ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

# Phenotypic and Symbiotic properties of Rhizobia on some *Acacia species* from Silti Zone, Lanfuro woreda, Southern Ethiopia

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## Symbols and Abbreviation

| | |
|---|---|
| ANOVA | Analysis of variance |
| BNF | Biological Nitrogen Fixation |
| BCP | Bromocreesol purple |
| BCP-YEMA | Bromocreesol purple- Yeast extract mannitol agar |
| BTB | Bromothymol blue |
| CR | Congo red |
| CR-YEMA | Congo red-Yeast extract mannitol agar |
| FG | Fast growing |
| g/p | gram per plant |
| LM | Large mucoid |
| LW | Large watery |
| Mo-Fe protein | Molybdenum iron protein |
| OD | Optical density |
| PGA | Peptone-glucose test |
| PGPR | plant growth promoting rhizobacter |
| PS | Phosphate solubilization |
| Rev/min | revolution/minute |
| SD | Small dry |
| SG | Slow growing |
| YEMA | Yeast extract mannitol agar medium |

# Abstract

*Acacia* is a multipurpose leguminous woody plant widely distributed in a wide range of habitats because of its ability to fix nitrogen and improve soil fertility. However, effectiveness in nitrogen fixation is variable, due to different factors. In this study, eleven isolates of root nodule bacteria from four *Acacia* species were isolated and evaluated for their effectiveness in nitrogen fixation under greenhouse conditions. The isolates were characterized based on their morphological, physiological and symbiotic characteristics. Accordingly, most of the isolates (80%) collected from *Acacia abyssinica*, *Acacia negrii* and *Acacia seyal* were fast growing rhizobia, whereas root nodule bacteria from *Acacia albdia* were slow growers. The isolates showed variations in morphological and physiological characteristics and most of them were tolerant to high concentration of NaCl (5-6%) and grew over a wider temperature ranges of ($4^0$C-$45^0$C) and they were capable of utilizing various types of carbohydrates. Three isolates of AURAaby21, AURAaby24 (both from *A. abyssinica*) and AURAsey41 (from *Acacia seyal*) were phosphate solubilizes. The symbiotic effectiveness of the isolates showed significant ($P \leq 0.05$) variation in mean shoot dry weight compared to their respective N+ and N- control groups. The isolates induced nodulation with average nodule number of 34 NN/plant (*Acacia albdia*) and 52 NN/plant with *Acacia seyal*, average nodule dry weight of 0.051 mg/plant (*Acacia negrii*) and 0.041 mg/p (*Acacia abyssinica*). The Seedlings accumulated shoot dry weight of 0.3 g/plant (*A. abyssinica*) to 0.43 g/plant (*Acacia negrii*). Accordingly, more than 70% of the isolates i.e. AURAal 11 (*Acacia albdia*), AURAaby22 (*A. abyssinica*), AURAneg31 (*Acacia negrii*) and AURAsey41 (*Acacia seyal)* induced highly effective, whereas, AURAaby21, AURAaby24 (both from Acacia .abyssinica), AURAneg32 (*Acacia negrii*) and AURAsey42 (*Acacia seyal*) induced effective nitrogen fixation on their respective host. Some acacia spp. nodulated only with fast growing rhizobia, while other acacia spp. nodulated only with slow growing rhizobia. In most case, acacia spp. that nodulated with both fast and slow growing can only develop an effective symbiotic with one type of symbiont.

**Key words:** *Acacia*, rhizobia, phosphate solubilization, salt tolerance

# 1. Introduction

Legumes are classified into the third largest family of flowering plants (Doyle and Luckow, 2003). That are very important in different agricultural system for the ability in fixing nitrogen (Biological Nitrogen Fixation, BNF) with root nodule bacteria, collectively referred to as rhizobia (Vance and Graham, 2003).

The genus *Acacia* belongs to the *Mimosoideae* and comprises over 1550 species and is widely distributed throughout the world, particularly in Africa, Asia and Australia (Maslin *et al.,* 2003). They are important components of many agro-forestry systems, and provide fire wood, pulp, timber, and forage for cattle, fruit and gum (Giller, 2001).
Many *Acacia* species are nodulated by fast and slow growing rhizaobia (Dommergues and Dryfus, 1981) and fix nitrogen ranging from 20 kg-200 kg/ha/yr (Sanginga *et al*, 1995). The selection of *Acacias* as important tree and shrub components in many agro forestry practices is attributed mainly to their capacity for nodulation and biological nitrogen fixation; even under adverse environmental conditions (Odee *et al.*, 1995; Zahran, 1999).

Rhizobia are agriculturally and environmentally important, because their symbioses with legumes are responsible for conversion of the atmospheric nitrogen into ammonia that can be utilized by plants as a nitrogen source. This process by which $N_2$ is converted into ammonia via legume-rhizobia symbiosis is known as Biological Nitrogen Fixation (BNF). A substantial portion of the world’s supply of organic nitrogen is fixed via the symbiosis between rhizobia and leguminous host plants (Postgate, 1998).

Originally, root nodule bacteria *Rhizobium* and *Brady rhizobium* were characterized as fast and slow growing rhizobia on the basis of their growth rate and production of acid or base on Yeast extract mannitol agar medium (YEMA)( Jordan, 1984).

According to Dreyfus and Dommergus (1981), *Acacia* species are nodulated either by *Rhizobium*, *Bradyrhizobium* or by both genera. Currently, the majority of the *Acacia* symbionts are further classified with in the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Leary *et al*., 2006).

*Acacia* is a cosmopolitan genus with diverse species adapted to wide range of extreme environmental conditions including higher acidity higher salinity and low soil fertility (Fassil Assefa, 1993; Zehari *et al*., 2000 and Brock well *et al*., 2005). These adaptation makes *Acacia's* species important to agro forestry and land reclamation under extreme environments. Yet environmental constraints such as soil moisture, salinity, nutrient deficiency or excess soil $P^H$ and soil temperature impose limitations to the effectiveness of acacia-rhizobia Symbiosis (Odee et al., 1995; Surange et al., 1997; Zahran, 1999 and Zehari et al., 2000).

