique character of forming nodules to fix atmospheric nitrogen (Biological nitrogen fixation; BNF) with soil bacteria, (root nodule bacteria) generally known as Rhizobia. They also form intimate association with the soil arbuscular mycoorhizal fungi (AMF) to enhance the availability of phosphorus and other useful nutrients to enable them colonize low nitrogen environments in natural ecosystems, agriculture and agroforestry systems (Graham and Vance, 2003).

The AM fungi directly take up inorganic nitrogen from the soil and transport it to the plants (Govindar-ajulu *et al*., 2005). Mycorrhizal association enhances the availability of N and P to host plants, and the nutrient improvement is related to the synergistic interaction between rhizobia and AM fungi (Barea *et al*., 2002; Zaidi *et al*., 2003). It has been verified that 88% of legume plants have the ability to form dual symbiosis with nitrogen fixing rhizobia and P acquiring AM fungi (Varennes and Goss, 2007).

Apart from rhizobia and mycorrhiza, other root microorganisms play a major role in biogeochemical cycle processes like solubilization mobilization, immobilization and mineralization of various plant nutrients mainly nitrogen and phosphorus (Bisht *et al*., 2009). These microorganisms are generally known as plant growth promoting microorganisms (PGPM) for they exert a beneficial effect on plant growth. Since 95% of these microorganisms are bacteria, they are known as Plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Some of the PGPR are*; Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* etc. (Bhattacharyya and Jha, 2012).

Somers *et al*. (2004) have classified PGPR based on their roles as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phyto-hormones production), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics, antifungal metabolites, fungal cell wall and its component degrading enzymes).

In Ethiopia, there are more than 600 species of leguminous plants (Thulin,1989) one of which is *Erythrina brucei* Schweinf. Emend. Gillett (Papilionoideae). This legume is a multi-purpose leguminous woody legume which is endemic to Ethiopia. It is widely distributed in different agro-ecologies (edges or open places of upland forests or woodland evergreen forest, etc.) in the central, southern, south western, northern and eastern parts of Ethiopia with altitudes between 2250 and 2520 m.a.s.l with rainfall about 1000 mm.

The legume is widely used by local farmers for fuel wood and construction, and is integrated as shade tree for coffee plantation soil fertility and other cropping systems to improve soil fertility and yield (Demel Teketay, 1994; Legesse Negash, 2002; Wassie Haile *et al.*, 2013). *E. brucei* has an attractive agro-forestry attributes, such as rapid establishment, tolerance to sunlight, high rate of litter production, fast decomposition and mineralization, vigorous re-growth after cutting and coppicing, as well as rapid recovery after a period of sustained drought (Powell and Westley, 1993).

Erythrina species produce up to 50kg fodder biomass per tree per year (Wassie Haile *et al.*, 2013), with annual litter fall production of about 929 gram per unit area of crown (Melese

Negash and Mike Starr, 2013). It has a fast decomposition rate of the plant biomass and releases nitrogen in the form of NH_4^+-N and NO_3^- -N within 2-3 weeks (Wassie Haile *et al*., 2013; Abebe Abay, 2018). The fast decomposition rate and mineralization of organic nutrients to accompanying crops is directly related to its high TN content and low lignin, cellulose and polyphenol content, and is considered as a high quality organic material (Wassie Haile *et al*., 2013).The authors also showed that application of 2.5 tons of *E.brucei* dry matter per hectare increased wheat grain and straw yield by 127% and 194% respectively.

Local farmers in southern Ethiopia plant *E. brucei* growth and leave in their farms, home gardens and as live fences around their land boundaries. The farmers prune and harvest the wood materials like nitrogen rich leaves, pods and green stems and use as mulch or green manure during land preparations just before sowing cereals (Legesse Negash, 2002).

E. brucei forms a symbiotic nitrogen fixing association with soil bacteria mainly of the genera *Rhizobium, Mesorhizobium and Bradyrhizobium* (Fassil Assefa, 1993; Endalkachew Wolde-meskel *et al*., 2004; Shasho Megersa and Fassil Assefa, 2011; Aregu Amsalu *et al.*, 2012). Some of the authors also recovered other non-rhizobial bacterial species that include *Rahnella aquatilis*, *Enterobacter*, *Agrobacterium* species from root nodules of *Erythrina brucei* (Endalkachew Wolde-meskel *et al*., 2004; Aregu Amsalu *et al.*, 2012, 2013). *E.brucei* also forms symbiotic association with arbuscular mycorrhizal fungi (AMF) (Shasho Megersa and Fassil Assefa, 2011; Beyene Dobo *et al*., 2016). Beyene Dobo *et al* (2016) have collected eight AMF genera from the rhizosphere soils of *E. brucei* from Sidama agro-forestry system, southern Ethiopia. The authors also recorded 80% AMF colonization of the roots of *E. brucei* which was comparable to highly mycorrhizal plants such as *Millettia ferruginea*, *Ensete ventricosum*, *Catha edulis*, and *Coffea arabica.*

It is established that the dual inoculation of legumes with rhizobia and AM fungi elicited a synergistic effect to improve plant growth, nutrient uptake and nitrogen fixation through reduction of symbiotic dependence, that is, reduce proportional dependence of legumes on atmospheric nitrogen (Xavier and Germida, 2002; Chalk *et al*., 2006; Stancheva *et al*., 2008). Dual inoculation of *E.brucei* with *Bradyrhizobium* species and AM fungi species in greenhouse exhibited 16.9% to 45.3% improvement in shoot dry weight and 41.6% to 75% increment in shoot total nitrogen (Shasho Megersa and Fassil Assefa, 2011). Similarly, the effectiveness of tripartite interaction among rhizobia-legume-AM fungal varies with host plant species, rhizobial strains, AMF species and soil conditions (Patreze and Cordeiro, 2005).

Although attempts were made to study the rhizosphere microbiology of *Erythrina brucei* in some parts of the country (Fassil Assefa, 1993), the information on its mycorrhizal status, phenotypic and genetic diversity of rhizobia and rhizobacteria and its tripartite interaction with rhizobia and AM fungi are not well explored. For this reason, there were scarcities of information on the arbuscular mycorrhizal fungi diversity, phenotypic and genetic diversity of rhizobia and rhizobacteria associated with *E.brucei*, the inoculums potential of AMF associated with *E.brucei* and effects of multiple inoculation of the host plant with *Rhizobium* and AM fungi.

Therefore, it is important to understand the rhizobiology of *E.brucei* so as to enhance its growth and improve its symbiotic association with rhizobium and AM fungi and as result improve N and P content in its biomass. Hence, this study was initiated to explore the phyto-beneficial traits of *E. brucei* associated indigenous bacteria and AM fungi.

1.1 Objectives

1.1.1 General objective

The general objective of the present study was to assess the diversity of symbiotic and non-symbiotic root nodule bacteria and other phytobeneficial microorganisms from the rhizosphere of *Erythrina brucei* to evaluate plant growth promoting traits in order to select potential candidate inoculants to enhance growth and development of the host plant.

1.1.2 Specific objectives

The specific objectives of the present study were to:

1) Investigate AM fungal diversity associated to *E. brucei* and their abundance among different land use systems in different geographic locations.

2) Assess the phenotypic and genotypic diversity of rhizobial and non-rhizobial bacteria isolated from the root nodules of *E. abyssinica* to evaluate their potential (in-vitro) tolerance to eco-physiological stress and plant growth promoting traits.

3) Isolate, screen and evaluate mineral phosphate solubilizing efficiency and assess multiple plant growth promoting traits of root nodule bacterial endophytes and rhizobacteria associated with *E. abyssinica*.

4) Investigate the effects of combined inoculations of *B. shewense (*AU27) and *Glomus* sp.1 (AMF1) and/or *Acaulospora* sp.1(AMF2) and /or *Acinetobacter soli(*RG6) on *E. abyssinica* growth,nodulation and its shoot nitrogen and phosphorous contents under greenhouse conditions.

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2. Literature Review

2.1 Rhizbium-Legume symbiosis

2.1.1 Legumes

Legumes are one of the most widely distributed flowering plants on earth and and are classified under the family Leguminoseae/Fabaceae which is the third largest family of higher plants (Sprent, 2009). There are over 20000 species of the family divided into three subfamilies, namely, *Papilionoideae*, *Mimosoideae*, and *Caesalpinioideae.*

Most of the legumes form nodules with soil bacteria (root nodule bacteria) generally known as rhizobia to fix atmospheric nitrogen (Biological nitrogen fixation; BNF). Although the majority of the legumes (90-96% species) from the subfamilies Papilionoideae and Mimosoideae form nodules and fix atmospheric nitrogen, only few members (22%) of the Subfamily Caesalpinioideae posess this characteristic (de Faria, 1989; Sprent, 2009).

2.1.2 Biological Nitrogen fixation (BNF)

Dinitrogen (N_2) is the major constituent (79%) of the Earth's atmosphere, representing the largest global nitrogen pool. However, atmospheric nitrogen cannot be utilized by organisms unless it is fixed or transformed into utilizable forms of NH_4 and NO_3. Nitrogen fixation is naturally mediated by prokaryotes (some bacteria and some archaea) and is known as Biological nitrogen fixation (BNF). It is undertaken at ambient temperature and pressure catalyzed by the enzyme nitrogenase present in the cells (Postgate, 1998). It has very important implication in the nitrogen cycle in global ecosystem. Nitrogen is also fixed industrially through the Haber-Bosch process using higher temperature and pressure in the presence of Fe catalyst to form ammonia.

There are three categories of Biological nitrogen fixation (BNF). These are free living, associative and symbiotic nitrogen fixation systems. The first one is fixed by free living bacteria in the soil such as *Azotobacter*, *Clostridium*, *Azomonas*, etc that do get their energy heterotrpically from the soil; whereas the associative nitrogen fixers represented by *Azosprillum* are adhered to the root surface, and endophytic nitrogen fixing bacteria living in the roots, stems, and leaves of plants that obtain their energy from root exudates and the rhizosphere (Chanway *et al*., 2014).. The symbiotic nitrogen fixing bacteria include the bacteria that induce legumes to form root/stem nodules and the actinomycete Frankia that form nodules with actinorhizal plants.

Biological nitrogen fixation is mediated by nitrogenase enzyme containing molybdenum/iron, which is found in most of the diazotrophs. However, some diazotrophic microorganisms alternatively contain vanadium and/or iron-only nitrogenase (Raymond *et al*., 2004). The process is coupled with the hydrolysis of 16 equivalents of ATP and is accompanied by the conformation of one molecule hydrogen. During the process of biological nitrogen fixation, the enzyme nitrogenase catalyzes the ATP-dependent reduction of dinitrogen to ammonia. The ammonium generated from the biological nitrogen fixation is assimilated into glutamate through glutamine synthetase/glutamate synthase pathway (Seefeldt *et al*., 2012).

$$N_2 + 8e^- + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

The nitrogenase enzyme is highly sensitive to oxygen and many bacteria stop production of this particular enzyme in the presence of oxygen. Some diazotrophs, exist only in anaerobic conditions while others having high respiration rates draw down oxygen levels (De Bruijn, 2015). Rhizobia solve the paradox between their strict aerobic status and the extreme sensitivity of nitrogenase to oxygen by inducing the nodules which regulate low free oxygen diffusion through a layer containing plant hemoglobin-leghemoglobin that reaches to bacteroids (nitrogen fixing states of rhizobia) (Raymond *et al*., 2004).

2.1.3 Events in Nodulation by legumes; the molecular dialogue in rhizobium-legume symbiosis

The establishment of legume-rhizobia symbiosis is a multifaceted process, which involves a coordinated exchange of multiple chemical signals between the plant partner and its microsymbiont (Janczarek *et al*., 2015). To start the symbiotic process, the host plants secrete a combination of phenolic molecules, primarily flavonoids which can diffuse across the bacterial membrane. The rhizobia receive flavonoid signals and reciprocate by the activation of bacterial nodulation (nod) genes, which encode the enzymes required for the synthesis of bacterial Nod factors, a family of lipo-chito-oligosaccharides essential for symbiotic development in most legumes. Nod factors are important for nodulation in legumes, except some photosynthetic rhizobia, which have the ability to nodulate their legume hosts without producing Nod factors (Giraud *et al*., 2007). The Nod factors of different diazotrophic bacteria share the same chitin-like N-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end (Wang *et al*., 2012). Such decorations on the ends of the Nod factor play a crucial role in determining whether a specific host can perceive the Nod factor. The products of nod ABC genes synthesize the core structure of Nod factor. Additional genes determine the specific decorations on the Nod factor core, and alterations in these genes often change specificity.

The symbiotic relationship is extremely specific and strongly regulated mainly by the plant partner, i.e a particular legume forms symbiotic interaction with only a restricted set of rhizobial species. The high level of specificity can occur at the early stages of molecular dialogue between the partners that influence bacterial infection and nodule development and nitrogen fixation (Wang *et al*, 2012). To increase their profit from symbiosis, legumes have developed a set of ‘host control’ traits that select for compatible rhizobia and protect against strains that offer little or no benefit (Boivin and Sachs, 2018).

The important components of the micro-symbiont cell wall such as bacterial surface exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS) and cyclic glucans are considered as symbiotically important molecules that enable them to interact directly with the hosts (Boivin and Sachs, 2018). Other studies also demonstrated that surface polysaccharides affect specificity of nitrogen-fixing phases of several legumes – rhizobial symbiosis (Kannenberg *et al*., 1992; Parniske *et al*., 1994). The authors also showed that a defect in surface polysaccharides may cause failures of symbiosis at either early or late stages.

The plant lectins also serve as receptors of the rhizobia surface polysaccharides and thus determine host range (Boivin and Sachs, 2018). These authors also showed that the binding of plant lectins to surface polysaccharides of rhizobia has been shown to play a role in triggering rhizobial attachment to root hairs, thereby enhancing the release of Nod factors to the root hairs for bacterial infection and nodule initiation.

In general, both the microsymbionts and host plants establish the first contact with each other at the surface of new and fine root hair tip, and the root hair tip infection process in most legume plants is initiated in epidermal root hairs (Gibson *et al*., 2008). If the initial molecular communication between the partners is successful, the root hair curls to catch a small number of bacteria. At the trap site, the root hair begins an inverse tip growth, forming a long and narrow passage, called the infection thread, in which the bacteria ‘travel’ to root cortex by continuously dividing at the leading edge.

The infection threads finally merge with the basal membrane to release the bacteria into the extracellular space and other infection threads are formed in cells of basic layers, repeating the process to convey the bacteria to their final destination, the root cortex (Wang *et al*, 2012). Following penetration by the bacteria, host cells in the root cortex give rise to a population of newly generated cells, which form the lateral organs nodules. When bacteria reach these nodule cells, they are internalized in an endocytosis-like process and individual bacteria are surrounded by a host membrane, symbiosome, the nitrogen fixing organelles, where bacteria differentiate into nitrogen-fixing stages called bacterioids. The fixed nitrogen from the bacteria and fixed carbon provided by the host are traded across the symbiosome membrane

2.2 Arbuscular Mycorrhizal Fungi (AMF)

The term ‘mycorrhiza’literally means ‘fungus roots’ form symbiotic relationships with plant roots in a fashion similar to that of root nodule bacteria within legumes. There are different types of mycorrhizas of which Arbuscular mycorrhiza (AM) is the ancestral and ubiquitous type of mycorrhiza among terrestrial plants (Brundrett, 2009). According to Helga son and Fitter (2009), arbuscular mycorrhizal fungi (AMF) are the principal types that are widely distributed in the tropics that form a symbiotic association with more than 80% of land plants so far studied covering 92% of the plant families which exist everywhere from small home gardens to large ecosystems. It is a mutualistic association where the host provides its 20% of the photosynthate to root microbial partners including mycorrhiza (Bago *et al*., 2000).

2.2.1 Arbuscular mycorrhizal development

Arbuscular mycorrhizal fungi are entirely biotrophic, which cannot complete their life cycle in the absence of symbiosis (Bonfante and Bianciotto,1995). This is because that the fungus lost some of its carbon fixating capabilities and/or the genetic machineries that support them, and become completely dependent on the host plant for fixed carbon supply in the long process of the evolution of symbiotic relationship with the host plants.

The AM fungi produce spores which constitute as the only autonomous phase of the microsymbiont in its life cycle. The spores are round-shaped structures consisting of a thick cell wall and average diameters between 50 and 100 µm. AMF spores have a very large number of nuclei, up to 2000 per spore (Becard and Pfeffer, 1992). The dormant spores may stay alive in the soil for one up to two years depending up on AM fungal species and genera (Giovanneti ,2000). The spores germinate under suitable water and temperature conditions and hyphae grow, with marked apical dominance and sporadic hyphal branching and stops growth after 2–4 weeks in the absence of a host plant (Requena *et al*., 2007). This stage of development in the absence of signals from the host plant is known as the asymbiotic stage.

2.2.2 Initiation of symbiosis; signaling and major molecules

AM symbiosis is initiated with a signal exchange between the fungal and the plant partners that leads to mutual understanding and development of symbiotic structures (Bonfante and Genre, 2008). Both the symbionts realize their presence by secreting some signal molecules, which can trigger the activation of the symbiotic genes (Smith and Read, 2008).The chemical dialogue between the fungal and plant partners involves molecules that are synthesized by the macro (plant) and micro (fungal) symbiont.

The host produce the signaling molecules known as the strigolactones; whereas the micro-symbiont (AMF) release the Myc factors that play an essential role in the mutual interaction (Aroca *et al.*, 2013). Apart from strigolactones, other signaling molecules such as flavonoids and hydroxy fatty acids have been reported as hyphal growth stimulators (Scervino *et al*., 2005; Nagahashi and Douds, 2011). Interestingly, strigolactones trigger a response only in AM fungi, but not in other beneficial fungal species such as *Trichoderma* and *Piriformospora indica* or in soil borne pathogens indicating that they are rather specific signaling molecules (Aroca *et al*., 2013).

The release of the strigolactones is recognized by AM fungal partner, and once the fungus receives the signal, the pre-symbiotic stage is induced which is characterized by the asymbiotic phase of spore germination, initial hyphal germ tube growth, and hyphal branching to increase the possibility of contact with the root and form the symbiosis (Aroca *et al.*, 2013). The fungal partners, in turn, produce signal molecules (Myc Factors) that are diffusible molecules, which are recognized by the plant to further induce molecular responses in the host root needed for a successful colonization (Puech-pages and Haouy, 2011).

Myc factors are not only symbiotic signals that initiate the formation of the AM symbiosis, but also act as plant growth regulators affecting root development by altering the root architecture to increase the number of colonization sites.After having received the Myc factors, the plant cells actively prepare the intracellular environment by carrying out an extensive and specific reprogramming of root tissues for the formation of the AM symbiosis (Bonfante and Genre, 2010).

2.2.3 The mechanisms of phosphorus and nitrogen transport in AMF-plant symbiosis

The nutrient trade between the symbiotic partners is all the more important when soil fertility and water availability are low that severely limit plant growth and production. The driving force behind the AM fungi-plant symbiosis is the exchange of important nutrients. In exchange to the carbon, the AM fungi provide the plant partner with essential nutrients such as phosphorous and nitrogen. A number of mechanisms have been proposed regarding uptake of phosphorous by AMF from soil microcosm. Van der Heijden *et al* (2006) have shown the density of mycorrhizal hyphae is 4.5–14.8 times higher than that of roots. Jakobsen *et al*. (2003) have reported that hyphae could scavenge nutrients in a given soil volume much more successfully than the plant roots. This can be explained in terms of kinetic parameters for phosphorous uptake is much higher by mycorrhizal hyphae than by roots, and this could explain why plant communities treated with AMF contained more phosphorous than non-AMF inoculated controls. The other mechanism is that AMF produce enzymes such as phosphatases that can mineralize organic Phosphorous from the soil (Joner and Johansen, 2000).

The AMF extra-radical hyphae accomplish the phosphate uptake at the AM fungal hypha-soil interface that enable them explore beyond the nutrient depletion zone that covers a large volume of soil (Bucher and Bucher, 2007). The fungal high-affinity phosphate transporters guide the phosphate transport from the interfacial apoplast to the plant cytosol across the periarbuscular membrane. After arrival in the plant cytosol, phosphate is translocated in to the vasculature for delivery to all parts of the plant (Javot *et al*., 2006).

One of the limiting factors that affect plant growth is the scarcity of nitrogen in the soil. Nitrogen is accessible in the soil in the form of ammonium (NH_4^+) and nitrate (NO_3^-)**.** Even though the concentration of ammonium in the soil is 10-1,000 times lower than that of nitrate, ammonium is the preferred form of nitrogen absorbed when plants are subjected to nitrogen scarcity (Gazzarrini *et al.*, 1999) or grown in water-logged or acidic soils (Marschner, 1995). Ammonium has low mobility in the soil and a depletion zone is formed in the vicinity of the roots in a fashion similar to that observed with phosphate.

The role of mycorrhizal nitrogen delivery to the plant root system is becoming better understood. The extra-radical mycelium of AM fungi absorbs ammonium, amino acids and nitrates (Hodge *et al*., 2001).The majority of nitrogen is thought to be in the form of ammonia through the action of fungal encoded ammonia transporters (Lopez-Pedrosa *et al*.,2006).

2.2.4 AMF enhances plant tolerance to stress

AM fungi can improve plant-water relations and enhance drought resistance by plants (Smith and Read, 2008). Although a number of mechanisms have been proposed by which the AMF-plant interaction improves drought tolerance, the principal mechanism is the extended mycorrhizal colonization that improve soil–hyphal contact, which creates more surface area and opportunity for scavenging water from the soil (Auge *et al*, 2001; Smith *et al*., 2010).

Augé (2001) have reported that inoculation of AM fungi in the field condition increased plant performance under drought condition, which could be due to combination of nutritional and non-nutritional effects of AM fungi. Furthermore, numerous studies revealed that mycorrhizal plants increased water use efficiency (Kaya *et al.*, 2003; Ruiz-Lozano and Aroca, 2010). AMF can increase water use efficiency by improving stomatal conductance (Augé *et al*., 2015) and increase antioxidant enzyme activity to reduce peroxidative damage (Duc *et al*., 2018).

Besides enhancing mineral nutrient and water up take, AMF also increases tolerance to stresses caused by high temperature, salinity, or transplantation (Porras-Soriano *et al*., 2009; Meddad-Hamza *et al*., 2010), induce resistance to plant pathogens, and develop tolerance to toxic substances in the soil (Smith and Read, 2008). It has been well established that mycorrhizal colonized plants exhibit more vigorous vegetative growth than non-mycorrhizal plants (Dag *et al*., 2010).

Although AMF colonized plants showed better performance over non-colonized plants under nutrient limiting conditions (Parniske, 2008), the relative benefits the plant partner gets from the fungal partner is highly reduced under nutrient saturated conditions, like in high input agricultural systems, because of the carbon cost accrued to the fungal partner (Janos, 2007). For this reason, the performance of the colonized plants can fall below the non-colonized plants under the circumstances.

2.2.5 AMF improves Soil Aggregate Stability

Soil aggregate stability is a very important soil property affecting soil sustainability, crop production, biological activity, soil carbon storage, and the movement and the storage of water (Amez keta *et al*., 1999). Soil aggregates influence microbial community structure and determine nutrient adsorption and desorption, thus contributing to soil fertility (Linquist *et al*., 1997). Stable soil aggregates are more resistant to detachment and loss by erosion (Barthes and Roose, 2002).

AM fungi are one of the important biotic factors affecting the formation and maintenance of soil aggregates (Rilling and Mummey, 2006). It has been well established that glomalin, a glycoprotein, produced by AM fungi, plays a very important role for soil aggregate stability (Feng *et al*, 2001). Mycorrhizal fungi influence charge, adhesiveness, and enmeshment mechanisms (Rilling and Mummey, 2006).

Fungal exudates form micro-aggregates (≤250 µm) by coating fine soil particles with the larger organic matter and by adhering the particles embedded and held on the surface. Fungal hyphae can physically bind and enmesh micro-aggregates into macro-aggregates (≥250 µm) (Graf and Frei, 2013). Mycorrhizal fungi also indirectly affect soil aggregate stability using their plant hosts, particularly by accelerating the development of their root network and by serving as a distributing vector for associated microorganisms

2.2.6 The impacts of fertilizer application on AMF symbiosis

Agricultural practices involve a variety of crop management methods that can affect the plant-AMF association. In general, farming activities have adverse effects on the plant-AMF symbiotic associations, particularly on its diversity and species composition (Gosling *et al*., 2006). The application of P fertilizers has significant impact on the symbiotic association between the plant and AMF. In intensive farming practices where there is a continuous application of P fertilizers, the P which is in excess of plant suppress the AMF-plant symbiosis (Jensen and Jakobsen, 1980;

Martensson and Carlgren, 1994; Gosling *et al*., 2006). Jensen and Jakobsen (1980) have indicated that AM root colonization was the highest at sites with the lowest soil available P and the increased use of P fertilizer reduced AM root colonization and spore density at all sites in Denmark. According to Martensson and Carlgren (1994), the application of even moderate amount of P fertilizer (45 kg ha^{-1} year^{-1}) reduced AMF spore number by 50% over five years.

Shukla *et al*. (2012) have conducted an experiment to study the P threshold for AMF colonization of crops and tree seedlings. They found that P application decreased the mycorrhizal dependency of the studied crops and tree species. Covacevich *et al.* (2007) have also reported that P supplied annually increased wheat growth and wheat shoot P content but soil available P suppressed the indigenous AMF root colonization. However, Alloush and Clark (2001) have shown that application of the organic nutrient sources, farmyard manure, compost and crop residues, and slow release mineral fertilizers such as rock phosphates do not suppress AMF and may even enhance their symbiosis.

2.2.7 Role of AM Biofertilizers in Sustainable Agro-ecosystems

Sustainable farming systems use natural processes to attain satisfactory levels of crop productivity and food quality, while reducing the use of chemical fertilizer, minimizing input costs and focusing on environmental friendly options (Siddiqui *et al*., 2008; Solaiman and Mickran, 2014). The importance of soil microbes for improvement of soil fertility and for increased crop productivity and plant protection is well recognized (Solaiman and Mickran 2014).

Currently, AMF is recognized as key components of the soil microbiota for sustainable management in agro-ecosystems, forestry and agroforestry system. According to Hamel and

Strullu (2006), development of improved inoculants to manipulate AM fungi is more important to attain productivity, stability and sustainability of different agro-ecosystems. Recent advances in knowledge also opened the way to AM fungi management in crop nutrition and production (Balzergue *et al*. 2013).

AMF management for sustainable agroecosystems starts from the use of AM fungi as biofertilizers for plant growth, production in agriculture, agro-forestry and forestry systems (Barea *et al.,* 2005; Gianinazzi *et al*., 2010). In order to achieve the benefits of the symbiosis, it is imperative to select the appropriate AM fungal species adaptable to the local conditions. Therefore, understanding the mechanisms controlling the plant root colonization, symbiotic efficiency and competence is critical to optimize mycorrhizal benefits (Lopez-Raez and Pozo, 2013).

It is a well-established fact that strigolactones are crucial for AM symbiosis development by inducing hyphal branching, thus increasing the probability of contact with the host plant (Akiyama *et al*., 2005). Likewise, the Myc factors are the fungal signal molecules which stimulate AM symbiosis development in leguminous and other mycotrophic plant species (Bouwmeester *et al*., 2007). Structural clarification of the chemical nature of the Myc factors can bring the AMF as the ideal candidates for application as bio-fertilizers (Maillet *et al*. 2011). Therefore, breeding for crop varieties that produce high strigolactone level appears an eye-catching strategy to get better mycorrhization of the host plant. On the other hand, enhanced mycorrhizal colonization could be obtained by the exogenous application of strigolactones or strigolactone analogues (Lopez-Raez and Pozo, 2013). With regard to Myc factors, efficient synthesis by bacteria has been already developed allowing their production on a large scale (Maillet *et al*. 2011).

2.3 The rhizosphere; a Microbial hotspot in the soil

In spite of the huge microbial diversity in soil, microorganisms are aggregated in small volume of soil, which constitute only 1% of the total soil volume (Young *et al*., 2008). These microhabitats wherein microorganisms are aggregated are characterized by faster rates of different biogeochemical processes than bulk soil.

Kuzyakov and Blagodatskaya (2015) have defined these soil volumes as “microbial hotspots” and identified four such hotspots in soil and these include (a) rhizosphere, the region of soil surrounding living roots which is under the influence of plant root exudates; (b) detritusphere, the soil region associated with decomposition of plant litter and turnover of soil organic matter; (c) biopores, formed by deep growing roots and burrowing fauna; and (d) the soil aggregate surfaces.

The localized availability of labile carbon and other readily utilizable nutrients in the rhizosphere is followed by different events like high rates of respiration, gas exchange, nutrient and moisture utilization, and other bioprocesses in the rhizosphere (Richter *et al*., 2011). This in turn, leads to rhizodeposition of root exudates, wherein plant roots secrete a wide range of low- and high-molecular weight compounds including sugars, organic acids, amino acids, polysaccharides, vitamins, and other secondary metabolites into the surrounding soil (Badri and Vivanco 2009).

These rhizodeposits account for approximately 11% of net photosynthetically fixed carbon and 10–16% of total plant nitrogen (Jones *et al*., 2009). These exudates alter soil chemistry in the vicinity of plant roots as a result of the growth of selected soil microorganisms (Yang and Crowley, 2000). Components of plant root exudates are qualitatively and quantitatively varied, depending on the nutritional status of the plant, growth stage, and time and space relative to the position of the root (Hartmann *et al*., 2009; Malusà *et al*., 2016). This creates a strong selective pressure in the rhizosphere leading to a plant-driven selection of specific rhizosphere microbial communities. Interestingly, only 2–5% of the rhizosphere microorganisms promote plant growth for they are beneficial microorganisms naturally selected by plants under nutrient constrained conditions (Antoun and Kloepper, 2001; Lareen *et al*., 2016),

The rhizosphere microorganisms may also enter the roots and migrate to the plant's aerial surfaces (phyllosphere) (Thapa and Prasanna, 2018) and internal tissues (endosphere). Hence, plants are often associated with communities of microorganisms, living on or within them, exhibiting mutually beneficial symbioses. This entire genome of microbial community, referred to as the microbiome, plays vital roles in host's nutrient uptake, metabolic capabilities and tolerance to biotic and abiotic stresses (Bulgarelli *et al*. 2013; Sessitsch and Mitter 2015). Generally, plants are able to shape their specific rhizosphere microbiome. Thus, the defining moment in the rhizosphere is the ability of the host plant to select a core distinct microbiome that supports its growth in a certain environment.

2.3.1 Plant growth promoting rhizobacteria (PGPR)

About 2–5% of rhizobacteria in a soil contain, competitive microflora that exert a beneficial effect on plant growth and are termed as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Somers *et al*. (2004) classified PGPR based on their roles as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones production), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics, antifungal metabolites, fungal cell wall and its component degrading enzymes).

Furthermore, in most cases, a single PGPR has often multiple modes of action including biological control (Vessey, 2003).

Gray and Smith (2005) have shown that the PGPR associations range in the degree of bacterial proximity and grouped them into extracellular (ePGPR) and intracellular (iPGPR). The ePGPR exist in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex. Some examples of ePGPR are like, *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* etc. (Bhattacharyya and Jha, 2012). The intracellular (iPGPR) exist inside root cells, generally in specialized nodular structures (Figueiredo *et al*., 2011) and examples of the iPGPR include *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium,Sinorhizobium* and *Rhizobium* of the family Rhizobiaceae (Figueiredo *et al*., 2011).

