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Nodulation Pattern and Biodiversity of Endosymbionts of Some Woody Legumes of Ethiopia

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By

Diriba Temesgen

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Abbreviations

ANOVA	Analysis of variance
BNF	Biological Nitrogen Fixation
BCP	Bromocresol purple
BTB	Bromthymol Blue
^{o}C	degree celicius
cfu	colony forming unit
CR	Congo Red
e^{-}	electron
Fig	Figure
IAR	Intrinsic Antibiotic Resistance
l	litre
log	logarithm
Mg ATP	Magnesium Adenosine Triphosphate
µg	microgram
mg	milli gram
ml	milli litre
MoFe protein	Molybdenum iron protein
N	Normal
OD	Optical Density
pH	Potential of Hydrogen
rev/min	revolution/minute
SPSS	Statistical Package for Social Science
UPGMA	Unweighed Pair Group Method with the Average
YEMA	Yeast Extract Mannitol Agar
YEM broth	Yeast Extract Mannitol broth
YLA	Yeast Lactose Agar
v/v	volume/volume
w/v	weight/volume

Abstract

A collection of 21 root nodule bacteria were isolated from some woody legumes that belong to 8 genera from various regions of Ethiopia. The isolates were presumptively tested to check contaminations followed by morphological and physiological characterizations, and numerical analysis of phenotypic data using a computer cluster analysis applying UPGMA (NT-SYS version 2.1). Isolates of *Erythrina brucei* and a species of *Crotalaria, Indigofera* and *Tephrosia* were selected as representatives and cross-inoculated on *Phaseolus vulgaris* and *Vigna unguiculata* for authentication and symbiotic effectiveness evaluation .The isolates showed a wide range of morphological and physiological characterstics. Most of the isolates were found to be slow growing, NaCl and streptomycin sulfate sensitive but able to utilize a wide range of carbohydrates, and grow over a wider temperature and pH ranges. The analysis of phenotypic data revealed that at a boundary level of 64.8% average similarity, all the isolates were grouped in to 3 clusters while at 92.2% the isolates were further grouped in to 5 clusters except 8 isolates. All the four cross-inoculated representative isolates elicited nodulation on *Phaseolus vulgaris* but one of them failed to nodulate *Vigna unguiculata*, which might be due to genetic incompatibility. An isolate of Crotalaria species showed the highest symbiotic effectiveness with both *Vigna unguiculata* and *Phaseolus vulgaris* suggesting that the isolate is worthy of further field testing and of great potential application in improving the yield of these grain legumes.

Declaration

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

Name: Diriba Temesgen

Place: Addis Ababa University

Signature……

Date…….

This thesis has been submitted for examination with my approval as

Advisor: Fassil Assefa, Ph.D

Signature……...

Date……...

1. Introduction

Leguminous plants are classified in to one of the largest families of flowering plants, Leguminosae (Fabaceae).This family consists of three subfamilies (Mimosoideae, Caesalpinoideae and Papilionoideae) with 670 to 750 genera and about 20,000 species of plants (Gepts *et al*., 2005).In Ethiopian flora, 621 species of leguminous plants have been recorded, of which 553 species are indigenous, 48 species are endemic and the rest are introduced(Thulin, 1989).The subfamily of Caesalpinoideae and Mimosoideae are represented by 55 and 80 species, respectively. The remaining are members of the subfamily of Papilionoideae (Thulin, 1989).

It is estimated that 30% of the species of the family of leguminosae are trees and shrubs of tropical and subtropical regions (Allen and Allen, 1981). These trees and shrubs are multipurpose plants associated with the welfare of the people of the regions. They provide high heat value fuel wood and charcoal, wood for poles, fence, pulp, construction, food, medicine, tannin, gumarabic and windbreak rows. Leguminous trees and shrubs constitute most of the main browse species for domestic and wild animals of inter tropical Africa. They are also used as a canopy cover and light shade for animals and understorey vegetation (example- coffee, tea and cocoa).

Leguminous trees and shrubs are widely distributed in all tropical soils displaying a wide range of tolerance to acidity, alkalinity, water logging, mineral deficiency and drought (Rengel, 2002).They are often fast growers, and constitute the key components of the pioneers of ecological succession in the rehabilitation of degraded and marginalized soils (Fassil Assefa, 1993; Herrera *et al*., 1993).

Leguminous plants are also important in low-input agroforestry and plantation forestry due to the fact that they form an endosymbiotic association with rhizobia and fix atmospheric nitrogen. The fixed nitrogen subsequently transfers to non-fixing associated plants as root exudate or as plant and animal residues after microbial transformations. However, due to some inherent genetic and ecological factors, a number of leguminous plants lack the capacity to nodulate and fix nitrogen. Among the legumes screened for nodulation so far, 23% of the species in Caesalpinoideae, 90% in Mimosoideae, and 97% in Papilionoideae have been found to nodulate (de Faria *et al*., 1989).

Although the Ethiopian high and low lands are relatively rich in legumes (Thulin,1989),the work on their nodulation status and nitrogen fixation potential is very scarce covering not more than 10% of the country's legumes(Fassil Assefa,1993). Moreover, the works were concentrated on food and forage legumes such as peas, beans, chickpeas, lentils, medics and alfalfa rather than woody legumes that offer several advantages over the food and forage legumes with regard to the effort of rehabilitating the degraded and marginalized soils (Fassil Assefa,1993).Trees and shrubs can persist heavy grazing, extract nutrients from deeper soil horizons and accumulate wind blown organic residues.

Recently, the nodulation analyses of some species of the Ethiopian leguminous trees and shrubs of agroforestry importance were made (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1997; Endalkachew Wolde-meskel *et al*., 2004; Endalkachew Wolde-meskel *et al*., 2005). Rhizobial isolates from tropical woody legumes were also found to promiscuously nodulate tropical pulse crops such as common bean (*Phaseolus vulgaris*), ground nut (*Arachis hypogea*), soybean (*Glycine max*), African yam bean (*Sphenostylis stenocarpa*) (Allen and Allen, 1981; Wang, 1989; Fassil Assefa and Kleiner, 1997).Some of the strains were found to be better nitrogen fixers under acidic conditions and high temperature regimes (Wang, 1989).

More work on the nodulation status and biodiversity of the endosymbionts of leguminous trees and shrubs of Ethiopia should be done since further screening for nodulation of wild legumes from their well-adapted indigenous environments serves not only as an additional reservoir to select high N-fixing strains for BNF technology in sustainable agriculture and environmental management but also as a source of more strains for taxonomic studies to clarify the hitherto blurred information regarding the phylogenetic relationships amongst the root nodule bacteria. Moreover, these legumes possess the ability to colonize marginalized and impoverished soils, and thus recommended for introducing agroforestry, aforestation and re-forestation programs (Dommergues, 1988) which Ethiopia needs to implement to rehabilitate its degraded land masses.

The success of the application of the rhizobial-legume symbiosis in sustainable agriculture and environmental management depends on the isolation, identification and characterization of strains of rhizobia to select those strains which are well adapted to the soil niche of the host legumes and

also compatible to broad-host range for nodule formation and efficient nitrogen fixation. Accordingly, in the current study, root nodule bacteria were isolated from some agronomically and ecologically important, widely distributed and most diversified woody legumes of Ethiopia: Crotalaria, Indigofera, Tephrosia, Aeschynomene, Erythrina, Lupinus, Dalbergia and Chamaecytisus with the following objectives.

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2. Objectives

2.1 General objective

- To identify the nodulation pattern of root nodule bacteria from some indigenous woody legumes of Ethiopia.

2.2. Specific objectives

- To study the morphological and physiological characters of the rhizobial isolates of the woody legumes
- To evaluate the cross-inoculation pattern and symbiotic effectiveness of some of the isolates on cow pea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*).

3. Literature review

3.1 Legumes and their multiple significances

Legumes are flowering plants broadly defined by their unusual flower structure, podded fruit and the ability of many of the examined species to form nodules with rhizobia (de Far*i*a *et al*., 1989). Legumes belong to the plant family, Fabaceae (Leguminosae) with about 20,000 species (Allen and Allen, 1981; Sprent, 1995; Moreira *et al*., 1998) distributed into three subfamilies: Mimosoideae, Caesalpinoideae and Papilionoideae. The subfamily papilionoideae includes grain legumes and species of trees, shrubs, herbs and climbers while the other two subfamilies include species of trees and shrubs and rarely herbs. Nodulation is estimated to occur in about 97% of papilionoideae, 90% of Mimosoideae and 23% of Caesalpinioideae (de Faria *et al*., 1989).

Legumes have multiple roles and have been used by mankind since antiquity as a source of food and forage (Howieson *et al*., 2000). Legumes play a critical role in natural ecosystems, agriculture and agroforestry where their ability to fix nitrogen in symbiosis with rhizobia makes them excellent colonizers of low-nitrogen environments(Graham and Vance, 2003).The importances of legumes are briefly described as follows.

Grain and Pasture production

Grain legumes (beans, peas, lentils, etc) alone provide 33% of the dietary protein needs of humans (Vance *et al*., 2000). Legumes, predominantly soybean and peanut, provide more than 35% of the worlds processed vegetable oils.

Forage legumes such as alfalfa (*Medicago sativa*), *Trifolium species* and vetches (*Vicia spp*.) are rich sources of proteins, fibers and energy so that they have been the foundation of the dairy and meat for centuries. Species from the genera *Aeschynomene, Desmodium, Arachis* and *Centrosema* offer promise for improved tropical pasture system (Thomas and Sumberg, 1995)

Agro forestry

Agro forestry is broadly defined as a farming practice that allows the cultivation of crops in close association to multipurpose trees (Palm, 1995). Food security issues, pressure on the land and increasing soil degradation have led to increasing research interest in tree fallow and alley-cropping systems for subsistence farmers in Africa and Asia (Buresh and Cooper, 1999).In tree fallows, species of *Sesbania, Leucaena, Tephrosia, Crotalaria, Glyricidia* and *Cajanus* are inter-planted into crops such as maize, and the wood is harvested while the N-rich leaves, pods and green stem materials are hoed into soil just before rainy season (Sanchez, 1999).

In alley cropping, crops are grown between hedgerows of trees and tree prunings are used as mulch or green manure resulting in higher crop yield. For example, *Phaseolus species* are grown between *Sesbania species*. *Sesbsnia sesban* is widely distributed in Sub Sahara Africa (Azene Bekele *et al*., 1993) and it is among the priority species identified for use in agroforesty system (Owino, 1992). *Erythrina brucei* is a leguminous tree endemic to Ethiopia with desirable agroforestry attributes such as fast establishment, presence of spreading canopy and production of large quantity of foilage after drought that decompose rapidly (Legese Negash, 2002).

Natural Ecosystem

Legumes, by virtue of their capacity to fix nitrogen, are widely distributed in all tropical soils displaying a wide range of tolerance to acidity, alkalinity, water logging, mineral deficiency and drought (Rengel, 2002). Consequently, they can colonize marginal lands and impoverished soils and thereby offer several advantages in rehabilitating degraded and marginalized soils (Fassil Assefa, 1993; Arianoutsou and Thanos, 1996).Legumes are environmentally friendly plants since all the nitrogen they fix in association with the rhizobia is assimilated by the plants with no leaching and ground water pollution.

Industrial and Medical uses

Industrially, legumes have been used to prepare biodegradable plastics, oils, gums, dyes and inks (Morris, 1997). Many legumes have been used in folk medicines. Isoflavones from soybeans have recently been suggested to reduce the risk of cancer and serum cholesterol (Kennedy, 1995) while phytoestrogen from soybean and soy food have been suggested as possible alternative to hormone replacement therapy for post menopausal woman (Graham and Vance, 2003).

Biological nitrogen fixation

The symbiotic association between rhizobia and legumes plays a significant role in world agricultural productivity by annually converting approximately 120 million metric tons of atmospheric nitrogen in to ammonia (Freiberg *et al.,* 1997) there by saving $ US 6.8 billion expenditure on nitrogenous fertilizers(Herridge and Rose, 2000). Moreover, organic production regulations prohibit the use of synthetic nitrogen fertilizers so that farmers are dependent on N_2- sources accepted by organic certification agencies. For example, the leguminous tree of the genus *Inga* is thought to be crucial in providing nitrogen to certified organic coffee production that commonly grow beneath its shade in the state of Chiapas, Mexico (Grossman *et al*., 2005).