Despite the importance of *Acacia* –rhizobia too many agronomic systems, little is known the specificity of symbiotic relationship between *Acacias* and rhizobia (Burdon *et al*., 1999). Recent works of some researchers on this area have proved that specificity for nodulation and $N_2$ fixation vary greatly among *Acacia* spp. and these variation in symbiotic effectiveness have been Documented (Fassil Assefa, 1993;Fassil and Kleiner, 1998 ; Burdon *et al*.,1999 and Thrall *et al*.,2000)

The nitrogen fixation in leguminous plants, in general, may involve one or more bacterial partners (Simms and Taylor, 2000). In some case, the ineffective strains may nodulate with no or little nitrogen accumulation that necessitates inoculation with more effective rhizobial partners. Simms and Taylor (2002) reported the need for inoculation of different *Acacia* species to achieve the desired outcomes in afforestation and land reclamation programs. Similarly, while working on the response of inoculation on seven African *Acacia* species, Sutherland *et al*., (2000), concluded that inoculation can result in substantial gain in nitrogen fixation on African *Acacia*; they reported that significant increase in dry weight and total nitrogen content was achieved with strain inoculum on *A. Karro, A. nilotica* and *A. tortilis.*

In Ethiopia, attempts were made to isolate and characterize some acacia rhizobia from some central and northern parts of the country (Fassil Assefa and Kleiner, 1998; Shishay Mesfin, 2008). They found that *Acacia* species are nodulated by either fast growing or slow growing rhizobia or by both partners, and can improve growth by selectively inoculated with effective rhizobia.

In the present study, attempt has been made to evaluate the Phenotypic and symbiotic properties of rhizobia on some *Acacia* species from Silti Zone, Lanfuro woreda, southern Ethiopia.

## 1.1. Statement of the problem

Previous legume-rhizobia symbiosis studies have concentrated on a few crop legumes, therefore leaving majority of woody legumes (*Acacia)* unstudied. Despite increasing researches on woody legumes have received no particular attention. This study investigated on the Acacia-rhizobia symbiosis through nodulation surveys, examination of Rhizobia diversity and selection of high effective or effective isolates in the greenhouse condition.

# 2. Objectives

## *2.1. General objective*

The general objective of the present study was to study the phenotypic and symbiotic characteristics of the root nodule bacteria of some *Acacia* species from some parts of the Rift valley area in Ethiopia.

## *2.2. Specific objectives*

The specific objectives of this study were:

- To isolate, characterize and authenticate the endosymbiont from *A. albida*, *A. abysinica, A. negrii and A. seyal*
- To evaluate the symbiotic effectiveness of the four acacia species

# 3. Literature review

## 3.1 legumes- the host plant

The legumes are classified into one of the largest groups of flowering plants, the *leguminosae*. This family comprises over 20,000 species, and it is the third largest family in the plant kingdom (Doyle and Luckow, 2003) and it is classified into three subfamilies including; *Caesalpiniodeae, Mimosoideae* and *Papilionoideae*. The legumes that are primarily tropical and subtropical are the sub families *Caesalpiniodeae* and *Mimosoideae* trees and shrubs and mainly distributed in lowlands, midlands and arid ecosystems (Dommerugus, 1988). They are multipurpose plants; with a number of significant functions in the use systems (Dommerugus, 1988; Young, 1996).

Legumes are very important both ecologically and agriculturally. This is mainly attributed to their substantial contribution to the global flux of nitrogen from atmosphere $N_2$ to biologically useful forms such as ammonia, and assimilates it into nitrogenous organic compounds (Young and Haukka, 1996). Although the vast majority of the *leguminosae* nodulate and fixes nitrogen; nevertheless some of them do not have this capacity. It is estimated that nodulation could occur in about 23% of the species in *Caesalpiniodeae,* 90% in *Mimosoideae* and 97% in *Papilionoideae* (de Faria *et al.,* 1989). Nodulation in legumes may not necessarily lead to effective nitrogen fixation (Vincent, 1987 in Fassil Assefa, 1993), and this could due to a number of factors; such as, the strains of the bacteria, the type of species of the host legume and the different biotic and abiotic environmental factors that affect the process of nitrogen fixation.

Several *Acacia* species have been used in land rehabilitation in Africa, Middle East, Vietnam and South America (Byrne and Broadhurst, 2003). Many Acacia species are multipurpose species with a potential to be utilized for many different uses. For example, *A. albida* has a principal uses in many agro forestry practices; such as terraces, dispersed trees, forages for livestock, shade tree, fuel and boosting soil fertility (Brockwell *et al.,* 2005).

Several Acacia species show enhanced soil fertility under their canopies and as a result increased crop yields. For example, yields of Sorghum and maize were over 50% greater beneath *A. albida* trees in eastern Ethiopia (Poschen, 1986) are important to add substantial N to the soil as a result of their leaf fall (Brock well *et al.*, 2005). Moreover, the litter of these *Acacias* could improve the soil environment by contributing to the retention of soil moisture through an increase in soil organic matter, improving soil structure, enhancing populations of soil micro fauna (Giller, 2001). *Acacia* planting has been recognized as an effective tool for contributing soil erosion starting from the early of 1960s because acacia can be readily established on degraded lands (Brock well *et al.*, 2005).

## 3.2. Biological Nitrogen Fixation

Biological Nitrogen Fixation (BNF) refers to the conversion of the inert atmospheric dinitrogen ($N_2$) in to biologically useful form such as ammonia. The process of biologically nitrogen fixation is carried out by bacteria that live freely or in symbiotic association with leguminous or non-leguminous plants (Postgate, 1998). These bacteria convert dinitrogen into ammonia with the aid of an enzyme complex system; nitrogenase (Zahran, 1999). In fact the ability to reduce $N_2$ is confined to bacteria and archaea, yet it is not found in eukaryotes.