Microbial inoculation in to soil influences the activity of indigenous microflora, ultimately having a bearing effect on their own survival (Ramos *et al*. 2003).The introduced microorganism must be able to colonize the rhizosphere, should adhere to the plant roots, compete for space and must be able to occupy the new niche in sufficient numbers so as to exert its effect on the host plant (Barriuso *et al.*, 2008). Often, the native inhabitants of soil, which are better adapted to the environmental conditions, out compete the inoculated population. Thus, production and development of an effective microbial inoculant requires the presence of multiple fitness traits that can facilitate its colonization and survival under harsh environmental conditions (Rana *et al*., 2011).

2.3.2 Plant growth promoting mechanisms of rhizobacteria

PGPR promote plant growth and support plant health by a variety of mechanisms in a wide range of plants. The mechanism by which PGPR promote plant growth can be classified into direct and indirect ones (Glick, 1995; Prashar *et al*., 2014). Direct plant growth promotion is based on either stipulation of the plants with favorable bacterial compounds or improving the nutrient uptake by the plant from the soil. The direct mechanisms are accomplished through processes like atmospheric nitrogen fixation (BNF), siderophore production and release, phosphate solubilization, synthesis and release of phytohormones. The indirect promotion of plant growth is primarily based on the reduction or prevention of the deleterious effects of phytopathogens, usually the fungi and the nematodes, thereby controlling the diseases and promoting plant health.

2.3.2.1 Nitrogen fixation and mineralization

Plants are able to use different N sources in both inorganic (i.e., nitrate [NO_3^-] and ammonium [$NH4^+$]) as well as organic (e.g., urea, amino acids, and peptides) forms. In soil, about 90% of total N is present in organic form (soil organic matter, SOM), and the biogeochemical cycle of the whole N pool (which includes also the N portion deriving from fertilization) is very important for the level of soil fertility (Jetten, 2008).

Microbial processes (SOM mineralization, atmospheric N_2 fixation, and denitrification) mediate the biogeochemical cycle of nitrogen. Considering the great extent of the organic N pool, it is evident that the mineralization, i.e., nitrification and ammonification, carried out by bacteria is crucial for plant mineral nutrition. In fact, microorganisms like mycorrhizal fungi and PGPRs mineralize organic matter (OM) by releasing hydrolytic enzymes and thus enhancing the nutrient availability in soil (Ollivier *et al*. 2011).

2. 3.2.2 Phosphorous

Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen, is abundantly available in soils in both organic and inorganic forms (Khan *et al*., 2009). Soils may contain large amounts of P but is very scarcely available for plant use (Bhattacharyya and Jha, 2012). Both inorganic and organic P forms are insoluble compounds (Pii *et al*., 2015). From 20 to 80% of P in soils is found in the organic form, of which phytic acid (inositol hexaphosphate) is usually a major component (Richardson, 1994).

The majority of the inorganic P present in soils is bound to Fe and/or Al in acidic soils, and/or Ca in alkaline soils that reduce its solubility, leading to precipitation and adsorption processes (Pii *et al*., 2015). To overcome the P deficiency in soils, there are frequent applications of phosphatic fertilizers in agricultural fields. Plants absorb fewer amounts of applied phosphatic fertilizers and the rest is rapidly converted into insoluble complexes in the soil (Mckenzie and Roberts, 1990). Bhardwaj *et al.* (2014) have estimated that 60–90% of total applied chemical fertilizer is lost mainly to insoluble and unavailable/fixed forms. Thus, the application of chemical fertilizers might not solve plant nutritional issues, since P can easily bind cations and become insoluble once applied to the soil (Adesemoye and Kloepper, 2009).

However, regular application of phosphate fertilizers is not only costly but is also environmentally undesirable. This has led to search for an ecologically safe and economically reasonable option for improving crop production in low P soils. In this context, microorganisms capable of phosphate solubilizing activity, often termed as phosphate solubilizing microorganisms (PSM), may provide the unavailable forms of P to the plants (Khan *et al*., 2006). Apart from that bacteria-derived phosphatases are able to mineralize P-containing organic molecules of soil (like phosphoesters, phosphodiesters [i.e., phospholipids and nucleic acids], and phosphotriesters) releasing consequently orthophosphate groups (Rodríguez *et al*. 2006). .

Phosphate solubilizing microorganisms release phosphorus from inorganic sources through several mechanisms the most important of which is the exudation of organic acids such as acetate, oxalate, succinate, citrate, and gluconate (Bhattacharyya and Jha 2012). Once released, organic acids can desorb Pi from soil adsorption sites by ligand exchange and thus solubilizing Pi from Ca/Fe/Al-Pi minerals (Tomasi *et al*., 2008). The most promising phosphorus biofertilizers are of the bacterial genera; *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* (Bhattacharyya and Jha, 2012).

2.3.2.3 Iron

Iron is a vital nutrient for almost all forms of life. In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to both plants and microorganisms (Rajkumar *et al.,* 2010). Commonly, bacteria acquire iron by the secretion of low-molecular mass iron chelators referred to as siderophores, which have high association constants for complexing with iron (Khan *et al*., 2009). Generally, rhizobacteria vary regarding the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores).

In both Gram-negative and Gram-positive rhizobacteria, iron (Fe^{3+}) in Fe^{3+}-siderophore complex on bacterial membrane is reduced to Fe^{2+} which is further released into the cell from the siderophore via a gating mechanism linking the inner and outer membranes (Rajkumar *et al*., 2010). During this reduction process, the siderophore may be destroyed/recycled. Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation.

2.3.2.4 Phytohormone production

Microbial synthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) has been known for a long time (Patten and Glick, 1996). It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Spaepen and Vanderleyden, 2011). These authors also showed that Tryptophan stimulates IAA production while, anthranilate, a precursor for tryptophan, reduces IAA synthesis. Hence, supplementation of culture media with tryptophan increases the IAA production by most of the rhizobacteria. Some of the important IAA producing rhizobacteria include; *Agrobacterium, Pseudomonas, Bradyrhizobium, Rhizobium, Azospirillum, Klebsiella*, and *Enterobacter* (Ahemad and Khan, 2012).

IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Glick, 2012). The different metabolic pathways for IAA synthesis are also essential for nodule formation. It is also reported that inoculation with *Rhizobium leguminosarumbv. viciae* wherein the IAA biosynthetic pathway had been introduced, produced potential nitrogen fixing root nodules containing up to 60-fold more IAA than nodules formed by the wild-type counterpart in *Vicia hirsute* (Camerini *et al*., 2008).

IAA produced by rhizobacteria likely supplement the above physiological processes of plants by changing the plant auxin pool. Moreover, bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients. In addition, rhizobacterial IAA loosens plant cell wall to facilitate an increase in root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick, 2012). Thus, rhizobacterial IAA is identified as an effector molecule in plant– microbe interactions, both in pathogenesis and phytostimulation (Spaepen and Vanderleyden, 2011).

2.3.2.5 Indirect PGP mechanisms of Rhizobacteria

The application of microorganisms to control plant diseases is an environment-friendly approach (Lugtenberg and Kamilova, 2009).The control of phytopathogens by using the plant growth promoting rhizobacteria is considered to be indirect plant growth promoting mechanism. The major indirect mechanism of plant growth promotion in rhizobacteria is through acting as biocontrol agents (Glick, 2012). In general, competition for nutrients, niche exclusion, induced systemic resistance and production of antifungal metabolites are the chief modes of biocontrol activity in PGPR (Lugtenberg and Kamilova, 2009). Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin (Bhattacharyya and Jha, 2012).

The interaction of some rhizobacteria with plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009). Moreover, ISR involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant’s defense responses against a variety of plant pathogens (Glick, 2012). Many individual bacterial components such as lipopolysaccharides (LPS), flagella, siderophores, cyclic lipopeptides, 2, 4-diacetylphloroglucinol, homoserine lactones, and volatiles like, acetoin and 2,3-butanediol induce ISR (Lugtenberg and Kamilova, 2009).

2.4 Microbial Inocula production and formulation

2.4.1 Methods of Arbuscular Mycorrhizal Fungi Mass cultivation

The application of AM fungi inocula is increasingly required in agriculture, horticulture, forestry programs, as well as for land reclamation, to increase crop yield, and health and to minimize the application of agrochemicals (Johansson *et al*., 2004). However, the obligate biotrophic nature of AMF has complicated the development of cost effective large scale production methods to obtain high quality AMF inocula (IJdo *et al*., 2011). The difficulty of growing AMF on culture media necessitated substrate based (sand/soil based), hydroponic (solution culture) or *in vitro*“root organ cultures” (ROC) inocula production.

The mass production and field utilization of AMF inocula highly depend on a suitability of the inocula, which should be very simple and easily inoculated in agricultural fields using conventional tools (Khaliq *et al*., 2010). Several techniques of inoculum production have been developed from time to time for practical application of AMF inocula in the field. Ijdo *et al.* (2011) have classified AMF production systems into three main categories; the substrate-based production system, the “substrate-free” cultivation systems (“true” hydroponics and aeroponics), and the “in-vitro” “root organ cultures” (ROC) production system.

2.4.1.1 Substrate-based production systems

The “classical”sand/soil and more advanced substrate based production system is the most common, standard and conventional method of producing AMF inocula around the world (Ijdo *et al*., 2011). Different substrates either separately or in mixture have been used to propagate and mass-produce AM fungi. For many years now, calcinated clay (Plenchette *et al.* 1982), sandy-soil reported (Sylvia and schenck 1983) as well as pure sand (Millner and Kitt 1992), peat (Lee and George 2005), vermiculite (Douds *et al*. 2006), perlite (Lee and George 2005), and compost (Douds *et al*. 2006), have been used for the preparation of AMF inocula.

The mass production of substrate based production system is most often initiated with a single identified species or consortium of selected identified AM fungal species. The starter inocula for AMF mass cultivation commonly consists of isolated and identified spores (Douds and schenck, 1990), a mixture of spores and mycorrhizal infected root segments (Gaur and Adholeya, 2002). According to Gaur and Adholeya (2002), mixed inocula can be obtained by drying and chopping infected host plant roots in to fine pieces while spores are most often obtained by wet sieving and decantation method (Gerdemann and Nicolson, 1963).

The soil containing AMF hyphae may also be used in mixed inocula. According to Biermann and Linderman (1983) and Klironomos and Hart (2002) have mixed inoculum is most attractive for cultivation of AM fungal species producing intraradical spores and vesicles. Besides direct inoculation of the host plants with isolated and identified AMF spores or mixed inocula, plantlets can also be pre-colonized before their transplantation to beds or containers (Douds *et al*., 2006).

The selection of the host trap plant species is very important for AMF inocula mass production. Plant species such as Onion and leek (*Allium* spp.), Maize (*Zea mays*) and Bahia grass

(*Paspalum notatum*) are commonly used for this purpose (Douds *et al*., 2006). These plant species are selected based on high mycorrhization (good colonization level by a large AM fungi), short life cycle, adequate root system development, and tolerance to relatively low level of phosphorous. Millner and Kitt (1992) have also included other desireable characteristics such as low susceptibility to pathogens, yellow appearance of colonized root and white appearance of un-colonized roots (leek and maize) and tolerance to a wide range of temperature.

Host-dependent sporulation of AM fungal species is an important determinant for inoculum production (Gaur and Adholeya, 2002; Douds *et al*. 2006). Gaur and Adholeya, (2002) have inoculated five different fodder crops with a consortia of indigenous AM fungal species and observed that the level of production of infective propagules was dependent on the host plant species.

2.4.1.2 Substrate-free cultivation systems

The “substrate-free” cultivation systems (“true” hydroponics and aeroponics) have been developed to produce relatively clean (sheared) AM fungal inoculum. However, these systems are limited to smaller-scale applications and research purposes because of higher costs incurred with inoculum production. The substrate-free AMF cultivation techniques, termed "solution culture techniques"are composed of static systems and nutrient flow technique (NFT) (Ijdo *et al*., 2011).

The major differences among the solution culture techniques are the mode of aeration and application of the nutrient solution. In static systems (in which the solution is not flowing), the nutrient solution needs to be aerated via an aeration pump. However, strong movement of nutrient solution and bursting of air bubbles might damage the development of the delicate AMF structures. The other technique, which could be an alternative to the static system, is the nutrient flow technique (NFT), in which a thin nutrient solution (film) flows in to the often-inclined channels (gulls) where the plant roots and the AMF develop. The use of a nutrient film that covers the roots increases the relative area for gas exchange and overcomes problems due to insufficient aeration (Lee and George 2005).

There are several host plants used for solution culture techniques, of which the most common host plant species recommended for static hydroponics are wheat (*Triticum aestivum* L.) and Lin seed (*Linum usitatissimum* L.) (Dugassa *et al*., 1995; Hawkins and George, 1997). Elmes and Mosse (1984) have tested several host plant species /AM fungus combination and found that the ones cultivated with maize (*Z. mays*) produced the most successful effective AM fungi inoculants in the NFT System. Bahia grass (*P. notatum*), Sudan grass (*S. sudanese Staph*.), and sweet potato (*Ipomoea batatas L*.) have been reported as adequate for aeroponic production of AM fungi (Jarstfer and Sylvia, 1995; Wu *et al*. 1995; Mohammad *et al*. 2000). The choice of the host plant may influence the colonization levels obtained with some AM fungal species.

2.4.1.3 In vitro production systems

The “in-vitro” cultivation systems are based either on excised roots, i.e “root organ cultures” (ROC) or on whole autotrophic plants. Despite their current high costs, these systems guarantee the contaminant-free production of pure AM fungi. *In vitro* cultivation of AM fungi is particularly adapted to the production of high-added-value crops (crops generated via micro-propagation techniques).

The *in vitro* large-scale production of AMF spores was first attempted on ROC (Chabot *et al*., 1992) and later expanded to plant systems (Voets *et al*., 2005). ROC is most often initiated on carrot (*Daucus carota L.*) roots. However, in the recent years, different excised roots, among which chicory (*Cichorium intybus L.*) and barrel medic (*Medicago truncatula Gaertn*.), have been successfully used to culture AM fungi (Boisson-Dernier *et al*., 2001; Fontaine *et al.,* 2004). Tiwari and Adholeya (2003) have shown that a change of plant root clone could impact AM fungal spore production. Voets *et al*. (2005) have used a potato plant (*Solanum tuberosum L.*) and obtained a production of ~12,000 spores in 12 weeks of cultivation. Other hosts such as banana (*Musa acuminate*) (Koffi *et al*. 2009) and grapevine (*Vitis vinifera*), were suitable for the association but less effective for the large-scale production of spores (Ijdo *et al*., 2011).

2.4.2 Bacterial Bio-inoculant production and formulation for woody useful plants

In several developing countries, farmers mostly practice low input farming owing to the unaffordable costs and low availability of chemical fertilizers and pesticides (Bashan, 1998). Bio-inoculants could offer significant economic importance: they can in part substitute other chemical fertilizers, which are becoming expensive and unaffordable (Herrmann and Lesueur, 2013). The development of bio-inoculants is a reply to increasing demands for more environmentally friendly farming practices (Herrmann and Lesueur, 2013).

Vessey (2003) has defined bio-inoculants as “a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant”. This is very important when there is scarcity in some of the growth limiting nutrients in the soil. When N supply is insufficient to satisfy the N demand of the growing legume, there is a need for effective nodulation (Herridge, 2008).

Production, formulation, and application of microbial bio-inoculants are the important steps in the biofertilizer technology. Bio-inoculants formulation is a very important process in biofertilizer production, which involves several steps. The steps are used in the production of a single or consortia of microorganism (s) mixed with suitable carrier and the carrier helps in withstanding adverse environmental conditions during the storage and secures survival and establishment after inoculation in soil (Bashan *et al*., 2014).

There are four major types of inoculant formulation techniques. These include: liquid, peat, granules, and freeze-dried powders, and success of inoculants in the soil depends on target plant, cost, market availability, environmental factors, and user-friendly nature of the inoculant (Herrmann and Lesueur, 2013; Arora *et al*., 2017).

The key challenges for the inoculant industries are to develop improved formulations that combine all-important characteristics, which are suitable for application under field conditions (Herrmann and Lesueur, 2013). To increase the possibilities of inoculation success, formulation of an inoculant should combine several characteristics; it should offer protection to the microorganisms in unfavorable environmental conditions, allow optimal growth of microorganisms and prevent the decline of microbial population (during storage and transport and after inoculation into soil); it should be user and environmentally friendly, and should be made of a cost-effective and readily available material.

Although a microbe can be potentially promising under laboratory and greenhouse conditions, formulating it into a commercial product under a wide range of field conditions is a difficult step (Stephens and Rask, 2000). Manufacturers prefer to produce consortia of microorganisms (e.g., rhizobia and AMF, rhizobia and PSB, diverse strains of AMF or PSB) in a single product, to maximize the benefits for the host plants (Suneja *et al*., 2007; Malusa *et al*., 2012).

2.5 An over view of the Rhizobia/PGPR and Mycorrhiza research on *Erythrina brucei*

In Ethiopia there are more than 600 species of leguminous plants (Thulin,1989) one of which is *Erythrina brucei* Schweinf. Emend. Gillett (Papilionoideae). It is a multi-purpose endemic leguminous tree found in Ethiopia, with different habitates (edges or open places of upland forests or woodland etc. evergreen forest) and widely distributed in the central, southern, northern and eastern parts of Ethiopia with altitudes between 2250 and 2520 m.a.s.l and rainfall about 1000 mm (Thulin, 1989). It is a multi-purpose plant used for fuel wood, construction material (Demel Teketay, 1994), traditional medicine (Tesfaye W/mariam *et al.*, 2009), for animal feed (AlemayehuYinnesu and Ajebu Nurfeta, 2012), shade for coffee plantations important component of different agro-forestery system (Beyene Dobo *et al*.,2016). Other studies were undertaken on the agronomy, phenology, and agroforestry attributes of the host (*Erythrina brucei*) in Ethiopia (Legesse Negash, 2002; Wasse Haile *et al*, 2013). These studies also showed that *E. brucei* is well known for soil fertility improvement and crop yield. The tree is characterized by rapid establishment, tolerance to sunlight, possession of spreading canopy, high rate of litter production, rapid litter decomposition, very soft woody nature, vigorous re-growth after cutting, as well as rapid recovery through profuse foliage production after a period of sustained drought.

The pioneer plant microbe interaction study on *Erythrina* was initiated on the nodulation status and effectiveness of the rhizobia in nitrogen fixation under greenhouse conditions (Fassil Assefa, 1993). The author reported that *Erythrina brucei* and *Erythrina burana* are nodulated by both fast growing (*Rhizobium sensu strictu*) and slow growing (*Bradyrhizobium*) root nodule bacteria. The latter was promiscuous to nodulate African yam bean (*Sphenostylis stenocarpa*) (Fassil Assefa and Kleiner, 1997)

Shasho Megersa and Fassil Aseffa (2011) also studied the phenotypic diversity of microorganisms in the rhizosphere of the legume that include root nodule bacteria (rhizobial), plant growth promoting rhizobacteria and mycorrhiza from limited sampling sites in Addis Ababa. They reported that root nodule bacteria of the genus *Bradyrhizobium* and rhizobacteria from the generan Bacillus and Pseudomonas were dominant in the rhizosphere of *Erythrina brucei*. The authors also showed that the mycorrhizal group Gigaspora, *Glomus* and *Acaulospora* were common and the multiple inoculations of the selected microorganisms significantly enhanced the growth of the host under greenhouse experiments.

Later, Endalkachew Wolde Meskel *et al* (2004) worked on the phenotypic and genetic diversity of different root nodule bacteria from different agroforestry important legumes *Erythrina brucei.* They reported that the root nodule bacteria were more diverse than previously reported that include *Agrobcterium radiobacter, Bradyrhizobium liaoningense, Bradyrhizobium sp., Rhizobium leguminosarum, Agrobacterium species (A. rhizogenes, A. rubi, A. tumefaciens).* Aregu Amsalu *et al* (2013) have also recovered root nodule bacteria such as *Rahnella aquatilis, Agrobacterium radiobacter, Rhizobium phaseoli, Rhizobium sp., Enterbacter sp., Mesorhizobium plurifarium* from the same host legume.

With regard to mycorrhiza, Beyene Dobo *et al*. (2016) have extensively studied the AMF association of *Erythrina brucei* together with other woody plant species from Sidama agroforestry system in southern,Ethiopia. They showed the occurrence of diverse group (n=8)

AMF genera such as *Acaulospora, Claroideoglomus, Dentiscutata, Funneliforms, Gigaspora, Glomus, Rhizophagus* and *Scutellospora.* The same genera were isolated and identified from agro-forestry systems that use combinations of *Erythrina brucei-Ensete ventricosum, Erythrina brucei-Coffea arabica, Ensete ventricosum-Erythrina brucei-Coffea arabica* based agro-forestry systems.

They also reported high mycorrhization (AMF root colonization level) ; 84% in *Millettia ferruginea*, 80% in *Erythrina brucei*, 86% in *Ensete ventricosum*, 85% in *Catha edulis*, 80% in *Coffea arabica*, 53% in *Rhamnus prinoides*, 52% in *Colocasia esculenta*. In the same study, they also reported high AMF spore populations (high spore population density) of 1066, 1054, 768 and 100 from the rhizospheres of *Croton macrostachyus, Catha edulis, E. brucei* and *Dioscorea alata* respectively per 100 gram dry soil.

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3. Arbuscular Mycorrhizal Fungi Status of *Erythrina brucei growing* in Different Land use Types in Ethiopia

Belay Berza[1], MarcelaClaudiaPagano[2], Prabavathy VR[3], Zerihun Belay[4], FassilAssefa[1]

1Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia
2Department of Biology, Federal University of Minas Gerais, Brazil. 3Microbiology Laboratories, MS Swaminathan Research Foundation, Tamil Nadu, Chennai, India
4Department of Applied Biology, Adama Science and Technology University, Adama, Ethiopia.

Abstract

Erythrina brucei is a leguminous tree commonly used in agro-forestry practices in the southern and southwestern Ethiopia. It has desirable agro-forestry attributes that makes it a good candidate in Agroforestry systems. However, there was scarcity of information on species diversity and spore density of Arbuscular Mycorrhizal Fungi (AMF) associated with *E. brucei* growing in different land use types. Therefore, we did this study to determine species diversity and richness in trap culture, spore density in field and trap cultures, and root colonization in trap culture of AMF associated with *E .brucei* growing in different geographic locations in various land use types. Soil samples were collected from different *E. brucei* growing areas with various land use types. Trap culture was established using a portion of each soil. AMF spore extraction was performed from field and trap culture soils. AMF species identification was done using spore morphology. *E. brucei* root colonization with AMF structures was studied using trap culture and AMF spore density in both field soils and trap culture soils was determined. Eleven AMF genera and 33 AMF species were recovered. The highest number of genera was recovered from forestland at Rebu Gebeya followed by Adiskdam, live fence land use types. *Glomus* and *Acaulospora* were the dominant AMF genera covering 73% of the samples while, contributing 10 and 9 AMF species respectively. All the soil samples contained indigenous AMF spore populations regardless of their origin and land use types. The highest mean AMF spore density in a field soil was recorded in farmland at Bodite (280 spores per 100 g dry soil) while the lowest was from Hossana (172 spores per 100 g dry soil).On the other hands , the highest mean AMF spore density in trap culture was also detected from farmlands such as Humbo (715 spores per 100 g dry soil) and the lowest was from Dhakallo (217 spores per 100 g dry soil). AMF direct extraction from field soils showed lesser AMF spore density compared to trap culture of the same soil sample. All the *E .brucei* root segments were colonized with all the AMF structures with colonization status ranged from 34.4% to 49.1%. Both geographic location and land use types significantly ($p<0.05$) affected AMF species richness associated with *E.brucei*. Undisturbed land use types were characterized by high species richness.The soil pH and available phosphate were negatively correlated to AMF spore density but AMF root colonization did not show any significant correlation. To explore more detailed AMF species diversity and richness across geographic location and land use types, more area coverage, combination of both direct field soil and trap culture techniques are recommended.

Keywords: AMF, Species diversity, spore density, trap culture, diversity studies

3.1 Introduction

Erythrina brucei Schweinf. Emend. Gillett (Papilionoideae) is a a multipurpose leguminous tree endemic to Ethiopia (Thulin , 1989). It is widely distributed with different habitats (edges or open places of upland forests or woodland etc. evergreen forest) and widely distributed in the central, southern, northern and eastern parts of Ethiopia with altitudes between 2250 and 2520 m.a.s.l and rainfall about 1000 mm. It is used by local farmers for fuel wood, construction material It is integrated as shade for coffee plantation and improves soil fertility and/or crop yield (Demel Teketay, 1994). *Erythrina brucei* has a desirable agro-forestry attributes such as rapid establishment, tolerance to sunlight, high rate of litter production and decomposition, vigorous re-growth after cutting and coppicing as well as rapid recovery after a period of sustained drought (Powell and Westley, 1993).

It is well known in the traditional agro-forestry systems of central, southern, and southwestern Ethiopia and grown in farmlands that grow important annual and perennial crops such as barley (*Hordeum vulgare* L.), Enset (*Ensete ventricosum)*(Welw.) , Cheesman, maize (*Zeamays L.*), sorghum (*Sorghum bicolour* (L.) Moench s.l.) (Legesse Negash, 2002; Wassie Haile and Abebe Abay, 2013). This is due to its prolific nodulation and nitrogen fixation with root nodule bacteria to improve soil fertility and increase in crop yield. (Allen and Allen, 1981; Fassil Assefa, 1993)

Apart from its nitrogen fixing potential to boost crop production, *Erythrina brucei* harbors soil microbiota such as arbuscular mycorrhizal fungi that are ubiquitous in soil and form symbiotic associations with most land plants including agricultural crops, cereals, vegetables, and horticultural plants (Read and Smith, 2008). Plant growth promoting rhizobacteria are soil microbiaota that also play a vital role in the ecology of natural ecosystems and sustainable agriculture (Hause & Schaarschmidt, 2009).

AM fungi can provide a considerable benefit to plants as a result of their ability to increase uptake of poorly mobile soil nutrients such as P, N, K and Mg (Cabrera and Rodriguez , 2006) , increase tolerance to water stress, induce greater resistance to pathogens and reduce sensitivity to toxic substances present in the soil (Siddiqui & Pichtel, 2008), formation and maintenance of soil structure and increase C input to soils (Rillig and Mummey, 2006), crop growth and yield (Gosling *et al.*, 2006). The presence of perennial mycorrhizal tree species with deep roots buildup and maintenance of mycorrhizal networks compared to the annual cropping systems characterized by regular disturbances (Andre *et al.*, 2010), increases the volume of soil to be exploited and thereby improves the efficiency of P cycling by AM fungi (Cardoso *et al.*, 2003; Diriba Muleta *et al.*, 2008)

Shasho Megersa and Fassil Assefa (2011) have reported the presence of three genera of AMF that included *Gigaspora*,*Glomus* and *Acaulospora* from the rhizosphere of *Erythrina brucei* from Addis Ababa. However, an extensive research on the agroforestry system of Sidama, southern Ethiopia was investigated later where the same woody legume constitutes as one of the agroforestry trees in the area (Beyene Dobo *et al.*, 2016). The authors showed that *Erythrina brucei* is a highly mycorrhizal agro-forestry plant with AMF colonization of 86% comparable to highly mycorrhized plants such as *Millettia ferruginea*, *Ensete ventricosum*, *Catha edulis*, and *Coffea arabica.* The authors also identified diverse groups of AMF with seven AMF genera from combinations of *Erythrina brucei-Ensete ventricosum, Erythrina brucei-Coffea arabica ,Ensete ventricosum-Erythrina brucei-Coffea Arabica* based agro-forestry systems.

The AMF symbiotic association in agro-forestry systems is a reasonable point to begin the evaluation of mycorrhizal functioning in low input sustainable agriculture system. The use of AMF as bio-fertilizers could be achieved through integrating trees, crop plants, horticultural plants and other plants to form mycorrhizal association and to increase population densities of AM fungi at field conditions (Godbold and Sharrock, 2003).

The local soil physicochemical characteristics may also act as selection pressure on fungal assemblages with reduced sporulation or that produce spores with low capacity to resist adverse conditions but with adaptation for a predominant vegetative life stage (Siqueira *et al.,* 1985), impairing their detection in only one growth cycle of trapping culture.

Even though *E. brucei* is playing important role in traditional agro-forestry systems and characterized by very important agro-forestry attributes and grows in various land use types, its mycorrhization is not well explored in different agro-ecology set up. Hence, there is still a dearth of information on the diversity of arbuscular mycorrhizal fungi that is associated with *E. brucei*, and the inoculums potential of AMF associated with *E. brucei*. Therefore, it is important to investigate the AMF diversity associated with *E. brucei* distributed in different geographical locations in Ethiopia. It is also very important to have knowledge about to dominant genera and species of AMF associated to this particular plant to select and/or develop AMF inocula.

Therefore, the objective of this work was to determine AM fungal diversity and abundance, community structure, percentage root length colonization, spore density and species richness among different land use systems associated to *E. brucei* in different geographic locations in Ethiopia.

3.2 MATERIALS AND METHODS

3.2.1 Sampling Locations and Soil sampling.

A total of 15 rhizospheric soil samples from various *Erythrin*a-based land use types (fields), farmlands, shade trees, land boundary fences and from small forests were collected from the north Amhara National Regional State (ANRS) and Southern Nations and Nationalities and Peoples Regional State (SNNPS). From each sampling site, 10 kg of *E. brucei* rhizospheric soil samples were collected from a depth of 10-30 cm. When the host plant was found in more than one place in a sampling site, the soil samples were pooled into one composite sample per location. The samples were collected in steam-sterilized polyethylene bags and transported to Applied Microbiology Laboratory, Addis Ababa University for analysis. Table 1 presents the distribution of soil sampling locations and land use types. The samples were collected between June and July 2015 and May and June 2016.

Table 3.1. Soil sampling Locations and Different land use types in *E. brucei* growing areas

	GPS Coordinates		Altitude (m.a.s.l)	Region	District	Land use type
	Latitude	**Longitude**				
Hossana	07^{0},32’,492’’	037^{0},50’,524’’	2354	South	Hadiya	Farmland
Teza Agara	07^{0},16’,435’’	037^{0},55’,123’’	2349	South	Kambata	Shade Trees
Bodit	06^{0},57’,429’’	037^{0},51’,341’’	2020	South	Wolayta	Farmland
Humbo	06^{0},43’,021’’	037^{0},46’,548’’	1637	South	Wolayta	Farmland
Sodo Town	06^{0},51’,178’’	037^{0},45’,462’’	2101	South	Wolayta	Shade Trees
Dhakalo	06^{0},12’,915’’	037^{0},19’,618’’	2598	South	Gamo Gofa	Farmland
Gidole	05^{0},38’,464’’	037^{0},21’,642’’	2158	South	Segen Area	Forest
Debre Markos	10^{0},19’,686’’	037^{0},44’,016’’	2387	Amhara	East Gojam	Shade Trees
AAU	09^{0},02’,082’’	038^{0},045’,964’’	2467	Addis Ababa	Arada	Shade Trees
Adiskdam	10^{0},33’,027’’	037^{0},74’,502’’	2424	Amhara	Awi	Land boundary fence
Injibara	10^{0},97’,589’’	036^{0},92’,776’’	2561	Amhara	Awi	Land boundary fence
Tilil	10^{0},86’,789’’	037^{0},00’,420’’	2453	Amhara	Awi	Land boundary fence
Burie	10^{0},71’,557’’	037^{0},06’,920’’	2122	Amhara	West Gojam	Forest
Rebu Gebeya	10^{0},71’,266’’	037^{0},06’,680’’	2087	Amhara	East Gojam	Forest
Enrata	10^{0},42’,846’’	037^{0},72’,838’’	2500	Amhara	East Gojam	Shade Tree

3.2.2 Soil chemical and physical characteristics

Soil samples were analyzed for their physicochemical characteristics. The total nitrogen (TN) was determined by using the Kjeldahl method (Hinds and Lowe, 1980) and the soil available Phosphorus was determined according to the method described by Olsen *et al.* (1954). The soil sample analysis was done at Horticoop Ethiopia, Soil and water analysis laboratory, Bishoftu, Ethiopia.