3.2. The Rhizobia

Rhizobia were first identified as a source of fixed nitrogen in root nodules of legumes in 1888 (Hirsch *et al*., 2001). Rhizobia are bacteria that establish symbiosis with legumes, forming root or stem nodules and fixing atmospheric nitrogen (Bala and Giller, 2001; Quatrini *et al.*, 2002). They are gram negative, motile rod shaped bacteria which are pleomorphic under adverse growth conditions (Jordan, 1984). Rhizobia promote plant development through nitrogen fixation and production of phytohormones such as auxins, cytokinins and abscisic acid (Matiru and Dakora, 2004).

Rhizobial strains form nodules with only specific species of legumes or with wide varieties of legumes. *Rhizobium leguminosarum* bv *trifoli* initiates nodules only on species of *Trifolium* while *Sinorhizobium meliloti* effectively nodulates species of *Medicago, Melilotus* and *Trigonella* (Hirsch *et al*., 2001). Rhizobium strain NGR 234 nodulates 232 species of legumes from 112 genera tested and even non-legumes, *Parasponia* and *Tremm*a (Akkerman and Van Dijk, 1981). The nodulation of *Parasponia* and *Tremm*a by both *Rhizobium* and *Bradyrhizobium* strains

provides an insight to a prospect and research for rhizobial nodulation of non-legume cereals (Matiru and Dakora, 2004).

A few aquatic legumes bear stem nodules in addition to the normal root nodules. Stem nodulation is peculiarly restricted to a few species of *Neptunia, Aeschynomene* and *Sesbania* (Subba Roa *et al*., 1981; Tomekpe *et al*., 1991). Stem nodulation is an important adaptation to avoid anoxia stress in flooded or wetland environments (Diekmann *et al*., 1996) and widely used in agroforestry involving paddy rice or other wetland crops (Subba Roa and Rodriquez-Barrueco, 1993).

Fig.1.Stem nodules on *Sesbania rostrata* (Goormachtig *et al*., 2004)

3.2.1 Rhizobial diversity

The strains of rhizobia inhabiting a particular soil may be diverse in symbiotic characters as well as in phenotypic and genotypic characters. Cropping history, the degree of disturbance of an environment and the range of legume species of an area can influence the diversity of rhizobial strains of an area (Brockwell *et al*., 1995).

Prior to the molecular era, rhizobial strain diversity studies were mainly based on cross-inoculation and phenotypic characters (Schwinghamer and Dudman, 1980). Phenotypic methods provide a valuable insight into rhizobial strain diversity but they have low discriminatory power compared to the molecular methods (Mullen and Wollum, 1989; Bottomley, 1992) and give a poor correlation between strain grouping (Roughley *et al*., 1992; Van Rossum *et al*., 1995). Thus, other methods

such as substrate utilization, protein profiling and multilocus enzyme elctrophoresis become prominent in rhizobial diversity study (Graham *et al*, 1994; Van Rossum *et al.,* 1995).

Genetic methods generally have higher discriminatory power and are faster than most phenotypic methods (Handley *et al*., 1998) but some of them especially polymerase chain reaction (PCR) based techniques are reported to have low reproducibility and greatly dependent on colony age, source and concentration of reagents and also on DNA purity and extraction protocol(Sato *et al.*,1999).

3.2.2. An overview of rhizobial taxonomy

Early classification of rhizobia was based on the cross inoculation group concept, which grouped rhizobia on the basis of their ability specifically to infect and fix nitrogen with a discrete group of legumes. This means, closely related legumes are nodulated by one species of rhizobium .This concept has gradually been disproven as more genetic information has been obtained and rhizobial classification should adjust to the general bacterial taxonomy and include a panel of genomic, phenotypic and phylogenic features instead of sole nodulation properties (Zakhir and Lajudie, 2001).

Until recently, inspite of the large diversity of leguminous hosts available for microsymbiont, all the symbiotic nitrogen fixing bacteria from leguminous plants were classified into a single genus, *Rhizobium* (Vincent, 1974). Moreover, only two genera (*Rhizobium* and *Bradyrhizobium*) were described in the first edition of Bergey's Manual of Systematic Bacteriology (Jordan, 1984; Rome *et al*., 1996).

Rhizobial taxonomy and systematics has progressed notably in the last decade mainly due to the characterization of new isolates together with the general use of 16s rRNA sequencing and polyphasic taxonomic approaches (van Berkum and Eardly, 1998; Zakhir and Lajudie, 2001). Since taxonomy of root and stem nodule bacteria has been expanding, recently six genera have been distinguished : *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium* and *Allorhizobium* (Rengel, 2002).These genera were once divided into three categories, the

Rhizobium, Sinorhizobium,Mesorhizobium and Allorhizobium branch,the Azorhizobium branch and the Bradyrhizobium branch.

A very interesting recent report is the finding that some highly specific nodule isolates from African *Crotalaria species* are found to be phylogenetically similar to the methylotrophic bacterial genus, *Methylobacterium* (Sy *et al*., 2001). These rhizobia grow facultatively on methanol and constitute a new discovered fourth phylogenetic branch of rhizobia (Fig.2) within the alpha – 2 – subclass of the proteobacteria.

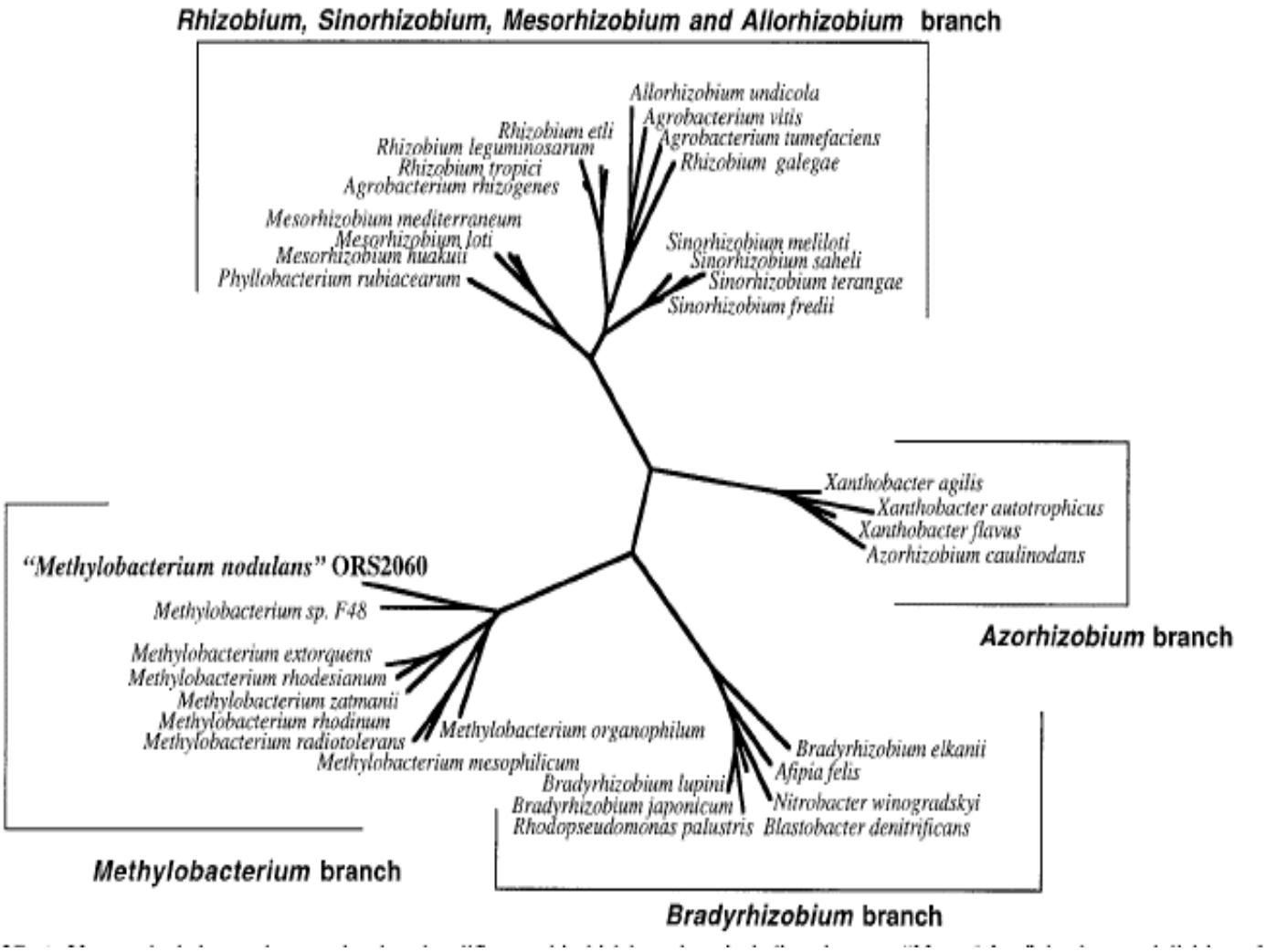


Fig.2. Unrooted phylogenetic tree showing different rhizobial branches in the α-subdivision of Proteobacteria (SY *et al*., 2001)

In the context of rhizobia, considerable changes in taxonomic status have come about during the past years so that Sahgal and Johri (2003) outlined the current status of rhizobial taxonomy and listed 36 species distributed among the seven genera (Table 1 *)* derived based on the polyphasic taxonomic approach.

Table 1. Current status of rhizobial taxonomy (Sahgal and Johri**,** 2003)

Genus	Species	Host
Allorhizobium	*A. undicola*	*Neptunia natans*
Azorhizobium	*Az. caulinodans*	*Sesbania rostrata*
Bradyrhizobium	*B. elkanii*	*Glycine max*
	B. japonicum	*G. max*
	B. liaoningense	*G. max*
Mesorhizobium	*M. amorphae*	*Amorpha fruticosa*
	M. chacoense	*Prosopis alba*
	M. ciceri	*Cicer arietinum*
	M. huakuii	*Astragalus*
	M. loti	*Loti*
	M. mediterraneum	*Cicer arietinum*
	M. plurifarium	*Acacia, Leucaena*
	M. tianshanense	*Glycyrrhiza, Sophora* and *Glycine*
Methylobacterium	*M. nodulans*	*Crotalaria and pedocarpa*
Rhizobium	*R. etli*	*Phaseolus vulgaris*
	R. galegae	*Galega*
	R. gallicum	*P. vulgaris*
	R. giardinii	*P. vulgaris*
	R. hainanense	*Centrosema, Desmodium, Stylosanthes, Tephrosia*
	R. huautlense	*Sesbania herbacea*
	R. leguminosarum	*Trifolium, Vicia*
	R. mongolense	*Medicago, ruthenica*
	R. phaseoli	*P. vulgaris*
	R. sullae	*Hedysarum hedysari*
	R. tropici	*Leucaena, P. vulgaris*
	R. trifolii	*Trifolium*
	R. yanglingense	*Amphicarpaea, Trisperma Corollina varia* and *Gueldenstaedtia multiflora*
Sinorhizobium	*S. arboris*	*Acacia senegal Prosopis chilensis*
	S. fredii	*G. max*
	S. kostiense	*A . senegal, P. chilensis*
	S. medicae	*Medicago* spp.
	S. meliloti	*Medicago sativa*
	S. saheli	*Sesbania*
	S. terangae	*Acacia, Sesbania*

3.2.3 Use of rhizobia as biofertilizers

The agricultural benefits possible from use of selected high N_2-fixing strains of rhizobia can be realized when farmers obtain and properly utilize high quality inoculum (Somasegaran and Hoben, 1994). Rhizobial inoculum can be produced using cheap and readily available substrates such as molasses, corn steep liquor and proteolyzed pea hask.

According to Brockwell *et al.* (1995) and Howieson (1999) rhizobial strains suitable as inoculant must have the following characterstics: able to colonize the soil and tolerate environmental stresses, able to compete with populations of back ground rhizobia to form nodules, able to have no deleterious effects on non-target hosts. Genetic stability and satisfactory growth and survival under inoculant manufacturing and broader edaphic conditions are also reported to be other characters of suitable rhizobial inoculum.