Although a wide range of organisms have the ability to fix nitrogen; however, the symbiosis between *Rhizobium* or *Bradyrhizobium* and legumes are a cheaper and usually more effective agronomic practices for ensuring an adequate supply of N for legume based crop and pasture production than the application of fertilizer (Zahran, 1999).

Certainly, much greater rates of N-fixation per hectare have been measured for legumes than for any of the free fixing organisms (Sprent *et al*., 2000). Only small number of organism possess the ability to fix nitrogen and they are all prokaryotes in either free living forms or symbiotic association. Free-living contributes to only 20% of the fixed nitrogen while the remaining 80% is fixed by diverse Symbiotic association (Ramos and Bisseling 2004) of the symbiotic association, the legume-rhizobia symbiosis is the highest of nitrogen fixation (Van Kammen 1997) and therefore, appears to be the most important association for terrestrial Ecosystem (Perreijn *et al.,2002*).

The nitrogen thus fixed by legumes subsequently passes as root exudates released directly and, as plant and animal residues, decomposed in a series of microbiological transformations to enrich the soil. It is consequently assimilated by associated non-fixing plant species. The process of $N_2$- fixation is the reduction of this $N_2$ to a biologically useful, combined form of $NH_3$-ammonia. Because $N_2$ is very stable, the reaction is costly energetically. The equation for the reaction is as follows:

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16P$$

**Biotechnological applications of rhizobia**

Bio fertilizers can help meet the demands of sustainable productive agriculture at low cost. Rhizobia inoculants have been applied to legumes crops for 120 years as bio fertilizers, and inoculants carrying plant growth promoting rhizobacteria (PGPR) have been used in agriculture over half of century (Bashan and Bashan 2005 ). A broad of beneficial effects has been reported for PGPR, including biological nitrogen fixation (Ashraf et al. 2011), phosphate solubilization and production of hormones, such as auxins Gibberelins, ethylene and control of pathogens (Rodriguez et al., 2004). Modern agriculture has increasingly focused on the use of microbial products as alternatives to chemical fertilizers. Benefits from this replacement includes, substantially lower costs for farmers, less pollution and land degradation and reduced concerns regarding adverse side effects on human health (Crews and Peoples 2004).

### 3.2.1. Mechanisms of $N_2$ reduction

The reduction of $N_2$ begins with the acquisition of electrons by the Fe proteins; of the nitrogenase-specific electron donors; Ferredoxins and Flavodoxins, which are known to be potential electron donors. Ferredoxin belongs to the clan of iron-sulphur proteins, which are capable of transferring single electron, while Flavodoxins are proteins that contain the prosthetic group; Flavin mononucleotide (FMN) and each FMN group can transfer two electrons ( Giller, 2001). The molybdenum (mo) nitrogenase is the most common type of nitrogenase found in root nodule bacteria ( Giller, 2001) and it consists of two components: component I contains the active site where $N_2$ is actually reduced, and is also known as the MoFe protein or dinitrogenase; while Component II provides electrons to Components I for $N_2$ reduction and is known as the Fe protein or dinitrogenase reductase and these two components form sort of two interlinked cycles that operate in a sequential manner in the reduction of $N_2$ in to two molecules of ammonia and $H_2$ .

### 3.2.2 Environmental Factors that affect the process of Nitrogen fixation

Several environmental conditions are limiting factors to the growth and activity of the $N_2$-fixing host legumes. A principle of limiting factors states that "the level of crop production can be no higher than that allowed by the maximum limiting factor." (Brock well *et al.,* 2005). If a similar principle is applied to the *Rhizobium-legume* symbiosis; in general or *Acacia-rhizobia* in particular, which is a nitrogen fixation system, the process of $N_2$ fixation would be strongly linked to the physiological state of the host legume.
Therefore, even a noble rhizobia strain, which is proved to be competitive and effective, is not expected to express its full capacity for nitrogen fixation; if environmental constraints; (Such as, salinity, soil temperature, unfavorable soil pH insufficient or excessive soil moisture) impose limitations on the vigor of the host plant (Zhang *et al.,* 1991; Zahran, 1999; Zehari *et al.,* 2000; Rockwell *et al.,* 2005).

The *legume-Rhizobium* symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Zahran, 1999). Symbiotic *Rhizobium* strains of the Moroccan that are isolated from *Acacia* species can grow up to 5% NaC1 concentration (Zehari *et al.,* 2000); and Zhang *et al.,* (1991), isolated rhizobial stains of the Sudanese soil that can normally grow in 3% NaC1 concentration and had a higher tolerance than the host acacia. Hence nodulation of *acacia* requires a much lower salt concentration than the tolerance level displayed by the *Rhizobia*.

Several reports have described that a number of symbiotic bacteria isolated from African Acacias that are indigenous to hot tropical climates, survive at temperatures greater than 40°c (Zhang *et al.,* 1991; Zehari *et al.,* 2000). Nevertheless, these high temperatures can severely affect the symbiosis; for example, In Kenyan soils, indigenous *Acacia* species do not nodulate at the hottest times of the year when temperature values reach 40°c (Odee *et al.,* 1995).

In the humid tropics, weathering and acidity are significant impediments to plant growth and productivity (Leary *et al.,* 2006). The failure of legumes to nodulate under acidic soil conditions is common, especially in soils of pH less than 5 (Zahran, 1999). Yet there are rhizobia isolates that can tolerate pH values of less than 5 (Zehari *et al.,* 2000). Hence, it seems that the host legume is the limiting factor for establishing *Rhizobium-legume* symbiosis under acidic conditions; and it has been noted that *Acacia* species differ greatly in their response to lower pH with regard to growth and nodulation (Brockwell *et al.,* 2005). Symbiotic $N_2$ fixation of legumes is also highly sensitive to insufficient or excessive soil moisture (Zahran, 1999); and Habish (1970) in Brockwell *et al.,* (2005), regarded 15% Soil moisture as optimal for growth and nodulation of *Acacia* species.