3.2.3 Establishment of trap cultures

The trap culture was carried out in triplicates for all 15 rhizospheric soil samples from various sampling sites according to (Morton *et al.*, 1993). Each pot was surface sterilized with 70% alcohol, filled with approximately 3 kg soil sample, and placed in a greenhouse for trap culture to obtain fresh and intact spores for identification of AM fungi and to induce sporulation of species that were present only as hyphae in field soil samples.

E.brucei seeds were collected from trees in College of Natural Sciences, AAU. The seeds were surface sterilized briefly with 70% alcohol for 1 minute and with 3% sodium hypochlorite solution for 8 minutes, and successively washed with distilled sterile water up to 6 times. After washing, seeds were immersed again in distilled water and left overnight at room temperature to remove the anti-nutritional factors**.** The seeds were transferred to 1% (w/v) water agar and incubated at 28°C for seven days for germination.

Five *E.brucei* seedlings were transferred into 3-kg capacity plastic pots filled with each soil sample thinned reduced down to three seedlings per pot after establishment. The seedlings were watered three times a week. All the seedlings were grown in the greenhouse under ambient light and temperature conditions (22-24 °C day/10-14°C night) for six months with marked reduction in watering in the final month from three to twice per week, then once per week and finally watering was completely stopped to initiate proper sporulation.

3.2.4 AMF Spore Extraction

AMF spores were extracted from both field and trap culture soil samples by sucrose density gradient centrifugation technique according to Brundrett *et al*. (1996). Quantification of AMF spores per 100 gram soil sample was carried out using a 100 g air dried sieved soil samples. A 50g subsample of each soil was suspended in a liter of tap water in 2-liter container and left for 2-4 hours. Soil samples were soaked in 6.3 mMol of sodium hexa-metaphosphate to disperse the clay fraction. The content was vigorously mixed to free the spores from the soil particles.

The soil suspensions were consecutively passed through four soil sieves with different mesh sizes (i.e., 500,212,106 and 45 µm). All the contents from 212 and 106 µm sieves were collected on to 45 µm sieve and mixed with water and centrifuged at 2000 rpm for 5 minutes. Each supernatant was discarded and the pellets were re-suspended in 50% sucrose solution and centrifuged at 2000 rpm for 1 minute. The supernatant was carefully collected in a 45 µm sieve and washed with tap water to remove the sucrose. The spores, spore clusters and sporocarps were transferred on to 90 cm plastic Petri dishes. Spore counting and morphotyping were performed under the dissecting microscope (ISO 1006; NOVEX,HOLLAND) at ×40. Enumeration of spores per 100-gram dry soil was done according to INVAM, http://invam.caf.wvu.edu.

3.2.5 Identification and characterization of AMF spores

The AMF spores were identified at the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia and Federal University of Minas Gerais, Brazil. The viable spores that were intact and with cytoplasm content, were picked up with forceps and mounted on slides in polyvinyl-lactic acid-glycerol (PVLG) (Omar and Bolland , 1979) or in PVLG mixed with Melzer's reagent (1:1 v/v) (Morton, 1991). Spores were crushed and examined under a compound microscope (OLYMPUS-BX51,Japan) at a magnification of ×400 and classification was based on a current species description and identification manual (Schenck and Perez, 1990) and according to online references of species description INVAM http://invam.caf.wvu.edu, West Virginia University, USA, University of Agriculture in Szczecin, Polandhttp://www.zor.zut.edu.pl/Glomermycota/, (Schüßler and Walker, 2010) and the Schüßler AMF phylogeny websitehttp://www.lrz.de/~schuessler/amphylo/

3.2.6 Determination of AMF spore density and species Composition

The AMF communities on different land use types across different sampling sites were determined and calculated as follows: Spore density (SD) was expressed as the number of AMF spores per 100 g dry soil. Species richness (S) was measured as the number of AM fungal species per sampling locations. The Shannon–Wiener index (H′) of diversity was calculated using the formula: $H' = -\Sigma\ (\frac{ni}{n})\ \ln(\frac{ni}{n})$, where: ni = number of individuals of species n and n=number of all individuals of all species. The Simpson's dominance index (D) was calculated using the formula $D = \Sigma\ (ni/n)^2$.

Frequency of occurrence (FO) was calculated by determining the percentage of samples from which spores of a particular species/genus were recovered, i.e. the number of samples in which a given species/genus was isolated divided by the total number of samples) ×100% (Fernandes *et al.*, 2016). The importance value (IV) was used to evaluate the dominance of AMF species based on incidendnce frequence (IF). An IV $\geq$ 50% indicates that a genus or species is dominant; 10% < IV < 50% applies to common genera or species; an IV $\leq$ 10% indicates that a rare genus or species(Chen, 2012).

3.2.7 Staining of Mycorrhizal Roots

The root samples (trap culture) were washed thoroughly under tap water to remove soil particles and other organic debris and stored at 4°C in 50% ethanol for the determination of the percentage AMF root colonization. Staining of mycorrhizal roots was conducted according to Vierheilig *et al* (1998). The fine roots were carefully washed with tap water and cut into 1 cm segment pieces. For each root sample representing a specific sampling location, a total of 2.5 g fine root samples were considered but for the ease of processing, about one gram root segments subsamples were cleared in 10 % KOH (w/v) at 121°C in an autoclave for 15-20 minutes.

Cleared roots were captured on a fine sieve and rinsed with water and were bleached with alkaline hydrogen peroxide [0.5%NH_4OH and 0.5% H_2O_2 (v/v) in water] for 3 minutes at room temperature. Roots were acidified with 1% HCl (v/v) for 5 minutes at room temperature and washed 4-5 times with tap water and boiled at 121°Cfor 3 minutes in a 5% (Pelikan Blue) ink-vinegar solution (v/v) with pure white household vinegar (5% acetic acid). Roots were destained by rinsing in pure household vinegar for 10 minutes and kept for two days to weeks for quantification of root colonization.

3.2.8 Quantification of AMF root colonization

AMF root colonization was assessed according to McGonigle *et al* (1990). Roots were aligned parallel to the long axis of the microscopic slides and mounted in PVLG and covered with cover slips and observed at magnification of X200 (OLYMPUS-BX51). Ten slides were considered for each sample (five to six root segments were mounted on a slide as subsample). Hundred to 150 intersections were taken for each subsample. The intersections were counted in the following categories; 'negative' (no fungal structures found in the root), 'arbuscules', 'vesicles', and 'hyphae only'. The arbuscular colonization (AC) and vesicular colonization (VC) were calculated by dividing the count for the 'arbuscules' and 'vesicles' categories, respectively by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections.

3.2.9 Statistical analysis

The number of spores per sample per 100-gram dry soil was log transformed in order to assess the relationship between the number of spores in the rhizosphere soils and AMF colonization of *E .brucei* roots grown in the same soils.The transformed values were used for one-way analysis of variance (ANOVA). The relationship between AMF spores parameters and soil chemical properties were evaluated by employing Pearson's correlation coefficient. ANOVA was employed to test differences between the density of AMF spores at each sampling site in both field soils and trap culture soils using SAS version 9.2. Duncan's multiple range test was conducted to test for mean separation ($p<0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1. Soil physicochemical characteristics

The *E. brucei* rhizosphere soil samples collected from different land use types were characterized by acidic pH regardless of the sampling sites (Table 3.1). The acidity was strong at Bodite (5.08) and slightly acidic at Dhakallo (6.89) farmlands (Table 3.2).

The highest available phosphate was recorded at Adiskdam (28.04 ppm) in live fence and the lowest was at Bodite (13.89 ppm) from farmland (Table 3.2). About 80% of the soil samples in the present study were categorized as medium in available P content (Landon, 2014). Similarily, the organic carbon ranged from 2.52% at Adiskdam (live fence) to 4.91% at Dhakallo (farmland). The highest total soil nitrogen (TN) was recorded at Dhakallo (0.43%) from farmland and the lowest value (0.17%) was recorded at Injibara (live fence) (Table 3.2). About 80% of the soil samples in this work were categorized as medium to high TN content and 93.3% were categorized as high in organic carbon content (Landon, 2014).

Table 3.2. Physicochemical characteristics of field soil collected from *Erythrina*. spp rhizosphere employing different land use practices in Southern and Northern Ethiopia.

Sampling Site	Soil Physicochemical parameters						Land use Types
	pH(H_2O)	Ava.P(ppm)	OC (%)	TN(%)	OM(%)	C:N	
Hossana	6.12	26.11	2.86	0.20	4.93	14.23	Farmland
Teza Agara	5.79	25.32	4.70	0.32	8.12	14.70	Shade tree
Bodite	5.08	13.89	3.03	0.26	5.22	11.69	Farmland
Humbo	5.11	14.99	2.93	0.22	5.03	13.32	Farmland
Sodo Town	6.32	25.12	3.19	0.28	5.58	11.54	Shade tree
Dhakallo	6.89	27.18	4.91	0.43	8.44	11.41	Farmland
Gidole	6.11	27.15	3.50	0.30	6.05	11.68	Forest
Debre Markos	6.38	26.12	3.57	0.29	6.16	12.30	Shade tree
Addis Ababa	6.88	27.05	3.42	0.33	5.88	10.36	Shade tree
Adiskdam	6.02	28.04	2.52	0.19	4.33	13.26	Land boundary fence
Injibara	5.75	27.21	2.67	0.17	4.59	15.70	Land boundary fence
Tilil	5.56	24.17	2.72	0.18	4.67	15.11	Land boundary fence
Burie	6.51	26.16	2.84	0.24	4.88	11.83	Forest
Rebu Gebeya	5.18	16.19	3.89	0.25	6.72	15.57	Forest
Enrata	5.73	18.69	4.27	0.31	7.37	13.77	Shade tree

Ava P= Available phosphorous, OC=Organic carbon, TN=Total nitrogen, OM=Organic matter, C:N= carbon to nitrogen ratio.

3.3.2 AMF species composition

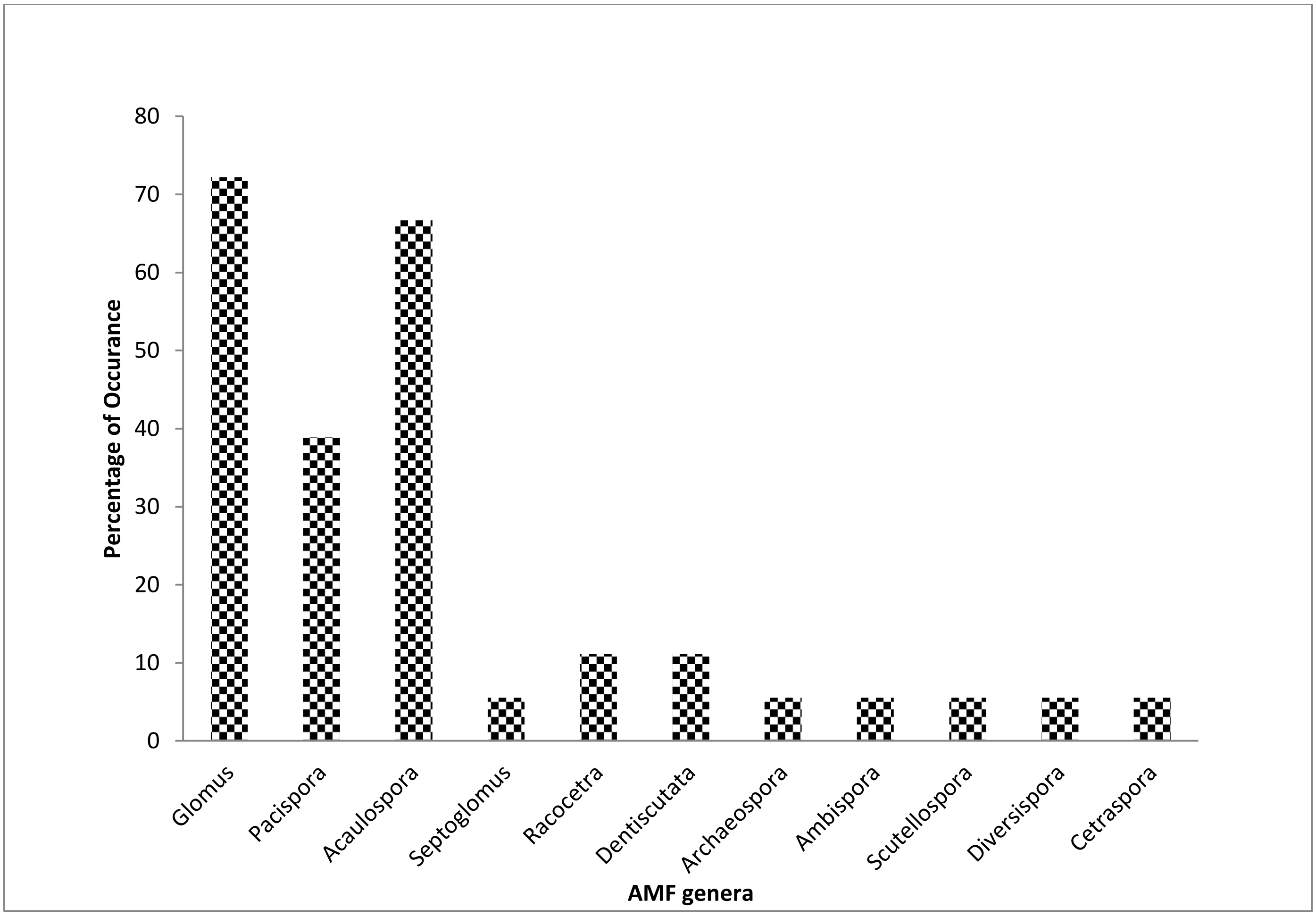
The AMF genera identified in this study are presented in Table 3.3. Eleven AMF genera were recovered from 15 different sampling sites, consisting of seven sampling locations each from south and north Ethiopia and one location from Addis Ababa. The highest AMF diversity was recorded from the rhizosphere soil of *E brucei* collected from the forest site at Rebu Gebeya (eight genera) which was twice more diverse than the AMF genera (4 genera) retrieved from the standing tree grown as live fence site collected from Adiskdam (both from North Ethiopia). The (40%) of the sampling locations (six sites) harbored two AMF genera, whereas Teza Agara, Bodite and Humbo sampling sites from southern Ethiopia contained only one genus each (Table 3.3). The land use types such as forests, shade trees and live fences comprised more AMF diversity compared to the farmland lands (Table 3.3). In this study, we recorded eleven AMF genera from the soil samples and land use types in the northern Ethiopia while four AMF genera from southern Ethiopia.

Table 3.3.Frequency of occurrence of AMF genera recovered by trap cultured from soils collected from *E. brucei* rhizosphere in the southern, central and northern Ethiopia.

Sampling sites	Region	Land use type	AMF Genera											No of AMF genera
			Glo	Pac	Aca	Sep	Rac	Den	Arc	Amb	*Scu*	*Div*	Cet	
Hosanna	S	FL		X	X									2
Teza Agara	S	ST			X									1
Bodit	S	FL	X											1
Humbo	S	FL	X											1
Sodo Town	S	ST	X	X	X									3
Dhakalo	S	FL	X	X										2
Gidole	S	FO	X		X			X						3
Debremarkos	N	ST	X		X									2
AAU	A.A	ST	X	X										2
AdisKidam	N	LF	X	X	X	X								4
Injibara	N	LF	X		X		X							3
Tilil	N	LF		X	X									2
Burie	N	FO	X		X		X							3
Rebu Gebeya	N	FO		X	X			X	X	X	X	X	X	8
Enrata	N	ST	X		X									2

S=South Ethiopia,A.A=Addis Ababa N= North Ethiopia, X=Present, Glo=*Glomus*,Pac=*Pacispora*,Aca= *Acaulospora*,Sep= *Septoglomus*,Rac= *Racocetra*,Den= *Dentiscutata*,Arc= *Archaeospora*,Amb= *Ambispora*,Scu= *Scutellospora*,Div= *Diversispora*,Cet= *Cetraspora*,FL=farmland, FO=forest, ST=shade tree, LF= land boundary fence.

Glomus and *Acaulospora* were recovered each from 11(73%) sampling sites (Table 3.3; Fig.3.1).The genus *Glomus* was recovered from five sampling locations in each northern and southern Ethiopia, whereas genus *Acaulospora* was identified from all sampling location in northern Ethiopia and four sampling locations in southern Ethiopia. The dominant AMF genera in this study were *Glomus* and *Acaulospora* followed by *Pacispora* which was recovered from 7(47%) sampling sites (three locations each from southern and northern Ethiopia) and one from Addis Ababa; whereas the genera *Racocetra* and *Dentiscutata* were isolated from two sampling sites. The genus *Racocetra* was identified only from two sampling sites (live fence and forest) in northern Ethiopia, while *Dentiscutata* was recorded in the southern and northern Ethiopia.



Figuge 3.1 The occurrence of AMF genera in the rhizosphere of *Erythrina brucei* grown at different land use types in the southern, central and northern Ethiopia from trap culture.

However, this genus (*Dentiscutata*) was restricted to forest land use types in both geographic locations. The genera *Septoglomus, Archaeospora, Ambispora, Scutellospora, Diversispora* and *Cetraspora* were limited to one sampling site (forest), Rebu gebeya (Table 3.3). The Rebu Gebeya forest was unique compared to other forests included in this study in that it is *Eucalyptus* tree dominated forest. In this study, seven AMF genera that include *Septoglomus, Racocetra, Archaeospora, Ambispora, Scutellospora, Diversispora*and *Cetraspora* were confined to northern Ethiopia.

In this study, the lowest number of AMF genera was recovered from south Ethiopia where four out of seven soil samples were from farmlands. At most two or at least one AMF genera were isolated from farmlands of Humbo, Bodite, Dhakallo and Hossana (Table 3.3). This data clearly indicates that agronomic practices or disturbance of soils reduced occurrence of AMF genera. The negative impact of conventional agricultural practices on AMF abundance and species diversity was reported previously by (Xiang *et al.,* 2014; Aguilera *et al.,* 2015). It was observed during sampling that in Humbo, Bodite and Hossana; maize crop was continuously cultivated under the canopy of *E. brucei,* while in Dhakallo barley was continuously cultivated in similar manner under the canopy of the host plant. Jefwa *et al.,* (2012) have showed gradual reduction in AMF diversity due to long term continuous monocropping practice and even absence of more AMF species in monocropping systems, which could be an indication that gradual removal of some AMF species from the original AMF community as a result of long term monocropping practices. Likewise, (Oehl *et al.*(2005) reported a decreased AMF diversity in conventional extensively farmed soils due to the conspicuously higher inputs of fertilizers, even though we did not have information on fertilizer application history in the present study.

Five species of *Glomus* were each recorded from north and south Ethiopia, whereas seven and four *Acaulospora* species were recorded from north and south Ethiopia, respectively. The dominance of this genera could be due to the facts that species in genus *Glomus* produce small sized but numerous spores (Bever *et al.,* 1996; Stutz *et al*, 2000),thus the species producing large number of spores usually had a wide distribution assisting in dominance (Dandan & Zhiwei, 2007). Moreover, AM fungal species producing small sized spores require less time to produce spores (Hepper, 1984) and these small sized spores are more adaptive in adjusting patterns of sporulation to varied environmental conditions (Stutz *et al*, 2000). Likewise, Oehl *et al*., (2010) have reported that *Glomus* species generally had a successional sporulation pattern, while many species of several non-*Glomus* genera had strongly seasonal sporulation pattern. According to Brundrett *et al*. (1999), the majority of *Glomus* species may germinate from spores as well as from mycorrhizal roots (especially from vesicles), while *Acaulospora* and *Scutellospora* and the relative genus (*Cetraspora*) are believed to germinate preferably or exclusively from spores after dormancy period. These characteristics exhibited by the species of genus *Glomus* could be a key to survival and dominance in different geographic locations. In addition,the dominance of genus *Glomus* could be attributed to its adaptability to varied soil conditions (Pande and Tarafdar, 2004), tolerance and survival in acidic soils and its host generalist nature (Börstler *et al.,* 2008; Mafaziya and Madawala, 2015), and production of large hyphal fragments (Zhao *et al.*,2017).

Shasho Megersa and Fassil Assefa (2011) have identified the AMF genera *Gigaspora*, *Glomus* and *Acaulospora* from the rhizosphere of *E. brucei* standing plant at College of Sciences, Addis Ababa University. In this particular study, we also identified the genera *Glomus* and *Acaulospora* from Addis Ababa. However, in the present study, we did not identify the genus

Gigaspora from the College of Sciences, Addis Ababa University, from the same land use type. This could be because of extensive soil sampling carried out by Shasho Megersa and Fassil Assefa (2011) compared to our soil samples (three pots) from Addis Ababa University. Moreover; the species in Gigasporaceae typically establish an extensive mycelium in soils and produce fewer spores compared to the members of Acaulosporaceae and Glomaceae (Hart & Reader, 2002;Piotrowski *et al.,* 2004) and hence may not be easily encountered in few soil samples and trap culture.

Beyene Dobo *et al* (2016) have reported seven AMF genera comprising of *Acaulospora, Glomus, Scutellospora, Claroidioglomus, Funneliformis, Gigaspora* and *Rhizophagus* from the rhizosphere of *E. brucei* from Sidama agroforestry systems in south Ethiopia, while we report in this work four AMF genera. The most probable explanation for such difference could be the soil used to obtain spores for identification. Beyene Dobo *et al* .(2016) obtained AMF spores directly from field standing trees rhizosphere for identification while in the present study AMF spores were obtained by trap culture technique. Chaturvedi *et al*. (2012) and Zerihun Belay *et al.,* (2015) have shown the roles of trap culture in enhancing AMF spore abundance. The difference between Beyene Dobo *et al*. (2016) and ours might be also attributed to the variations in land use types used in the studies. These authors recorded AMF genera from mixed agro-forestry systems which is the combination of *Erythrina brucei-Ensete ventricosum, Erythrina brucei-Coffea arabica ,Ensete ventricosum-Erythrina brucei-Coffea arabica* based agro-forestry systems. However, 57.1(%) of our samples from southern Ethiopia were from farmlands. Similarly, Zerihun Belay *et al.,* (2015) have identified less AMF species diversity from farmlands in Shewa robit, Central, Ethiopia. The impact of land use type on AMF diversity is well established. (Vega-fraga *et al* (2011) have reported 50% less AMF species in maize field compared to Avocado plantation in Mexico. Likewise, Oehl *et al*. (2010) have also recorded very less AMF genera and species from arable land compared to grassland land use type. Moreover; Bedini *et al.*, (2007) exhibited only genus *Glomus* from three land use types: maize mono-culture, grassland and poplar grove. Even though Beyene Dobo *et al* (2016) have covered a few sampling locations compared to the present study, they extensively sampled the soils to recover the reprehensive AMF species from that land use type. However, in this study, we collected one sample from each sampling location. The other reason for the difference in the number of genera in these two studies could be difference in sampling season. Beyene Dobo *et al.* (2016) have collected soil samples during dry season, in November and December while we collected our soil samples during wet season in May, June and July. Excess water availability has been previously reported to reduce the amount of spores in the soil (Pomar *et al.*,2008).

The community structure and isolation frequency of the AMF genera and species are presented in Table 3.3. The genera *Glomus* and *Acaulospora* constituted the highest number of 10 and 9 species, respectively followed by the genus *Pacispora* which was represented by five species (Table.3.4). The isolation frequency (IF) of AMF species ranged from 6.7% to 40% from which *Acaulospora* sp.1*. was the most frequently recovered species. However, ten of the AMF species belonging to 8 different genera were not identified to the species level. The unidentified species were assigned into *Glomus* and *Acaulospora* each with 2 species, and one species each from*Pacispora, Racocetra, Scutellospora, Ambispora, Diversispora* and *Cetraspora* (Table 3.4).

Table 3.4. The community structure and isolation frequency of AMF genera and species in a trap cultures from soils collected from different *Erythrina brucei* growing geographic locations employing various land use types in Ethiopia

AMF Genera	Genera IF(%)	AMF Genera Status	AMF species	Sampling locations	no of samples	Species IF (%)	Species status	Land use Type
Glomus	73.3	Dominant	*Glomus tortuosum*	AD	1	6.7	Rare	LF
			Glomus spinuliferum	AD	1	6.7	Rare	LF
			Glomus claroideum	AAU	1	6.7	Rare	ST
			Glomus geosporum	AAU	1	6.7	Rare	ST
			Glomus caledonium	AAU	1	6.7	Rare	ST
			Glomus glomerulatum	BU,DH,AAU	3	20	Common	FO,F,S
			Glomus sp.1	IB	1	6.7	Rare	LF
			Glomus rubiforme	HU	1	6.7	Rare	FL
			Glomus sp.1*	AD,B,DM,EN,GH	5	33.3	The most common	LF,FL,ST,FO
			Glomus sp.2*	AAU,B,GH,ST	4	26.7	Common	ST,FL,FO
Pacispora	40	The most common	*Pacispora boliviana*	AD,TL	2	13.3	Common	LF
			Pacispora sp.	ST	1	6.7	Rare	ST
			Pacispora robiginia	HO	1	6.7	Rare	FL
			Pacispora dominikii	RG	1	6.7	Rare	FO
			*Pacispora sp.1**	AAU,ST,HO,TL	4	26.7	Common	ST,FL,LF
Acaulospora	80	Dominant	*Acaulospora scrobiculata*	AD,IB,EN,TL	4	26.7	Common	LF,ST
			Acaulospora bireticulata	IB	1	6.7	Rare	LF
			Acaulospora reducta	ST,RG	2	13.3	Common	ST,FO
			Acaulospora foveate	ST	1	6.7	Rare	ST
			Acaulospora sieverdingii	TA,GH	2	13.3	Common	ST,FO
			Acaulospora denticulat~~e~~a	ST	1	6.7	Rare	ST
			Acaulospora excavate	GH	1	6.7	Rare	FO
			*Acaulospoara sp.1**	AD,BU,DH,DM,EN,HO	6	40	The most common	LF,FO,FL,ST

			*Acaulospora sp.2**	GH,DH	2	13.3	Common	FO,FL
Septoglomus	6.7	Rare	*Septoglomus constrictum*	AD	1	6.7	Rare	LF
			Septoglomus fuscum	AD	1	6.7	Rare	LF
Racocetra	13.3	Common	*Racocetra sp.1**	BU,IB	2	13.3	Common	FO,LF
Denticutata	13.3	Common	*Dentiscutata scutata*	GH,RG	2	13.3	Common	ST,FO
Archaeospora	6.7	Rare	*Archaeospora undulata*	RG	1	6.7	Rare	FO
Scutellospora	6.7	Rare	*Scutellospora sp.1**	RG	1	6.7	Rare	FO
Ambispora	6.7	Rare	*Ambispora sp.1**	RG	1	6.7	Rare	FO
Diversispora	6.7	Rare	*Diversispora sp.1**	RG	1	6.7	Rare	FO
Cetraspora	6.7	Rare	*Cetraspora sp.1**	RG	1	6.7	Rare	FO

*=species not identified to species level, AD=Adiskdam, AAU=Adis Ababa U, BU=Burie, DH=Dhakalo, IB=Injibara, HU=Humbo, B=Bodit, DM=Debre Markos, EN=Enrata, GH=Gidole, ST=Sodo Town, TL=Tilil, RG=Rebu Gebeya, Ho=Hossana, TA=Teza Agara, FL=farmland, FO=forest, LF=land boundary fence, ST=shade Tree.

Even though 33 AMF species were recovered in this study, i.e., the AMF species identified in the present study may not fully represent the total AM fungal species diversity due to the fact that the diversity measurement was based on spores collected from trap culture, and the soil samples were collected for one season.

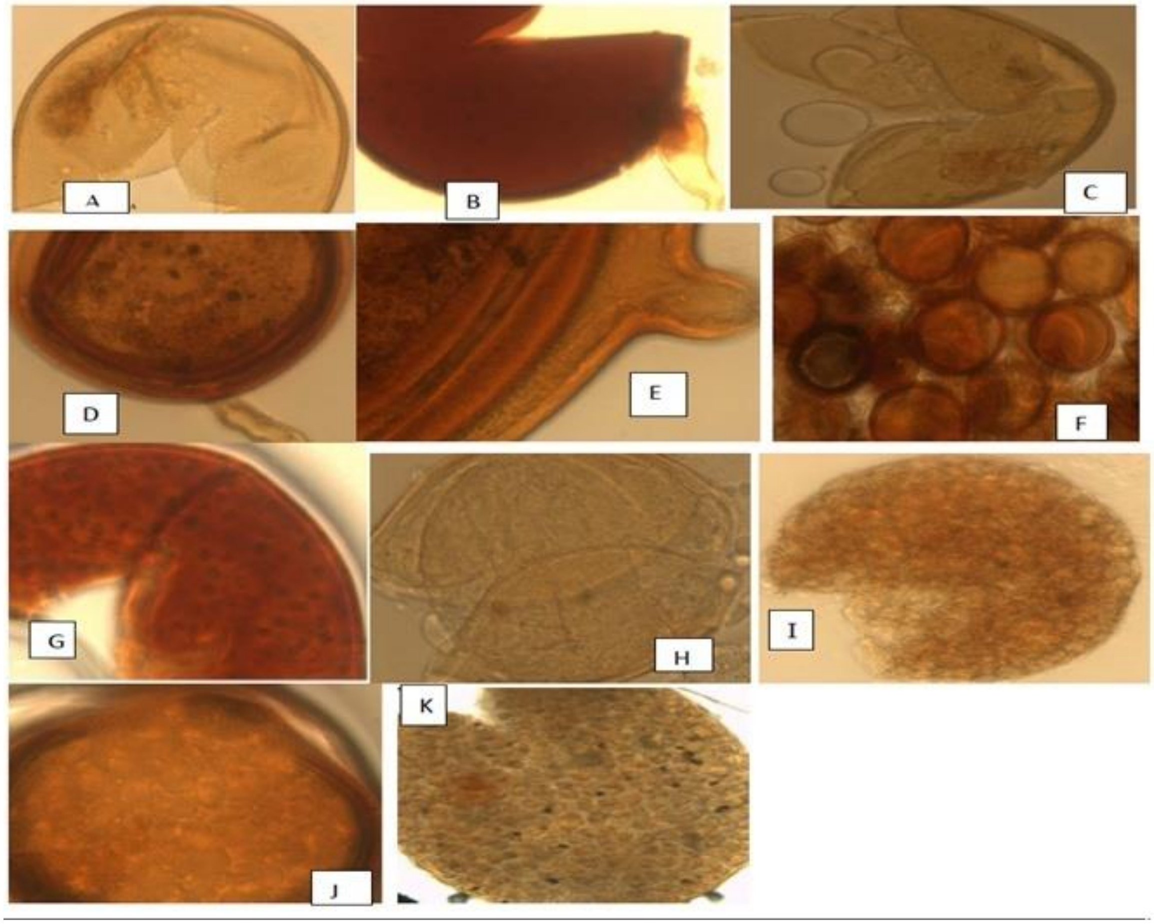


Figure 3.2. Some AM fungal species identified from the soil samples collected from the rhizosphere of *Erythrina brucei*. All photos are taken from slides prepared in PVLG. A) *Acaulospora scrobiculata B) Septoglomus constrictum C) Glomus claroideum D) Glomus geosporum E) Glomus caledonium F)* Sporocarp of *Glomus glomerulatum G) Acaulospora foveata H) Acaulospora sieverdingii I) Archaeospora undulata J) Pacispora dominikii K) Acaulospora excavata.*

The AMF species belonging to genus *Glomus* and *Acaulospora* were isolated from samples in all land use types (Table 3.4). However, AMF species belonging to genus *Glomus* were not recorded from four locations encompassing all the land use types distributed in the southern and northern Ethiopia. The species belonging to genus *Acaulospora* were not recovered in soil samples from Bodite, Humbo, Dhakallo all farmlands from south Ethiopia and Addis Ababa (shade trees) (Table 3.4).