Most rhizobial inoculants are mixed with finely milled, neutralized carrier materials such as peat, Vermiculite, polyacrylamide and charcoal (Somasegaran and Hoben, 1994). Somasegeran and Hoben (1994) also listed desirable properties of rhizobial inoculant carrier materials as non-toxicity to rhizobia, good capacity of moisture absorption, pH buffering and adhesion to seed, inexpensiveness and availability in adequate amount and easy to process and sterilize.

3.3 Biological nitrogen fixation

Biological nitrogen fixation (BNF) is a complex biochemical reaction where by the inert atmospheric N_2 is enzymatically reduced into utilizable form for plants by nitrogenase enzyme complex. BNF is carried out by some species of microorganisms, all which belong to a biological group known as prokaryotes (Zahran, 1999). A wide rang of organisms have the ability to fix nitrogen and about 87 species in the genera of archae, 38 genera of bacteria and 20 genera of

cyanobacteria have been identified (Zahran *et al*., 1995).Biological nitrogen fixers may be symbiotic or non-symbiotic (Tilak *et al.*, 2005).

Non-symbiotic nitrogen fixers may be free-living or associative N_2-fixers. The free-living nitrogen fixers such as cyanobacteria, Azospirillum, Azotobacter fix N_2 independently of other organisms using chemical energy in the case of non-photosynthetic forms or light energy in the case of photosynthetic forms. The associative N_2 fixers live in closer to or in side the plant (present in rhizosphere, rhizoplane or grow endophytically) for their energy source to the energy intensive nitrogen fixing process (Tilak *et al*., 2005).

Due to the mutualistic dependence of the partners in the relationship, associative N_2 fixation has some characteristics of symbiotic relationship (Rengel, 2002). The associative N_2 - fixing organisms make a modest contribution of fixed nitrogen to agriculture and forestry while the free living diazotrophs contribute little fixed N_2 to the agricultural crops. Some important non-symbiotic nitrogen-fixing bacteria are *Azotobacter, Azospirillum, Acetobacter, Alcaligenes, Arthrobacteria, Azomonas, Bacillus, Clostridium* and *Pseudomonas* (Saxena and Tilak, 1998).

Symbiotic N_2 fixers include two groups of N_2 fixing bacteria: Rhizobia and Frankia. Frankia are gram-positive bacteria (nitrogen fixing actinomycetes) that form root nodules on more than 280 species of non-leguminous wood plants (Schwintzer and Tjepkema., 1990). Species of *Alnus* and *Casuarina* are globally known to form effective symbiosis with Frankia (Huss-Danell,1990). Frankia-nonlegume symbiosis (actinorhizal interactions) is a major contributor of nitrogen inputs in forests, wetlands and disturbed sites of temperate and tropical regions (Tate, 1995). The contribution of fixed N_2 to native as well as managed ecosystems by actinorhizal symbioses is comparable to that of the more extensively studied rhizobial-legume interactions. What makes the rhizobia and frankia species different from associative nitrogen- fixing microbes is that most of the rhizobia or frankia species fixed N_2 is transferred to and assimilated by the plant for the plant growth (Hirsch *et al,* 2001).

Molybdenum nitrogenase (Mo nitrogenase) is the most common type of nitrogenase found in root nodule bacteria (Fisher and Newton, 2002). This enzyme has two distinct metalloproteins, known as MoFe Protein (component I or dinitrogenase) and Fe protein (Component II or dinitrogen

reductase) (Peters *et al*., 1995; Howard and Rees, 1996). The Fe protein contains two Mg ATP-binding sites (Benton *et al*., 2002).

Anaerobic environment and adenosine triphosphate (Mg ATP) are the two requirements that have to be met for nitrogenase to catalyze the biological nitrogen fixation process .Nitrogenase is inactivated when exposed to oxygen because oxygen reacts with the iron component of the protein. Although this is not a problem for anaerobic bacteria, aerobic species have various mechanisms to over come the problem (White, 1995). Azotobacter species produce large amount of extra cellular polysaccharide that limits the diffusion rate of oxygen in to the cells and also have high rate of respiratory metabolism to maintain very low level of oxygen in their cells. Root nodules can contain oxygen-scavenging molecules such as leghaemoglobin in the symbiotic nitrogen-fixing organisms such as rhizobia. In cyanobacteria, nitrogen fixation occurs in special cells (called heterocysts) that possess only photo system I (generates ATP by light mediated reaction) where as other cells have both photo system I, and Photo system II (generates oxygen when light energy is used to split water to supply H_2 for the synthesis of organic compounds).

Biological nitrogen fixation is a high energy demanding reaction which can be indicated as follows.

$$\mathbf{N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16Pi}$$

Two interconned processes, namely the Fe-protein cycle and the MoFe protein cycle operate in the sequential delivery of electrons to MoFe protein and then to the substrate for its reduction (fig.3)

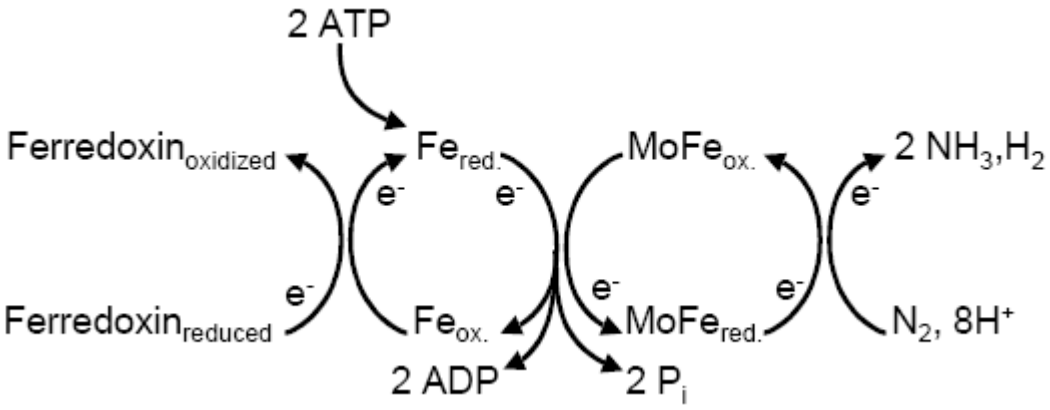


Fig.3.The Fe-protein cycle and the MoFe protein cycle

3.4 .Environmental factors that affect BNF

Symbiotic nitrogen fixation depends on the host plant genotype, rhizobia strain and the interaction of these symbionts with other environmental conditions (Bordeleau and Prevost, 1994).This is due to the fact that the establishment of an effective rhizobia-legume symbiosis requires colonization and survival of rhizobia in the soil as saprophytes competing with other indigenous microbes and genetic compatibility with the host legume, and favourable environment to allow maximum N_2 fixation. Typical environmental stresses faced by the legume nodules and symbiotic rhizobia may include photosynthetic deprivation, water stress, salinity, soil nitrate, soil acidity and alkalinity, temperature, heavy metals and biocides (Walsh, 1995).

Salinity

Salinity is a serious threat to agriculture in arid and semi-arid regions. Increased salt concentration may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate,1995).Effects of salt stress include inhibition of initial step of rhizobia-legume symbiosis such as curling of root hairs, reduction of nitrogen fixing activity attributed to reduction in nodule respiration (Delgado *et al.*, 1994 ;Walsh,1995), reduction in nodule cytosolic protein production specifically leghaemoglobin (Delgado *et al.*, 1994) and reduction in photosynthetic activities and growth of the plant.

In contrast to their host legumes, some rhizobia can survive in the presence of high level of salt both in the culture and soil (Bordeleau and Prevost, 1994). Many species of bacteria adapt to saline conditions by intracellular accumulation of low molecular weight organic solutes (example-trehalose) called osmolytes (Csonke and Hanson, 1991).

Water Stress

Water stress reduces N_2 fixation, nodule respiration, and cytosolic protein content of the nodule (Bordeleau and Prevost, 1994). Soil moisture deficiency reduces the rhizobial population density in the soil (Tate, 1995), rhizobial migration, nodule number and size (Williams and De Mallorea,1984) and has a pronounced effect on N_2-fixation since nodule initiation, growth and activity are more sensitive to water stress than are generally root and shoot metabolism (Albrecht *et al.*, 1994). In dry soil, infection of root hairs is restricted because they become short, stubby and inadequate for rhizobial infection (Lie, 1981).

According to Eaglesham and Ayanaba (1984), excess water is detrimental to N_2 - fixation because it lowers oxygen diffusion for nodule functions and may lead to a build up of CO_2 (which inhibits nodule formation) and ethylene (which restricts nodulation at lower concentration).

Temperature

Temperature affects legumes non-specifically and symbiosis ceases when it is exposed to extreme temperature (Bordeleau and Prevost, 1994). Temperature affects root hair infection, bacteroid differentiation, nodule structure and functioning (Roughley, 1970).High soil temperature in tropical and subtropical areas is a major problem for biological nitrogen fixation of legume crops (Michiel *et al*., 1994). Elevated temperatures may delay nodule initiation and development (Graham, 1992; Bordeleau and Prevost, 1994).Low temperature delays root hair infection and decreases nodulation and nitrogenase activity (Waughman, 1977).

Soil Acidity and Alkalinity

Most leguminous plants require a neutral or slightly acidic soil pH (Bordeleau and Prevost, 1994). The failure of legumes to nodulate under acidic-soil conditions is common, especially in soils with pH less than 5 (Raza *et al*., 2001). Soil acidity limits the rhizobial survival and persistence in soils (Ibekwe *et al*., 1997). Slow growing bradyrhizobia are generally more acid tolerant than fast-growing strains of rhizobia but the basis for the differences in pH tolerance among strains of rhizobium and bradyrhizobium is still unclear (Correa and Barneix, 1997).

The principal effects of soil acidity may be resolved in to higher hydrogen ion concentration, and excessive quantity of aluminium and manganese but deficiency of calcium, phosphorus and molybdenum in the soil. This nutritional disorder affects legume-rhizobia symbiosis (Lie,

1981).Two strategies have been adapted to solve the problem of soil acidity: liming the soil acidity and selecting tolerant varieties of plants and strains of rhizobia. Very few data have been documented on the effects of high pH on rhizobial growth, nodulation or legume growth (Bordeleau and Prevost, 1994).

4. Materials and Methods

4.1 Study sites, legume variety and nodule collection

Root nodules were obtained from eight genera of woody legumes collected from various regions of Ethiopia (Table.2) in October and November 2005, and brought to the Applied Microbiology Laboratory in sealed vials containing a desiccant (silica gel) covered with 1 cm layer of cotton wool (Lupwayi and Haque, 1994) except for those collected from *Erythrina brucei* at the Science Faculty and used immediately. Voucher legume specimens were collected and brought to the Natural Herbarium, Biology Department, for identification.

Table.2 Woody legumes and the study sites

Woody legume	Site	Geographic location
Dalbergia lactea	Belete forest	$7^0$8'22" N and $36^0$7'3" E
Indigofera species	Belete forest	$7^0$8'22" N and $36^0$7'3" E
	Fenchir	$8^0$30.2' N and $37^0$58.5' E
	Harawe	not determined
	Ganda Chero	not determined
	konso	$5^0$20'5' N and $37^0$25"39' E
	Hammer	$5^0$11' 19" N and $36^0$32' 24" E
Crotalaria species	Gojeb	$7^0$24' N and $36^0$22'29" E
	Gelogalana	not determined
	Errer Valley	$9^0$14' N and $42^0$15' E
	Grawa	not determined
	Alamaya University Campus	$9^0$24' N and $42^0$1' E
Crotalaria spinosa	Konso	$5^0$20'5" N and $37^0$25'39" E
Crotalaria spinosa	Afer town	$5^0$31'20" N and $36^0$43'51" E
Chamaecytisus palmensis	Dangla	not determined
Lupinus albus	Finote Selam	not determined
Tephrosia species	Addis Kidam	not determined

Aeschynomen schimpe r	Fogera field	not determined
Aeschynomen elaphroxylon	Konso	$6.5^0$12'63" N and$33^0$41'93" E
Erythrina brucei	AAU, Science Faculty	$8^0$59'57" N and 38^0 4'18.5" E

4.2 Isolation of rhizobia from root nodules

Dried, undamaged root nodules were selected and allowed to reimbibe water by immersing in sterile distilled water over night in labelled sets of Petri dishes as described by Vincent (1970). Then, the nodules were transferred in to sterile empty petridishes using forceps sterilized by dipping in alcohol and flaming. The reconstituted nodules were exposed to 95% ethanol for 10 seconds and then surface sterilized by immersing in 0.1% acidified mercuric chloride (1g $HgCl_2$ added to one litre of distilled water containing 5ml of concentrated HCl) for 4 minutes after tipping out the ethanol according to Lupwayi and Haque (1994).