## 3.3. Rhizobia and legume symbiosis

The symbiosis between rhiziobia and the angiosperm family *leguminosae* is perhaps the most well- known and best studied association due to its economic and ecological importance (Doyle 1994). As in all nitrogen fixing has the microbial partner accommodate inside plant cells. The rhizobia partner infects root tissue of the host legume to form root nodules (Occasionally stem nodules) where the rhizobia are housed and reduce atmospheric nitrogen. The plant provides the rhizobia with accommodation and food (sprent 2000)

### 3.3.1. Nodule forming processes

The processes of forming symbiotic association between the host legume and the rhizoibia are complex and involve many biochemical changes regulated by many genes and cell differentiation in both partners (Treva skis *et al* 2002). These steps are now well documents and detailed review was published by Hirsh (1992). Nodule development involves three basic stages:

1. **initial pre-infection stage**- the rhizobia attach to root hair of the host plant causing curling of root hairs
2. **infection and nodule organogenesis stage-** the rhizobia enter root hairs leading to the formation of nitrogen fixing bacteroids and
3. **Differentiation and functioning stage**- the nodule fix nitrogen for plant use (Hirsch 1992; Huang 2001).

The complex interactions begin with exchange and recognition of chemical signals between compatible partners. The root of the host legume secretes compounds such as lectins and flavonoids which induce the expression of nod genes in rhizobial.

The nod genes function together to produce rhizogial lip chitin oligosaccharides known as nod factors (Denarie *et al* .1996). These nod factors diffuse in to the plant root and stimulate cell division in the nodule primoridum. At the same time, the rhizobia enter the root and infect the nodule primordium cells. Once inside plant cells, the rhizobia are surrounds by plant derived membrane and different in to nitrogen-fixing units called bacteroids (Oke and long 1999).

The nodule premodium eventually develops in to a nodule in which the bacteriods fix atmospheric nitrogen in to ammonia. Newly-formed ammonia is transported in to the plant cell cytoplasm where it is transformed into amides for pant use (Huang 2001).

### 3.3.2. Host specificity

Although the fundamental processes of nodule formation are similar in all legumes – rhizobia association, in some symbiotic association both the legume and rhizobia show certain degree of specificity which means that certain types of rhizobia only colonize certain types of legumes plants (Postagate 1998). For example *Rhizobium leguminosarum* by *viciae* forms nodules with pea and vetch but another crop legume soy bean is nodulated by *Bradyrhizobium japonicum* (Stougaard 2000). Host-specificity is genetically controlled and accurse in different steps of nodule formation (Sharma *et al*.1993).

### 3.3.3. Nodulation ability

Traditionally, it was through that all legumes were able to form root nodules because nodulation studied increase particularly on those non-crop species, it was found that legumes vary in their abilities to form root nodules. The position of the legumes in the three sub-families particularly are able to forms nodules. The most primitive sub-family of legume *Caesalpinioiodeae* has only 23% of these species which form nodules (Sprent 2000). In this sub-family, nodulation is restricted to a few tribes.

The most notable one is cassieae which is a big tribe containing both nodulating and non-nodulating species (Sprent, 2000).

Within this tribe, all members of the genera cassia and senna are unable to nodulate, but all species of the closely related genera chamaecrista form nodules.

At most all members in the sub-family *Mimosoideae* (90%) and *Papiliioideae* (97%) can form root nodules, Non-nodulating members *Papilionoideae* are largely found in primitive tribes, such as Dipterygeae (Franco and de Faria 1997). Despite the trend described above, nodulation in a legume species, as remarked by many researchers, is an extremely variable event. (Sprent 2000). This is because formation of nodules is not only controlled by genetic factors but also environmental factors, since nodules are organs that fix nitrogen in legumes (Giller 2001).

Environmental stresses can be expressed through the host legumes, the rhizobia symbionts and the process of symbiotic association. Phosphorus limitation is well documented to reduce nitrogen fixation in legumes (Christiansen and Granam 2002). Variation in salinity affects legumes to heavy metal pollution (Giller and McGrath 1988) the infection process itself appears to be particularly sensitive to calcium deficiency (Gille2001).

## 3.4. Acacia

One of the most studied genera of woody legumes is Acacia. The current classification of the genus Acacia lists 1352 species, thus making it the second largest genus in the *leguminosae* family and the largest of the *Mimosiodeae* sub family (Maslin *et al*.2003). *Acacia* consists of three sub- general including *Acacia* (161 species), *A culeifrum* (231 species) and phyllodineae (960 species) (Maslin et al. 2003; Vassal, 1972). It is distributed throughout the world, particularly in Australia (>940specees), Africa (>140 species), Asia (>90 species) and the Americas (>180 species). They are rarely found in Europe. *Acacia* range from herbs (rare) to enormous trees, but most are shrubs and small trees. Their habitats range arid areas of low or seasonal rain fall to moist forests and river banks (Allen and Allen 1981). They are found on soil types. *Acacia* are valued as foliage, green manure, soil coverage to reduce erosion and in gardening land scalping (Martin and Sherman, 1992 and Giller, 2001).

*Acacia* forms symbiotic associations with strains of at least six genera including (*Rhizobium*, Bradyrhizobium, *Allorhizobium*, *Mesorhizobium*, *Azorhizobium* and

*Sinorhizobium)* of nodulating symbiotic bacteria (Leary et al., 2006). Many of the associations fix nitrogen from the atmosphere; nevertheless there is variation in there nodulation pattern in that some acacia are highly specific i.e. They fix nitrogen with only a small number of rhizobial strains, while others show promiscuous nodulation pattern. More surprisingly, species of non-nodulating acacias still display pre-infection event similar to that of nodulating ones, which include root curling and nod gene induction in rhizobia (Shaw *et al.,* 1997; Harrier *et al.,* 2000).The results of several pot studies using a variety of methods have confirmed that acacias have the capacity for symbiotic $N_2$fixation. Nevertheless, there are variations with respect to the symbiotic effectiveness for $N_2$fixation (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998 and Thrall *et al.,* 2000).