Likewise, AMF species belonging to *Pacispora* were not detected from more than 50% of the sampling sites although it was the most common genus next to *Glomus* and *Acaulospora*. Only one *Racocetra* sp, was recovered from the forest site at Burie and Injibara (live fence). Moreover, *Dentiscutata* sp. was recovered only from forest at Gidole and Rebu Gebeya. S*eptoglomus* (two species) were recorded only at Adiskdam (live fence) from northern Ethiopia. Similarly, one species from each of the following genera: *Archaeospora, Ambispora*, *Scutellospora*, *Diversispora* and *Cetraspora* were recovered from Rebu Gebeya (forest) northern Ethiopia.

The fact that different AMF taxonomic groups were recovered from the same host plant at different locations indicated that soil physicochemical properties and climatic properties at different geographic locations had strong influences on AM fungal species assemblage (Oehl *etal*., 2010; Jansa *et al.*, 2014). Hazard *et al*. (2013) have revealed that the distribution of AM fungi was driven by local environment rather than geographic distance, which was supported by Baas-Becking’s Hypothesis that says “Everything is everywhere but the environment selects”. Van Der Gast *et al*.(2011) have also revealed that farming practices and geographic distance affected AM fungal community composition of a bait plants from nine locations.

3.3.3 AMF Spore Density

AMF spore density was recorded from all the field and trap culture soil samples from all the land use types at different geographic locations (Fig.3.3). The highest mean AMF spore density from the field soil (280 spores per 100 gram dry soil) was recorded at Bodite (farmland) (southern Ethiopia) followed by Humbo (272 spores per 100 gram dry soil) (farmland) from southern Ethiopia (Fig. 3.3), while the lowest mean AMF spore density was detected at Hossana (173 spores per 100 gram dry soil) (farmland) is (southern Ethiopia).

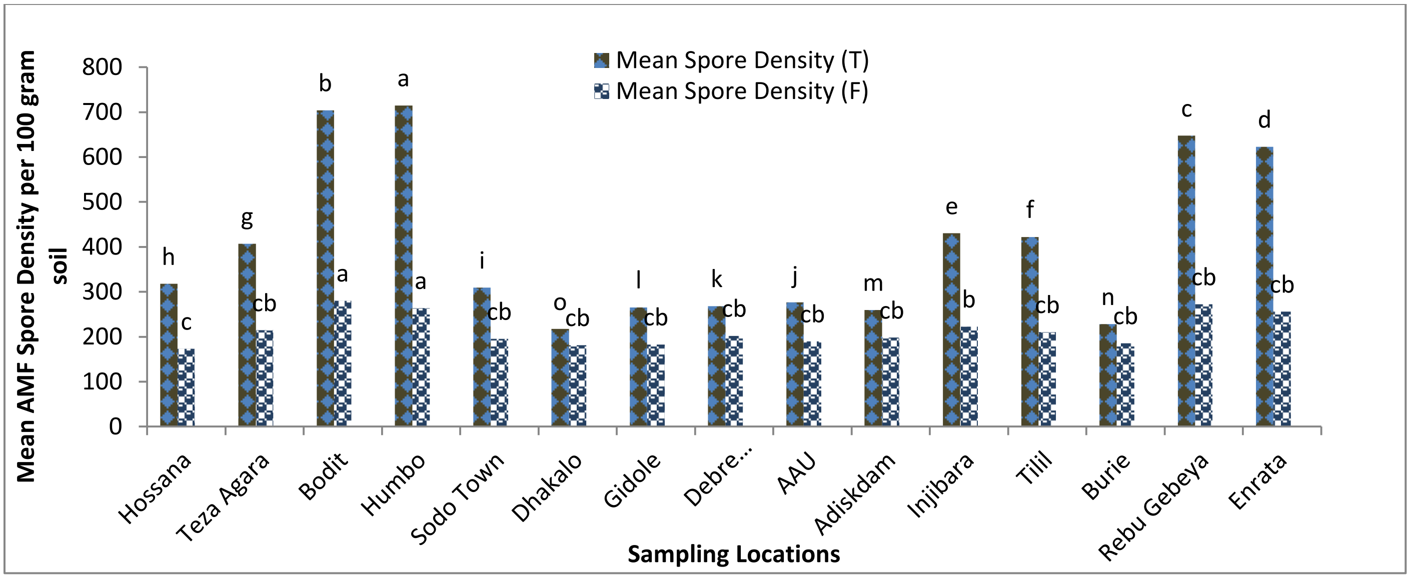


Figure 3.3 .AMF spore density recovered from field soils (F) and trap culture (T)

There was significant difference in mean AMF spore density among field soils from different geographic locations ($p<0.05$).

AMF spore density also varied (p<0.05) among the same land use types (Fig.3.3). From our observations during sampling in Bodite and Humbo there was continuous and extensive maize cultivation that might cause stressful condition. Compared to the other forest land use types in this study, Rebu gebeya (northern Ethiopia) exhibited high AMF spore density in field soil samples. This could be due to domination of *Eucalyptus* trees in the forest in Rebu Gebeya that might have created stressful environment. Likewise, Pande and Tarafdar (2004) also reported variations in AMF spore population under Neem plants from Rajistan, India, which may be attributed to variations in edaphic and climatic factors.

The highest mean AMF spore density in the trap culture was recorded from Humbo (715 spores 100 g^{-1} of dry soil) and Bodite (704spores 100 g^{-1} of dry soil) both farmlands (South Ethiopia) followed by Rebu gebeya (647spores 100 g^{-1} of dry soil) forest and Enrata (622spores 100 g^{-1} of dry soil) shade tree both from North Ethiopia. The lowest mean AMF spore density in trap cultures was detected at Dhakallo (217spores 100 g^{-1} of dry soil) farmland (South Ethiopia). There was significant difference in the mean AMF spore density among trap culture soil samples collected from different geographic locations (Fig. 3.3) at ($p<0.05$). Our results also exhibited variations in AMF spore density in both field and trap cultures within the same land use types.

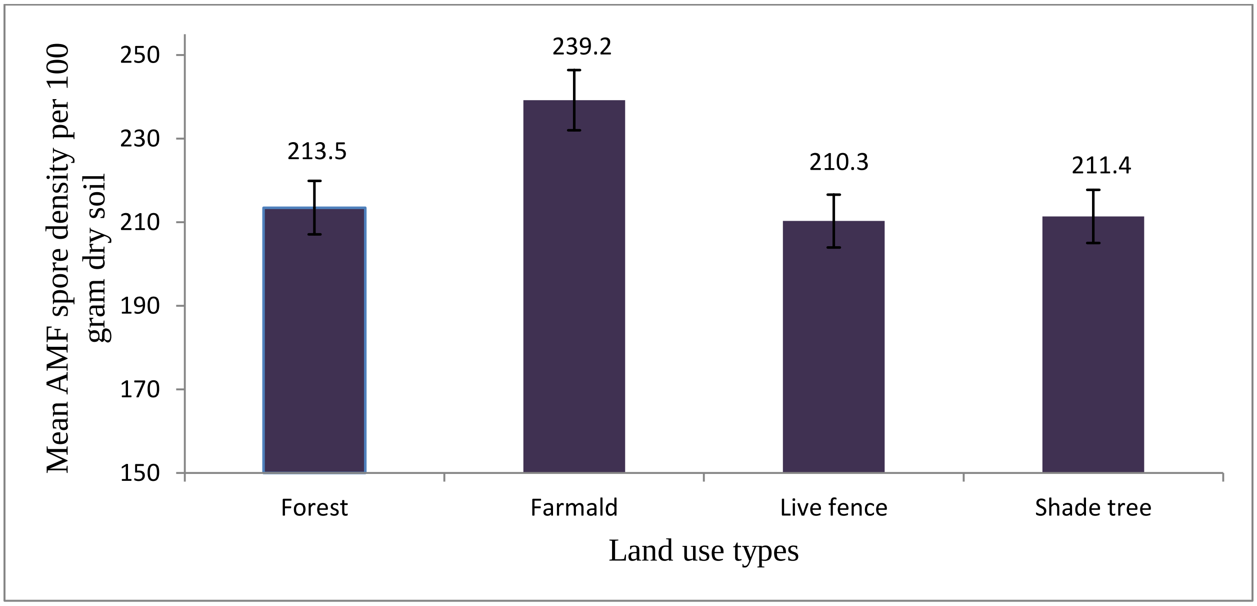


Figure 3.4 Mean AMF spore density in the field soils obtained from *Erythrina brucei* rhizosphere in different land use types.

The mean number of spores (173-280 spores 100 g^{-1} of dry soil) recorded in this study in field soil from the rhizosphere of *E. brucei* was much lower than 768 AMF spore population in 100 g dry *Erythrina brucei* rhizosphere soil collected from Sidama agro-forestry system in south Ethiopia by Beyene Dobo *et al. (*2016). This a higher AMF spore density in Beyene Dobo *et al* (2016) that could be most probably attributed to sampling season, that is, Beyene Dobo *et al.* (2016) collected their soil samples during dry season which triggers AMF sporulation, while in the present study we collected soil samples during rainy season.

The mean AMF spore density in trap cultures was greater than the field soils at all sampling locations regardless of the land use type. This is because trap culture favors greater number of AMF spores than field soils (Zerihun Belay *et al.,* 2015; Fernandes*et al.,* 2016). The density of AMF spores varied with geographic location. There was significant difference ($p<0.05$) in mean AMF spore density among trap cultures from various geographic locations (Fig.3.3). Humbo (farmland) harbored significantly ($p<0.05$) higher AMF spore density in both trap culture and field soil (Fig.3.3). Even though there was no significant ($p<0.05$) difference in AMF spore density in field soils at various land use types (Fig.3.4). However; the highest mean AMF spore density was recorded in farmlands. Likewise, Zerihun Belay *et al* (2013) have reported varied AMF spore population from the same land use type from the rhizosphere of acacia tree species in Ziway and Debrezeit, Ethiopia. Zerihun Belay *et al* (2015) have also reported AMF spore density in the range between 280 to 610 spores in 100 g dry soil from field soils and 250 to 1140 spores in 100 g dry soil from trap culture.

3.3.4. AMF root colonization

All *E. brucei* root segment in the trap culture experiment were colonized with different AMF structures; hyphae, arbuscules and vesicles (Table 3.5). The hyphal root colonization ranged from 34.4% to 49.1%. The highest value was recorded in soils from Teza agara (shade tree) followed by Hossana (farmland) while the lowest was recorded from Injibara (live fence). Teza agara (shade), Hossana (farm land), Adiskdam (live fence) and Tilil (live fence) showed the highest root colonization by arbuscules and vesicles. Beyene Dobo *et al* (2016) recorded 80.23% AMF root length colonization of *E.brucei which* is more than recorded in this study. This could be primarily due to differences in time of exposure of the AMF and the host plant roots. Beyene Dobo *et al* (2016) determined AMF root colonization from field standing aged plant roots. However, in this study we used six month aged plant roots from trap culture.

Table 3.5 AMF percentage root colonization of *E. brucei* plants grown in trap culture in soils collected from southern, central and northern Ethiopia

Sampling Location	% Root length colonization by AMF structures		
	AC %	VC %	HC %
Hossana	14.78a	8.08a	48.84 b
Teza agara	13.91a	7.42b	49.05 a
Bodite	9.21ed	3.22e	34.56 l
Humbo	12.66b	6.46c	40.97 f
Sodo Town	11.53c	5.96c	40.45 g
Dhakallo	8.42ef	2.72f	34.40 n
Gidole	9.46ed	2.99f	35.44 k
Debre markos	9.42ed	4.64d	34.56 m
AAU	11.35c	6.70c	41.29 e
Adiskdam	14.08a	8.21a	40.00 d
Injibara	9.44ed	4.35d	34.38 o
Tilil	14.18a	8.03a	48.22 c
Burie	10.17d	3.71e	35.56 i
Regu gebeya	9.11ed	4.19d	39.32 h
Enrata	9.08f	3.89d	35.49 j

Ac=Arbuscular colonization, VC=Vesicular colonization and HC=Hyphal colonization, Mean values with the same lowercase letter in the in the same column for each AMF structure do not differ among themselves in the ANOVA Duncan test (p<0.05).

The other difference could be due to effects of available P concentration on the AMF root length colonization, thus, the lower available P concentration (9.10 mg/kg) in Beyene Dobo *et a*l. (2016) enhanced a higher root colonization compared to the available P concentration in the present study (13.89-28.04 mg/kg). On the other hand, Zerihun Belay *et al* (2013) have recorded comparable AMF root colonization (12 to 67.3 %) from field standing acacia woodland in Debrezeit and Ziway, Central, Ethiopia. In general, AMF hyphal root colonization did not show significant (*p*<0.05) difference among different land use types and within the same land use types (Fig.3.5).

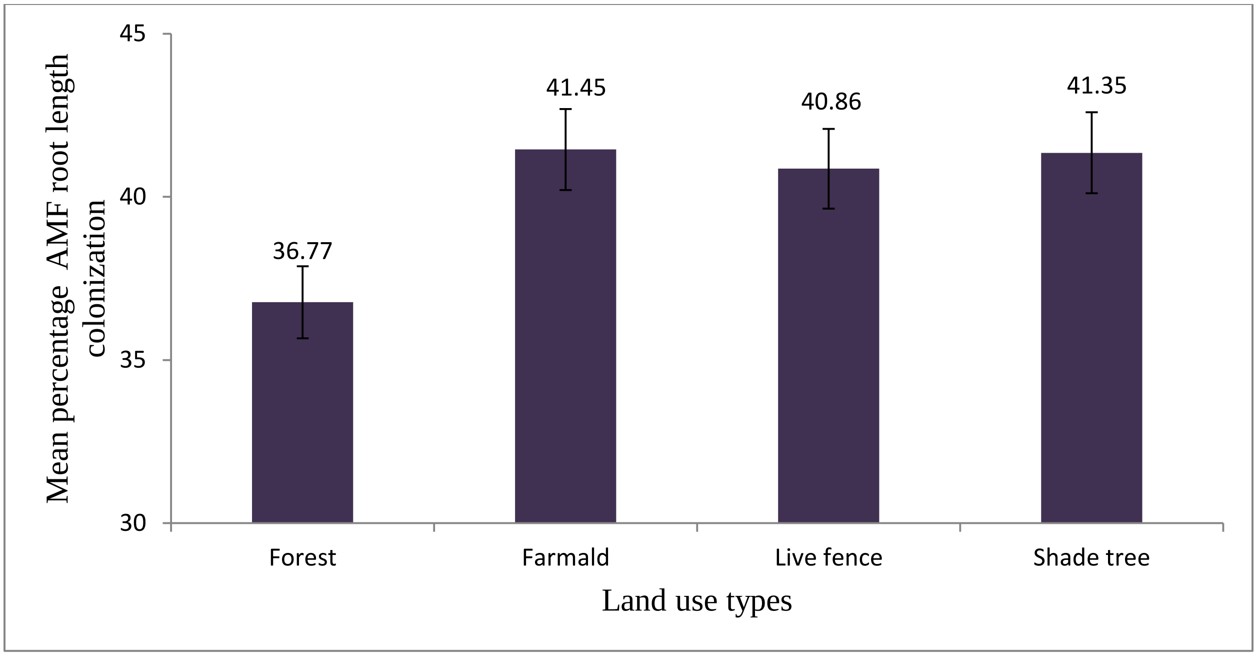


Figure 3.5. Mean AMF root colonization of *E. brucei* in trap culture with soils from different land use types.

3.3.5 AM fungal species richness

AM fungal species richness significantly varied ($p<0.05$) both among sampling sites distributed in the southern and northern parts of the country (Table 3.6) and among different land use types (Table 3.7).

Table 3.6 AMF species richness, Shannon index and Simpson's dominance index associated to *E. brucei* grown in trap culture in soils from different sampling sites and land use types.

Sampling Location	Land use type	AM fungal Species richness(S)	Shannon and wiever index (H')	Simpson's dominance index (D)
Hosanna	FL	3	0.15d	0.0026d
Teza Agara	ST	1	0.07f	0.0003f
Bodit	FL	2	0.11e	0.0011e
Humbo	FL	1	0.07f	0.0003f
Sodo Town	ST	6	0.23b	0.0103b
Dhakalo	FL	3	0.15d	0.0026d
Gidole	FO	6	0.23b	0.0103b
Debre markos	ST	2	0.11e	0.0011e
AAU	ST	6	0.23b	0.0103b
AdisKidam	LF	8	0.27a	0.0184a
Injibara	LF	4	0.18c	0.0046c
Tilil	LF	3	0.15d	0.0026d
Burie	FO	3	0.15d	0.0026d
Rebu Gebeya	FO	8	0.27a	0.0184a
Enrata	ST	3	0.15d	0.0026d

Species richness ranged from one species each from Teza agara (shade) and Humbo (farmland) to eight species each from Adiskdam (live fence) and Rebu Gebeya (forest) both from north Ethiopia. With regard to land use type, the mean AM fungal species richness ranged from 2.25 in farmland to 5.7 in forest (Table 3.7). Zerihun Belay *et al.*(2015) have reported different AMF species richness among different land use types in Central Ethiopia. These authors identified 6 to 31 species from field soils, while 11 to 23 species from trap culture indicating less species richness in trap culture compared to field soils. Likewise, Chaturvedi *et al.*(2012) and Leal *et al.* (2016) have also detected less AMF species richness in trap culture as compared to direct field soils. The mean species richness recorded in forest and live fence was twice greater than that recorded from farmland.

Table 3.7 AMF species richness, Shannon index and Simpson's dominance index associated to *E. brucei* grown in trap culture in soils from different land use types.

Land use type	Mean AM fungal Species richness (S)	Shannon and wiever index(H')	Simpson's dominance index(D)
Forest (FO)	5.7	0.23a	0.009a
Farmland(FL)	2.25	0.12d	0.001d
Live fence(LF)	5	0.21b	0.007b
Shade tree(ST)	3.6	0.17c	0.003c

Mean values with the same lowercase letter in the in the same column for each AMF index do not differ ($p<0.05$).

AM fungal diversity expressed by the Shannon and Wiever diversity index, and Simpson's dominance index was significantly varied ($p<0.05$) among the different sampling locations as well as among land use types (Tables 3.6 &3.7). Both the highest Shannon diversity and Simpson's dominance indices were recorded from forest soil samples, whereas the lowest values were recorded from farmland soil samples. This study exhibited also that both AMF species richness and AMF diversity were the highest in forest land use types and lowest at farmlands. This indicates none or less disturbed land use types (forest, live fence and shade) support higher AMF species richness and diversity compared to the disturbed or stressed land use types (farmlands). Fernandes *et al* .(2016) in their report support this observation in that intensity of land use like farming reduced AMF species richness.

The AMF spore counts in the field soils collected from *E. brucei* rhizosphere were negatively related to the soil pH ($n=15$, $R^2=0.719$, $p<0.05$) indicating 71.9% of the sporulation was explained by pH stress. Acidic soils produced higher AMF spore counts compared to slightly acidic soils evidencing that the pH stress induced sporulation. Coughlan *et al*.(2000) have reported larger AMF spore population but lower taxonomic diversity in acidic soils. AMF spore density was also negatively correlated to soil available phosphate (n=15, $R^2=0.896$, $p<0.05$) indicating that 89.6% of the spore formation was related to soil available phosphate. Such inverse relation between AMF spore density and soil available phosphate was reported by Tadesse Chanie & Fassil Assefa (2013) in Bonga forest in south west Ethiopia. In this study, we did not observe significant correlation between AMF root colonization and AMF spore counts (n=15, $R^2=0.0274, p>0.05$). Daniell *et al* (2001) and Mafaziya and Madawala (2015) have shown that AMF spore abundance does not directly reflect the AMF colonizing potential of plant root.

In conclusion, the host plant is found to be associated with eleven AMF genera of which *Glomus* and *Acaulospora* are dominant. Both geographic location and land use types significantly affect AMF species richness. The soil pH and available phosphate were negatively related to AMF spore density; however, AMF root colonization did not show significant relation to soil pH. Since, *E. brucei* is commonly planted and used in traditional agro-forestry practices in southern Ethiopia, understanding its AMF status will help in enriching the plant for better agro-forestry use. This research is limited to a few geographic locations, and hence we recommend more detailed AMF species diversity and richness across geographic location need study using molecular approaches.

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4. Phenotypic and Genetic diversity of rhizobial and non-rhizobial bacteria from the root nodules of *Erythrina abyssinica* grown under different land use types in southern and northern, Ethiopia

Belay Berza[1],Jegan Sekar[3], Prabavathy VR [3], Marcela C Pagano[2] Fasil Assefa[1]

1Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia, 2Federal University of Minas Gerais, Brazil, 3Microbiology Laboratory,MS Swaminathan Research Foundation, Chennai-600113, Tamil Nadu,India

Abstract

Erythrina abyssinica is a leguminous tree endemic to Ethiopia and grows on different land use types. It is a multipurpose tree used by local farmers mainly in traditional agro-forestry systems in southern Ethiopia. Despite the crucial agro-forestry attributes and wider distribution in the country, there was limited information on phenotypic and genetic diversity, and properties of stress tolerance of root nodule bacteria of *E. abyssinica* to fully realize their significance in the growth and health of the host plant. Therefore, this work reports the phenotypic and genetic diversity and eco-physiological stress tolerance traits of rhizobial and non-rhizobial obtained from the root nodules of *E. abyssinica.* To this end**,** root nodule bacteria were isolated from soil samples collected from different parts of the country using standard methods. The isolates were identified based on their phenotypic and genetic characteristics. The result showed that 67% of the soil samples induced nodules from which 30 root nodule bacteria were recovered. The isolates showed generation time with mean doubling time of between 1.5-6.6h. The 16S rDNA RFLP analysis grouped the bacterial isolates into twelve clusters at 70% similarity level. Although *NifH* gene was detected in 15 (50%) of the isolates, only three isolates (10%) induced nodules upon reinoculation studies. The 16S rRNA gene sequence analysis grouped the root nodule bacterial into nine genera: *Bradyrhizobium* (n=3), *Paenibacillus* (n=2), *Bacillus* (n=2), *Staphylococcus* (n=2), *Enterobacter* (n=2), *Achromobacter* (n=1), *Acinetobacter* (n=3), *Gluconobacter* (n=4) *and Stenotrophomonas* (n=2). The ones that formed root nodules were *Bradyrhizobium cajani* and *Bradyrhizobium cytisi* and *Bradyrhizobium shewense.* With regard to stress tolerance test, fourteen isolates (69%) were tolerant to 2.5% NaCl concentration and eleven (52%) of the isolates were able to grow in the pH range of 5.0-9.5, while six (29%) of them were able grow at 40°C. Fifteen strains (50%) were resistant to Chloramphenicol (30 µg). *Acinetobacter soli* (AU4 and RG6) and *Gluconobacter cerinus* (TL3) were able to catabolize all the carbon and nitrogen substrates tested. The research indicates that root nodules of *E. brucei* harbored phenotypically and genetically diverse rhizobacteria.

Keywords: *Acinetobacter*, *Bradyrhizobium*, 16S rRNA gene sequence, stress tolerance

4.1 Introduction

Erythrina abyssinica (Papilionoideae) is an endemic woody leguminous tree widely distributed in different parts in Ethiopia (Thulin, 1989). It grows on the edges, farmlands, home gardens, open places, upland forests, woodland and evergreen forests. The plant is a multipurpose tree used by local farmers for fuel wood ,construction material, shade for coffee plantation (Demel Teketay, 1994; Beyene Dobo e*t al.*, 2016),

It is very common to observe *E. abyssinica* on farmlands with important annual and perennial crops, such as barley (*Hordeum vulgare* L.), *Ensete ventricosum* (Welw.) Cheesman, maize (*Zeamays L.*), sorghum (*Sorghum bicolour* (L.) Moench s.l.) and coffee (*Coffea arabica* L.) for it improves soil fertility and crop yield (Thulin M, 1989; Legesse Negash, 2002; Wassie Haile, 2013)). This is because the woody legume forms symbiotic association with different groups of nitrogen fixing root nodule soil bacteria (Fassil Assefa and Kleiner,1997; Endalkachew W/meskel *et al*.,2004) and arbuscular mycorrhizal fungi (Shasho Megersa and Fassil Assefa, 2011; Beyene Dobo *et al*., 2016).

Although previous reports showed that *Erythrina abyssinica* is mainly nodulated by *Bradyrhizobium* spp. in Ethiopia (Fassil Assefa and Kleiner,1997), recent genetic studies showed several groups of root nodule bacteria can nodulate the woody legume (Endalkachew W/meskel *et al*.,2004; Aregu Amsalu *et al*, 2012, 2013). These authors reported that the legume not only harbors *Bradyrhizobium* spp., but also other root nodule bacteria such as, *Rhizobium phaseoli*, *Rhizobium* sp., *Rhizobium leguminosarum*, *Enterobacter* sp., *Mesorhizobium plurifarium*, different *Agrobacterium* species and *Rahnella* sp were isolated from the legume tree.

It is interesting to note that some of the isolates recovered from the root nodules bacteria such as *Enterobacter* sp., and *Agrobacterium* species were not typical root nodule bacteria of legumes that fix inorganic nitrogen from the atmosphere. Despite high specificity of the legume-Rhizobium interaction and the selective nodule environment, the presence of non nodulating endophtes has been reported; for example, *Shinella* from *Milletia ferruginea* (Tulu Degefu *et al*., 2013), and non-symbiotic endophytic bacteria from several legumes growing in Ethiopia (Aregu Amsalu *et al*., 2013), and non-nodulating endophytes from the nodules of chickpea (Negash Demissie *et al.* 2018). These non-nodulating strains used to be considered as contaminants based on authentication tests (Vincent, 1970), and root nodule bacteria that lost their nitrogen fixing characteristics (Berlinger, 1974).

These days, however, it has been hypothesized that these endophytes are important components of the nodule system that may have a supportive role of the symbiosis. Several studies also showed that these rhizosphere and rhizoplane bacteria are similar to the endophytes that have multiple plant growth promoting properties and support plant health (Tariq *et al*., 2014; Subramanian *et al*.,2015; Etesami & Maheshwari, 2018). All the hitherto phenotypic and genetic diversity studies on endosymbionts of *E. abyssinica*, notwithstanding, there is still limited information about the true picture of the existence of different endophytic (non-nodulating) and nodulating bacteria isolated from the nodules of the plant.

Thus, it is imperative to study the nitrogen fixing and plant growth promoting properties of these nodule rhizobacteria. This, together with the study on their ability to stress tolerance, would help select the best isolates for the development of inoculants to improve growth and productivity of the legume in the restoration of degraded land and as important component in agro-forestry practices and for other soil fertility improvements schemes such as green manuring and mulching to enhance the productivity of important crops in low input agriculture.

This study was therefore, conducted to study the phenotypic and genotypic diversity of rhizobial and non-rhizobial bacteria isolated from the root nodules of *E. abyssinica* and to evaluate their potential (in-vitro) tolerance to eco-physiological stress and versatility to various carbon and nitrogen substrates.

4.2 MATERIALS AND METHODS

4.2.1 Sampling Locations and Soil sampling

The soil samples were collected from different locations in the Southern, Addis Ababa and northern parts of Ethiopia where *E. abyssinica* has been growing for different purposes, i.e., agro-forestry, live fence around land boundaries, shade trees and forests. The soil samples were obtained from fifteen different locations characterized by different land use types. The details of routine sampling procedures are presented in section 3.2.1 (chapter 3) and the geographic locations and GPS coordinates are also presented in Table 3.1 (chapter 3**).** The soil samples were collected from June to July 2015 and May 2016.

4.2.2 Induction of nodulation (the trap culture experiment)

Induction of nodulation of rhizobia on the host plant was undertaken in plastic pots according to Vincent (1970). The details and routine procedures in the establishment of the trap cultures were described in section 3.2.3 (chapter 3). The seeds were surface sterilized with 70% alcohol for 1 min and 3% Sodium hypochlorite for 8 mins and repeatedly washed with sterile water and four to five *E. abyssinica* seeds were sown in four kg capacity sterile plastic pots containing the respective soil samples (Fig.4.1). After germination, the seedlings were thinned down to three plants per pot (Appendix 1). The plants were watered regularly three times a week either in the morning at 12 am or in the evening 12 pm for three months. The seedlings were grown in the greenhouse at the College of Natural Sciences, Addis Ababa University. The trapping experiment was conducted in triplicates.

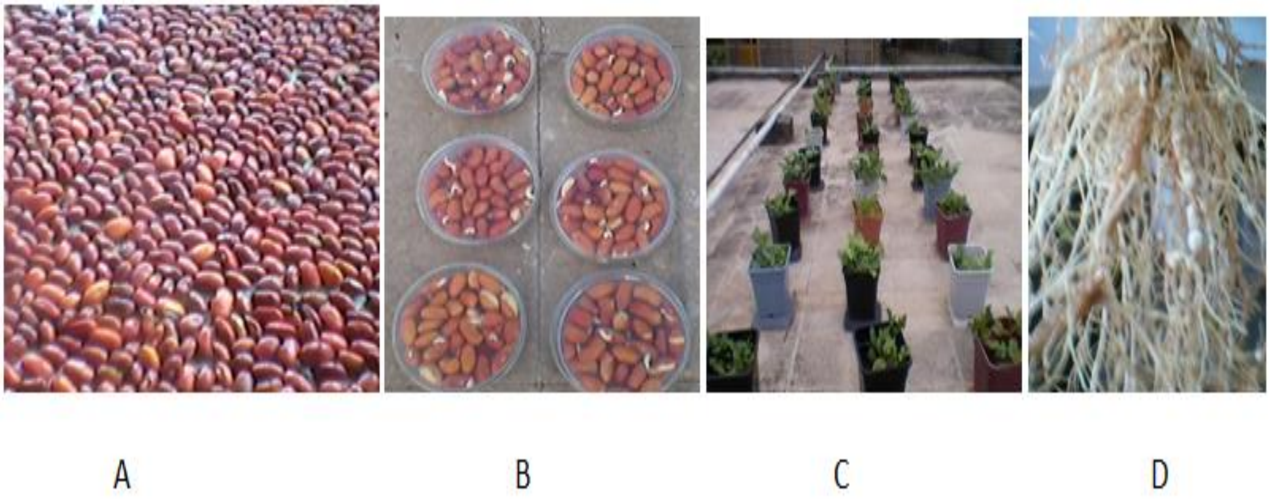


Figure 4.1 Plant infection .A-*Erythrina abyssinica* seeds, B-Seeds germination, C- trapping root nodule bacteria, D-root nodules.