The surface sterilized nodules were washed with sterile distilled water at least six times according to Vincent (1974). Finally, the surface sterilized nodules were transferred in to empty sterile petridish and individual nodule was crushed with alcohol flamed sterile glass rod in a drop of sterile distilled water aseptically in a laminar flow hood. One loopful of each nodule suspension was streaked on to sterilized plates of yeast extract mannitol agar (YEMA) containing 0.5 g of KH_2PO_4, 0.5g of K_2HPO_4. 0.5g of yeast extract (OXOID), 0.2g of NaCl, and 0.2g of $MgSO_4.7H_2O$, 10g of mannitol and 16g of agar in a litre of ditilled water as described by Lupwayi and Haque (1994). The pH of the medium was adjusted to 7.0 with 1N NaOH and HCl befor autoclaving at 121 ^oC for 15 minutes. Then, the streaked plates were incubated at 28 ^0C in an inverted position for 4 to10 days until colonies appear along the lines of spreading.

4.3 Purification and preservation of the isolates

After incubating for 4 to 10 days according to the growth rates of the isolates, all the plates were carefully examined and single colony of each isolate was picked with alcohol flamed sterile inoculating loop and transferred in to 10 ml of sterilized yeast extract mannitol broth (the same to YEMA but with out agar) in test tubes, vortexed and then placed on rotary shaker at room

temperature. After four days, when the yeast extract mannitol broth became cloudy due to bacterial multiplication, streaking was performed on sterile plates of YEMA, and then the plates were incubated at 28 ^{o}C. The purity and uniformity of colony type was carefully examined in the re-streak, and a single well isolated colony was picked to yeast extract mannitol agar slant and incubated at 28^{o}C.When sufficient growth occurred, the culture slant was preserved (stored) at 4^{0}C(Lupwayi and Haqua,1994).

4.4 Presumptive tests of the isolates

Even after surface- sterilizing the nodules, it can not be guaranted that all the bacteria that grow on YEMA are rhizobia (Lupwayi and Haque, 1994) so that the following checks were made to confirm identity.

Gram Reaction

All the isolates were gram stained, and their shapes and gram reaction were observed microscopically.

Congo red absorption

Stock solution of congo red (CR) was prepared by dissolving 0. 25g of CR in 100ml sterile distilled water (Vincent, 1970) and then 10ml of the CR stock solution was added to one litre of YEMA before autoclaving. Finally, the yeast extract mannitol broth culture was streaked on YEMA plates with CR and incubated at 28 ^{o}C wrapped with aluminium foil to provide a dark condition.

Peptone - glucose test

The peptone-glucose medium was prepared according to Lupwayi and Haque (1994) by dissolving 5g of glucose, 10g of peptone, 15g of agar and 10ml of Bromcresol purple (BCP, prepared by dissolving 1g of BCP in 100 ml of ethanol) in a liter of distilled water and the pH was adjusted to 6.7 with 1N NaOH and HCl. Three days old Yeast extract mannitol broth culture was streaked on to the peptone- glucose medium to observe the presence of growth by incubating at 28 ^{o}C.

Ketolactose test

In this test, the rhizobial isolates were streaked on sterile yeast lactose agar (YLA), which is YEMA in which mannitol is replaced with the same concentration of lactose, and finally the YLA surface was covered with 10-15 ml of Benedict reagent for 10 minutes after colony formation to detect the production of 3- ketolactose as described by Lupwayi and Haque (1994).

4.5. Bacterial designation of the isolates

AURA - Addis Ababa University rhizobia isolated from *Aschynomene species*

AURC - Addis Ababa University rhizobia isolated from *Crotalaria species*

AURD - Addis Ababa University rhizobia isolated from *Dalbergia species*

AURE- Addis Ababa University rhizobia isolated from *Erythrina brucei*

AURI – Addis Ababa University rhizobia isolated from *Indigofera species*

AURL – Addis Ababa University rhizobia isolated from *Lupinus albus*

AURT – Addis Ababa University rhizobia isolated from *Tephrosia species*

AURCp- Addis Ababa University rhizobia isolated from *Chamaecytisus palmensis*

Table 3. Summary of the isolates, host woody legume study sites and regional states

No.	Isolate	Host woody legume	Study sites	Regional state
1	AURD2	*Dalbergia lactea*	Belete forest	SNNP*
2	AURI6	*Indigofera species*	Belete forest	SNNP
3	AURI14	*Indigofera species*	Fenchir	SNNP
4	AURC22	*Crotalaria species*	Gojeb	SNNP
5	AURC23	*Crotalaria species*	Gelogalana	Oromiya
6	AURE2	*Erythrina brucei*	AAU, Science Faculty	Addis Ababa
7	AURI30	*Indigofera species*	Harawe	Oromiya
8	AURI32	*Indigofera species*	Ganda Chero	Oromiya
9	AURC36	*Crotalaria species*	Errer Valley	Oromiya
10	AURC42	*Crotalaria species*	Grawa	Oromiya
11	AURC48	*Crotalaria species*	Alamaya University Campus	Oromiya
12	AURCp2	*Chamaecytisus palmensis*	Dangla	Amahara
13	AURL2	*Lupinus albus*	Finote Selam	Amahara
14	AURT2	*Tephrosia species*	Addis Kidam	Amahara
15	AURA13	*Aeschynomen schimperi*	Fogera field	Amahara
16	AURI21	*Indigofera spinosa*	Konso	SNNP
17	AURC24	*Crotalaria species*	Konso	SNNP
18	AURA28	*Aeschynomen elaphroxylon*	Konso	SNNP
19	AURI34	*Indigofera spinosa*	Hammer	SNNP
20	AURI36	*Indigofera spinosa*	Hammer	SNNP

21	AURC37	*Crotalaria species*	Afer town	SNNP

* Southern Nations, Nationalities and peoples`

4.6. Growth rate determination

Stored culture of each isolate was inoculated into 10 ml YEM broth in test tubes, vortexed and shaken on orbital shaker at 150 rev/min for 3 days at room temperature. Then, 1ml of each broth culture (10^9cells) was inoculated into 100ml sterilized YEM broth in 250ml Erlenmeyer flask and placed on orbital shaker at 150 rev/min at room temp and optical density (OD^{540}) of the YEM broth cultures was taken just at the time of inoculation (0hr) and then at every 6 hours interval by using spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer) after calibrating it to zero with sterile un inoculated YEM broth as blank. Samples were simultaneously taken, serially diluted (10^{-1} to 10^{-10} with sterile distilled water), 0.1ml sample from each dilution was dispensed on to sterilized YEMA plates and spread by using alcohol flamed glass rod spreader bent in to a hockey stick to determine the colony forming units, cfu according to Somasegaran and Hoben (1994).Finally the generation time (g) was calculated from the logarithmic phase according to White (1995) using the formula

$$g = \frac{\log 2\,(t)}{\log X - \log Xo}$$

, where **g** is generation time

t is time elapsed

Xo is first OD reading in logarithmic phase

X is second OD reading in logarithmic phase

4.7. Characterization of the isolated rhizobial strains

4.7.1. Cultural Characterization

The cultural characterization of the isolates was performed according to Lupwayi and Haque (1994) as follows.

Acid and alkali production

The production of acid or alkali by a given strain was detected by streaking a loopfull of 72 hours old culture (10^6 cells) into 0.05% of bromthymol blue (BTB)-incoroporated YEMA (Prepared by dissolving 0.5g of BTB in 100ml of ethanol and then adding into a liter of YEMA and adjusting the pH of the mixture to 6.8 using 1N NaOH and HCl before autoclaving).YEMA containing BTB is green at pH 6.8.

Colony size and shape

The shape and diameters of colonies of each strain were determined by streaking a loop full of 72 hours old YEM broth culture (10^6 cells) on to sterile YEMA plates and incubating at 28^oC for 5-7 days. In each case, the diameters of 5 colonies were measured and the mean was taken.

Colony texture

The colonies of the isolates were touched with a wire loop and lifted to see whether they were elastic or buttery.

Colony appearance

The appearance of colonies of each strain was examined and recorded as shiny (translucent) or opaque.

Gum production

The colonies of each strain were examined and noted whether they were dry (little gum) or soft and gelatinous (much gum).

4.7.2. Physiological characterization of the rhizobial strains

4.7.2.1 Growth at different pH

Growth of the rhizobial strains at different pH values was evaluated by using Keyser-defined medium prepared according to Lupwayi and Haque (1994) as follows.

A. Micronutrient	gram per litre
$MnCl_2.4H_2O$	504
$ZnSO_4.7H_2O$	0.227
$CuCl_2.2H_2O$	0.034
$NaMoO_4.2H_2O$	0.008
B. Phosphate stock solution	
KH_2PO_4	1.36
C. Vitamin stock solution	
Thiamine HCl	0.4000
d-Pantothenic acid (Ca)	0.4000
Biotin	0.0001
Medium	**per litre**
Glycerol	5 ml
K_2SO_4	0.131g
Na glutamate	0.220g
$MgSO_4.7H_2O$	0.074g
$CaCl_2.2H_2O$	0.007g
Fe-EDTA	0.035g
Solution A	0.5 ml
Solution B	1.0 ml
Solution C	1.0 ml
Agar	20 g

The pH of the media was adjusted to the desired levels (4, 4.5, 5, 5.5, 6, 7.5, 8, 8.5, 9, 9.5, 10 and 10.5) by using 1N HCl or NaOH before autoclaving. The media were autoclaved at 121^0C for 15 minutes and then poured in to different plates. Each plate was streaked with a loopful of 72 hours old YEM broth culture (10^6 cells) and incubated at 28 ^{0}C. For each strain, duplicate plates were used at the tested pH levels. Control plates with pH adjusted to 7.0 were also used. Finally, the

growth of the rhizobial strains was qualitatively evaluated using (-) for no growth and (+) for positive growth.

4.7.2.2. Growth at different temperature levels

YEM broth culture of each isolate was streaked on to sterile YEMA plates in duplicates and kept at room temperature for 2 hours. Then, the plates were incubated at various temperature levels (5, 10, 15, 20, 35, 40 and 45 ^{0}C) and the growth of the isolates was evaluated qualitatively as (-) for no growth and (+) for positive growth as described by Lupwayi and Haque (1994).Streaked plates of each isolate were also incubated at 28^{o}C as control.

4.7.2.3. NaCl tolerance

The ability of the rhizobial isolates to NaCl tolerance was tested by streaking a loopful of YEM broth culture on sterilized YEMA plates containing 1, 2,3,4,5 and 6% NaCl (w/v) and incubating at 28^{0}C. Duplicates of plates were used for each isolate at each salt concentration and subsequently the bacterial growth was evaluated qualitatively as (-) for no growth and (+) for positive growth according to Lupwayi and Haque (1994). Control plates with 0.02% NaCl were used as control for each isolate.