### 3.4.1 Acacias in agro forestry

Agro forestry is a collective name for land use systems in which woody perennials (trees, shrubs and bamboos) are deliberately cultivated in association with agricultural crops and/or pasture and livestock, and in which there is both an economic and ecological interactions between the tree and non-tree components of the system (Nair, 1985; Young, 1999 and Giller, 2001). Agro forestry systems take advantage of trees for many uses; such as, for food, fodder for livestock, fuel, often fibers, to provide shade, construction materials, protection of crops or animals by live fences, increase soil fertility through nitrogen fixation, or through bringing minerals form deep in the soil and depositing them leaf-fall (Young, 1999). Many $N_2$- fixing trees are chosen to from the tree component of agro-forestry systems; because they can grow rapidly on poor soils, due to at least partly to their ability to fix $N_2$ (Giller,2001). They may be selected either because they produce a needed product -timber, fuel wood or fodder or simply because of their overall contribution to soil fertility. The genus acacia has a large number of species that are adapted in wide range of habitats in both tropical and temperate regions throughout the world, making it an important tree and shrub component in agro forestry systems; and they are valued as forage, foliage green manure, wood, fuel, fodder for livestock, Shade, soil fertility etc. (Young, 1999; Martin and Sherman, 1992; Byrne and Broadhurst, 2003 and Brockwell *et al.,* 2005)

**Table 1.Utilization of some acacia species in agro forestry systems**

| *Acacia species* | Uses |
|---|---|
| *A. acuminate* | Charcoal, wood production |
| *A. albida* | Fodder, soil enrichment |
| *A. aneura* | Fodder, bush food |
| *A. auriculiformis* | Environmental rehabilitation, soil stabilsation, fuel wood, pulpwood |
| *A. baileyana* | Foliage, pollen, gum |
| *A. berlandieri* | Gum |
| *A. cambagei* | Posts, bush wood |
| *A. catechu* | Fuel wood |
| *A. crassicarpa* | Tolerance of high water tables |
| *A. Cyclops* | Salinity tolerance |
| *A. dealbata* | Pulpwood, gum, foliage, oils, pollen |
| *A. decurens* | Pulpwood, timber, fuel wood |
| *A. elata* | Pulpwood |
| *A. excels* | Fodder |
| *A. gerrardii* | Fodder |
| *A. harpophylla* | Fuel wood, charcoal |
| *A. hebeclada* | Gum |
| *A. homalophylla* | Turning |
| *A. imbricate* | Foliage |
| *A. implexa* | Fuel wood, turning, pollen |
| *A. irrorata* | Fuel wood, tannin |
| *A. kempeana* | Fodder |
| *A. mangium* | Timber, pulpwood |
| *A. mearnsii* | Tannin, fuel wood, charcoal, pulpwood |
| *A. melanoxylon* | Pulpwood |
| *A. mellifera* | Fodder |
| *A. nilotica* | Wood |
| *A. notabilis* | Bush food |
| *A. papyrocarpa* | Bush food |
| *A. parramattensis* | Pulpwood, tannin |
| *A. pendula* | Fodder, turning, fuel wood |
| *A. pycnatha* | Tannin, gum, bush food |
| *A. recinodes* | Salinity tolerance, foliage |
| *A. salicina* | Salinity tolerance, soil stabilization |
| *A. saligna* | Salinity tolerance, soil stabilization, tannin, fodder, bush food, gum, fuel wood |
| *A. Senegal* | Gum |
| *A. seyal* | Wood |

Source  adopted from Brockwell et al., 2005

### 3.4.2. Rhizobia –the microbial partner

Rhizobia usually refer to produce (occasionally stem) root nodules on leguminous plants. Rhizobia are also known as root nodule bacteria (Zakhia *et.al*. 2004). When free-living rhizobia occur as normal components of the soil microbial population. Morphologically rhizobia are motile by flagella, non-spore forming, gram negative and rod shaped bacteria. They are easily cultured on yeast-mannitol medium at the optimal condition of 25-39 0c, pH 6.0-7.0 (Jordan 1984; Somasegaran and Hoben 1994). Initially all legumes were considered to be infected by single species of rhizobia.

More studies and advanced molecular techniques have, however, revealed that there are many species of root nodule bacteria and some of them are host-specific which only induce nodules on specific legume hosts (Vance 1996).

Recent studies on legume tree species in the tropics have described various new rhizobia species based on their growth characteristics. The fast- growing group (Colonies appear 3-7 days after incubation) were acid-producing and were assigned to the genus *Rhizobium*. Whereas the slow- growing group (colonies appear 5-10 days after incubation) were alkaline-producing and were assigned to the genus *Brady rhizobium* (Jordan 1984). All currently known *Rhizobium* are in the phylum Proteobacteria, mostly in the sub class Alpha-protobacteria, which contains six rhizobia families in single order Rhizobiales (Garrity *et al* .2004).

### 3.4.3. Micro-Symbionts of Acacia

A majority of the acacia micro-symbionts are classified with in the genera *Brady rhizobioum, Mesorhizobium, Rhizobium, and Sinorhizobium* (Lafay and Burdon, 2001). There is a wide diversity with respect to their host range symbiotic effectiveness, and geographic origins. Originally, *Rhizobium* and *Bradyrhizobum* were characterized as fast and slow-growing *Rhizobia*, respectively. Dreyfus and Dommergues (1981) *Acacia* spp. that nodulate with both fast and slow growing rhizobia can only develop an effective symbiosis with one type of symbiont (Fassil Assefa and Kleiner, 1998). Variation in effectiveness can be found even among strains with in a single genus of bacteria (Thrall *et al*., 2000; Woldemeslkel and Sinclair, 1998). Thus, many Acacia species lack symbiotic specificity for nodulation, but appear to have specificity for nitrogen fixation.

### 3.4.4. Environmental factors that influences Acacia symbiosis

Acacia is a cosmopolitan genus with diverse species adapted to a wide range of extreme Environmental conditions. Factors that influence symbiosis of Acacia include soil temperature, salinity and acid pH (Lippi *et al*.2000). These naturally occurring adaptations makes *Acacia* important to Agro forestry and land reclamation under extreme-environments.