4.2.3 Nodule collection, bacteria isolation and cultural characterization

The *E. abyssinica* plants were uprooted to detect nodulation and collect representative pinkish nodules following standard routine procedures described in Somasegaran & Hoben (1994). Nodules were preserved in a refrigerator at 4^0C until used for isolation of root nodule bacteria.

Fresh and healthy root nodules were surface-sterilized with 70% ethyl alcohol for 1 minute and in 1% sodium hypochlorite for 3 minutes. They were washed about five times with sterile water and crushed, serially diluted and spread plated on YEMA (Yeast Extract Mannitol Agar) with Congo red comprised of (g/l) [yeast extract, 0.5; D-mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$,0.2; NaCl, 0.1 ; Congo red 0.025 and agar-agar,15] in distilled sterile water (Somasegaran and Hoben, 1994). The plates were incubated at 28°C for 5-10 days. Representative colonies were randomly taken and sub-cultured to obtain pure cultures. The isolates were stored at −20◦C as stock cultures in Yeast Extract mannitol (YEM) broth containing 25% (v/v) glycerol. Isolates were designated by taking the first two letters of soil sampling locations followed by numbers.

4.2.4 Phenotypic Characterization of root nodule bacteria

The isolates were grown on YEMA for 5-10 days to evaluate their colony characteristics, i.e., color, texture and size (Smibert and Krieg, 1981). Growth rate of each isolate was determined according to the methods described in Somasegaran and Hoben(1994). Each isolate was grown in100 ml sterile YEM broth in 250 ml Erlenmeyer flask on rotary shaker (Thermo Fisher scientific, Germany) and incubated at 28°C at 100 rev. min^{-1} for 72 h. Samples were taken every two hours to measure optical density (OD600; Jenway, 6405 Uv/vis spectrophotometer, England) and determine viable colony forming units (Cfu) on YMA according to Somasegaran and Hoben, (1994). The generation time (g) was calculated from the logarithmic phase according to White (1995) using the formula $g=\frac{[Log2\ (t)]}{[LogX-LogX0]}$, where g stands for generation time, t stands for time elapsed,x0 stands for initial OD600 reading and x stands for second, OD600 reading at the logarithmic phase.

4.2.5 Genetic characterization of root nodule bacteria

4.2.5.1 Extraction of Genomic DNA and Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The genomic DNA of each bacterial isolate (n=30) was extracted according to the methods described in Kumar &Nair (2009). The quality and quantity of the extracted genomic DNA was confirmed by Nano-drop and gel electrophoresis. The genetic diversity of rhizobial and non-rhizobial *E. abyssinica* root nodule bacteria was conducted as follows. The 16S rDNA gene amplification was carried out by using universal primers described in Weisburg *et al*.(1991). The fD1 (5’-AGTTTGATCCTGGCTCAG-3’) and rP2 (5’-ACGGCTACCTTGTTACGACTT-

3') primers were used.

The amplification was carried out in a 20 µl reaction volume and each reaction mixture contained; 2 µl 10X PCR buffer, 2 µl 2.5mM dNTP mix, 2 µl forward primer (30 ng/ µl), 2 µl reverse primer (30 ng/ µl), 0.3 µl Taq DNA polymerase, 2 µl template DNA (25-50 ng/µl) and 9.7 µl double distilled sterile water to make final volume 20 µl. The amplification reaction condition was as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1min; elongation at 72°C for 1:30 min and final extension at 72° C for 10min. A 3 µL PCR product was loaded in to 1.2% (w/v) agarose gel stained with EtBr and run in 0.5X TBE buffer at a constant voltage of 60V for 2 h and visualized under UV transilluminator, followed by image capturing by using BioRad, Gel documentation, USA. A1kb DNA ladder (bio tools) was used as a DNA size standard.

A 10 µl aliquot of the amplified 16S rDNA genes of each bacterial isolate was digested by two restriction endonucleases simultaneously. A 2U of each of the restriction endonucleases (HinfI and MspI) (Roche, Germany) were added into each isolate amplified 16S rDNA and incubated at 37°C for 2 h according to the recommendations made by the manufacturers. The 16S rDNA gene restriction fragments were separated by electrophoresis in 3% (w/v) agarose gel containing 0.5 µg ethidium bromide mL^{-1}. Three independent restriction experiments were performed and those with consistent banding patterns were used.

The genetic diversity among isolates was established by using the data from the restriction fragment length polymorphism that adequately differentiated the isolates through banding patterns. A binary scoring system(1,0) was applied [where (1) stands for presence of a band and (0) for absence of a band)] to generate an input matrix, which was analyzed by using unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal, 1973), and a dendrogram was constructed from the matrix using NTSYS PC soft ware.

4.2.5.2 Screening for nifH gene using genomic DNA

The isolates (n=30) were screened for the presence of symbiotic (nifH gene) using the genomic DNA by applying the primers PolF (-5'TGCGAYCCSAARGCBGAC-3') and PolR (5'-ATSGCCATCATYTCRCCG-3') described in Poly *et al*. (2001). The PCR amplification was carried out in 10 µl reaction volume containing: 1 µL of 1.5mM PCR buffer;1 µL of 2.5 mM dNTP mix; 0.5 µL of each forward and reverse primer; 0.1 µL Taq DNA polymerase; 1 µL of template DNA and the final volume was made to 10µl using sterile HPLC water.

The PCR amplification was performed using Biorad T100 thermal Cycler (Germany) programmed at PCR conditions as follows: 95 °C for 5.00 minutes of preheating followed denaturation at 95°C for 1 min ,annealing at 52°C for 1 min , extension at 72°C for 1 min and the final extension at 72°C for 10 min (Poly et al., 2001). The PCR amplification product (3 µl) was run electrophoresis in 1.2 % (w/v) agarose gel stained with ethidium bromide. The loaded gel was run in 0.5X TBE buffer at constant voltage of 80V for 1 h. The PCR profile was visualized under UV trans-illuminator followed by image capturing (BioRad, Gel documentation, USA).

4.2.5.3 Amplification and sequencing of 16S rRNA genes

The phylogenetic position of *nifH* plus *E. abyssinica* root nodule bacterial isolates was determined using the 16S rRNA gene sequence analysis. The 16S rRNA gene amplification was carried out by using the primers described in Weisburg *et al*. (1991). The primers fD1 (5'-AGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') were used. The amplification reaction was carried out in a 50 µl reaction volume. Each reaction mixture contained 5 µl 10X PCR buffer, 5 µl 2.5mM dNTP mix, 5 µl forward primer (30 ng/ µl), 5 µl reverse primer (30 ng/ µl), 0.75 µl Taq DNA polymerase, 5 µl template DNA (25-50 ng/µl) and 24.25 µl double distilled sterilized water to make final volume 50 µl.

The amplification condition was as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min; elongation at 72°C for 1:30 min and final extension at 72° C for 10 min. A 3 µL PCR product was loaded into 1.2% (w/v) agarose gel stained with EtBr and run in 0.5XTBE buffer at a constant voltage of 60V for 2 h and visualized under UV transilluminator followed by image capturing (BioRad, Gel documentation, USA). The 1-kb DNA ladder (bio tools) was used as a DNA size standard ladder. For the construction of the phylogenetic tree, the closest 16S rRNA sequences and others belonging to type strains were obtained from NCBI Gen bank. The sequences of the *nifH* positive isolates were deposited in the Gen bank database under accession numbers (MK370568-MK370578). For sequence comparison, the alignment of sequences in this study and those obtained from Gen bank were included. The ClustalW algorithm was used for alignment, and the Neighbor-Joining method was employed for the phylogenetic tree construction. The phylogeny was tested by the bootstrap values with 1,000 replications. The sequence alignments and tree construction were made by using MEGA 7.0 software.

4.2.6 Eco-physiological Stress Tolerance

Tolerance to varied NaCl concentrations by each isolate was evaluated at different NaCl concentration levels (1%,1.5%, 2%, 2.5%, 3% ,3.5%,4%; w/v) on YEMA; the ability of each isolate to grow in acidic and/or alkaline conditions was also evaluated at different pH levels (4.0,4.5 , 5.0, 8.0 , 9.0, 9.5, 10, 10.5) on YEM broth or YEMA and incubated at 28◦C for 72-120 h as described by Wdowiak and Malek, (2000). Each isolate was also tested for its tolerance to different temperature ranges by growing on YEMA and incubating at different levels of temperature. i.e., 5◦C, 10◦C, 15◦C 37◦C, 40◦C and 45◦C.

Each plate was wrapped with parafilm to minimize moisture lose. All the tests were carried out in triplicates.

4.2.7 Intrinsic resistance to antibiotics (IAR)

Each isolate was evaluated for its inherent antibiotic resistance (IAR) by using some selected antibiotics (Josey *et al*., 1979). All the isolates were inoculated on YEMA plates containing (µg mL^{-1}): Chloramphenicol (20,25,30), Streptomycin sulfate (5,10,15), Nalidixic acid (90,100,120), Penicillin G(150,200,250), Tetracycline (5,10,15), and Ciprofloxacin (5,10,15) to test their inherent antibiotic resistance (IAR) according to Amarger *et al*.(1997). Each antibiotic was filter-sterilized (0.22 µm) and mixed with YMA just before pouring. A loopful (10 µL; 10^8 cells mL^{-1}) isolate was individually streak plated and incubated at 28°C for 7-10 days. Growth of the isolates was qualitatively described as (-) for no growth/ susceptibility and as (+) for positive growth/resistance in all cases.

4.2.8 The carbon and nitrogen substrate utilization

The ability of each isolates to catabolize various carbon and nitrogen sources was evaluated using basal medium consisted of (g/L): K_2HPO_4, 1.0; KH_2PO_4, 1.0; $FeCl_3.6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $(NH_4)_2SO_4$, 1.0; agar-agar, 15.0; pH, 7.0. During tests for nitrogen source utilization, $(NH_4)_2SO_4$, 1.0g was substituted by mannitol, 1.0 g. The sole carbon sources tested were lactose, D-sorbitol, dextrin, D-mannose, fructose, cellulose, sucrose, galactose, glucose and mannitol. Each of the carbon sources was used in 1.0 g final concentration. The sole nitrogen sources tested were L-Tyrosine, L-Tryptophan, DL-Phenylalanine, DL-Proline, DL-Serine, L-Glutamic acid, DL-Lysine, DL-Methionine, DL-Threonine and L-Arginine. Each of the nitrogen sources was supplied in the final conc. of 0.5 g.

Later, 10 µl of each isolate was individually inoculated into 5mL sterile YEM medium and incubated for 72-96 h at 28°C. A 1 mL of YEM broth (10^8 cells mL^{-1}) of each isolate was separately centrifuged at 12,000 rpm for 10 minutes. The pellet was suspended in 20 µl distilled sterile water and 10 µl of each isolate was individually spot inoculated on each basal medium supplemented with sole carbon or nitrogen source and incubated for 72 h at 28°C (Amarger *et al*., 1997). Heat labile carbon sources (D-mannose and D-sorbitol) and amino acid (DL-tryptophan and DL-glutamic acid) were filter sterilized (0.22 µm) and included into autoclaved basal medium just before pouring. The results were confirmed by performing three independent experiments. Growth of each isolate was qualitatively scored as (-) for no growth or non-utilization and as (+) for positive growth or indicative of substrate utilization in all cases.

4.3 RESULTS AND DISCUSSION

4.3.1 Bacteria trapping, Nodulation, and Isolation

A total of fifteen rhizosphere soil samples obtained from *Erythrina abyssinica* growing areas in the southern and northern Ethiopia and Addis Ababa were used for the trap culture experiment. The data showed that ten of the soil samples (67%) induced nodules on *Erythrina abyssinica* plant, whereas five soil samples from Teza agara, Bodite, Sodo Town and Dhakallo (South Ethiopia), and Adiskdam (north Ethiopia) did not induce nodules (data not shown). This could be attributed to the absence of compatible indigenous rhizobia in the top 30 cm soil depth. Endalkachew W/meskel *et al.*(2004) have also reported the absence of nodules from soils where *E. abyssinica* and other woody legumes *Albizia gummiferea* and *Millitia ferruginea* and other acacia species in southern Ethiopia. Lack of nodulation was also observed from indigenous acacia species grown in Kenya (Odee *et al*., 1995).

4.3.2 Cultural characteristics of the *E. brucei* root nodule bacteria

Thirty (30) *E. abyssinica* root nodule bacteria were isolated using YEMA medium (Table 4.1). The isolates varied in their cultural and growth characteristics (Table 4.1). They displayed colony sizes from 1.5 mm to 6 mm after 7-10 days growth on YEMA medium and showed differences in their colony texture; smooth wet/elevated and mucoid and creamy white on the growth medium.

The isolates also showed mean doubling time between 1.5 h -6.6 h **(**Table 4.1**)**. Most of them (90%) showed fast growth generation time of with 1.5-4 h with large colony diameter of 3 mm-6 mm. Three isolates AU27, HO2, and HU3 displayed slow growth with doubling time of (6.0-6.6 h) having smaller colony diameters (1.5 mm-2 mm) (Table 4.1). According to Jordan (1984) all the above features were characteristics of fast growing *Rhizobium* spp. and slow growing *Bradyrhizobium* spp.

Table 4.1 Cultural and growth characteristics of the bacterial isolates grown on YEMA medium at 30°C for 5-10 days

Serial No	Isolate code	Colony characteristics				Growth characteristics	
		appearance	Color	Shape	Size (mm)	Growth rate	MGT(h)
1	AU4	We	Wo	RE	3.0	fast	3.5
2	AU22	M	CW	FIM	6.0	fast	1.5
3	AU24	S	CW	CE	4.0	fast	2.0
4	AU27	We	Wo	RE	1.5	slow	6.0
5	AU37	S	CW	CE	4.0	fast	2.0
6	BU1	S	CW	CE	4.5	fast	2.5
7	BU2	We	Wo	RE	2.0	fast	3.4
8	BU4	M	CW	RE	4.5	Fast	3.0
9	DM27	M	CW	RE	3.7	fast	2.5
10	EN2	M	CW	RE	3.5	Fast	3.0
11	EN4	S	CW	CE	4.0	fast	2.0
12	EN7	M	CW	FIM	6.0	fast	2.8
13	EN8	M	CW	RE	4.2	Fast	2.4
14	GH6	M	CW	RE	3.5	Fast	3.0
15	DM17	S	CW	CE	4.0	fast	2.0
16	GH10	M	CW	CE	4.0	Fast	3.0
17	HO2	We	Wo	RE	2.0	slow	6.6
18	HO3	M	CW	FIM	6.0	fast	2.0
19	HO5	S	CW	CE	4.0	fast	2.2
20	HO6	We	Wo	RE	3.5	fast	2.7
21	HU3	We	Wo	RE	1.5	slow	6.3
22	HU5	We	Wo	RE	3.5	fast	3.1
23	HU6	M	CW	FIM	6.0	fast	2.0
24	EN5	M	CW	FIM	6.0	fast	2.0
25	IB5	M	CW	CE	3.0	fast	3.6
26	EN6	We	Wo	RE	3.5	fast	3.0
27	IB9	We	Wo	RE	3.5	fast	3.0
28	RG5	M	CW	FIM	6.0	Fast	2.0
29	RG6	M	CW	FIM	6.0	Fast	2.0
30	TL3	M	CW	FIM	6.0	Fast	2.0

S-smooth, M- mucoid, We-wet/elevated, CW-Creamy white, Wo-white/opaque, CE-circular with entire margin, FIM-Filamentous with irregular margin -RE-Round with entire margin.

4.3.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16S rDNA was amplified and resulted in a characteristic single band of 1427 bp (data not shown).The restriction digestion produced bands between 2 and 6, and the majority of the isolates exhibited four bands (Appendix 2). The dendrogram constructed based on ARDRA grouped the root nodule bacterial in to twelve (12) clusters or genotypic groups (Fig.4.2) at 70% similarity level.

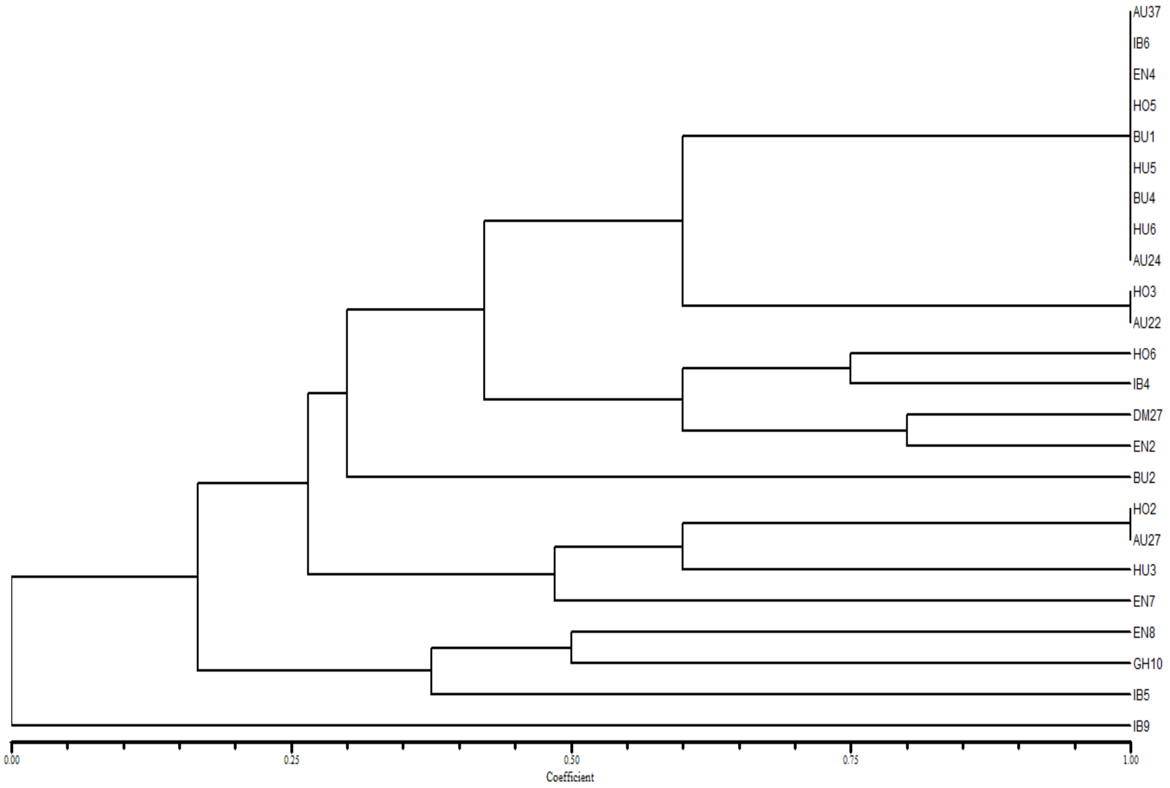
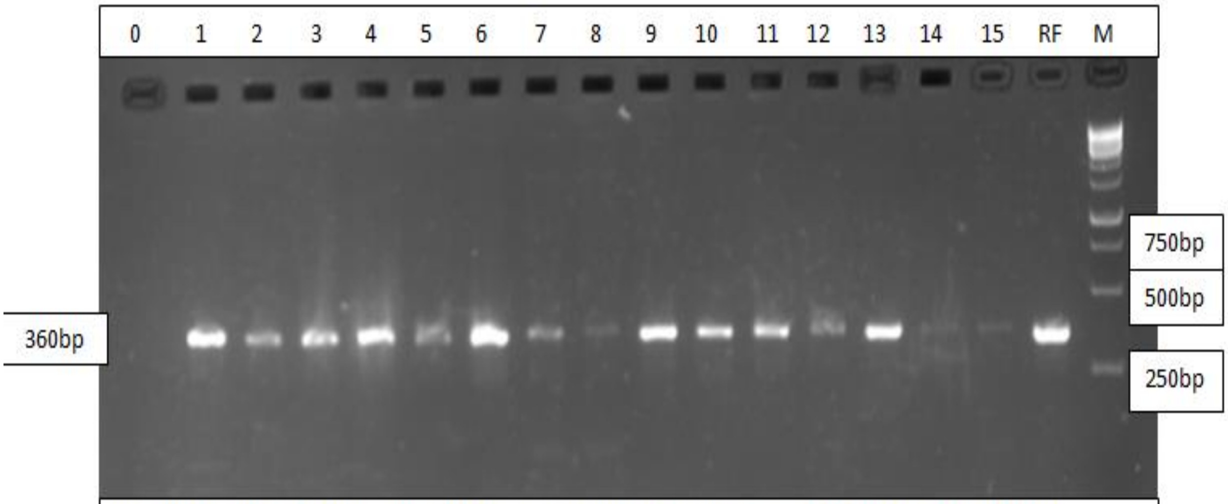


Figure 4.2 a dendrogram constructed for the indigenous *E. abyssinica* root nodule bacteria based on the 16S rDNA gene restriction fragment length polymorphism using restriction enzymes *HinfI* and *MspI*.

The slow growing root nodule bacteria (AU27, HO2 and HU3) were grouped into two cluster. However, the fast-growing isolates were distributed into ten different clusters (Fig.4.2).

4.3.4 Screening for nifH genes and Taxonomic position

After having observed the genetic diversity of the isolates in the 16S rDNA restriction fragment length analysis, 30 isolates were screened for *nifH* gene using primers PolF and PolR. *NifH* gene was detected in 15 (50 %) of the isolates (Fig.4.3). In all these isolates, approximately about 360 bp bands were detected on agarose gel electrophoresis. The size of the band was equivalent to the size of the *nifH* gene amplified by similar primers (360bp) (Poly *et al.*, 2001).



0-negative control,1- HU3, 2-HO3,3-AU27 ,4-EN2 ,5-EN7,6-EN8,7-IB5,8-DM27,9-HO2,10-AU37,11-IB9,12AU22,13-GH10,14-BU1,15-HO6,Rf-Reference strain,M-marker

Figure 4.3 the *NifH* gene positive indigenous *E. abyssinica* root nodule bacteria amplified using primers PolF and PolR.

The *nifH* gene detection and amplifications grouped the isolates in to two broad classes. (1) Those with the ability of symbiotic nitrogen fixation and (2) those non-symbiotic root nodule endophytes. The detection and amplification of *nifH* gene from these fifteen isolates revealed possible symbiotic/endophytic nitrogen fixing capability of the isolates.

The atmospheric nitrogen-fixing ability is previously demonstrated in the non-nodulating non-rhizobial isolates such as *Paenibacillus* (Liu *et al.*, 2019*), Enterobacter* (Lin *et al.*, 2012), *Bacillus* (Rilling *et al.*, 2018) *Staphylococcus* (Holguin *et al.*, 1992) and *Stenotrophomonas* (Wang *et al.*, 2018). Therefore, the possession of *nifH* genes by these root nodule bacterial isolates may imply potential symbiotic/endophytic nitrogen-fixing capability of the bacteria in their associations with E. *abyssinica*.

The phylogenetic position of the isolates using partial 16S rRNA gene sequencing isolates were compared with annotated bacterial sequences in NCBI data base (Table 4.2**)**.

Table 4.2 Taxonomic position of the different root nodule endophytes isolated from *Erythrina abyssinica*.

Isolate	Query length(bp)	Isolate identified to	Accession number	NCBI Best match ID	Query coverage (%)	Identity (%)
AU4	1422	*Acinetobacter soli*	MK370560	APPU01000012	99	99
AU22	1437	*Paenibacillus peoriae*	MK370573	AJ320494	100	98
AU 27	1363	*Bradyrhizobium shewense*	MK370570	Jgi.1052898	100	99
AU37	1401	*Staphylococcus edaphicus*	MK370576	KY315825	100	100
BU1	1401	*Staphylococcus cohnii*	MK370575	AB009936	100	99
BU2	1367	*Gluconobacter cerinus*	MK370563	BEWM01000030	100	100
DM17	1427	*Bacillus thuringiensis*	MK370566	ACNF01000156	100	100
DM27	1414	*Enterobacter ludwigii*	MK370572	JTL001000001	100	99
EN5	1367	*Gluconobacter cerinus*	MK370564	BEWM01000030	100	100
EN6	1367	*Gluconobacter cerinus*	MK370565	BEWM01000030	100	100
EN7	1422	*Bacillus luti*	MK370567	MACI01000041	100	100
EN8	1427	*Stenotrophomonas maltophilia*	MK370577	JALV01000036	99	96
GH6	1414	*Achromobacter xylosoxidans*	MK370558	CP006958	99	98
GH10	1421	*Stenotrophomonas maltophilia*	MK370578	JALV01000036	99	96
HO2	1363	*Bradyrhizobium cajani*	MK370568	KY349447	100	99
HO3	1424	*Paenibacillus peoriae*	MK370574	AJ320494	100	98
HU3	1363	*Bradyrhizobium cytisi*	MK370569	EU561065	100	99
IB5	1414	*Enterobacter ludwigii*	MK370571	JTL001000001	100	99
RG5	1422	*Acinetobacter soli*	MK370559	APPU01000012	99	99
RG6	1422	*Acinetobacter soli*	MK370561	APPU01000012	99	99
TL3	1367	*Gluconobacter cerinus*	MK370562	BEWM01000030	100	100

Thus, the phylogenetic tree grouped the isolates into six clades represented by the genera *Bradyrhizobium* (AU27, HO2 and HU3), *Paenibacillus* (AU22 and HO3), *Enterobacter* (IB5 and DM27), *Bacillus* (EN7), *Staphylococcus* (AU37 and BU1) and *Stenotrophomonas* (EN8 and GH10) (Fig. 4.4).

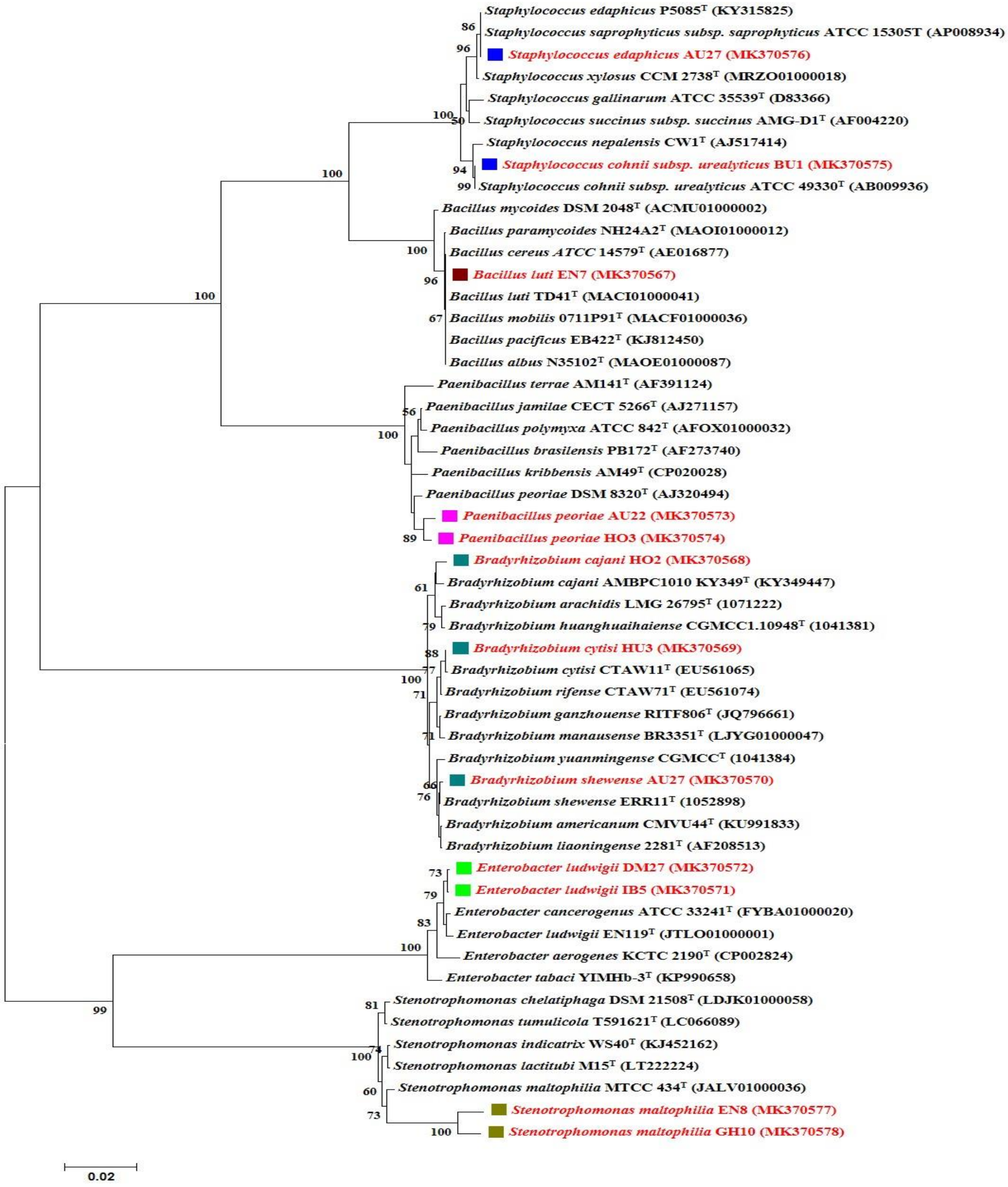


Figure 4.4 a Phylogenetic tree constructed using 16S rRNA gene partial sequences of *nifH* gene positive indigenous *E. abyssinica* root nodule bacteria.

Each genus consisted of at least two isolates except for *Bacillus* that includes EN7 (*Bacillus luti*). The isolates AU27, HO2 and HU3 were closely related to *Bradyrhizobium shewense*, *Bradyrhizobium cajani,* and *Bradyrhizobium cytisi*, respectively that were recovered from soil collected from three different geographic locations in Addis Ababa and southern Ethiopia (Fig.4.4).

The *Bradyrhizobium* species identified in this study were related to type strains *Bradyrhizobium cytisi* from Morocco (Chahboune *et al*.,2011), *Bradyrhizobium cajani* from Dominican Republic (Araújo *et al*.,2017) and *Bradyrhizobium shewense* from Ethiopia isolated from *E. abyssinica* (Aregu Amsalu *et al*., 2017).

The isolates AU37 and BU1 were closely related to *Staphylococcus edaphicus* and *Staphylococcus cohnii subsp urealyticus,* respectively (Fig.4.4). Isolates AU22 and HO3 were affiliated with *Paenibacillus peoriae*. Likewise, isolates DM27 and IB5 were closely related to *Enterobacter ludwigii*. Aregu Amsalu *et al*. (2013) have also isolated *Enterobacter spp*. from root nodules of *E. abyssinica* collected from Harar and Addis Ababa. Even though DM27 and IB5 were closely related to each other and to *Enterobacter ludwigii*, they were isolated from two sampling locations in northern Ethiopia (Debre Markos and Injibara), respectively.

Isolates EN8 and GH10 were clustered together with species *Stenotrophomonas maltophilia*. Similarly, these two isolates were isolated from very different geographic locations, GH10 from Gidole (southern Ethiopia) and EN8 from Enrata (northern Ethiopia). Several reports showed the dominance of *Bradyrhizobium* spp. from root nodules of *Erythrina abyssinica* (Fassil Assefa and Kleiner, 1997; Endalkachew W/Meskel *et al*.,2004a,b; Shasho Megersa and Fassil Assefa, 2011; Aregu Amsalu *et.al,* 2012*;*Aregu Amsalu *et al*., 2013).