4.7.2.4. Intrinsic antibiotic resistance (IAR)

Seven antibiotics (chloramphenicol, streptomycin sulfate, rifampcin, erythromycin, kanamycin monosulfate, neomycin sulfate and ampicillin sodium sulfate) were used to test the natural resistance of the strains to the antibiotics. The stock solution of each antibiotic was prepared according Lupwayi and Haque (1994) by dissolving 2.0 g of chloramphenical and rifampcin in 100ml absolute ethanol while distilled water was used as a solvent for each 2.0g of the remaining five antibiotics. The stock solution of each antibiotic was filter sterilized using a milipore filter (0.22µm) and aseptically added to autoclaved YEMA (kept at 50^{0}C in water bath) at the final concentrations of 10,20 and 30µg/ml,which is 50,100 and 150µl of antibiotic solution per 100 ml medium, respectively, and finally poured separately in to plates. Duplicate plates were used for each antibiotic with the mentioned concentrations and antibiotic free plates were used as control. All the plates were kept at room temperature for 24 hours before use. Finally, culture of each

rhizobial isolate was streaked on YEMA plates supplemented with each antibiotic of test and incubated at 28^0C for 10 days and rhizobial growth was scored by visual inspection as (+) for positive growth and (-) for no growth.

4.7.2.5. Carbon source utilization

For carbon source utilization tests, 16 carbon sources were taken. They were monosaccharides (D-glucose, D-fructose, D- galactose, D-arabinose, D-mannose and xylose), disaccharides (maltose, lactose, trehalose, D- sucrose and cellobiose), sugar alcohols (mannitol, glycerol and sorbitol) and organic salts (Na-citrate and Na- glutamate).The test was carried out according to Somasegaran and Hoben (1994). Ten percent distilled water solution of each carbhydrate (w/v but v/v for glycerol) was prepared and temporarily stored in a refrigerator after filter sterilizing the heat labile carbohydrates (D-arabinose, D- mannose, sorbitol, D-galactose, maltose, Na-citrate, Na.glutamic acid, xylose, trehalose, cellobiose and glycerol) using a milipore filter (0.22μm). Then carbohydrate-free basal medium (essentially similar to YEMA except for the reduction of yeast extract from 0.5g/l to 0.05g/l) was prepared. Ninty milli litres of the carbohydrate-free basal medium together with each test carbohydrate was dispensed in to several 250ml Erlenmeyer flasks. The media were then poured in to sterilized plates and kept inverted for 24 hours in a laminar flow hood to check for contamination. Finally, a loop full of 72 hours old YEM broth culture of each rhizobial isolate(10^6 cells) was streaked on the plates of incorporated carbohydrates under test and incubated at 28^oC for 10 days and growth was recorded as (+) for positive growth and (-) for no growth in relation to the positive control YEMA plates.

4.7.2.6. Phosphate solubilization

The phosphate sulubilizing ability of the isolates was tested on the Pikovskaya's medium accrding to Tewari *et al*. (2004) with the composition indicated below. Pikovskaya's medium was prepared and autoclaved at 121^0C for 15 minutes. Finally, a loopful of 72 hours old YEM broth culture of each isolate (10^6 cells) was streaked on the sterile Pikovskaya's medium plates, incubated at 28 ^{o}C and examined for the formation of clear zones around the colonies when sufficient growth was observed.

Pikovskaya's medium components	**amounts gl^{-1} of distilled water** (Tewari *et al*., 2004)
Glucose	10
$Ca_3 (PO_4)_2$	5
$(NH4)_2SO_4$	0.5
NaCl	0.2
$MgSO_4.7H_2O$	0.1
KCl	0.2
Yeast extract	0.5
$MnSO_4.H_2O$	0.002
$FeSO_4.7H_2O$	0.002
Agar	15
pH	7.0

4.8. Numerical Analysis

Phenotypic variability of the isolates was analyzed using a computer cluster analysis using a similarity coefficient. A phenotypic dendrogram tree was constructed by the unweighed pair group method with the average, UPGMA (NT-SYS version 2.1) soft ware based on the results of carbohydrate utilization, pH, antibiotic, NaCl and temperature tolerance.

4.9 Cross-inoculation and symbiotic effectiveness test

Experimental design

Four different isolates (AURE2, AURI30, AURC36 and AURT2) of different host woody legumes were selected as representative and tested for their ability to form nodules and effective symbiosis with common bean (*Phaseolus vulgaris) and* cowpea *(Vigna unguiculata).*The representative isolates were selected on the basis of the diversity and agronomic importance of their host plants

in the country. Cowpea and common bean were also selected due to their symbiotic promiscuity as well as due to their significances as grain commodities, and source of food. Three replicates were used for each treatment and also three replicates of N+ (Nitrogen added) and N- (nitrogen free) treatments were used as controls for each grain legume species. The treatments were arranged in a randomized complete block design according to Somasegaran and Hoben (1994).

Preparation of sand and pots

River sand was thoroughly washed six times with tap water, drained and autoclaved at 121^{o}C for 1 hour. About 1.5kg of the autoclaved sand was added to surface sterilized normal plastic pots with lids and saturated with sterile distilled water prior to sowing seeds.

Seeds: source, preparation and sowing

The seeds of *P.vulgaris* (Awash I Variety) and *V.unguiculata* (white wonder trealing Variety) were obtained from Ethiopian Institute of Agricultural Research, Melkasa Research Center.

The seeds were rinsed in 95% ethanol for 10 seconds and surface sterilized by soaking them in 0.2% mercuric chloride solution for 3 minutes as described by Lupwayi and Haque (1994). After draining of the mercuric chloride solution, the seeds were washed seven times with sterile distilled water. Then, the seeds of common bean were plated into sterilized 0.75 %(w/v) water agar and incubated at 28^{o}C while the seeds of cowpea were directly sown into the sterile potted sand at a depth of 1.5cm since they failed to show good germination on water agar. The germinated seeds of common bean were transferred by using sterile forceps into sterile potted sand at a depth of 1cm after two days. Four seeds were planted into each pot and thinned down into three after a week.

Inocula: Preparation and inoculation

Starter cultures of the selected test strains(AURE2, AURI 30, AURC36 and AURT2) were grown in test tubes containing 10ml YEM broth on orbital shaker at 150 rev/min at room temperature (average 25^{o}C) for 3 days. One ml YEM broth culture of each test strain (10^{9}cells) was transferred in to 100ml sterilized YEM broth in 250ml Erlenmeyer flask and placed on orbital shaker at 150 rev/min at room temperature for 4 days.One ml of each undiluted 4 days old YEM broth culture (about10^{9}cells, 0.93OD^{540}) was inoculated by pipetting on to the base of the seedlings of

V.unguiculata when they emerged and to seedlings of *P.vulgaris* seven days after the emerging of the sown seedlings according to Vincent (1970).

Green house conditions, nutrient and watering

The green house is located on Science Faculty Campus (Addis Ababa University) and its average day temperature was 31^{o}C during the work. One hundred twenty ml of 0.05% KNO_3 solution was added to plus-nitrogen (positive control) treatments per each pot once every week (Maatallah *et al*., 2002).The nitrogen-free nutrient solution for grain legumes was prepared according to Broughton and Dilworth (1970) as indicated below.

Stock solution	**Chemical**	**g/liter**
1	$CaCl_2.2H_2O$	294
2	KH_2PO_4	136.1
3	Fe $C_6H_5O_7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.\ 2H_2O$	0.048

After preparing the stock solutions, 10 liters of full strength nitrogen-free plant nutrient solution was prepared by mixing 5 ml of each stock solution with 5 litres of distilled water in a container and further diluting it to 10 liters by adding another 5 litres of ditilled water. The pH of the solution was adjusted to 6.8 with 1N NaOH and HCl. Hundred ml of quarter-strength N-free nutrient solution was supplied to each pot once every week. All the pots were watered every two days (sterilized distilled water was used).

Harvesting the plants and assessing the symbiotic effectiveness

The plants were harvested 7 weeks after inoculation, with in the time range (6-8 weeks) indicated by Lupwayi and Haque (1994).The shoots were collected by cutting the plants at the level of the sand. The shoots from each growth unit were placed in paper bags and dried at 70^0C for 48 hours as described by Somasegaran and Hoben (1994) and their dry weight was determined. The roots and adhering sand were dislodged in to a coarse sieve (0.76mm) and were washed with a gentle tap water and observed for nodules. The nodules were collected, counted and their dry weight was determined in the same way as the shoots.

After determining the shoot dry weight of each growth unit, the shoots were finely ground using pistil and mortar followed by sieving with 0.5mm size. Then, the total nitrogen content of the shoots was determined by modified "Wet" Kjeldahl method according to Sahlemedhin Sertsu and Taye Bekele (2000).

Modified "Wet" Kjeldahl method makes use of apparatus (Digestion block, Digestion tubes and Distillation unit) and reagents: concentrated H_2SO_4, 30% H_2O_2 , selenium powder, salicyclic acid, sulfuric acid - selenium mixture (made by mixing 3.5g selenium powder with 1 litre of H_2SO_4 by heating to 300^0C until the colour of the solution became light yellow), digestion mixture (made by dissolving 7.2g of saliciclic acid in 100ml of sulfuric acid-selenium mixture), mixed indicator (contained mixture of 0.5g bromocresol green and 0.1g methyl red dissolved in 100 ml of 95% ethanol and the pH was adjusted to 4.5 using 1N NaOH and HCl), 40% NaOH (made by dissolving 400g of NaOH in 800ml distilled water in one litre volumetric flask and bringing the volume to one litre with distilled water after cooling in stoppered flask), 2% boric acid solution (made by dissolving 20g boric acid in 600ml distilled water in a one litre volumetric flask and making to the volume with distilled water), and 0.1N H_2SO_4 solution (made by pipetting 2.82 ml of 96% H_2SO_4, in to distilled water in a litre of volumetric flask and making to the volume with distilled water).

Ground shoot sample (0.3g) was transferred into digestion tube, 2.5ml of the digestion mixture was added to each digestion tube, swirled carefully to moisten the ground shoot samples and let stand for 2 hours. Then, the tubes were placed on heating block and heated at 100^0C for 2 hours. After two hours, the tubes were removed from the block and allowed to cool and three 1ml of 30% H_2O_2

was added successively in to each digestion tube and mixed thoroughly. The digestion tubes were again placed on the preheated block and heated at 300^0C until the digest turned to colorless or light yellow. Then the tubes were removed from the block, cooled to room temperature and 48.3ml of distilled water was added to each tube, mixed and then let stand overnight. On the next day, the content of each digestion tube was mixed again by shaking, filtered on a 100ml volumetric flask, brought to the volume with distilled water. Each 100 ml of the acid digest was transferred into a macro-Kjeldahl tube and 20ml of boric acid solution was measured from a dispenser flask into 250 ml Erlenmeyer flask corresponding to the number of samples and 2 drops of mixed indicator solution were added to each 20ml boric acid solution, mixed thoroughly and placed under the condenser. After adding 75ml of 40% NaOH solution to each digestion tube containing the digest, it was fitted to the corresponding holder and distillation was started. When the distillation was completed, that is, when about 80ml of the distillate had been collected to boric acid, the flask was removed and distillation process of another sample was continued. Titration was then performed by using 0.1N H_2SO_4 until the colour of the distillate turned from green to pink at the end point and the utilized H_2SO_4 for titration was recorded volumetrically. Finally the percent of N_2 content of the samples were calculated after correcting for the blank as described by Sahlemedhin Sertsu and Taye Bekele (2000).

$$\%N = \frac{(a-b) \times N \times 0.014 \times mcf}{S} \times 100$$

Where:

A = ml of H_2SO_4 required for titration of sample

B = ml of H_2SO_4 required for titration of blank

S = air-dry sample weight in grams

N = normality of H_2SO_4 (0.1N)

0.014 = meq weight of nitrogen in g

mcf = moisture correction factor.

Data Analysis

One way analysis of variance (ANOVA) of the data was done using version 10 SPSS statistical program. Mean separation was calculated using the Turkey's value when the F-test was significant

at p = 0.05 Percentage. Symbiotic effectiveness of the tested isolates was calculated according to Lupwayi and Haque (1994) using the following formula

$$\% \text{ Symbiotic effectiveness} = \frac{\text{shoot dry weight of plants inoculated with test strain}}{\text{shoot dry weight of plants supplied with nitrogen}} \times 100$$

5. Results

5.1 Presumptive tests of the bacterial isolates

All the isolates were found to be gram negative - rods and did not absorb congo red except isolate AURI34 (from *Indigofera spinosa*) which absorbed it very slightly. The isolates showed no growth on the peptone-glucose agar medium. No yellow colour was formed when all the isolates were grown on the yeast extract lactose agar medium with Benedict reagent, indicating the absence of 3-ketolactose production.