Several reports have showed African strains, indigenous to hot tropical climates that survive at temperatures greater than $40^0$c Zerhari (2000). However, these high temperatures can still have a negative effect can symbiosis.
For example *A.Mellifera* is unable to nodulate at temperature> 40c$^0$ (Habish, 1970). *Rhizobium* has been described to have higher salt tolerance than *Brady rhizobium,* among the strains isolated from Africa (Zou *et al*., 1995). In Sudan, symbiotic rhizobia can grow normally in 3% NaCl and have a higher tolerance than the host *Acacia* (Zhang *et al*, .1991). Thus nodulation of *Acacia* requires a much lower salt concentration than the tolerance levels displayed by the Rhizobia.

**Brief descriptions of the test *Acacia* species**

***Acacia albida (Faidherbia albida)***

*A. albida* is large tree up to 30m high; with straw colored or whitish brachletes and yellowish-white flowers and falcate or coiled pods (Azene Bekele *et al.*, 1993). *A.albida* is now called *F. albida*, because many of its parts are not closely related to any other *A. albida* is widely spread in Tigray, Gondar, Shewa, Arsi, Harerge, Sidama and Gamo-Gofa regions of Ethiopia (Azene Bekele *et al*., 1993).
This species is well recognized by farmers in the sub-humid and semi-arid zones of Africa for its favorable effect on both crops and pastures growing beneath it (Young, 1988). *A. albida* is very important in many agro forestry practices; such as, in the provision of firewood, fuel, timber, forage for livestock, soil improvement, shade, fence etc. (Martin and Sherman, 1992; Azene Bekele *et al*., 1993).

***Acacia abyssinica***

*A.abyssinica* is flat-tapped tree, to 16cm. bark reddish-brown on older trees; pale yellowish-brown, peeling off in papery wads, on younger trees. Young twigs softly hairy. Torn in straight pairs at nodes. Inflorescence of white spherical heads. Pods dehiscent. Habitat: woodland and mountain grassland; in Zimbabwe confined to the Eastern Highland. Frequently planted in gardens in Harare. Flowering time: October-December (Timberlake, Fagg and Barnes, 1999)

***Acacia negrii***

*A. negrii* is a shrub or tree up to 6m a flattened crown which grows in upland wooded *grasslands, mountain woodland, and hilly grassland with scattered Acacias, and rocky grazed area* with Eucalyptus-stands, open dry hillside in a low scrub and in disturbed habitats such as along roadsides. The seed of *A.negrii* very small in relation to pod width. The species is used as fuel wood and bee forage.A.negrii endemic to Ethiopia (Gonder, Wellow, Gojam, Shewa, Harege region).

***Acacia seyal***

*A.seyal* is tree up to 9m tall, sometimes reaching 17m with flattened, spreading crown. The slash is bright red, exuding yellowish gum, and has reddish bark with a straight thorns in pairs, up to 8cm. Leaves are pip innate with 2-12 pairs of pinnae each with 10-22 pairs of leaflets. Flowers bright yellow, in large round heads, 2-3 flower heads together in the leaf axils. The dehiscent pods are light brown, slightly curved, 7-22cm long, with fine longitudinal veins and slightly constricted between the seeds (Thulin, 1983). There are 6-10 seeds/pods. Normally found in areas with 500-1200mm rain/yr. and distinct dry season.

### 3. 4.5. The $N_2$-fixation diversity of Acacia trees

The results of several pot studies using a variety of methods have confirmed that *Acacias* Have the capacity for symbiotic $N_2$-fixation. Nevertheless, several workers have reported that there are variations with respect to the symbiotic effectiveness for $N_2$-fixation (Fassil Assefa and kleiner, 1998; Thrall *et al.*, 2000). For example, among nine Ethiopian *Acacia* species, Fassil Assefa and Kleiner (1998) have classified *A. abyssinica, A.negrii* and *A.etbaica* as high-nitrogen fixing legumes.

The works of Thrall *et al.* (2000), on the performance of Australian acacias indicated that there was a significant variation with regard to host performance among the test *Acacia* species. Besides, they have demonstrated that there was a wide range in host specificity with respect to performance with different rhizobial isolates; for example, they found that *A.melanoxylon* performed quite well with most isolates, whereas *A.mearnsii* was much more variable in its response to isolates from different species.

# 4. Materials and methods

## 4.1 Description of the Study Area

Lanfuro Woreda is located in Silte zone of the SNNPR state.Astronomical location of the study area is located between 7035’00’’N-- 7056’15’’ N of latitude and 38014’30’’E - 38032’00” E of longitude.

In terms of relative location Lanfuro Woreda is found east of Dalocha Woreda, west of Adami Tulu, north of Alaba Woreda and south of Silti Woreda. The study area is far away 54km from Werabe (the capital city of silte zone), 154km from Hawassa and 181km from Addis Ababa. The area is within kola and Woina- Dega climate zone and has clay soil types. It has a mean maximum and minimum temperature of $22^0$c and $18^0$c respectively. The area’s total annual rainfall ranges from 700mm to 900 mm. (LanfuroWoreda finance and economic development office, 2017).