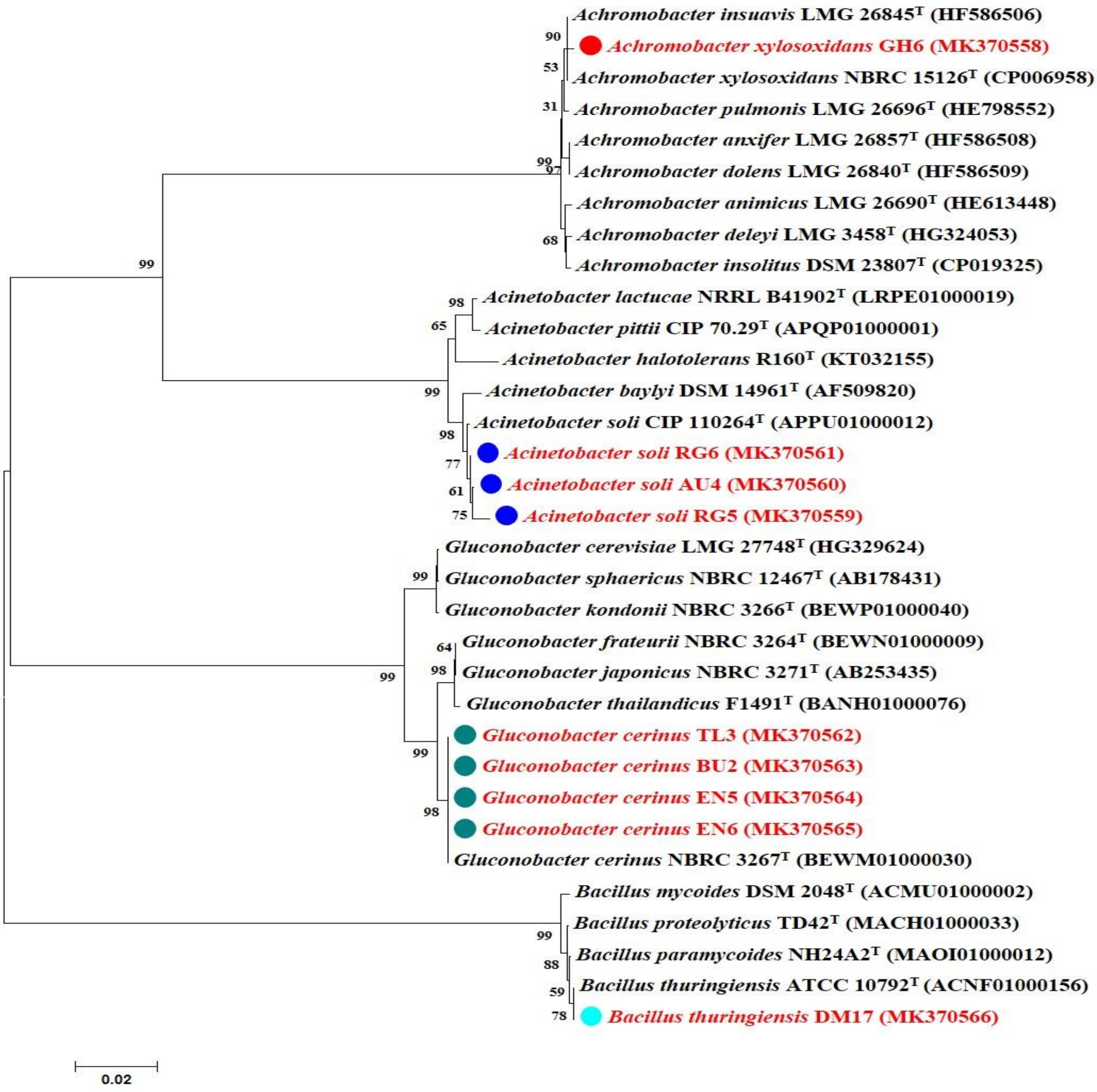


Figure 4.5 the phylogenetic relationship between *nifH* gene negative strains and reference strains from genbank based on 16S rRNA gene sequences. The genbank accession numbers of the strains are presented in the parentheses.

Isolate GH6 is clustered to genus *Achromobacter* and closely related to *Achromobacter xylosoxidans* sharing 98% sequence identity (Table 4.2). This isolate was recovered from root nodules obtained from Gidole, south Ethiopia. Likewise, isolates AU4, RG5 and RG6 were grouped into *Acinetobacter* and closely related to *Acinetobacter soli* sharing 99 % sequence identity. The other four isolates BU2, EN5, EN6 and TL3 were grouped into the genus *Gluconobacter* and closely related to *Gluconobacter cerin*us sharing 100% sequence identity (Table 4.2**)** and 98 % bootstrap value (Fig.4.5). Moreover, isolate DM17 was clustered to the genus *Bacillus* and closely related to species *Bacillus thuringensis* with sequence identity of 100%. This isolate was root nodule endophyte recovered from Debre Markos, north Ethiopia.

In general, the study showed non-root nodulating endophytes; *Staphylococcus edaphicus, Staphylococcus cohnii subsp urealyticus, Paenibacillus peoriae, Stenotrophomonas maltophilia, Bacillus loti*; and the nodulating *Bradyrhizobium cajani,* and *Bradyrhizobium cytisi* were identified for the first time from root nodules of *E. abyssinica* in Ethiopia. In addition, the nine isolates which demonstrated eco-physiological competitiveness and multiple plant growth promoting traits belonged to four genera; *Achromobacter, Acinetobacter, Gluconobacter and Bacillus (*Fig.4.5) that were the first time report from the root nodules of *E. brucei*. Endophytic bacteria such as *Paenibacillus, Bacillus, Pseudomonas,* and *Enterobacter* were frequently isolated from root nodules of other legume species (Liu *et al*., 2010; Palaniappan *et al.*,2010; Deng *et al*., 2011).

4.3. 5 Tolerance to Eco-physiological parameters

The isolates differed in their tolerance to NaCl supplemented to YEMA medium (Table 4.3). They exhibited varied tolerance ranging from 1% to 3.5% (w/v) (Table 4.3). However, 14 (67%) of the isolates were the most salt tolerant that were able to grow at NaCl of 2.5% and above. All the *Bradyrhizobium* strains; *B. shewense* (AU27), *B. cajani* (HO2) and *B. cytisi* (HU3) were unable to grow at NaCl concentration above 2.5% while the fast growers varied in their tolerance to NaCl concentrations. Fassil Assefa and Kleiner (1997) reported *Bradyrhizobium* sp.

(AUEB20) from *E. abyssinica* root nodule was tolerant to 3% salt (NaCl) concentration on YEMA medium. Salinity affects rhizobia, endophytic non-nodulating root nodule bacteria and host plant by inducing ionic stress through high concentration of ions and osmotic stress due to the change in osmotic concentration around cells causing desiccation and water deficit (El-Akhal *et al.*, 2013).

Table 4.3 Eco-physiological tolerance properties of different root nodule endophytes isolated from *Erythrina abyssinica* grown on YEMA medium at 30°C for 72-120 h.

Isolate	Isolate identified	%NaCl(w/v)	pH	Temp	IAR tolerated
AU4	*Acinetobacter soli*	0.5-3.5	5.0-10.0	10-40	Chl,Nal,Tet,Str,Cip
AU22	*Paenibacillus peoriae*	0.5-2.5	5.0-10.0	10-40	Tet,Str
AU 27	*Bradyrhizobium shewense*	0.5-2.0	5.0-10.0	10-37	Chl,Nal,Cip
AU37	*Staphylococcus edaphicus*	0.5-1.5	5.0-10.0	15-37	Chl,Tet,Pen
BU1	*Staphylococcus cohnii*	0.5-3.5	5.0-9.5	15-40	Nal,Tet,Str,Pen
BU2	*Gluconobacter cerinus*	0.5-3.5	5.0-9.5	15-35	Chl,Tet,Str,Cip
DM17	*Bacillus thuringiensis*	0.5-3.5	5.0-10	15-37	Chl,Cip,Pen
DM27	*Enterobacter ludwigii*	0.5-2.0	5.0-10.0	10-35	Nal,Str,Cip,Pen
EN5	*Gluconobacter cerinus*	0.5-3.5	5.0-9.5	15-35	Chl,Nal,Tet,Cip
EN6	*Gluconobacter cerinus*	0.5-3.0	5.0-9.5	15-35	Chl,Tet,Cip,Pen
EN7	*Bacillus luti*	0.5-2.0	5.0-9.5	15-40	Chl,,Cip,Pen
EN8	*Stenotrophomonas maltophilia*	0.5-2.5	5.0-9.5	10-35	Chl,Tet,Pen
GH6	*Achromobacter xylosoxidans*	0.5-3.5	5.0-10.5	15-37	Nal,Tet,Pen
GH10	*Stenotrophomonas maltophilia*	0.5-1.5	5.0-10.0	10-35	Chl,Nal,Str,Pen
HO2	*Bradyrhizobium cajani*	0.5-2.5	5.0-10.0	10-37	Chl,Nal,Cip,Pen
HO3	*Paenibacillus peoriae*	0.5-3.0	5.0-10.0	15-35	Chl,Nal,Str
HU3	*Bradyrhizobium cytisi*	0.5-2.5	5.0-9.5	15-40	Chl,,Str,Cip,Pen
IB5	*Enterobacter ludwigii*	0.5-1.5	5.0-9.5	10-35	Nal,Tet,Str,Pen
RG5	*Acinetobacter soli*	0.5-3.5	5.0-9.5	15-37	Chl,Nal,Str,Cip
RG6	*Acinetobacter soli*	0.5-3.5	5.0-9.5	10-40	Chl,Nal,Tet,Str,Pen
TL3	*Gluconobacter cerinus*	0.5-3.0	5.0-9.5	15-35	Nal,Tet,Cip,Pen

Chl-Chloramphenicol, Nal-Naldixicacid, Tet-Tetracycline, Str-Streptomycin,Cip-Ciprofloxacin Pen G-Penicillin

The isolates also varied in their tolerance to grow at different pH (Table 4.3). All the bacterial isolates were able to grow at pH values that range 5.0-9.5. More than half (52.4%) of the isolates were tolerant to pH between 5.0 and 9.5, but failed to grow at pH 4.5. According to Jordan (1982), the pH ranges for the growth of members of the family *Rhizobiaceae* are between 4.5 and 9.5. As pH decreases, structures like the import-export systems on the outer and inner membranes, periplasmic proteins, flagella, exo-polysaccharides, and cell walls may be adversely affected. On the other hand, rhizobial symbionts that induce growth under low pH can have effective symbiosis and nitrogen fixation with their leguminous partners in the soil (Marschner *et al*,1995).

With regard to temperature tolerance, eleven (52.4%) isolates were able to grow at incubation temperatures between 15-35 °C. five fast growing isolates (29%) *A. soli* (AU4 and RG6), *Paenibacillus peoriae* (AU22), *Staphylococcus cohnii subsp urealyticus* (BU1) and *Bacillus luti* (EN7) were tolerant to a temperature of 40°C (Table 4.3). The slow growing nodulating isolates also varied in their growth at different temperature ranges. *Bradyrhizobium shewense* (AU27) and *Bradyrhizobium cajani* (HO2) were able to grow with in temperature ranges between 10°C-37°C, while *Bradyrhizobium cytisi* (HU3) showed a wide range of temperature tolerance between 15°C and 40°C.

The optimum temperature range of the most rhizobia strains is 28-31°C (Graham, 1992). Likewise, Fassil Assefa and Kleiner (1997) have reported a promiscuous *Bradyrhizobium* sp (AUEB20) isolated from root nodule of *E. abyssinica* which exhibited growth at temperature between 5°C and 42°C.

Higher temperature adversely affects symbiotic effectiveness of rhizobia and reduces host legume growth and development (Hungria and Kaschuk, 2014). According to Hungriaa *et al.* (2001) the ability of rhizobial bacterial isolates to survive and grow at elevated temperature has strong correlation with their symbiotic performance under high temperature stressed soil conditions. Therefore, selection of high temperature tolerant rhizobia and other endophytic bacteria with plant growth promoting traits from the root nodules of plants is very vital for selection of bacterial inoculants for tropical agriculture.

4.3.6 Intrinsic Antibiotic Resistance (IAR)

The isolates also varied in their intrinsic antibiotic resistance (IAR) with reference to six selected antibiotics tested (Table 4.3). Over 70% of the isolates were resistant to Chloramphenicol (25 µg/mL) and 12 (57 %) of the isolates were resistant to Penicillin G (200 µg/mL), Nalidixic (120 µg/mL) and Tetracycline (15 µg/mL). The slow growing isolates *(Bradyrhizobium* species) in general, were resistant to at least 50% of the antibiotics tested. The isolates *Bradyrhizobium shewense* (AU27), *Bradyrhizobium cajani (*HO2) and *Bradyrhizobium cytisi* (HU3) were resistant to 50%, 67% and 67% of the antibiotics tested, respectively. The fast growing isolates *A. soli* (AU4 and RG6) were the most antibiotic resistant species that were able to grow on the medium containing most (83%) of the antibiotics tested (Table 4.3**)**.

IAR test is vital in view of its importance to identification and taxonomic classification of bacterial strains (Amarger *et al.* 1997; Hungria *et al*., 2001), and as one of the methods to screen for ecological competitiveness of the rhizobial strains in the soil (Kremer and Peterson, 1982). The multiple antibiotic resistances by the rhizobial and other root nodule endophytic bacteria in the present study could be attributed to previous exposure of these strains to the antibiotics tested in the soil.

4.3.7 Versatility in the utilization of carbon and Nitrogen substrates

The root nodule bacteria showed versatility in the utilization of different carbon and nitrogen sources (Table 4.4**).** All the isolates (100%) were able to utilize mannose, glucose and galactose as a sole carbon source and glutamic acid as sole nitrogen source. Three strains of *A. soli* (AU4 and RG6) and *G. cerinus* (TL3) utilized all the carbon substrates; and strains from *B.*

thuringiensis (DM17), *G. cerinus* (EN5 and TL3), *A. soli* (RG5 and RG6) utilized all the nitrogen substrates tested. The strains from *A. soli* (AU4 and RG6) and *Gluconobacter cerinus* (TL3) catabolized the carbon and nitrogen substrates (100%) tested. The slow-growing isolates (*Bradyrhizobium* species) were able to catabolize a wider range of carbon sources (80-90%) and nitrogen (60-70 %).

Table 4.4 Carbon and nitrogen source utilization patterns of root nodule endophytes isolated from *E. abyssinica*

Isolate	Isolate identified to (16S rDNA gene sequences)	Carbon Substrates									Nitrogen Substrates										
		Lactose	Sorbitol	Dextrin	Mannose	Fructose	Cellulose	Sucrose	Galactose	Glucose	Mannitol	Tyrosine	Tryptophan	Phenylalanine	Proline	Serine	Glutamic acid	Lysine	Methionine	Threonine	Arginine
AU4	*Acinetobacter soli*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AU22	*Paenibacillus peoriae*	+	-	-	+	-	+	-	+	+	+	+	-	+	-	-	+	-	+	-	+
AU 27	*Bradyrhizobium shewense*	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-
AU37	*Staphylococcus edaphicus*	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
BU1	*Staphylococcus cohnii*	-	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+
BU2	*Gluconobacter cerinus*	+	-	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
DM17	*Bacillus thuringiensis*	+	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
DM27	*Enterobacter ludwigii*	-	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+
EN5	*Gluconobacter cerinus*	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EN6	*Gluconobacter cerinus*	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
EN7	*Bacillus luti*	-	-	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+
EN8	*Stenotrophomonas maltophilia*	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	+	-	+	+	-
GH6	*Achromobacter xylosoxidans*	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
GH10	*Stenotrophomonas maltophilia*	-	-	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	-
HO2	*Bradyrhizobium cajani*	+	+	+	+	-	+	-	+	+	+	-	-	+	+	-	+	+	+	+	-
HO3	*Paenibacillus peoriae*	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+
HU3	*Bradyrhizobium cytisi*	+	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+	-	+	-	+
IB5	*Enterobacter ludwigii*	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
RG5	*Acinetobacter soli*	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RG6	*Acinetobacter soli*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TL3	*Gluconobacter cerinus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+=utilization;-=non-utilization

Hungria *et al*. (2001) have showed a correlation between the carbon content of the soil and their ability to utilize a wide range of carbon compounds i.e., the lower the soil carbon of the sampling locations, the broader the ability of the isolates to catabolize the carbon compounds. In this study, however, the soil organic carbon ranged between 2.52% and 4.91% which is high according to Landon (2014) and the isolates were able to catabolize a wide range of carbon substrates. Therefore, soils with medium to higher organic carbon content can also harbor bacteria with metabolic diversity utilizing a wide range of carbon and nitrogen substrates.

The ability of rhizobia and root nodule endophytic bacteria to metabolize a wide range of carbon and nitrogen substrates could be considered as an important strategy for survival and growth during their saprophytic lifestyle in the soil. Therefore, the versatility of bacterial strains in utilizing various C and N substrates could be a vital issue in selecting competitive rhizobial/other root nodule bacterial inoculants as biofertilizer.

4.3.8 Conclusion and recommendation

This study showed the occurrence of diverse groups of many non-symbiotic and few symbiotic root nodule bacteria in the nodules of *E. abyssinica.* Moreover, *B. cajani* and *B. cytisi* are the first report both from *E. abyssinica* root nodules and Ethiopia as well. Eco-physiological stress tolerant, nutritionally versatile and potential bio-inoculant candidate strains are reported. Further exploration of the rhizosphere of *E. abyssinica* in general, and those locations from which the rhizosphere soils were failed to induce nodulation in particular is needed.

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4.3.9 References

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5. Evaluation of inorganic phosphate solubilizing and other plant growth promoting traits of root nodule bacteria isolated from *Erythrina abyssinica*

Belay Berza[1], Jegan Sekar[3] ,Prabavathy VR[3], Marcela C Pagano[2] ,Fassil Assefa[1]

1Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia 2Department of Biology, Federal University of Minas Gerais, Brazil.3 Microbiology Laboratories, MS Swaminathan Research Foundation, Tamil Nadu, Chennai, India

Abstract

Soil bacteria that dominate the zone under the influence of the root are generally known as rhizobacteria. These organisms support plant growth through various means and hence they are known as plant growth promoting microorganisms (PGPM). Some of them selected and applied to enhance the productivity of the host plant. Thus, the objective of this study was to assess plant growth promoting properties of root nodule bacteria isolated from *Erythrina abyssinica*. The bacteria were primarily screened for inorganic phosphate solubilizing ability on Pikpvskaya agar (PA) medium followed by evaluation of their ability to produce Indole Acetic Acid (IAA), Hydrogen cyanide (HCN), Ammonia (NH_3) production and to evaluate synthesis of hydrolytic enzymes such as chitinase, lipase and protease. The selected isolates were further evaluated for insoluble inorganic phosphate solubilization efficiency in liquid National Botanical Research Institute Phosphate (NBRIP) medium supplemented with tricalcium phosphate (TCP), aluminium phosphate (Al-P) or iron phosphate (Fe-P) by quantitative colorimetric method. Out of 21 isolates, 62%, 76%, 48%, 33% and 19% were inorganic phosphate solubilizers, IAA, NH_3, HCN and chitinase producers, respectively. All the selected isolates were potential solubilizers of inorganic phosphates in liquid NBRIP medium. Hence, *Acitenobacter soli* (AU4) solubilized the inorganic phosphate sources and released free phosphate of 109, 87 and 84 mg L^{-1} from TCP, Al-P and Fe-P sources, respectively. Likewise, *B. thuringiensis* (DM17) produced the highest amount of IAA (0.313 mg mL^{-1}). *A. soli* (AU4) could be potential bio-inoculant candidate for the growth enhancement of the host plant for better agro-forestry practices in acidic and alkaline soils in Ethiopia.

Keywords/phrases: *Acitenobacter soli* (AU4), aluminium phosphate, *B. thuringiensis* (DM17), IAA, tricalcium phosphate (TCP)

5.1 Introduction

Phosphorous (P) is the second important key plant nutrient which limits plant growth and with no atmospheric reservoir that can be made biologically available. Phosphorous is commonly exists in the soil both in organic and inorganic forms. The majority of inorganic phosphorous in the soil is fixed and hence, unavailable to plant nutrition in soil for it forms complex with Al, Fe, and Mn in acidic soils while in neutral and alkaline soils it reacts strongly with Ca (Khan *et al*.,2014).

The existence of naturally soil inhabiting rhizospheric phosphate solubilizing microorganisms has been recognized since 1903 (Khan *et al.*, 2007). These microorganisms could convert inorganic forms of Al, Fe and Ca phosphates through various mechanisms , mainly by producing organic acids that chelate cationic partners of P ions and release PO_4^{-3} directly in to soil solution (Khan *et al*., 2014).

Among the whole microbial population in the soil, phosphate solubilizing bacteria constitute 1 to 50% (Chen *et al*., 2006). The dominant effective phosphate solubilizing rhizobacteria are of the genera *Pseudomonas, Bacillus, Serratia, Erwinia* (Diriba Muleta *et al.*, 2013) , *Achromobacter* (Ma *et al*., 2008), *Acinetobacter* (Ogut *etal*.,2010), *Aerobacter, Agrobacterium, Flavobacter, Micrococcus* (Rodríguez & Fraga, 1999) and the endosymbiotic *Rhizobium and Burkhoderia* (Aregu Amsalu , *et al*., 2013).

Apart from phosphate solubilization, these rhizobacteria and endophytes have additional characters to enhance growth and health of the plant hosts and hence are known as Plant growth promoting microorganisms. Plant growth promoting microorganisms enhance plant growth through several mechanisms like IAA synthesis (Tariq *et al*., 2014), NH_3 and HCN production (Dinesh *et al.*, 2015), production of hydrolytic enzymes (Kumar *et al*., 2012) siderophore production (Viruel *et al*., 2011), antibiotics synthesis (Taurian *et al*., 2010) and inhibit phytopathogens (Singh *et al*., 2010).

The bio-control of fungal diseases by chitinolytic enzyme producing rhizobacteria is also well established (Ajit *et al*., 2006; Indris *et al.,* 2007; Kishore and Pande, 2007; Kamil *et al.*, 2007). Production of different hydrolytic enzymes by rhizobacteria was reported by several scholars (Kumar *et al*., 2012; Rashid *et al*.,2012; Dinesh *et al*., 2015). Similarly, production of fungal cell wall and cell lytic enzymes such as chitinase, protease or lipase by rhizobacteria was also reported (Prasad *et al.*, 2011).

Erythrina abyssinica (Papilionoideae) is an endemic woody leguminous tree widely distributed in different land use types in Ethiopia (Thulin, 1989). The contribution of *E. abyssinica* in cropping and traditional agro-forestry systems/practices in different parts of Ethiopia is well established (Legesse Negash, 2002; Wassie Haile *et al*., 2013; Beyene Dobo *et al.*, 2016). A few studies were also undertaken on the host-microbe interaction of *E. abyssinica.* Shasho Megersa and Fassil Assefa (2011) have isolated a few rhizobacteria from the host to study their plant growth properties. However, the work was limited to one sampling site and a few phenotypically identified bacteria.

In order to enhance the growth and development of the host plant for better plant production for agro-forestry practices, investigating its rhizosphere and root nodule bacteria for multiple plant growth promoting traits is very necessary. This study, therefore, aimed to assess plant growth promoting properties of E. *abyssinica* root nodule bacteria and evaluated inorganic phosphate solubilizing efficiency of selected root nodule bacterial endophytes.

5.2 MATERIALS AND METHODS

5.2.1 Sources of microorganisms

The root nodule bacteria that were identified genetically and screened for other eco-physiological traits were maintained in a culture collection at the College of Science, Addis Ababa University and are shown in Table 4.2.

5.2.2 Screening for inorganic phosphates solubilization potential

The root nodule bacteria were screened for ability to solubilize sparingly soluble inorganic phosphates using tri-calcium phosphate ($Ca_3(PO_4)$, ferric phosphate ($FePO_4$) and aluminium phosphate ($AlPO_4$) on Pikovskaya's solid media (PA) containing of (g/L): yeast extract, 0.50; glucose,10.0; $Ca_3(PO_4)_2$,5.0; $(NH_4)_2SO_4$,0.50; NaCl ,0.20; KCl,0.20; $MgSO_4.7H_2O$, 0.10; $MnSO_4.2H_2O$, 0.002; $FeSO_4.7H_2O$, 0.002 and agar-agar, 15.0 (Pikovskaya, 1948). In FePO4 or AlPO4 solubilization studies, the quantity of tri-calcium phosphate (Ca_3 $(PO_4)_2$ was substituted with FePO4 or $AlPO_4$.

Ten (10 µl) 2-6 days old (log phase) culture (10^8 cells mL^{-1}) of each isolate was spot inoculated on the centre of the respective pre-prepared PA and incubated at 28 ◦C for 7-10 days. A clear halo zone around the colonies was considered as an indication of phosphate solubilization. The phosphate solubilization index (PSI) was calculated according the methods described in Premono *et al*.(1996).

$$PSI = \frac{colonydiameter + Halozonediameter}{Colonydiameter}$$

5.2.3 Production of Indole acetic acid (IAA) by the isolates

Indole acetic acid (IAA) production was detected and estimated according to Gordon and Weber, (1951). The root nodule bacteria were inoculated 1 mL (10^8 cells mL^{-1}) of 3 to 6 days old (log phase) YEM broth grown bacterial culture into 5ml Luria Bertani (LB) broth composed of (g/L); tryptone, 10.0; yeast extract, 5.0; NaCl, 2.5, and supplemented with L-tryptophan (0.1 g L^{-1}). They were incubated at 28°C for 72 h with gentle shaking (120 rpm). Each culture was individually centrifuged at 12,000 g for 10 minutes and the supernatant was filtered by using Whatman No-1 filter paper. Two (2) mL filtrate was gently mixed with 4 mL Salkowski reagent and incubated for 20-30 min in dark. IAA production was indicated by the appearance of pink color. A separate un-inoculated broth medium was served as a control. Color intensity was indicated as +++-deep pink; ++- red; +-pale red; - no color change.

Based on the colimetric analysis, nine isolates were selected and the amount of IAA produced was quantified by measuring absorbance at 535 nm using a spectrophotometer **(**Jenway, 6405 Uv/vis spectrophotometer, England) against a standard curve prepared with known concentrations of analytical grade IAA (Appendix 6).

5.2.4 Hydrogen cyanide (HCN) and Ammonia (NH_3) production

Production of HCN was measured qualitatively according to the method described in Dinesh *et al*. (2015). Log phase bacterial culture (25 µL) was inoculated into 5 mL of King's B broth composed of (g/L); protease peptone, 20; glycerol, 10 mL; K_2HPO_4, 1.5; $MgSO_4$, 1.5; pH 7.2; supplemented with 4.4 g L^{-1} of glycine. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3in 1L distilled H_2O) were inserted into the test tubes and tightened with screw cap and additionally sealed with parafilm. The test tubes were incubated in a shaker at 120 rpm at 28°C for 72 h. Production of HCN was indicated by the change in color of the filter paper strips from yellow to brown to red. The intensity of the color changes was recorded qualitatively as (+ production, - no production).

The production of NH_3 was determined by the method described in Cappuccino and Sherman,(1992). Briefly, 50 µL of 72 h grown bacterial cell suspension was inoculated in to 30 mL of peptone water (4%) (W/v) and incubated at 28◦C for 72 h to which 1 mL Nessler's reagent was added. The formation of yellow to brown precipitate indicated the presence of NH_3 in the culture. The concentration was indicated qualitatively based on precipitate color change (+, brown, -, yellow).

5.2.5 Synthesis of Hydrolytic enzymes

Chitinase production was tested according to the method described by Saima *et al*.(2013). Ten microliter of a 72 h old bacterial culture was spot inoculated on the agar medium amended with colloidal chitin consisted of (g/L): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar-agar, 15 and colloidal chitin 1% (w/v), and incubated at 28◦C for 72 h. The bacterial colonies with clearance zones with a creamish background were considered as chitinase-producing bacteria.

The test for the production of protease enzymes was carried out following the procedures described in Dinesh *et al*. (2015). One loopful of 72 h old bacterial cell suspension was spot inoculated on skim milk agar plate consisted of (g/L); skim milk ,100; peptone , 5; agar ,15) and incubated at 28◦C for 72 h. Plates were observed for clear zone around the colonies after 72 h .

The ability of the root nodule bacteria to produce Lipase was detected according to the methods described in Smibert *et al*. (1994). A loopful of 72 h old exponential growth phase bacterial culture was individually spot inoculated on basal medium that consisted of (g/L) (bacto peptone, 10 ; NaCl ,5 ; $CaCl_2 \cdot H_2O$,0.1 ; and agar-agar ,9) supplemented with Tween 80 (1%; w/v) and incubated at 28◦C for 72 h. A production of opaque halo zones around colonies indicated lipolytic activity.

5.2.6 Quantification of inorganic phosphate solubilizing potential using liquid media

The inorganic phosphate solubilizing potential of selected strains was determined using National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). The strains were selected based on their overall performance of eco-physiological stresses tolerance, nutritional versatility and multiple plant growth promoting traits (Table 5.3). Each isolate was grown to 72 h in YEM broth from which 10 µl (10^8 cell mL^{-1}) suspension was inoculated into 20 mL NBRIP liquid medium in fifty (50) mL flasks. The medium contained (g/L) ; glucose, 10 ; $Ca_3(PO_4)_2$, 5 ; $MgCl_2.6H_2O$, 5 ; $MgSO_4.7H_2O$, 0.25; KCl, 0.2 and $(NH_4)_2SO_4$, 0.1. With regard to ferric and aluminium phosphates solubilization studies, they substituted $Ca_3 (PO_4)_2$ .The pH of each medium was adjusted to neutral before the experiment. The un-inoculated controls were included as control. All flasks were incubated at 28◦C with gentle shaking at 120 rpm for three consecutive days.

On the third day, 10 mL culture was removed and centrifuged at 12,500 rpm for 10 minutes and the supernatant was used for determining the pH and the amount of phosphate released in the medium. The released phosphate was determined using colorimetric methods as described by Murphy &Riley, (1962). In brief, the supernatant obtained by centrifugation was filtered through a 0.45 µM millipore filter and 0.1 ml of the supernatant was mixed with 0.25 ml of Barton's reagent and the volume was made to 5 ml with distilled water. After 10 min, the intensity of yellow color was read using spectrophotometer (Jenway, 6405, UV–VIS

Spectrophotometer, England) at 430 nm and the amount of P-solubilized was extrapolated from the standard curve (Appendix 7).

The concentration of phosphate released in the culture medium was quantified using a standard curve prepared from known analytical grade of KH_2PO_4 concentrations (Appendix 7). The amount of solubilized phosphate was quantified by subtracting the phosphate released in un-inoculated control (probably phosphate released as result of autoclaving) from the inoculated cultures. The experiments were conducted in triplicates and values were expressed as their mean.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Isolation and Screening inorganic phosphate solubilizing bacteria

Twenty-one bacterial strains were screened for the solubilization potentials of sparingly soluble inorganic phosphates (Table 5.1). Thirteen (62 %) of the species exhibited clearly visible halos around colonies on PA medium supplemented with $Ca_3 (PO_4)_2$. All the 13 isolates were able to grow on PA medium supplemented with $AlPO_4$ or $FePO_4$ without showing visible clear halos. Other authors also showed the same pattern of growth of rhizobacteria on the medium with no halo zone formation from the desert plant in Mexico (Puente *et al*. 2004), from bacteria naturally colonizing limonitic crust in Venezuela (Pérez *et al*., 2007), and from endophytic bacteria from the root nodules of French bean (Dinic *et al*., 2014).