5.2 .Growth rate determination

The mean generation time of the isolates was found to range from a minimum of 1.3 hours for isolate AURA13 (from *Aeschynomene schimperi*) to a maximum of 11.4 hours for isolate AURD2 (from *Dalbergia lactea*) (Table 2).

5.3. Cultural Characterization

The isolates showed diverse cultural characterstics indicated by variations in colony size, shape, texture and appearance, and acid or alkali production (Table 4).The majority of the isolates produced alkali and formed gelatinous, shiny and dome-shaped colonies. Three isolates, AURD2 (*Dalbergia lactea),* AURC36 (*Crotalaria species)* and AURT2 (*Tephrosia species)* showed elastic colony texture while all the rest formed buttery colonies.

Table 4.Cultural characterization of the rhizobial isolates

Host woody legume	Rhizobial isolates	Mean generation time (hr)	Colony size(mm)	BTB reaction
Dalbergia lactea	AURD2	11.4	1.4	blue
Indigofera species	AURI6	6.2	1.0	blue
Indigofera species	AURI14	5.9	1.0	blue
Crotalaria species	AURC22	9.6	2.4	yellow
Erythrina brucei	AURE2	6.2	2.4	blue
Crotalaria species	AURC23	5.1	2.0	blue
Indigofera species	AURI30	3.7	4.0	yellow
Indigofera species	AURI32	4.1	1.0	blue
Crotalaria species	AURC36	9.6	3.0	yellow
Crotalaria species	AURC42	10.3	1.0	blue
Crotalaria species	AURC48	5.0	2.0	blue
Chamaecytisus palmensis	AURCp2	4.2	1.5	blue
Lupinus albus	AURL2	5.7	2.0	blue
Tephrosia species	AURT2	3.5	3.0	yellow
Aeschynomene schimperi	AURA13	1.3	5.0	yellow
Indigofera spinosa	AURI21	4.8	1.5	blue
Crotalaria species	AURC24	11.0	1.4	blue
Aeschynomene elaphroxylon	AURA28	3.9	2.0	yellow
Indigofera spinosa	AURI34	10.5	2.4	blue
Indigofera spinosa	AURI36	6.1	3.2	blue
Crotalaria species	AURC37	5.1	1.8	blue

5.4. Physiological characterization of the rhizobial isolates

5.4.1. Growth of the isolates at different pH levels

All the isolates failed to grow at pH 4 and 4.5 except AURA13, which grew well at pH values ranging from 4.0 to 10.5. All the strains were able to grow at acidic pH (5.5) and basic pH up to 9.5. At pH 5.0, 33% of the isolates were inhibited to grow while only 19% of the isolates failed to grow at pH 10.5 (Table 5).

Table 5. Growth of the isolates at different pH levels

Rhizobial isolates	PH level											
	4	4.5	5	5.5	6	7.5	8	8.5	9	9.5	10	10.5
AURD2	-	-	+	+	+	+	+	+	+	+	+	-
AURI6	-	-	+	+	+	+	+	+	+	+	+	+
AURI14	-	-	+	+	+	+	+	+	+	+	+	+
AURC22	-	-	+	+	+	+	+	+	+	+	+	+
AURE2	-	-	+	+	+	+	+	+	+	+	+	+
AURC23	-	-	-	+	+	+	+	+	+	+	+	+
AURI30	-	-	-	+	+	+	+	+	+	+	-	-
AURI32	-	-	+	+	+	+	+	+	+	+	+	+
AURC36	-	-	-	+	+	+	+	+	+	+	+	+
AURC42	-	-	-	+	+	+	+	+	+	+	+	+
AURC48	-	-	-	+	+	+	+	+	+	+	+	+
AURCp2	-	-	+	+	+	+	+	+	+	+	-	-
AURL2	-	-	+	+	+	+	+	+	+	+	+	-
AURT2	-	-	-	+	+	+	+	+	+	+	+	+
AURA13	+	+	+	+	+	+	+	+	+	+	+	+
AURI21	-	-	+	+	+	+	+	+	+	+	+	+
AURC24	-	-	+	+	+	+	+	+	+	+	+	-
AURA28	-	-	+	+	+	+	+	+	+	+	+	+
AURI34	-	-	-	+	+	+	+	+	+	+	+	+
AURI36	-	-	+	+	+	+	+	+	+	+	-	-

AURC37	-	-	+	+	+	+	+	+	+	+	+	+

(+) = growth and (-) = no growth

5.4.2. Growth of the isolates at different temperature levels

Only three isolates, AURI14 (*Indigofera species*), AURI30 (*Indigofera species)* and AURA13 *(Aeschynomene schimperi*) were able to tolerate and grow at 5^{o}C while 5 of the 21 isolates grew at 45 ^{o}C (Table 6). Most of the isolates grew at 15 and 35 ^{o}C, and more of them grew at 35 than 10 ^{o}C. All of the isolates managed to grow within a range of 20 to 30 ^{o}C. Isolate AURI14 was able to grow at tested temperature levels and AURA13 also grew over a wider range of temperature (5-40 ^{o}C).

Table 6. Growth of the isolates at different temperature levels

Rhizobial isolates	Temperature(^{o}C)						
	5	10	15	20	35	40	45
AURD2	-	+	+	+	-	-	-
AURI6	-	+	+	+	+	-	-
AURI14	+	+	+	+	+	+	+
AURC22	-	+	+	+	+	-	-
AURE2	-	+	+	+	+	-	-
AURC23	-	-	+	+	+	+	+
AURI30	+	+	+	+	-	-	-
AURI32	-	-	+	+	+	+	-
AURC36	-	-	+	+	+	+	-
AURC42	-	-	+	+	+	+	+
AURC48	-	+	+	+	+	-	-
AURCp2	-	+	+	+	+	-	-
AURL2	-	-	+	+	+	-	-
AURT2	-	-	+	+	+	-	-
AURA13	+	+	+	+	+	+	-
AURI21	-	-	+	+	+	+	-
AURC24	-	-	-	+	+	+	+
AURA28	-	+	+	+	+	+	-
AURI34	-	-	+	+	+	+	+

AURI36	-	+	+	+	+	-	-
AURC37	-	-	+	+	+	-	-

(+) = growth and (-) = no growth

5.4.3. NaCl tolerance of the isolates

All isolates failed to tolerate the minimum tested 1% NaCl (w/v) except AURA13 (*Aeschynomene schimper*), AURI21 (*Indigofera spinosa*) and AURC24 (*Crotalaria species*) that tolerated up to 6% NaCl, and AURI34 (*Indigofera species*) which tolerated up to 3% NaCl (Table7).

Table7. NaCl tolerance of the isolates

Rhizobial isolates	Percent of NaCl(w/v)					
AURD2	1	2	3	4	5	6
AURI6	-	-	-	-	-	-
AURI14	-	-	-	-	-	-
AURC22	-	-	-	-	-	-
AURE2	-	-	-	-	-	-
AURC23	-	-	-	-	-	-
AURI30	-	-	-	-	-	-
AURI32	-	-	-	-	-	-
AURC36	-	-	-	-	-	-
AURC42	-	-	-	-	-	-
AURC48	-	-	-	-	-	-
AURCp2	-	-	-	-	-	-
AURL2	-	-	-	-	-	-
AURT2	-	-	-	-	-	-
AURA13	+	+	+	+	+	+
AURI21	+	+	+	+	+	+
AURC24	+	+	+	+	+	+
AURA28	-	-	-	-	-	-
AURI34	+	+	+	-	-	-
AURI36	-	-	-	-	-	-
AURC37	-	-	-	-	-	-

(+) = growth and (-) = no growth

5.4.4. Intrinsic antibiotic resistance (IAR) of the isolates

The evaluation of intrinsic antibiotic resistance (IAR) of the isolates revealed that most of the isolates are resistant to chloramphenicol, rifampicin, erythromycin, neomycin sulfate and ampicillin sodium sulfate but sensitive to streptomycin sulfate followed by Kanamycin monosulfate (Table 8).Two isolates of *Crotalaria species* (AURC22 and AURC37) resisted all the tested antibiotics at concentration of 30μgml^{-1} while isolate AURI34 (from *Indigofera spinosa*)was inhibited by all antibiotics at the indicated concentrations(Table. 8).

Table 8. Intrinsic antibiotic resistance (IAR) of the isolates

Rhizobial isolates	Antibiotics and their concentrations(μg ml^{-1})																				
	Chloramphenicol			Streptomycin sulfate			Rifampicin			Erythromycin			Kanamycin monosulfate			Neomycin sulfate			Ampicillin sodium salt		
	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
AURD2	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
AURI6	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI14	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURE2	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
AURC23	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI30	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+
AURI32	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC36	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC42	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC48	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURCp2	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+

AURL2	+	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+
AURT2	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+
AURA13	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	-	+	+	+
AURI21	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
AURC24	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
AURA28	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AURI36	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+
AURC37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) = growth and (-) = no growth

5.4.5. Carbon source utilization

Most of the isolates were able to utilize all the tested carbon substrates except sodium citrate (Table 9). AURI21 (from *Indigofera species*) was found to grow on all the tested 16 carbon sources including sodium citrate. Although AURA13 (from *Aeschynomen schimperi)* was found to grow on sodium citrate, it failed to catabolize D-arabinose, cellobiose and glycerol (Table 9).

Table 9.Carbon source utilization of the isolates

Rhizobial isolates	Carbon source															
	D-glucose	D-fructose	D-galactose	D-arabinose	D-mannose	xylose	maltose	lactose	trehalose	D-sucrose	Cellobiose	mannitol	glycerol	Sorbitol	Na-citrate	Na-glutamate
AURD2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURE2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURCp2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURL2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURT2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURA13	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+
AURI21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

AURA28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURI36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
AURC37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+

(+) = growth and (-) = no growth

5.4.6. Phosphate Solubilization

On testing the phosphate solubilizing ability of the isolates on Pikovskaya's medium, isolate AURI30 and AURA13 formed relatively wider clear zones around their colonies. Isolate AURT2, AURCp2, AURL2 and AURI21 formed narrower clear zones while all the remaining 15 isolates did not form any clear zones.

5.5 Numerical Analysis

At a boundary level of 64.8% average similarity, all the isolates were grouped in to one major cluster and two minor clusters (Fig .4).The major cluster contained 16 of the 21 isolates which were isolated from root nodules of *Lupinus albus, Aeschynomene elaphroxylon, Chamaecytisus palmensis, Erythrina brucei* and species of *Dalbergia, Indigofera, Crotalaria* and *Tephrosia*. One of the minor clusters contained two isolates of *Indigofera species(AURI34 and AURI30)* while the other minor cluster contained three isolates of different species of woody legumes(*Aeschynomene, Crotalaria and Indigofera*). At a boundary level of 85% similarity, all the isolates were grouped in to four clusters except isolate AURI34, AURI30, AURA13, AURT2 and AURL2 which were not clustered with any of the other isolates. At 92.2% level of similarity, the isolates were further grouped into five clusters except 8 isolates.