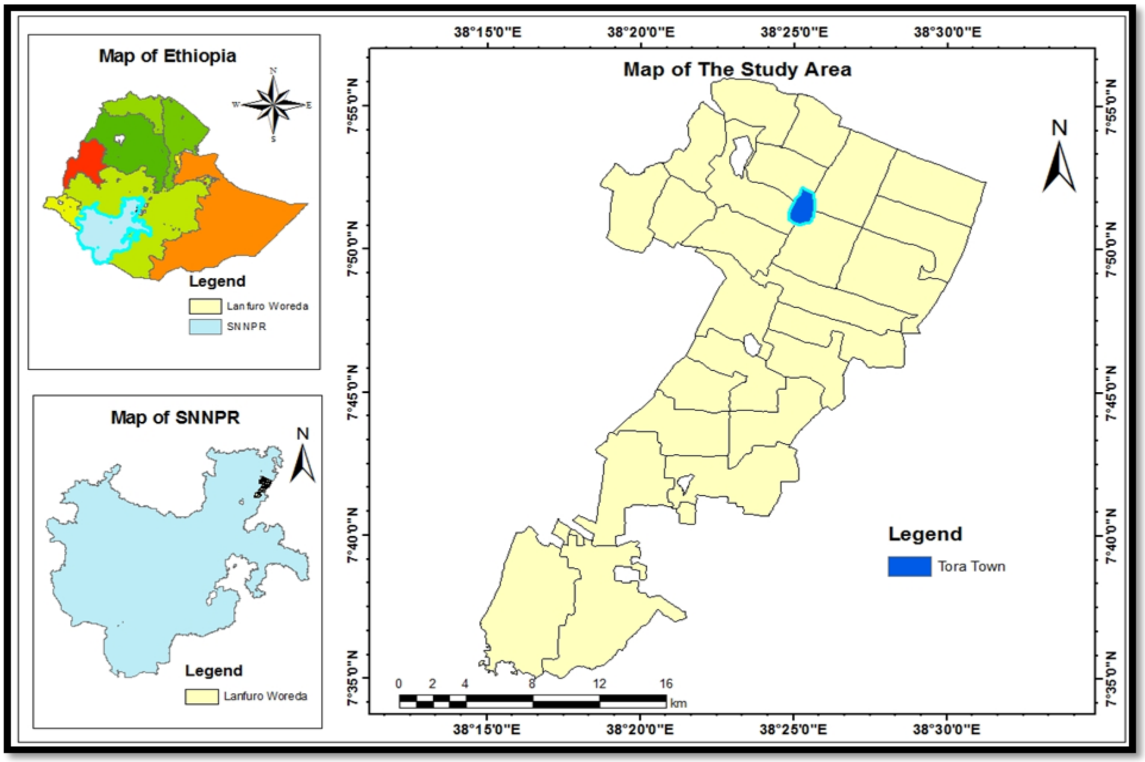

Figure 1: Absolute Location of the study area

Source: Auther’s computation on GIS and CSA data,2016

## 4.2. Sample Collection site

In this study, all sample collection were carried out around Tora town Lanfuro Woreda Silte Zone, in south part of Ethiopia (Rift Valley).

## 4.3. Soil and seed Sample collection

Seeds and soil samples for acacia species were collected from around Tora town in March 2008 E.C. Mature and healthy seeds of the same mother trees were collected, air dried and preserved in refrigerators. Soil samples for each *Acacia* spp. were collected from the rhizosphere part of the host acacia species in plastic pouches for nodule induction.

## 4.4. Induction of nodulation

Nodulation was induced by the ‘plant trap’ method of Barnet *et al.,* (1985). To ensure rapid and uniform seed germination, hard seeded *Acacia* species (*A. abyssinica*, *A. negrii* and *A. seyal*) were scarified with concentrated sulphuric acid for 30 min. whereas, *A. albdia* was surface-sterilized first briefly with 95% alcohol with sodium hypochlorate for 3-5 min (Vincent,1970). Six up to seven seeds were soaked overnight in sterile water. Small and bigger ones were aseptically transferred to Petri dishes filled with 1 % (w/v) sterile water-agar medium. All were kept 3-4 days at room temperature for germination.

Six germinated seeds were then transferred into surface sterilized plastic pots (70% alcohol) filled with soil samples collected from the rhizosphere of the parent *Acacia* spp. The soil samples were supposed to provide the host legume plant with inocula (root nodule bacteria) and other important nutrients required for plant growth. Seedlings were later thinned down to four plants per pot. Plants were watered three times a week and were grown for twelve weeks in a greenhouse (in a School Compound) under natural illumination with 12h photoperiod.

## 4.5. Isolation of root nodule bacteria

For *Rhizobium* isolation, root nodules from the acacia test plants were picked, washed several times with sterile water, surface sterilized as before. After being washed several times with sterile water, each nodule crushed in sterile test tube contained a drop of sterile water with a sterile glass rod. Streak one loopful of crushed materials (nodules suspension) were transferred onto YEMA medium and incubated at $28^0$C for seven days (Vincent, 1970). Single colonies were picked and purified several times on Yeast Extract Mannitol Agar (YEMA) and Congo Red Yeast Extract Mannitol Agar (CR-YEMA) media and finally preserved in YEMA slants at $4^0$C. YEMA contains (Vincent, 1970):

| | g/ml |
|---|---|
| $Mg SO_4. 7H_2O$ | 0.092 |
| NaCl | 0.046 |
| $K_2HPO_4$ | 0.23 |
| Yeast extract | 0.23 |
| Manitol | 4.6 |
| Agar | 9.2 |
| Congo red | 0.0115 |
| Dist $H_2O$ | 460 ml |

## 4.6. Presumptive tests of the isolates

**Gram reaction**

All isolates were gram stained, to observe their shapes and gram stain reactions of rhizobia under light microscopes.

**Procedures of gram stain**

1. Make thin smear of the various bacteria provided and heat fix
2. Stain the smear with solution I (crystal violet) for 1 min
3. Wash lightly with water and flood with solution II (iodine)
4. Drain immediately and flood again with solution II for 1 min
5. Drain solution II and decolorized with solution III (95% alcohol) for 15-30 sec.
6. Wash with water and blot dry carefully
7. Counter stain with solution IV (safranin) for 1 min
8. Wash with water and air dry, observe the preparation under the microscope

**Congo red absorption**

Stock solution of Congo Red (CR) was prepared by dissolving 0.25 g of CR in 100 ml sterile distilled water (Vincent, 1970) and then 10 ml of the CR Stock solution was added to one liter of YEMA before autoclaving. Then the bacterial isolates were streaked on CR-YEMA medium and incubated at 28$^0$C for 2-3 days. Here the plates were wrapped with aluminum foil to provide darkness.