However, very low solubility of $AlPO_4$ or $FePO_4$ may be a result of structural complexity of the compounds and the particle size of the phosphates (Nautiyal *et al*. 2000)

Table 5.1 Plant growth promoting properties of root nodule bacteria isolated from *E. abyssinica*

Isolate	Isolate identified	$Ca_3(PO4)_2$ *	IAA production	HCN production	NH3 production	Hydrolytic Enzymes			Sum of Exhibited traits
						Chitinase	Protease	Lipase	
AU4	*Acinetobacter soli*	6.0	++	+	+	-	+	+	6
AU22	*Paenibacillus peoriae*	0.5	++	-	-	-	-	-	2
AU 27	*Bradyrhizobium shewense*	1.0	-	-	-	-	-	-	1
AU37	*Staphylococcus edaphicus*	-	-	-	-	-	-	-	0
BU1	*Staphylococcus cohnii*	2.0	++	-	-	-	-	-	2
BU2	*Gluconobacter cerinus*	4.12	++	+	+	-	-	+	5
DM17	*Bacillus thuringiensis*	5.0	+++	+	+	+	-	+	6
DM27	*Enterobacter ludwigii*	0.5	+++	-	-	-	-	-	2
EN5	*Gluconobacter cerinus*	4.2	++	+	+	+	-	+	6
EN6	*Gluconobacter cerinus*	4.4	++	+	+	-	+	+	6
EN7	*Bacillus luti*	-	+	-	-	-	-	-	1
EN8	*Stenotrophomonas maltophilia*	-	+	-	-	-	-	-	1
GH6	*Achromobacter xylosoxidans*	5.4	++	-	+	+	+	+	6
GH10	*Stenotrophomonas maltophilia*	-	+	-	+	-	+	+	4
HO2	*Bradyrhizobium cajani*	-	-	-	-	-	-	-	0
HO3	*Paenibacillus peoriae*	-	+	-	-	-	-	-	1
HU3	*Bradyrhizobium cytisi*	-	-	-	-	-	-	-	0
IB5	*Enterobacter ludwigii*	-	-	-	-	-	-	-	0
RG5	*Acinetobacter soli*	4.0	+++	-	+	+	+	-	5
RG6	*Acinetobacter soli*	4.3	+++	+	+	-	+	+	6
TL3	*Gluconobacter cerinus*	4.3	++	+	+	-	+	+	6
Total		13	16	7	10	4	7	9	

+++-deep pink; ++- red; +-pale red/production; - no color change/no production; * Phosphate solubilization index.

The rhizobacterial species showed significant differences in clear halos zone formation and phosphate solubilization indices (PSI) (Fig 5.1). The isolates induced solubilization with PSI between 0.5 and 6.0 on PA medium supplemented with tricalcium phosphate (Table 5.1). On the basis of PSI values, the strains were grouped into high and low category of phosphate solubilization. Thus,, nine of the strains produced PSI values ≥4.0 which is considered as high and four (31 %) of the strains produced PSI values less than 2.0 which is taken as low (Marra *et al.*,2012).

Diriba Muleta *et al*. (2013) have reported PSI values between 2.05 and 5.85 from phosphate solubilizing rhizobacteria isolated from natural coffee forest in south western Ethiopia. This preliminary observation revealed the presence of potential phosphate solubilizing bacterial population in the root nodules of E. *abyssinica* across different areas.

Among the isolates, *Acinetobacter* soli (AU4) displayed the highest PSI of 6 followed by *Bacillus thuringensis* (5.0) and *Achromobacter xylosoxidans* with PSI 5.4. It is also interesting to note that most of the phosphate solubilizing isolates (69%) showed higher PSI ranging from 4 to 6 (Table 5.1). The isolates were from the genera *Acitenobacter*, *Achromobacter* and *Gluconobacter*.

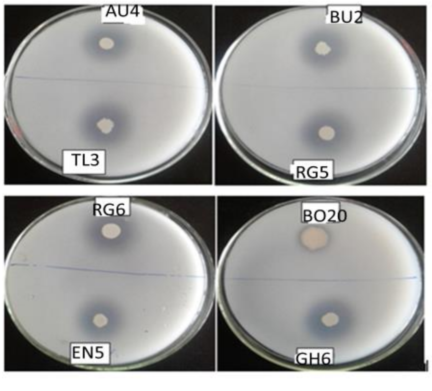

Figure 5.1 some of the bacterial strains exhibited clear halos around colonies on Pikovskaya agar medium supplemented with tricalcium phosphate within five days incubation at 28°C.

5.3.2 Screening phosphate solubilizing bacteria for multiple PGP traits

The data showed that sixteen (16) strains produced IAA as indicated through production of pink coloration (Table 5.1; Appendix 3). Nine of the selected isolates also produced high IAA ranging from 75-314 μg mL^{-1}**.** Thus, *B. thuringiensis* (DM17) produced the largest amount of IAA (314 μg mL^{-1}) followed by *Gluconobacter cerinus* (EN6) that released 266 μg mL^{-1} of IAA in the medium. The smallest amount of IAA (75 μg mL^{-1}) was produced by G. *cerinus* (BU2). This indicates that the *B. thuringiensis* (DM17) was four times more effective in IAA production than G. *cerinus* (BU2). *B. thuringiensis* (DM17) and *Gluconobacter cerinus* (EN6) can be used in the enhancement of growth and development of the host plant for improved agroforestry practices.

The effectiveness of the isolates in IAA production was not, however, as effective as the one reported by Andrade *et al.*(2014) who reported that produced IAA between 0.05 μg/L to 47.88 mg/L. Tariq *et al.*(2014) have also reported that bacterial root endophytes isolated from pea plant synthesized IAA between 0.86 mg/l to 16.16 mg/L which is much higher than ours.

The data also showed that seven and ten of the rhizobacteria produced HCN (Appendix 5) and NH_3 (Appendix 4), respectively (Table 5.1). All the strains of *Acinetobacter soli,* G. *cerinus* (BU2), and *B. thuringiensis* (DM17) were capable of producing both HCN and NH3; whereas *Achromobacter xylosoxidans* (GH6) and *Stenotrophomonas maltophila* (GH10) produced only ammonia (Table 5.1).

Furthermore, out of the rhizobacteria screened for production of hydrolytic enzymes, only 43 %, 33 % and 19 % of the strains produced lipase, protease and chitinase respectively (Table 5.1**)**. The only isolates that showed chitinase activity, which is the most important enzyme with antagonistic property against phytopathogens were *B. thuringiensis* (DM17), *Achromobacter xylosoxidans* (GH6), *Gluconobacte rcerinus* (EN6) and *Acinetobacter soli (RG5)*. All taken together, among the rhizobacteria, all the members of *Acinetobacter soli, Gluconobacter cerinus*; *B. thuringiensis* (DM17) and *Achromobacter xylosoxidans* (GH6) exhibited multiple growth promoting properties tested (Table 5.1).

5.3.3 Determination of inorganic phosphate solubilization efficiency in liquid media

5.3.3.1 Evaluation of inorganic phosphate solubilizing efficiency

The rhizobacteria showed statistically significant ($p<0.05$) differences in the amount of phosphate released from inorganic phosphate sources (Table 5.2). Thus, *Acinetobacter soli* (AU4) produced the highest amount of solubilized phosphate (108.96 mg L^{-1}) from calcium phosphate followed by another strain A. *soli* (RG6) (107.48 mg L^{-1}). However, the smallest amount of solubilized phosphate (100.82 mg L^{-1}) was recorded by *G. cerinus* (TL3) showing significant difference in phosphate solubilization from tricalcium phosphate among the selected species.

Phospate solubilization was closely related to pH changes, and the isolates exhibited a sharp medium pH reduction throughout sampling periods day 0 to day 3 (Table 5.2). The maximum pH drop was recorded by strain *A. soli* (AU4) which had initially 7.00 and reduced to 5.92 followed by strain *A. soli* (RG6) which reduced its medium to pH of 5.93, indicating that phosphate solubilization is associated with medium acidification and drops in the pH values. This could be due to the solubilization of inorganic phosphates by medium acidification is associated with either proton extrusion or organic acid secretion by the bacterial isolates (Diriba Muleta *et al*., 2013; Matos *et al*., 2017).

Table 5.2 Quantification of IAA and solubilized phosphate in NBRIP liquid medium using selected root nodule bacteria isolated from *E. abyssinica*

Isolate	Bacterial taxonomic group	IAA (μg/mL)	$Ca_3(PO4)_2$			$AlPO_4$			$FePO_4$		
			Net P (mg/L) Day 3	Net P (mg/L) increased by	pH Day 3	Net P (mg/L) Day 3	Net P (mg/L) increased by	pH Day 3	Net P (mg/L) Day 3	Net P (mg/L) increased by	pH Day 3
AU4	*Acinetobacter soli*	171.65c	108.96a	4.50	5.92	87.33a	12.40	5.79	84.08a	19.55	5.80
BU2	*Gluconobacter cerinus*	75.07i	106.76cd	4.41	6.02	84.23b	11.96	5.90	80.95b	18.83	6.05
DM17	*Bacillus thuringiensis*	313.61a	104.62ed	4.32	6.04	77.78c	11.05	5.95	78.50d	18.26	5.89
EN5	*Gluconobacter cerinus*	112.57f	102.87f	4.25	5.98	77.32c	10.98	5.92	82.05b	19.08	5.96
EN6	*Gluconobacter cerinus*	266.29b	104.71ed	4.33	6.13	77.10c	10.95	6.0	81.38b	18.93	6.04
GH6	*Achromobacter xylosoxidans*	147.28d	103.73ef	4.28	6.09	64.18e	9.12	6.01	81.10b	18.86	5.94
RG5	*Acinetobacter soli*	120.65e	105.60cd	4.36	5.86	27.12f	3.85	6.03	40.46e	9.41	6.17
RG6	*Acinetobacter soli*	104.55h	107.48b	4.44	5.93	84.12b	11.95	5.82	79.68c	18.53	5.85
TL3	*Gluconobacter cerinus*	106.37g	100.82g	4.16	6.11	74.97d	10.65	5.84	81.71b	19.00	5.98

PVK, Pikovskaya; PSI, Phosphate solubilization index; NBRIP, National Botanical Research Institute Phosphate .Values are expressed as means of three independent experiments. Means sharing the same letters in the same column do not differ significantly at $p \leq 0.05$ by ANOVA Duncan test

All the isolates were able to solubilize $AlPO_4$ in the liquid NBRIP medium (Table 5.2). The largest amount of solubilized phosphate (87.33 mgL-1) was recorded by *A. soli* (AU4) followed by (84.23 mgL-1) exhibited by G. *cerinus* (BU2) and (84.12 mgL-1) by *A. soli* (RG6) (Table 5.2).

The strains exhibited higher inherent $AlPO_4$ solubilization potential (27.12 mg/ml up to 87.33 compared to Chung *et al*.(2005) who reported that the bacterial isolates solubilized (3.7-13.8mg/L) phosphate. Likewise, Son *et al*.(2006) have also reported bacterial isolates from soya bean rhizosphere in Korea which solubilized small amount of phosphate from $AlPO_4$ (19 mg/L) after five days of incubation compared to ours.

The pH of the NBRIP medium supplemented with $AlPO_4$ showed sharp pH reduction (Table 5.2**)**. The largest medium pH drop was recorded by *A. soli* (AU4) that changed the pH from 7.00 to pH 5.79 followed by A. soli (RG6) which dropped the medium pH from 7.00 to pH 5.82. Similar pH dropping patterns were reported by several authors (Son *et al.,* 2006; Diriba Muleta *et al*., 2013; Andrade *et al*., 2014; Dinic *et al*., 2014; Matos *et al*., 2017). There was an inverse relation between drops in the medium pH and the amount of solubilized phosphate ($p<0.05$).

All of the nine isolates were able to grow well in PA medium supplemented with $FePO_4$ as sole phosphate source. The data showed that *A. soli* (AU4) and *G .cerinus* (EN5) produced the highest amount of phosphorus of (84.08 mg L^{-1}) and $^{(}$82.05 mg L^{-1}), respectively (Table 5.2).

The strains exhibited greater solubilized phosphate from $FePO_4$ compared to report by Chung *et al*.(2005) which was in the range between 4.5 and 49.5 µg mL^{-1} and Son *et al.* (2006) who reported 28 mg L^{-1}.

Similarly, the highest pH drops were recorded from A. soli (AU4) from pH 7.00 to pH 5.80. Interestingly, there was no significant correlation between pH drops and the amount of solubilized phosphate from of $FePO_4$. Son *et al*. (2006) tested $FePO_4$ solubilization in NBRIP medium and did not find any relationship between phosphate solubilization and drops in pH values. Other studies also showed the relationship between $FePO_4$ solubilization and Medium acidification with pH drops (Andrade *et al*., 2014; Dinic *et al*., 2014;;Matos *et al*., 2017). The medium acidification could be due to synthesis of diverse organic acids by the strains by utilizing the original carbon sources in the medium. In general, this study did not show correlations between phosphate solubilization indices obtained on the agar plates and the estimated amounts of solubilized phosphate released in liquid medium supplemented with $Ca_3(PO_4)_2$. Based on multiple plant growth promoting properties, isolates A. *soli* (AU4, RG6 and RG5), *A. xylosoxidans* (GH6) and *G. cerinus* (TL3) were selected as effective plant growth promoting rhizobacterria for further studies in the future (Table 5.3). These isolates can be potential candidates for use in the enhancement of growth and improvement of nutritional status of E. *abyssinica* in the greenhouse and field conditions.

Table 5.3 Evaluation of the overall performance of root nodule endophytes for strain selection as potential bioinoculants for growth and production of *E. abyssinica*.

Taxonomic group	Identified to	pH(9)*	NaCl (8)	Temp ºC(7)	C sources (10)	N sources(10)	Antibiotics(6)	Phosphate (3)	IAA(1)	Total (out of 54)	Rank
AU4	*Acinetobacter soli*	4	5	4	5	5	5	2	5	35	1
AU22	*Paenibacilluspeoriae*	4	4	4	4	3	2	2	5	28	8
AU 27	*Bradyrhizobiumshewense*	4	3	3	5	4	3	2	0	24	11
AU37	*Staphylococcus edaphicus*	4	2	3	5	5	3	0	0	22	12
BU1	*Staphylococcus cohnii*	3	5	3	4	4	4	2	5	30	6
BU2	*Gluconobactercerinus*	3	5	2	4	5	4	2	5	30	6
DM17	*Bacillus thuringiensis*	4	5	3	7	5	3	2	5	32	4
DM27	*Enterobacter ludwigii*	4	3	3	4	4	4	2	5	29	7
EN5	*Gluconobactercerinus*	3	5	2	5	5	4	2	5	31	5
EN6	*Gluconobactercerinus*	3	4	2	5	5	4	2	5	30	6
EN7	*Bacillus luti*	3	3	3	4	3	3	0	5	24	11
EN8	*Stenotrophomonas maltophilia*	3	4	3	3	3	3	0	5	24	11
GH6	*Achromobacterxylosoxidans*	5	5	3	5	5	3	2	5	33	3
GH10	*Stenotrophomonas maltophilia*	4	2	3	4	4	4	0	5	26	10
HO2	*Bradyrhizobiumcajani*	4	4	3	5	4	4	0	0	24	11
HO3	*Paenibacilluspeoriae*	4	4	3	3	5	3	0	5	27	9
HU3	*Bradyrhizobiumcytisi*	3	4	3	4	4	4	0	0	22	12
IB5	*Enterobacter ludwigii*	3	2	3	4	5	4	0	0	21	13
RG5	*Acinetobacter soli*	3	5	3	5	5	4	2	5	32	4
RG6	*Acinetobacter soli*	3	5	4	5	5	5	2	5	34	2
TL3	*Gluconobactercerinus*	3	4	3	5	5	4	2	5	31	5

*Numbers in the parenthesis represent the number of tests made for each character, and number 5, 4, 3, 2, 1, and 0 represented that isolates demonstrated 80-100%=5, 60-79%=4, 40-59%=3, 20-39%=2, 1 -19%=1 and 0%=0 of the tested traits.

5.3.4 Conclusion and recommendations

E. abyssinica root nodules harbored a great diversity of soil bacteria with reference to plant growth promoting properties. The drops in the medium pH were concomitant with the increased amount of solubilized phosphate regardless of the phosphate sources. The isolates *A. soli* (AU4 and RG6) exhibited better phosphate solubilization potential. Isolate *A. soli* (AU4) can be selected as potential candidates for bio-inoculant production for the host plant growth enhancement in alkaline and acidic soils. Further and detailed exploration of bacteria with multiple plant growth promoting traits from root nodules and rhizosphere of E . *abyssinica* across its growing ecology is recommended.

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6. The effect of multiple Inoculations of *Bradyrhizobium shewense,* Arbuscular mycorrhizal fungi and rhizobacteria on *Erythrina abyssinica* growth in greenhouse conditions

Belay Berza [1], Marcela C Pagano [2], Prabavathy VR[3] Fassil Assefa [1]

[1]Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia,[2] Department of Biology, Federal University of Minas Gerais, Brazil, 3 Microbiology Laboratory, 3 MS Swaminathan Research Foundation, Tamil Nadu, Chennai, India

Abstract

Nitrogen (N) and phosphorous (P) are the major plant growth limiting nutrients. The legume-rhizobium symbiosis provides N to plants, while Legume-AMF symbiosis enhances P availability. This requires a selection of effective symbiosis to fully realize the potential of such a dual symbiosis in plant growth and production. Therefore, this research was conducted to evaluate the symbiotic effectiveness of different *Bradyrhizobium* spp. in a sand culture and the effects of combined inoculation of the most effective *Bradyrhizobium shewense* (AU27), *Glomus* sp.1 (AMF1), *Acaulospora* sp.1 (AMF2) and *Acinetobacter soli* (AU4) on growth and increase in N and P content of *Erythrina abyssinica* under greenhouse conditions. The experiment was carried out in a completely randomized design in three replications. The results showed that the *Bradyrhizobium* species were symbiotically effective, but *B. shewense* (AU27) was the most effective and selected for multiple inoculation studies. The inoculated plants showed increased shoot length and dry weight by (140%) and (268%), respectively upon multiple inoculation with *B. shewense* (AU27) + *Glomus* sp.1(AMF1) + *Acaulospora* sp.1 (AMF2) + *A. soli* (AU4) compared to the un-inoculated control plants. Similarly, inoculation treatments involved *B. shewense* (AU27) + *Acinetobacter soli* (AU4) increased shoot TN by 260 %, whereas 1200% increment in shoot P content was recorded from the inoculation treatments that consisted of *B. shewense* (AU27)+ *Glomus* sp.1 (AMF1) compared to un-inoculated control plants. The highest nodule number per plant (18.0) was recorded from the inoculation of all the endosymbionts. The same inoculation treatment exhibited the maximum AMF root colonization (54.93%). In general, the combined inoculation of *E. abyssinica* with *B. shewense* (AU27) and/or AMF species and/or *A. soli* improved shoot TN and P and the result showed that microbial inputs could be potential candidates for growth enhancement of *E. abyssinica.*

Keywords: *Acaluspora***;** AMF**;** *B. shewense;* dual inoculation; multiple inoculations

6.1 Introduction

Nitrogen (N), phosphorous (P) and Potassium (K) are the three most important nutrients that determine soil fertility and limit plant growth. However, it is an established fact that plant growth and health is not only determined by the availability of these nutrients, but also by the presence of consortia of microorganisms in the vicinity of the root surface known as the rhizosphere. About 2–5% of rhizosphere microorganisms in a soil contain competitive microflora that exert a beneficial effect on plant growth and are termed as plant growth promoting microorganisms (PGPM) (Kloepper and Schroth, 1978). The plants are engaged in symbiotic relationships with a multitude of above- and belowground beneficial microbes including rhizobia, mycorrhizal fungi, and endophytic microbes.

Most of these microorganisms are bacteria and are known as rhizobacteria. Somers *et al*. (2004) classified these microorganisms based on their roles as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones production), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics, antifungal metabolites, fungal cell wall and its component degrading enzymes). The plant growth promoting rhizobacteria have the potential to produce different types of metabolites that help the host plant to improve minerals such as phosphorus and iron, promote growth and protect them from phytopathogens, and enhance also their tolerance to abiotic stresses (Rodríguez & Fraga, 1999; Bai *et al*., 2017).

The symbiotic association between the leguminous plants and rhizobium leads to biological nitrogen fixation (BNF) (Wu *et al.*, 2005). Arbuscular mycorrhizal fungi (AMF) are present in nearly in all soils by forming a symbiotic association with roots of approximately more than 80%

of plant species (Smith and Read, 2008). AMF provide up to 70% of total P uptake by plants and other immobile nutrients to their plant hosts by producing hyphae that grow out from roots and effectively increase exploratory areas of roots and help in harnessing fixed/immobile P (Suri & Choudhary, 2013). .

The symbiotic association involving rhizobium, AMF and the legume plant is referred to as tripartite symbiosis, and is a mutually beneficial process and plays a pivotal role in natural ecosystems by influencing plant productivity, nutrition, and community structure (Bai *et al.*, 2017). Dual inoculation of leguminous plants with rhizobium and AMF is being suggested as a possible solution to improve legume plant growth, nodulation and to increase shoot N and P content (Zhang *et al.*, 2010; Bai *et al.*, 2017). However, the effectiveness of the Rhizobium-AMF-plant interactions varies with host species, Rhizobium strains, fungus species and soil conditions (Patreze & Cordeiro, 2005; Choudhary & Suri, 2013).

E. abyssinica is a legume woody tree characterized by very important agroforestry attributes such as rapid establishment, tolerance to light, possession of spreading canopy, high rate of litter production, rapid litter decomposition and very soft woody nature (Legesse Negash, 2002; Wassie Haile, 2013). It has been planted in the farmlands in agroforestry systems in southern Ethiopia. Its biomass has been used as crop growth enhancement green manure material. The farmers commonly plant *E. abyssinica* inside their farmlands while cultivating crops such as barley, wheat and maize. The farmers used to prune the *E. abyssinica* plant branches and leaves and buried under soil and sow their grains. The legume is well integrated in Sidama agro-forestry system (Beyene Dobo *et al*, 2016). The plant fixes atmospheric nitrogen to improve soil fertility with root nodule bacteria (Fassil Assefa, 1993; Endalkachew W/Meskel *et al*, 2004). Shasho Megersa and Fassil Assefa (2011) have also shown that the dual inoculation of root nodule bacteria and AMF (*Gigaspora* and/or *Glomus*) improved its production threefold compared to the control plants under greenhouse conditions.

Despite the agro-forestry importance of *E. brucei,* there is still a dearth of information regarding enrichment of *E. abyssinica* biomass with N and P through multiple inoculation with multiple selected and effective endosymbionts to improve growth. Therefore, this study was conducted to investigate the effects of combined inoculation of *B. shewense (*AU27) and *Glomus* sp.1(AMF1) and/or *Acaulospora* sp.1(AMF2) and/or *Acinetobacter soli* (AU4) on nodulation and growth of *E. abyssinica* under greenhouse conditions.

6.2 MATERIALS AND METHODS

6.2.1 Nodulation and Evaluation of Symbiotic effectiveness of *Bradyrhizobium* species

6.2.1.1 Sources of microorganisms

*Bradyrhizobium shewense (*AU27), *Bradyrhizobium. cytisi* (HU3), *Bradyrhizobium. Bradyrhizobium cajani* (HO2), *Glomus* sp.1 (AMF1), *Acaulospora* sp.1 (AMF2), *Acinetobacter soli* (AU4) were obtained from the culture collection of the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. Seeds of *Erythrina abyssinica* were obtained from the Botany Department, Addis Ababa University. The microorganisms were collected and characterized (chapters 3, 4&5). The *Rhizobium* spp and other potent bacteria were deposited in the Genbank data base of NCBI.

6.2.2 Evaluation of symbiotic effectiveness of the three Bradyrhizobium spp**.**

The greenhouse pot experiment was conducted to evaluate nodulation and symbiotic effectiveness at College of Natural Sciences, Addis Ababa University. Seeds were briefly surface sterilized using 70% ethanol for 1 min , followed treated with 3% sodium hypochlorite for 8 mins ans repeatedly washed with water and germinated on 1% water agar (w/v) (Somesegaran and Hoben, 1994). Four to five healthy seedlings were transplanted in to surface sterilized 3 kg capacity plastic pot filled with acid washed coarse river sand**.** The seedlings were thinned down to three per pot after successful establishment. *Bradyrhizobium shewense* (AU27), *Bradyrhizobium cajani* (HO2), and *Bradyrhizobium cytisi (HU3)* were grown into exponential phase on YEM broth at 28°C for 6 days (Vincent, 1970) from which 2 ml (approximately 10^6 cells mL^{-1}) of the suspension was inoculated to each seedling a week after transplanting. Pots without bacterial inoculation were included as negative control and nitrogen fertilized (0.05% KNO3) pots as positive control, respectively.

The experiment was carried out in triplicates and in a completely randomized design in a greenhouse with 12 h photo period and 22^{o}C and 15^{o}C day and night temperatures, respectively. Each pot was fertilized with 50 ml of N-free nutrient solution (Broughton and Dilworth, 1970) at 15 days interval. The N-free nutrient solution consisted of (g/l); KH_2PO_4, 0.136.1; $CaCl_2.2H_2O$, 294.1; $MgSO_4.7H_2O$, 123.3; $FeC_6H_5O_7.3H_2O$, 6.7; K_2SO_4, 87.0; $MnSO_4.H_2O$, 0.338; H_3BO_3, 0.247; $ZnSO_4.7H_2O$, 0.288; $CuSO_4.5H_2O$, 0.1; $CoSO_4.7H_2O$, 0.056; $Na_2MoO_2.2H_2O$, 0.048. The solution pH was adjusted to 7.0 with 1 N NaOH). The positive control pots were fertilized with KNO_3 (0.05%) (W/v) every week.

All pots were watered regularly at two days interval with tap water. Plants were up rooted 90 days after planting (DAP) to collect root nodules and measure plant growth parameters such as nodule number, nodule dry weight, shoot length and shoot dry weights. The relative symbiotic effectiveness (SE) of the each *Bradyrhizobium* species was computed using the formula:

$$SE(\%) = \frac{shootdryweightoftheinoculatedplants}{shootdryweightofNsuppliedplants} x100$$

Where the relative symbiotic effectiveness (SE) values were rated as highly effective (HE) (if % SE value > 80), effective (E) (if % SE value is between 50-80) and ineffective (I) (if % SE value < 35) (Beck *et al*., 1993).

6.2.3 Multiple inoculations Experiment

6.2.3.1 Compatibility test

The compatibility between *B. shewense* (AU27) and *Acinetobacter soli* (AU4) was conducted following cross streaking method (Santiago *et al*., 2017).

6.2.3.2 Microbial inocula preparation

The mycorrhizal species *Glomus* sp.1 (AMF1) and *Acaulospora* sp.1 (AMF2) were selected based on their most common occurrence in the rhizosphere of *E. abyssinica* (Appendix 8). The mycorrhizal inocula were multiplied using maize (*Zea mays*) host plant grown on 4 kg capacity plastic pots filled with sterile soil-sand (2:1) mixture for 90 days (Appendix 9; Fig.6.1**)**. AMF spore density 100 g^{-1} of dry soil was counted using sucrose density gradient centrifugation technique according to the methods described in Brundrett *et al*. (1996). AMF root colonization maize plant was also checked and determined according to the methods described in McGonigle *et al.* (1990). The AMF colonized maize plant roots and the soils were air dried, and roots were chopped into 1cm length and mixed well with the soil-sand mixture (2:1) and served as crude inocula. The crude inocula consisted of 120 spores 100 $g^{-1}$of dry soil.

Figure 6.1 AMF inocula preparation using maize plant.

The *Bradyrhizobium shewense* (AU27) was selected based on its symbiotic effectiveness, whereas the root nodule endophyte *Acinetobacter soli* (AU4) was selected based on its multiple plant growth promoting properties, eco-physiological stress tolerance and versatility in C and N substrate utilization. The two inoculants were grown in YEM broth and nutrient broth, at 28°C for 6 days and 3 days, respectively adjusted to 10^9 cells mL^{-1}.

6.2.3Multiple inoculation Experiment in the greenhouse

The seeds of *E. abyssinica* were surface sterilized and germinated as before and five seedlings were transferred into ten (10) kg capacity pots filled with mixture of soil: sand ratio of 2:1. The soil used in this greenhouse experiment was obtained from Menagesha; Gallica Flower P.L.C farms 20 kms from Addis Ababa. The physicochemical characteristics of the soil were; pH (H_2O) (1:2.5) 7.02; EC 0.13 (us/cm); available P 36.04 mg/kg; K^+ 865.76 mg/kg ; Ca^{++} 4170.80 mg/kg; Mg^{++} 765.93 mg/kg; TN (%) 0.3; OM(%) 5.48. The soil sample nutrient analysis was outsourced to CROPNUTS Laboratory services, Nairobi Kenya.

The seedlings were thinned down to three after establishment to which 150g crude AMF inocula (consisted *Glomus* sp1. And/or *Acaulospora* sp.1) were inoculated in each pot depending upon the treatment.

The greenhouse experiment was arranged in a completely randomized design (CRD) with the following treatments: (T1), Un-inoculated control; (T2), *Bradyrhizobium shewense (*AU27) + *Glomus* sp.1 (AMF1); (T3), *Bradyrhizobium shewense (*AU27) + *Acaulospora* sp.1 (AMF2); (T4), *Bradyrhizobium shewense (*AU27) + *Acinetobacter soli* (AU4); (T5), *Bradyrhizobium shewense (*AU27) + *Glomus* sp.1 (AMF1) + *Acinetobacter soli* (AU4); (T6), *Bradyrhizobium shewense (*AU27) + *Acaulospora* sp.1 (AMF2) + *Acinetobacter soli* (AU4) and (T7), *Bradyrhizobium shewense (*AU27) + *Glomus* sp.1 (AMF1) + *Acaulospora* sp.1 (AMF2) + *Acinetobacter soli* (AU4).

The pots were watered with tap water as required 2-3 times a week for 90 days and also fertilized with N-free nutrient solutions (Broughton and Dilworth, 1970), once in two weeks from N-free nutrient solution excluding the phosphate source (KH_2PO_4). This solution contained the nutrients in the following final concentration (gL^{-1}):$CaCl_2.2H_2O$, 294.1; $FeC_6H_5O_7.3H_2O$, 6.7; $MgSO_4.7H_2O$, 123.3; K_2SO_4, 87.0; $MnSO_4.H_2O$, 0.338; H_3BO_3, 0.247; $ZnSO_4.7H_2O$, 0.288; $CuSO_4.5H_2O$, 0.100; $CoSO_4.7H_2O$, 0.056 and $Na_2MoO_2.2H_2O$, 0.048.

Plants were harvested to collect data on shoot and root length and root dry weight and shoot dry weight, nodule number and AMF root length colonization. The total shoot nitrogen content was determined according the Kjeldahl method described in Hinds & Lowe (1980) and shoot phosphorous was determined according to the method described in Olsen *et al*. (1954). The number of nodules per plant was determined for each treatment with *B. shewense* inoculation and

AMF root colonization was assessed for AMF species inoculated treatments according to McGonigle *et al* (1990).

6.2.4 Statistical analysis

ANOVA was employed to test significant differences in different parameters within and between inoculation treatments using SAS version 9.2. Duncan's multiple range test was conducted to test for mean separation ($p<0.05$).