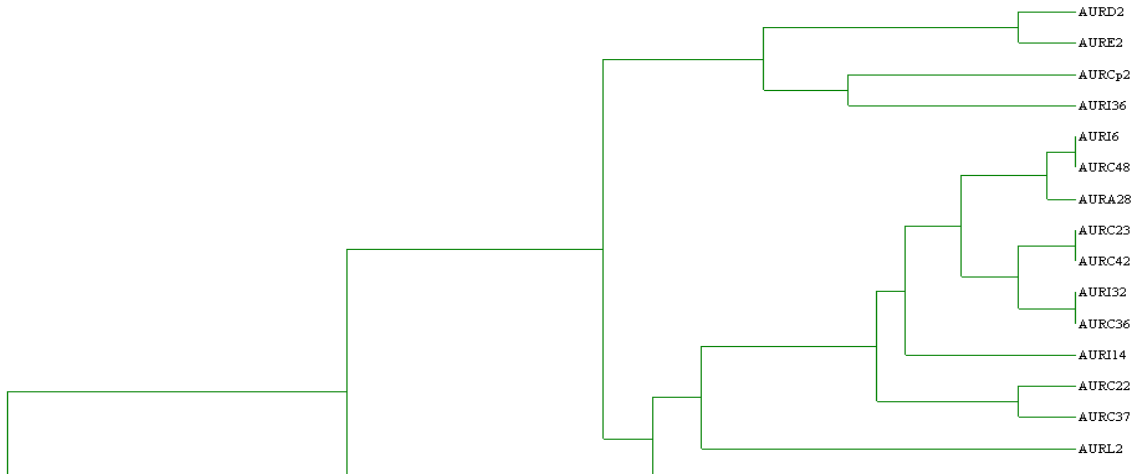


Fig .4 Dendrogram highlighting phenotypic similarity of the isolates

5.6. **Cross-inoculation and symbiotic effectiveness test**

Of the four inoculated isolates, AURE2 (from *Erythrina brucei*), AURC36 (from *Crotalaria*) and AURT2 (from *Tephrosia*) were found to induce nodulation on cowpea (*Vigna unguiculata)* while AURI30 (from *Indigofera*) failed to do so. The cowpea plants nodulated by AURE2 and AURC36 were found to be taller, well grown with darker green leaves than even those nitrogen-treated control plants(Fig .5A).Those cowpea plants inoculated with isolate of *Tephrosia* (AURT2) showed poor growth compared to the ones inoculated with isolates *Erythrina brucei* and *Crotalaria species.*

*A*ll of the four inoculated isolates elicited root nodules on *Phaseolus vulgaris.*The nodulated plants showed better growth and possessed greener leaves compared to the uninoculated control plants (Fig.6A).Root nodules induced by the isolates on *P.vulgaris* were found to be smaller than those induced on *V.unguiculata* (Fig.5B and Fig.6B)


V. ungulata
AURE
17-8-98


V. ungulata
AURC36
17-8-98


V. ungulata
AURI30
17-8-98


V. ungulata
AURTe
17-8-98


V. ungulata
N+
17-8-98


V. ungulata
N-free
17-8-98

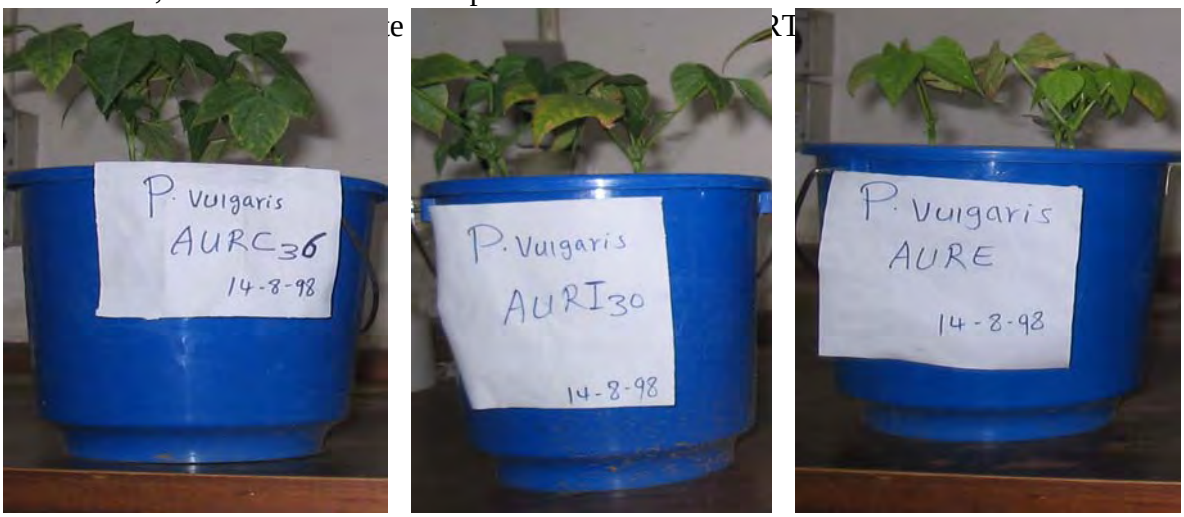
B

B

AURC36 AURE2 AURT2

Fig 5.A. *Vigna unguiculata* plants inoculated with isolate AURE2, AURC36, AURI30 and AURT2; and N+ and N- control plants

A



B

AURC36 AURE 2 AURT2 AURI30

Fig .6 A. *Phaseolus vulgaris* plants inoculated with isolate AURC36, AURI30,AURE2, and AURT2; and N+ and N- control plants
B. Nodules induced by isolate AURC36, AURE2 and AURT2 and AURI30 on *Phaseolus vulgaris*

The cross-nodulating isolates of woody legumes on *V.unguiculata* and *P.vulgaris* showed variations in nodule number, nodule dry weight, shoot dry weight and total nitrogen as well as symbiotic effectiveness(Table 10 and 11).AURC36(*Crotalaria*) accumulated the maximum shoot dry weight of 473 mg/plant on *V.unguiculata* and 409 mg/plant on *P. vulgaris*. AURI30 (*Indigofera*), which totally failed to nodulate *V.unguiculata,*showed an increase in shoot dry weight of 398 mg/plant on *P.vulgaris*.Similarly,AURE2(*Erythrina brucei*) displayed an increase of shoot dry weight of 356 mg/plant on *V.unguiculata* and 256 mg/plant on *P.vulgaris.*AURT2(*Tephrosia*) managed to increase shoot dry weight by 284 mg/plant, and 225 mg/plant on *P.vulgaris* and *V.unguiculata,* respectively.

The maximum total nitrogen (1.91%) was accumulated by isolate of *Erythrina brucei* (AURE2) on *V.unguiculata* followed by AURC36 (from *Crotalaria*) on *P.vulgaris*(1.63%).AURT2(from *Tephrosia*) accumulated total nitrogen of 1.2% and 1.0% on *V.unguiculata* and *P.vulgaris* , respectively. The least total nitrogen (0.65%) was accumulated by AURI30 (from *Indigofera*) on *P.vulgaris*. Shoot dry weight was found to be positively correlated with total nitrogen (Pirson`s correlation, r = 0.6) at 0.01 significant level (2-tailed) both in the case of *P.vulgaris* and *V.unguiculata*.

AURC36 displayed symbiotic effectiveness of 112% and 87% on *P.vulgaris* and *V.unguiculata*, respectively (Table 10 and 11). Similarly, AURE2 showed 70% and 65% symbiotic effectiveness on *P.vulgaris* and *V.unguiculata,* respectively. AURT2 showed symbiotic effectiveness of 78% on *P.vulgaris* and 41% on *V. unguiculata.* Although it failed to nodulate *V.unguiculata,* AURI30 displayed 109% symbiotic effectiveness on *P.vulgaris*

Table10. Evaluation of symbiotic effectiveness of the nodulated isolates with *Vigna unguiculata*

Treatment	NN/Plant	NDW(mg/plant)	SDW(mg/plant)	TN%	Symbiotic effectiveness%
AURC36	111^{a}	54^{ab}	473^{ab}	1.57^{b}	87
AURE2	132^{a}	65^{a}	356^{bc}	1.91^{a}	65
AURT2	25^{b}	40^{b}	225^{cd}	1.20^{c}	41
N+	-	-	543^{a}	1.36^{bc}	-
N-	-	-	89^{d}	0.16^{d}	-

.NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, TN = total nitrogen
.Numbers are the means of variables of three replicates of three plants per pot
.Letters in the columns (a, b, c and d) are ranks of the means
.Numbers in the same column raised to different letters are significantly different at 0.05 level (Turkey HSD).

Table11 .Evaluation of symbiotic effectiveness of the nodulated isolates with *Phaseolus vulgaris*

Treatment	NN/Plant	NDW(mg/plant)	SDW(mg/plant)	TN%	Symbiotic effectiveness%
AURC36	120^{a}	77^{a}	409^{a}	1.63^{a}	112
AURE2	63^{b}	28^{b}	256^{ab}	1.11^{ab}	70
AURT2	79^{b}	50^{ab}	284^{ab}	1.00^{abc}	78
AURI30	73^{b}	54^{ab}	398^{a}	0.65^{bc}	109
N+	-	-	365^{ab}	1.61^{a}	-
N-	-	-	160^{b}	0.20^{c}	-

NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, TN = total nitrogen
.Numbers are the means of variables of three replicates of three plants per pot
.Letters in the columns (a, b, c and d) are ranks of the means

.Numbers in the same column raised to different letters are significantly different at 0.05 level (Turkey HSD).

6. Discussion

All the isolates were found to be gram negative rods with white colonies, failed to absorb congo red(except AURI34), produce 3-ketolactose and grow on peptone glucose agar, revealing the characterstics of rhizobia (Lupwayi and Haque, 1994).

The rhizobial isolates of the woody legumes included in this study (Table 3) showed variations in the morphological and physiological characteristics. Most of the isolates formed dome-shaped colonies with shiny appearance and buttery texture. All of the isolates produced gelatinous colonies due to the production of much exopolysaccharides except AURC42.

The mean generation time of the isolates ranged from a minimum of 1.3 hours to a maximum of 11.4 hours (Table 4). Strains with colony diameter of 2-4 mm within 3-5 days on YEMA (Jordan, 1984) and mean generation time of less than 4 hours (Zerhari *et al*., 2000) were designated as fast growing rhizobia while those strains with mean generation time of greater than 4 hours (Zerhari *et al*., 2000) and colony size that does not exceed 1 mm within 5-7 days (Jordan, 1984) were classified as slow growing rhizobia. Four isolates (AURI30, AURT2, AURA13 and AURA28) achieved the mean generation time of less than 4 hours with colony diameter of 2-5 mm after 3 days. Thus, they can be grouped as fast growing rhizobia. The remaining 17 isolates displayed mean generation time of greater than 4 hours and colony diameter of 1-2.4 mm after 7 days so that they can be grouped as slow growing rhizobia on the basis of their mean generation time.

All of the fast growing isolates and two slow growing isolates (AURC36 and AURC22) produced acid while all the remaining slow growing isolates produced alkali. The production of acid and alkali were confirmed by the conversion of BTB incorporated green YEMA (pH = 6.8) into yellow (pH = 6) and blue (pH = 7.6) respectively. Although acid production and alkali production are typically the characteristics of fast growing and slow growing rhizobia, respectively, Odee *et al*. (1997); Endalkachew Wolde-Meskel *et al*. (2004); Endalkachew Wolde-Meskel *et al*. (2005) isolated some fast growing alkali producing and some slow growing acid producing rhizobia from woody legumes. However, fast growing alkali producing rhizobia were not encountered in this study.

The nodulation pattern of the isolates showed that *Indigofera* is nodulated by both slow growing and fast growing root nodule bacteria; *Crotalaria*, *Dalbergia*, *Chamaecytisus palmensis Erythrina brucei* and *Lupinus albus* are nodulated by slow growing rhizobia where as *Tephrosia* and *Aeschynomene* are nodulated by fast growing rhizobia. Similar to the result of the current work, there are reports on the isolation of slow growing rhizobia from root nodules of plants of the genus *Erythrina* (Fassil Assefa, 1993;Moriera *et al*.,1998), *Dalbergia*(Moriera *et al*.,1998),*Chamaecytisus*(Vinuesa *et al*.,1998) and *Lupinus*(Trujillo,2005).Isolation of strains of *Rhizobium* from *Tephrosia* and *Indigofera* was also reported by Sahgal and Johri(2003),and Wei *et al*.(2002),respectively.

The nodulation pattern clearly indicated that tree legumes are nodulated by both fast growing and slow growing rhizobia in agreement with several investigators (Turk and Keyser, 1992; Fassil Assefa, 1993; Moriera *et al*., 1998; Endalkachew Wolde-Meskel *et al*., 2004; Endalkachew Wolde-Meskel *et al*.,2005) that showed tree legumes are as often nodulated by fast growing rhizobia as by slow growing rhizobia contrary to early reporters that argued tree legumes are exclusively nodulated by slow growing rhizobia.