6.3 RESULTS AND DISCUSSION

6.3.1 Nodulation and symbiotic effectiveness *Bradyrhizobium* species

In this study, the three *Bradyrhizobium* species were evaluated for their symbiotic effectiveness based nodulation and growth parameters (Table 6.1).

Table 6.1 **N**odule number (NN), nodule dry weight (Ndwt), shoot length (SL), and shoot dry weight (Sdwt) and relative symbiotic effectiveness (SE) of *Bradyrhizobium* species from *E. abyssinica* root nodule after 90 days of growth in a greenhouse

S. No	Strains	Taxonomic position	NN	Ndwt (g/ plant^{-1})	SL(cm)	Shdwt (g/plant^{-1})	SE (%)	SE Status
1	HU3	*B. cytisi*	30.00a	0.43b	7.56b	4.05c	94	HE
2	AU27	*B. shewense*	26.33a	0.65a	7.83ba	5.45a	126	HE
3	HO2	*B. cajani*	6.33b	0.28c	6.50ba	3.16d	73	E
4	+ control	-	-	-	8.90a	4.32b	-	-
5	- control	-	-	-	2.53c	1.36e	-	-

HE-Highly effective, E-Effective. Values are expressed as means of triplicate experiments. Means with the same letter in the same column are not significantly different $p \leq 0.05$.

The highest number of nodules per plant (30) was recorded from plants inoculated with *B. cytisi* (HU3) followed by 26 nodules induced by *Bradyrhizobium shewense* (AU27) (Table 6.1). Nodules were big, round and concentrated on tap roots and evenly distributed on lateral roots. The inoculated plants also varied in nodule dry weight ranging from 0.18 g/plant to 0.65 g/plant. Thus, *B. shewense* (AU27) induced the highest nodule dry weight per plant (0.65 g^{-1} plant) followed by *B. cytisi* (HU3) with 0.43 g per plant. In all cases, the two *Bradyrhizobium* spp. performed better than *Bradyrhizobium cajani* (HO2).

The shoot length of the inoculated plants was between 6.5 and 7.83 cm/plant which was much lower than the N-fertilized control plants (8.90 cm). All of them showed 2-3 fold increase in shoot length more than the un-inoculated control plants (Table 6.1). All the inoculants produced significantly (p<0.05) higher shoot dry weight 3.16-5.45 g/plant that were much higher than the un-inoculated and unfertilized control plants (1.36 g/plant). Inoculation with *B. shewense* (AU27) produced significantly higher (p<0.05) shoot dry weigh (5.45 g $plant^{-1}$) followed by *B. cytisi* (HU3) (4.05 g/plant), and *B. cajani* (HO2) (3.16 g/plant).

Based on shoot dry matter accumulation, B. *shewense* (AU27), and *B. cytisi* (HU3) were highly effective (HE); for they accumulated shoot dry matter more than 80% of the N-fertilized plants (Beck *et al*, 1993). However, *B. cajani* (HO2) enabled the host plant to accumulate 73% of the shoot dry matter of the positive control plants, and was rated as effective (E). Other researchers also emphasized that symbiotic nitrogen fixation is directly related to shoot dry weight produced as a result of rhizobium inoculation (Somesegaran and Hoben, 1994; Mwenda *et al*., 2018).

It is interesting to note that inoculation with *B. shewense* (AU27) accumulated shoot dry matter more than the N-fertilized control plants (126%) (Table 6.1), indicating that the inoculant not only fixed nitrogen, but also enhance growth with a possible production of different types of hormones by the *Rhizobium* species. The accumulation of 3.16 -5.45 g/plant by the different bradyrhizobial species in this study, was much higher than the shoot dry weight of 2.49 g per plant obtained from the study by Shasho Megersa and Fassil Assefa (2011). Apart from the inherent effectiveness of the inoculants, the big difference can be attributed to the long growing period (90 days) of the plants in this experiment compared to the 60 days of planting by the other study. Although all the strains were effective and highly effective, *B. shewense* (AU27) and *B. cytisi* (HU3) strains could be used as potential candidates for field trial to enhance the growth of the host plant.

6.3.2 Multiple inoculations Experiment

6.3.2.1 Effects of multiple inoculations on nodule number

The greatest number of nodules per plant (18.0) was recorded from treatments with all inoculants (T7) followed by (17.00) nodules per plant produced with treatment T5 i.e. triple inoculation with *B.shewense* (AU27) + *Glomus* sp.1 (AMF1)+ *Acinetobacter soli* (AU4) (Table 6.2). Between 25 and 50 nodules per plant were recorded during isolation of root nodule bacteria from *E. brucei* rhizosphere soil samples. However, in this multiple inoculation experiment recorded few nodules per plant that could be attributed to the absence of competing ineffective native *E. abyssinica* nodulating bacteria as shown by the lack of nodules in negative controls.

Shasho Megersa and Fassil Assefa (2011) have reported 125-143 root nodules per plant in greenhouse experiment that involved single, dual and multiple inoculations in soil. The number of root nodules produced may be determined by the host variety of the plant and the type of the *Bradyrhizobium* strain (Patreze & Cordeiro, 2005). Hence, the number of nodules produced could depend on the host plant growth stage, the demand of fixed nitrogen and nitrogen fixing efficiency or symbiotic nitrogen fixing efficiency of nodulating rhizobia species.

6.3.2.2 Effects of microbial inoculation on shoot dry weight and shoot length

The effect of dual, triple and multiple inoculations of the selected root nodule bacteria and arbuscular mycorrhiza on growth and production of the host plant were tested in a soil-sand culture under greenhouse conditions (Table 6.2; Appendix 10). The inoculation showed statistically significant (p<0.05) difference in shoot dry weight among treatments involved in dual, triple or multiple inoculations. The highest shoot dry weight (57.67 g) per plant was recorded from the treatment with all inoculants (T7) followed by (51.80 g) with treatment (T5) inoculated with all, but the *Acaulospora* inoculant (Table 6.2).

Dual inoculations with *B. shewense* and AMF (*Glomus* sp.1) (T2) or *Acaulospora* sp.1 (T3) exhibited significantly higher (p<0.05) shoot dry weight compared to dual inoculations with *B. shewense* (AU27) and *Acinetobacter soli* (AU4) (T4). This difference could be due to the fact that AMF infection often results in increased allocation of C to the root system, implying increased root biomass, respiration and mycelia biomass. These root and mycelia mass could explore larger soil volume, beyond the root depletion zone for nutrients, resulting in a higher nutrient uptake rates (Bisht *et al.*, 2009)

In general, dual, triple and multiple inoculations increased shoot dry weight by 66-267.8 % compared to un-inoculated control. The multiple inoculations (T7) and triple inoculations in (T5) increased the shoot dry weight of the host plant by more than twice compared to un-inoculated control. However, Shasho Megersa and Fassil Assefa (2011) have reported 16.9-45.3 % increment in shoot dry weight as result of single, dual and multiple inoculations compared to un-inoculated control in the greenhouse experiment.

The disparities in shoot dry weight in the present study could be attributed to several factors. The soils used in this greenhouse experiment was characterized by a higher organic matter content (5.48%) compared to soil used in Shasho Megersa and Fassil Assefa (2011) which was (1.53%). The higher soil organic matter content has a multifaceted strategy to improve soil quality such as increment in bioavailability of soil nutrients like P (Cui *et al*., 2015). The soil organic matter provides substrates and energy and therefore, increases bioavailability macro and micro nutrients, allowing the maintenance of soil quality and ecosystem functionality (Lal, 2009; Guimaraes *et al*., 2013). These could enhance symbiotic nitrogen fixation and photosynthetic rates. The enhanced nitrogen fixation and photosynthetic rate could be expressed in terms of biomass accumulation as indicated in this particular work by higher shoot dry weight. In addition, relatively higher AMF root colonization in this study might have contributed to the enhanced nitrogen fixation and photosynthetic rate compared to Shasho Megersa and Fassil Assefa (2011).

Inoculations with *B. shewense* + *Glomus* sp.1 (T2) and *B. shewense* + *Acaulospora* sp.1 (T3) increased plant shoot length by 113% and 111% respectively compared to un-inoculated control after 90 DAP. Likewise, the inoculation with *B. shewense* + *A. soli* (T4) increased the shoot length by 103% compared to un-inoculated control at 90 DAP. This indicates that *Glomus* sp.1 or *Acaulospora* sp.1 co-inoculations enhanced plant growth by 40.63% compared to *Acinetobacter soli* co-inoculations. The triple inoculation with *B.shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp.1 (AMF1) (T5) increased shoot length by 130% compared to un-inoculated control at 90 DAP. The highest increase in shoot length of 140% was recorded from the treatment with all inoculants (T7) (Appendix 11).

Table 6.2 The mean shoot and root length, shoot dry weight, nodule number and AMF root length colonization of *E. brucei* inoculated with *B. shewense* (*AU27)* and *Glomus* sp.1(AMF1) and/or *Acaulospora* sp.1(AMF2) and/or *A. soli* (AU4) grown for 90 days in greenhouse conditions.

Treatments	Treatment code	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Nodule number	Root length colonization (%)
Un-inoculated control	T1	32d	21.00e	15.68f	0.00e	25.30c
AU27+AMF1	T2	68.00ba	58.53cb	38.17d	11.00c	37.4b
AU27+AMF2	T3	67.67ba	55.73cd	35.70d	10.67c	38.47b
AU27+AU4	T4	55c	63.00b	26.04e	7.33d	27.20c
AU27+AU4+AMF1	T5	73.67a	76.00a	51.80b	17.00ba	52.10a
AU27+AU4+AMF2	T6	59.67bc	51.67d	44.33c	15.00b	41.73b
AU27+AMF1+AMF2+AU4	T7	76.67a	73.67a	57.67a	18.00a	54.93a

Means with the same letter in the same column are not significantly different $p \leq 0.05$.

In general, the data showed variations in the role of AMF co-inoculation on shoot growth compared to root nodule bacteria co-inoculation. This could be due to differences in rhizosphere function between AMF and root nodule bacteria, i.e., AMF are well known for their absorption and translocation of available P, macro/micronutrients and water beyond the root depletion zone of roots besides organic phosphate solubilization (Bai *et al.*, 2017; Raklami *et al*., 2019). The treatment with all inoculants highly enhanced the growth of *E. abyssinica* growth that may be attributed to the synergetic interaction among *B. shewense*, AMF species and the *Acinetobacter soli*.

Vafadar *et al.* (2014) have indicated that legumes benefit very much from dual inoculations to improve their relative growth, biomass and nutrient assimilation. In the present experiment, the increased shoot length could be as result of inoculation with AMF species and phosphate solubilizing *Acinetobacter soli* which improved plant growth parameters and nutrient uptake.

6.3.2.3 Root length colonization

Mean percentage root length colonization was recorded from dual, triple and multiple inoculation in a greenhouse pot culture (Table 6.2). The percentage of AMF root length colonization varied significantly (p<0.05) among treatments. The average AMF root colonization ranged between 25.3 % in un-inoculated control (T1) plants and 54.93% treatment inoculated with *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus sp.1* (AMF1) + *Acaulospora* sp.1 (AMF2) (T7) (Table 6.2**).** However, there was no significant difference in root length colonization between dually *B. shewense* and AMF inoculated treatments (T2 & T3). However, significant (p<0.05) differences were recorded in root length colonization between triple inoculated treatments (T5 & T6). Statistically significant differences were not observed between triple inoculations that involved *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) + *Acinetobacter soli* (AU4) (T5) and multiple inoculations that involved *B. shewense* (AU27) + *Glomus sp*.1 (AMF1) + *Acaulospora* sp.1 (AMF2)+ *Acinetobacter soli* (AU4) (T7).

Shasho Megersa and Fassil Assefa (2011) have recorded 25-28 % AMF root length colonization in the single, dual and multiple inoculation studies carried out in greenhouse using the same host plant. The higher AMF root length colonization in this study could be attributed partly to the soil physicochemical properties such as available P and soil organic carbon. The soils used in this experiment were characterized by an available P concentration of 27.05 (mg/kg) which falls in medium soil available P range (Hazelton and Murphy, 2007; Landon, 2013). Shasho Megersa and Fassil Assefa (2011) used soils with 37(mg/kg) available P which falls in higher soil available P range (Hazelton and Murphy, 2007; Landon, 2013). In addition, the higher soil organic carbon content (3.18 %) in this experiment might have enhanced AMF root infection compared to soil organic carbon content (0.88 %) in Shasho Megersa and Fassil Assefa (2011).

The root nodule bacterial inocula in this experiment might have assisted the germination of AMF spores, thus leading to higher infection percentage (Wu *et al.*, 2005). Mycorrhize helper bacteria (MHB) could promote mycelia growth, improve host recognition and change root system architecture, improve receptivity of roots (Tarkka and Frey-Klett, 2008). The root nodule bacteria, *Acinetobacter soli* (AU4) inoculum applied in the present study was IAA producer. Hence, this strain might have enhanced *E. abyssinica* root growth and development, which in turn stimulated the AMF root colonization (Tarkka and Frey-Klett,2008).

6.3.2.4 Root length

The effects of inoculating *E. abyssinica* with different inoculants on root length showed that the longest root length (76.0 cm) was recorded from treatment (T5) followed by 73.67 cm root length due to multiple inoculation treatment with all inoculants (T7) (Table 6.2).

The dual inoculations of the host plant with *B. shewense* and *A. soli* (T4) produced longer roots compared to dual inoculations that consisted of *B. shewense* and *Glomus* sp.1 (T2) or *Acaulospora* sp.1 (T3). This difference could be attributed to the phytohormone production by *Acinetobacter soli* that probably enhanced root extension, growth and development. The triple and multiple inoculations in treatments (T5) and (T7) increased the root length by 261.9% and 250.8 % respectively compared to un-inoculated control plants. The stimulation of root hairs growth and lateral roots elongation by secreting IAA provides more active sites and access for symbiotic association with rhizobia and AMF to improve root architecture, length and dry weight (Driss *et al.,* 2015).

6.3.2.5 Shoot nitrogen and phosphorous

The dual, triple and multiple inoculations resulted in statistically significant (p<0.05) difference in shoot nitrogen and phosphorous contents (Table 6.3**).** The shoot total nitrogen (TN) content was varied between 2.72% in *B. shewense* and *Acaulospora* sp.1 (T3) inoculated treatments and 4.50% in *B. shewense* and *Acinetobacter soli* treatments (T4).

Table 6.3 Shoot total nitrogen (TN) (%) and phosphorus (P) (%) of *E. abyssinica* dual, triple and multiple inoculated with *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) and/or *Acaulospora* sp.1 (AMF2) and /or *A. soli* (AU4) and grown in greenhouse for 90 days.

Treatments	Treatment code	Shoot nitrogen	Shoot phosphorus
Un-inoculated control	T1	1.25g	0.12f
AU27+AU4	T4	4.50a	1.25c
AU27+AU4+AMF1	T5	3.35d	1.04e
AU27+AU4+AMF2	T6	3.93b	1.04e
AU27+AMF1	T2	2.82e	1.56a
AU27+AMF2	T3	2.72f	1.49b
AU27+AMF1+AMF2+AU4	T7	3.72c	1.18d

Means with the same letter in the same column are not significantly different $p \leq 0.05$.

Dual and triple inoculations of *E. abyssinica* with *B. shewense* + *A. soli* (T4) and *B. shewense* + *Acaulospora sp.1* + *A. soli* (T6) increased the shoot TN content by 260 %, and 214.4 % respectively compared to un-inoculated control.

The higher shoot TN content in the dual inoculated treatments might be due to a higher nitrogenase activity as a result of better P nutrition, further corroborating enhanced nitrogen fixation (Bai *et al.*, 2017). The higher shoot TN and P content could allow for increased shoot growth and photosynthetic rates and lead to increased TN and P levels resulted from the improved biological nitrogen fixation (Vafadar *et al.*, 2014).

Likewise, shoot P content significantly ($p<0.05$) differed among dual, triple and multiple inoculated treatments (Table 6.3). The largest shoot P content (1.56%) was recorded in treatment (T2) followed by 1.49% in treatment (T3).

The inoculation treatments that involved *B. shewense* +*Glomus* sp.1 (T2) and *B. shewense* + *Acaulospora* sp.1 (T3) increased the shoot P content by 1200 % and 1141.6 % respectively, compared to un-inoculated control. Though, *Acintobacter soli* (AU4) is good inorganic phosphate solubilizer strain, inoculations with *Glomus* sp.1 (AMF1) or *Acaulospora* sp.1 (AMF2) enhanced better shoot P accumulation compared to inoculation with *B. shewense* (AU27) and *A. soli* (AU4). Therefore, inoculation of AMF contributed for the higher shoot P accumulation. This difference could be attributed to increased available P exploration beyond the depletion zone and translocation to the plant roots by AMF compared to PGPR which played role in inorganic P solubilization (Bai *et al.*, 2017; Raklami *et al.*, 2019).

6.3 Conclusion and recommendation

In this experiment, all the three *Bradyrhizobium* species were effective nitrogen fixers, however, AU27 (*B.shewense*) is the best performer. Synergetic interactions among the microsymbionts and macrosymbiont were observed. The triple and multiple inoculations of *E. brucei* with *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) and *Acaulospora* sp.1 (AMF2), and *Acinetobacter soli (AU4)* improved shoot length and dry weight. Moreover, TN content was enhanced and the plant shoot was enriched as result of dual, triple or multiple inoculations. Similarly, shoot P content was improved as a result of dual, triple or multiple inoculations. The evaluation of the performance of the microbial inputs; B. *shewense* + *A. soli* and *B.shewense* + *A. soli* + *Acaulospora* sp.1 in the case nitrogen fixation and B. *shewense* +*Glomus* sp.1 and B. *shewense* + *Acaulospora* sp.1 for P accumulation in the field condition is recommended.

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7. General Conclusions and Recommendations

7.1 Conclusions

- In this study, eleven AMF genera consisted of 33 species associated to *E. brucei* are recovered.
- Higher AMF spore density is recorded 100 g $^{-1}$ dry soil from trap cultures compared to field soils.
- AMF species richness is affected by both land use types and geographic locations
- Phylogenetically and functionally diverse soil bacteria are inhabited in the root nodules of *E. abyssinica*.
- Nine bacterial genera comprised of 13 species are recovered from the root nodules of *E. abyssinica* from which seven genera are the first reports from the root nodules of the host plant. *Bradyrhizobium* species *B. cajani* and *B. cytisi* are also the first reports from this plant and Ethiopia as well.
- E. brucei root nodules harbored ecologically competitive and nutritionally versatile bacterial species.

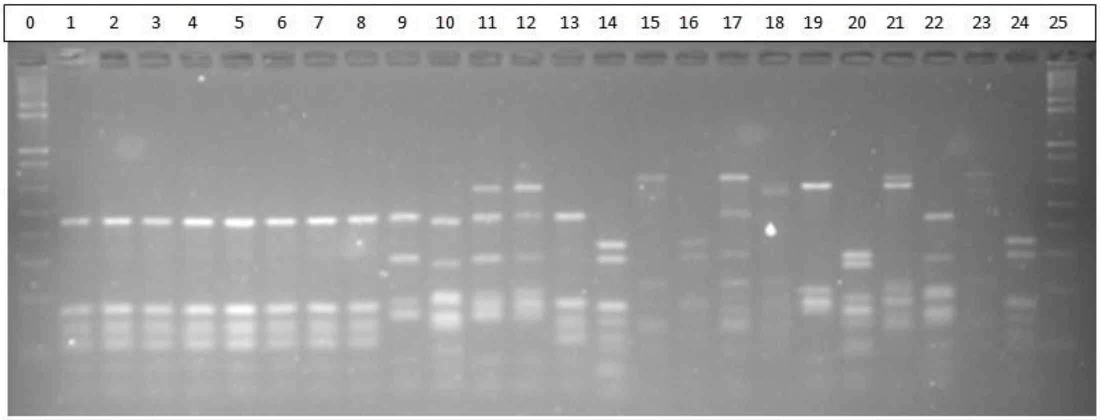
- *B. shewense* is symbiotically highly effective compared to *B. cajani* and *B.cytisi*.
- Synergistic interaction is observed among the microsymbionts and the host plant.
- Multiple inoculations enhanced *E. abyssinica* growth and development, biomass production and nitrogen fixation. It also improved phosphorus uptake by the host plant.

7.2 Recommendations

- More detailed exploration of AMF species diversity and richness consisting of other *E.brucei* growing locations including different seasons is needed.
- More studies are required to explore the rhizosphere of *E.* spp particularly, in those places where nodule induction is not occurred.
- Evaluation of the performance of the microbial inputs which exhibited potential performance in greenhouse needs evaluation in the field condition.

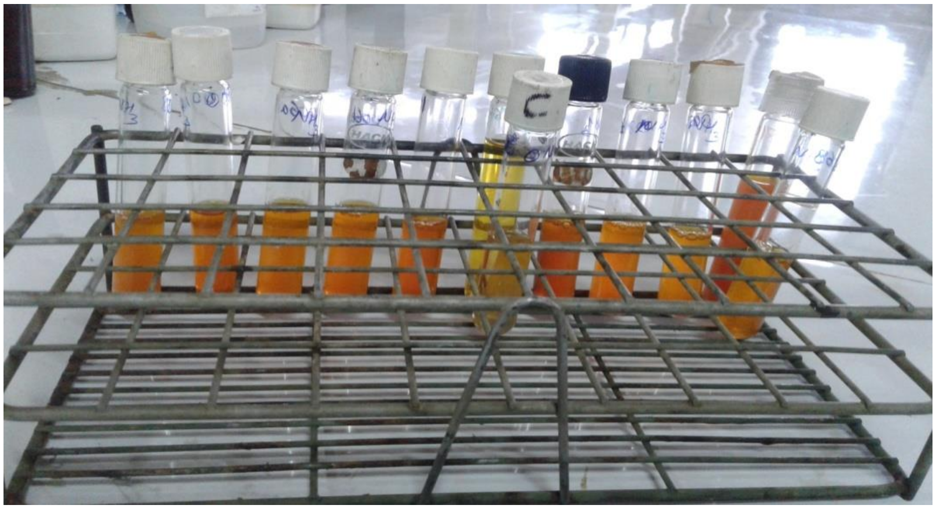
Appendices

Appendix 1 Trap culture experiment using plant infection method

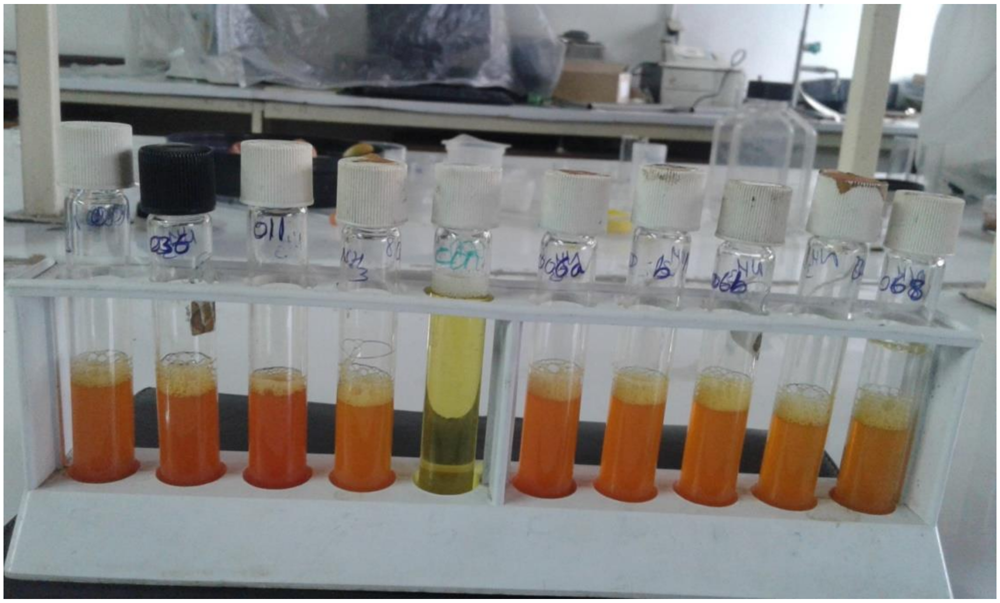


0-, Marker(1kb) ,1-IB4 , 2-IB6, 3- EN4, 4-HO5, 5- BU2 ,6-HU5, 7-BU4 , 8-HU6 ,9-AU27 , 10- HO3, 11- HO2, 12-BU1 ,13-AU24 , 14- DM27,15-EN2, 16-HO6 ,17-GH10 , 18- IB9 , 19-AU37 , 20- HU3 , 21- EN7 , 22- AU22 ,23- EN8 , 24- IB5 , 25-,Marker (1kb)

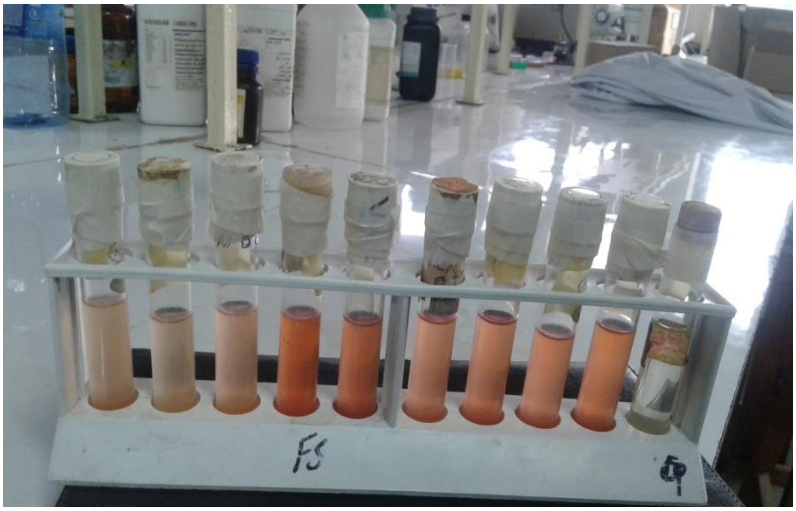
Appendix 2 Amplified Ribosomal DNA Restriction Analysis (ARDRA) of *E. brucei* root nodule bacteria



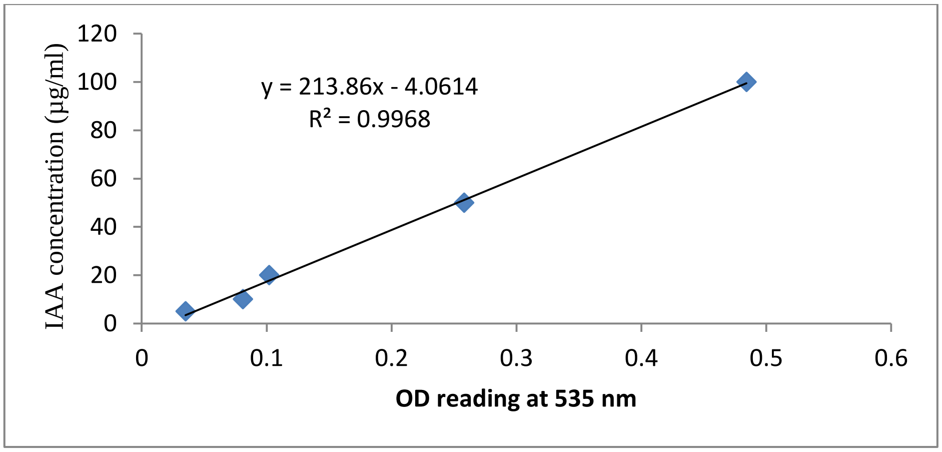
Appendix 3 IAA production evaluation experiment by using *E.brucei* root nodule bacteria



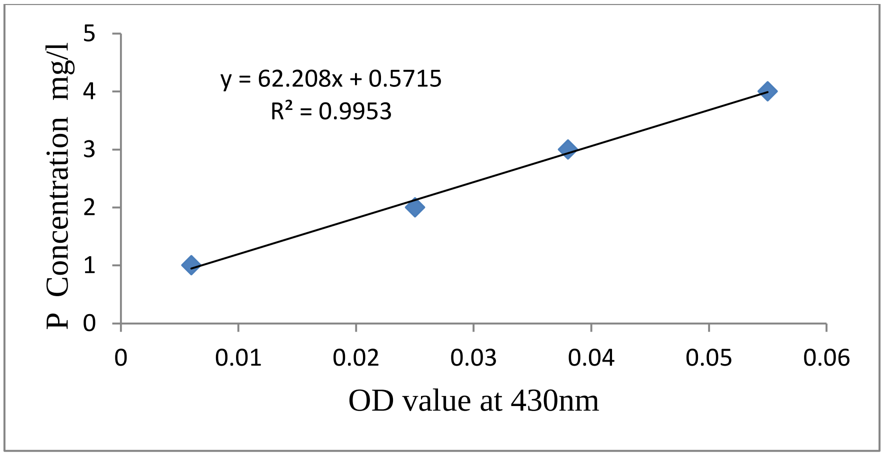
Appendix 4 Ammonia production test by using *E.brucei* root nodule bacteria



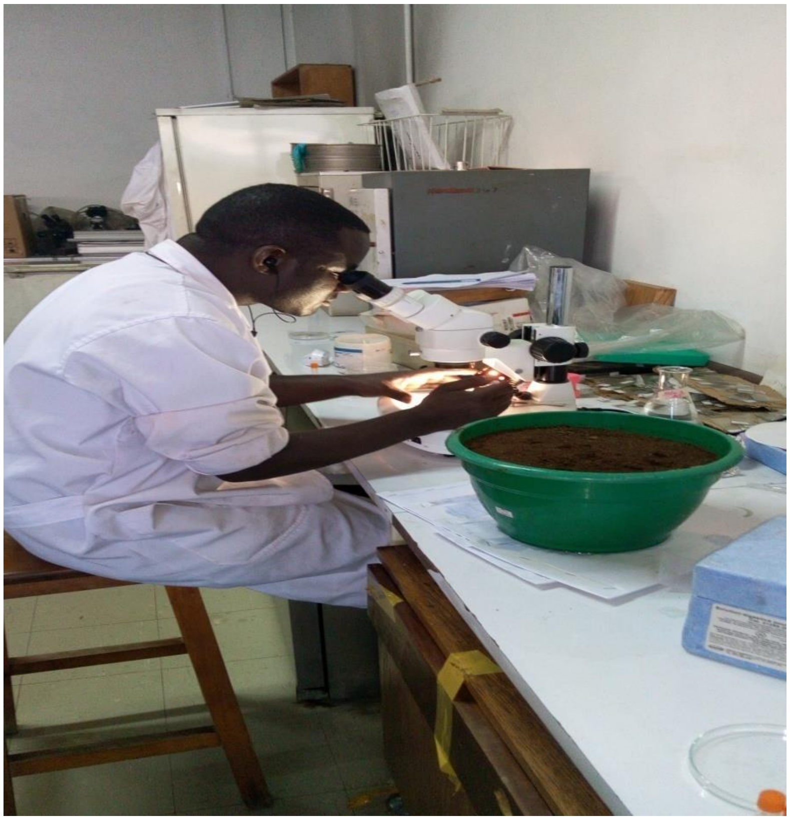
Appendix 5 HCN production experiment by using *E.brucei* root nodule bacteria



Appendix 6 Standard curve for IAA quantification



Appendix 7 Standard curve for quantification of solubilized inorganic phosphate



Appendix 8 Inoculation of maize plant with AMF spore inocula for AMF multiplication

Appendix 9 AMF multiplication using maize plant

Appendix 10   multiple inoculation experiment using the host plant at 30 days after planting.

Appendix 11 multiple inoculation experiment using the host plant at 90 days after planting.