All isolates were able to grow at pH levels ranging from 5.5 to 9.5 (Table 5). The slow growing alkali producing isolates grew better at alkaline pH levels (7.5-9.5) rather than at lower pH levels (4-5).Similarly, Grossman *et al*. (2005) reported the high acid susceptibility of slow growing strains of rhizobia isolated from leguminous tree of the genus *Inga*. However,the slow growing bradyrhizobial isolates of *Acacia saligna* were found to be alkali-sensitive and acid-

tolerant(Marsudi *et al*.,1999). AURA 13, a fast growing acid producing isolate was able to grow at pH 4 and 4.5 contradicting with the general conclusion: slow growing bradyrhizobia are generally more acid tolerant than fast growing rhizobia (Cooper *et al*., 1985).Fassil Assefa (1993) demonstrated that it is difficult to prove the authenticity of the relationship between pH tolerance and acid or base production. Changes in protein expression, cytoplasmic potassium and glutamate levels (Aarons and Graham, 1991) as well as the composition and structure of outer membrane (Graham *et al.*, 1994) were reported to involve in pH tolerance.

Only 3 isolates were able to grow at 5 ^{o}C (Table 6) as opposed to Fassil Assefa (1993) who reported the growth of all of his rhizobial isolates from some woody legumes of Ethiopia at 4 ^{o}C. Over 90% and 47% of the isolates tolerated and grew at 35 and 40 ^{o}C, respectively. Thus, most of the isolates can over come high soil temperature which is one of the major problems for biological nitrogen fixation in tropical and subtropical areas (Michiel *et al*., 1994).

Eighty percent of the isolates failed to tolerate 1.0% NaCl (w/v).Three isolates (AURA13, AURI21 and AURC24) tolerated up to 6% NaCl (Table 7) similar to root nodule bacteria from woody legumes such as *Acacia, Prospis* and *Leucaena,* which were found to be tolerant to 5% NaCl (Lal and Khanna, 1995).

The majority of the isolates were found to be more sensitive to streptomycin sulfate followed by kanamycin monosulfate than the other antibiotics, all tested at concentrations of 10, 20 and 30 µg/ml (Table 8). On the contrary, all the isolates were tolerant to ampicillin sodium sulfate except AURI34, which failed to tolerate all the tested antibiotics at the indicated concentrations. Similarly, Odee *et al*. (1997) found that most rhizobia isolated from woody legumes in Kenya were more sensitive to streptomycin than kanamycin monosulfate and ampicillin. Two isolates of *Crotalaria species* (AURC22 and AURC37) tolerated all the tested antibiotics at a final concentration of 30µg/ml. These isolates might have developed resistance to antibiotics due to co-existence with antibiotic producing organisms or harbour plasmids that carry genes for antibiotic resistance. Similar results of IAR may indicate a similar genetic background of the isolates irrespective of their sites of isolation (Raza *et al*., 2001).

All isolates catabolized and grew on monosaccharides (D-glucose, D-fructose, D- galactose, D-mannose and xylose), disaccharides (maltose, lactose, trehalose and D-sucrose) and sugar alcohols (D-mannitol and sorbitol) as sole carbon sources (Table 9). However, only two isolates (AURA13 and AURI21) grew on Na-citrate. The ability of all the slow growing isolates to catabolize all the tested disaccharides conflicts with other reports that indicate the inability of most bradyrhizobia to utilize disacchrides (Marsudi *et al*.,1999). Although it is a fast growing isolate, AURA13 failed to grow on D-arabinose, cellobiose and glycerol on which all the slow growing isolates grew. The utilization of 13-15 carbon sources by fast growing islates(AURI30,AURT2,AURA13 and AURA28) and 14-16 carbon sources by slow growing isolates (Table 9) shows that both rhizobial groups are more or less equally efficient in catabolizing the various carbon sources. This result could not show the taxonomic diversity of the isolates when compared to the result of IAR. The higher discriminating power of IAR profile was also indicated by Alexandre *et al.* (2005).

The formation of relatively wider clear zones by isolate AURI30 (Indigofera) and AURA13 (Aeschynomene) around their colonies indicates their better phosphate solubilizing ability.Tewari *et al.*(2004) and other authors described the phosphate solubilizing ability of rhizobia though rhizobia were not considered as the main phosphate solubilizing bacteria (Bacillus and Pseudomonas) by Tilak *et al*.(2005)

Numerical analysis of the phenotypic data of the isolates also revealed their diversity. The isolates were grouped in to different clusters at different similarity levels and even some of the isolates failed to form clusters with others at higher similarity levels (Fig.4). Ingeneral, the isolates did not cluster on the basis of site or host of isolation similar to the finding of Odee *et al*. (1997) in contrast to the early grouping of rhizobia on the basis of the host.

The nodulation of common bean (*Phaseolus vulgaris*) by all of the four tested isolates (AURC 36, AURE2, AURT2 and AURI30) and cowpea (*Vigna unguiculata*) by three of the isolates (AURC36, AURE2 and AURT2) indicates the symbiotic promiscuity of these grain legumes that has been reported by several researchers (Allen and Allen, 1981; Bala and Giller, 2001).It also indicates the ability of rhizobial isolates from tropical woody legumes to promiscuously nodulate tropical pulse crops(Allen and Allen, 1981; Wang, 1989; Fassil Assefa and Kleiner, 1997). For example, Fassil Assefa and Kleiner (1997) demonstrated the ability of *Bradyrhizobium species*

isolated from *Erythrina brucei* to induce nodulation on African yam bean (*Sphenostylis stenocarpa*), and Somasegaran and Hoben (1994) described *Crotalaria species* as a notable example of promiscuous legumes in the legume-rhizobial association. Isolate AURI30 (from *Indigofera*) elicited nodulation on *P. vulgaris* but failed to do so on *V. unguiculata* under the same greenhouse conditions. This failure is best explained interms of genetic incompatibility of the isolate and the heterologous host legume.

The inoculation response of the nodulated *P. vulgaris* and *V. unguiculata* plants was expressed by the well developed appearance with greener leaves(Fig.5A and Fig.6A), the higher accumulated shoot dry weight and total nitrogen than uninoculated control plants in all cases and the nitrogen fertilized plants in some cases(Table 10 and 11).

With regard to symbiotic effectiveness, which was calculated interms of the ratio of the shoot dry weight of the nodulated plants to that of the nitrogen fertilized plants, isolate AURC36 (*Crolataria*) performed best on both *P. vulgaris* (112 %) and *V. unguiculanta* (87 %). Of the three isolates that induced nodulation on *V. unguiculata,* isolate AURT2(*Tephrosia*) produced the least nodule number and dry weight, shoot dry weight and accumulated the least total nitrogen and displayed the least symbiotic effectiveness(41%). However, in the case of *P. vulgaris*, isolate AURT2 (from *Tephrosia*) revealed almost similar symbiotic effectiveness (78%) with AURE2 (70 %). Isolate AURI30(from *Indigofera*), which failed to induce nodulation on *V. unguiculata*, showed higher symbiotic effectiveness (109%) on *P. vulgaris* than isolate from *Erythrina brucei*,AURE2(70%) and *Tephrosia*,AURT2(78%).

Variation in the symbiotic effectiveness of the isolates suggests the presence of specific host-strain requirements. AURC36 (*Crotalaria*) was found to be the most effective isolate with both *V. unguiculata* and *P. vulgaris*. The ability of such a single strain to nodulate and fix N_2 effectively with different legumes offers potential for identification of a single inoculant strain for several legume species (Bala and Giller, 2001).

Although shoot dry weight was positively correlated with the total N_2 ($r = 0.6$) both for *V. unguiculata* and *P. vulgaris* at 0.01 significant level (2-tailed), higher shoot dry weight was not necessarily associated with higher total nitrogen percent. For instance, *V. unguiculata* plants which

were fertilized with N_2 and acquired a mean shoot dry weight of 543 mg/plant or those inoculated with AURC36 and acquired a mean shoot dry weight of 473 mg/plant resulted in a significantly ($\alpha = 0.05$) lower total N_2 percent than those inoculated with AURE2 and acquired a mean shoot dry weight of 356 mg/plant. Such observation was explained by Bala and Giller (2001) in terms of the nitrogen use efficiency to produce dry matter per unit nitrogen accumulated.

7. Conclusion and recommendations

7.1. Conclusion

The diversity of the rhizobial isolates of the woody legumes was revealed interms of variations in their morphological, physiological and symbiotic characteristics. The bulk of the isolates were slow growing with mean generation time of greater than 4 hours. The ability of the isolates to utilize a wide range of carbon sources may give a competitive advantage for the isolates when they live as saprophytes in the soil. The growth of the isolates over a wider range of pH and temperature implies they can be good candidates for inoculation under varied physical conditions. The majority of the isolates failed to tolerate 1%NaCl (w/v) irrespective of their host species and site of collection. Of the tested antibiotics, streptomycin sulfate was found to be the most potent antibiotic against the isolates.

Both *V.unguiculata* and *P.vulgaris* exhibited inoculation responses expressed in terms of greener leaves, higher shoot dry weight and accumulated total N_2 possessed by the inoculated plants than the uninoculated control plants. Symbiotic effectiveness varied from a minimum of 41% in the *V.unguiculata*-AURT2 (from Tephrosia) association to a maximum of 112% in *V.unguiculata*-AURC36(from Crotalaria) association. The symbiotic effectiveness revealed that the rhizobia from *Crotalia species* was best matched with both *P.vulgaris* and *V.unguiculata.* This matching of rhizobial strain to host legume is one of the most important factors in maximizing productivity and full expression of N_2 fixation capacity.

7.2. Recommendations

Based up on the results of this work, the following points are recommended.

- Isolation and characterization of endosymbionts of woody legumes should be encouraged to obtain isolates that can form effective symbiosis with a broader host range under a wide range of environmental conditions.
- The effective isolates that have been tested with *V.unguiculata* and *P.vulgaris* at green house level need to be tried in the field since greenhouse findings should not be necessarily extrapolated.
- Rhizobia inoculum production and utilization should be encouraged to improve agricultural yield and avoid the draw backs of chemical fertilizers.
- Further taxonomic study of the isolates should be carried out on the basis of polyphasic approach by taking as many morphological, biochemical and genetic characters as possible to have a more complete picture about their taxonomy and evolutionary relationship.

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Appendices

Appendix 1.ANOVA of shoot dry weight, nodule number and dry weight, and total nitrogen common bean plants

parameter	Source of variation	Sum of squares	Mean of squares	df	F value	Sig.
Nodule number	Between groups Within groups Total	6658 25498 32156	2219 1821	3 14 17	1.3	0.34
Nodule dry weight	Between groups Within groups Total	5099 6810 11908	1610 486	3 14 17	3.5	0.044
Shoot dry weight	Between groups Within groups Total	149045 177737 326782	29809 9874	5 18 23	3.0	0.038
Total nitrogen	Between groups Within groups Total	4.6 2.4 7.0	0.93 0.14	5 18 23	6.9	0.001

Appendix 2.ANOVA of shoot dry weight, nodule number and dry weight, and total nitrogen of cowpea plants

parameter	Source of variation	Sum of squares	Mean of squares	df	F value	Sig.
Nodule number	Between groups Within groups Total	57943 15235 73179	28972 635	2 24 26	45.6	0.00
Nodule dry weight	Between groups Within groups Total	2745 7864 10609	1373 328	2 24 26	4.2	0.03
Shoot dry weight	Between groups Within groups Total	1216434 622604 1839039	304109 15565	4 40 44	19.5	0.00
Total nitrogen	Between groups Within groups Total	16 2 18	3.90 0.06	4 40 44	67.0	0.00

Appendix 3.Correlation test of shoot dry weight and total nitrogen percent of common bean (A) and cowpea (B) plants.

A

		Shoot dry weight	Total nitrogen percent
Shoot dry weight	pearson correlation Sig.(2-tailed) N	1 . 24	0.6** 0.002 24
Total N-percent	pearson correlation Sig.(2-tailed) N	0.6** 0.002 24	1 . 24

B

		Shoot dry weight	Total nitrogen percent
Shoot dry weight	pearson correlation Sig.(2-tailed) N	1 . 45	0.6** 0.0 45
Total N-percent	pearson correlation Sig.(2-tailed) N	0.6** 0.0 45	1 . 45

** Correlation is significant at the 0.01 level (2-tailed)