**Peptone glucose test**

For this test, a 72 hours old rhizobia culture was streaked on to the peptone glucose medium and incubated at 28$^0$c for ten days to check if growth and color change occurred. The peptone glucose medium contains (Vincent, 1970):

| | g/ml |
|---|---|
| Glucose | 5.0 |
| Peptone | 10.0 |
| Agar | 20.0 |
| Bromocreesolpurple (% in ethanol) | 10 ml |
| Dist. $H_2O$ | 1000 ml |

## 4.7. Bacterial designations of the rhizobia isolates

AURAaby- Addis Ababa University Rhizobial strains isolated from *A. abyssinica*

AURAal- Addis Ababa University Rhizobial strains isolated from *A. albdia*

AURAneg- Addis Ababa University Rhizobial strains isolated from *A. negrii*

AURAsey- Addis Ababa University Rhizobial strains isolated from *A. seyal*

## 4.8. Characterization of the rhizobial isolates

All tests for characterization of the isolates were undertaken on YEMA medium; adjusted to pH 6.8 and inoculated with 10$^6$ cells/ml after growing the isolates for 48 h (fast growing) and 72 h (slow growing). They were incubated at 30$^0$C for 5-10 days, unless stated otherwise.

### 4.8.1. Cultural characterization

**Acid or alkali production (color on BTB-YEMA)**

Acid or alkali production by the isolates was detected by streaking the isolates on Bromothymol Blue Yeast Extract Mannitol Agar (BTB-YEMA), after dissolved 0.5 g of Bromothymol blue in 100 ml of ethanol and then added into one litter of YEMA and the pH was adjusted to 6.8 as described by Lupwayi and Haque (1994).

**Colony characteristics**

Colony characteristics were observed on CR-YEMA. Individual colonies were described on the basis of characters such as absorption of Congo red, transparency, colony size and the production of a watery or mucilaginous gum as indicated in Ahmed *et al.,* (1981).

### 4.8.2. Physiological characteristics

#### 4.8.2.1. pH tolerance

The ability of isolates to grow in acid and alkaline media was tested by inoculating them in to liquid YEMB in which the pH was adjusted to 4, 5, 8 and 9 respectively.

#### 4.8.2.2. Sodium chloride tolerance

The ability of the isolates to grow at different NaCl concentrations was tested by streaking a loop full YEM broth on YEMA plates containing 2, 3, 5 and 6 % NaCl (W/V).

#### 4.8.2.3. Temperature tolerance

The ability of the rhizobial isolates to grow at different temperature ranges was monitored by incubating them on solid YEMA medium at 4, 25, 37 and $45^0$C respectively.

#### 4.8.2.4. Utilization of carbohydrates

Carbon utilization of the isolates was determined by streaking them on basal media plates containing (g/L): $K_2HPO_4$, 1; $KH_2PO_4$, 1; $FeCl_36H_2O$, 0.01; $MgSO_4.7H_2O$, 0.02; $CaCl_2$, 0.1; $(NH4)_2SO_4$, 1; and 15 g of agar (Amarger *et al*., 1997).

Final concentration of different carbon sources (1g/ml) monosaccharide; D glucose, D-fructose, D-galactose, D-arabinose D-mannose and D-Xylose, disaccharides; maltose, lactose, trehalose, D-sucrose and cellobiose, sugar alcohol; glycerol and sorbitol, and organic salts; Na-citrate).

The heat sensitive carbohydrates; (D-arabinose, D-mannose, sorbitol, D-galactose, Maltose, Na-citrate, Xylose, trehalose, cellobiose and glycerol) were sterilized using a Millipore filter (0.22 µm).

#### 4.8.2.5. Phosphate Solubilization

The ability of the isolates as phosphate solubilizers was examined by streaking a loopful of 72 hours old YEM broth cultures of the rhizobial isolates on Pikovaskaya's medium and incubated at 28 $^{0}$c for seven days according to Tawari *et al.* (2004).

| | (g/ml) |
|---|---|
| Glucose | 8.0 |
| Ca ($PO_4$) | 4.0 |
| $(NH_4)_2SO_4$ | 0.4 |
| $MgSO_4.7H_2O$ | 0.08 |
| NaCl | 0.16 |
| KCl | 0.16 |
| Yeast extract | 0.4 |
| $MnSO_4.H_2O$ | 0.0016 |
| $FeSO_4.7H_2O$ | 0.0016 |
| Agar | 12.0 |
| Distilled Water | 1000 ml |

### 4.8.3. Authentication of the isolates on their hosts

Colonies that had been presumptively tested for, and tentatively classified as root nodule bacteria were examined for authentication since their definitive diagnostic test is their nodulation ability on a host plant (Vincent, 1970).

### Seed germination and sowing:

Viable and healthy seeds for the four *Acacia* species were selected treated, and surface sterilized as before (Vincent, 1970). All procedures for planting were similar to induction of nodulation. Six geminated seeds were then planted in each pot that were later thinned down to four.

### Preparation of sterile sand

River sand was thoroughly washed many times with tap water, drained and autoclaved at $121^0$c/15mmHg for 1 hour. Then 2 kg of this sterile sand was added into the surface sterilized plastic pots (3-kg capacity).

### Inoculation

Isolates from YEMA agar slants were grown on YEMA for 3 days. A 1 ml YEMB culture of each isolates ($10^9$ cells) was transferred in to100 ml sterilized YEMB in 250 ml Erlenmeyer flask and put on rotary shaker at 150 rev/min. for four days. Each Seedling was then inoculated with 1 ml inoculants (approximately $10^9$ cell/ml) immediately after planting and were also re-inoculated 10 days later.

### Watering plants with water and N free media

Plants were fertilized every three days with N-free nutrient solutions according to (Vincent, 1970). Distilled water was supplied every other day to avoid any salt accumulation in the pot. The positive control group was fertilized with 0.05 % (w/v) $KNO_3$, in addition to the N- free nutrient solutions, once every week.

### Determination of symbiotic efficiency of rhizobia isolates

**Symbiotic effectiveness (SE) of each isolates can be calculated as fallows**

**% of SE =** N in the shoot dry matter of inoculated plants over N in the shoot dry
Matter of N fertilized plant, the result multiplied by 100%

Symbiotic effectiveness (SE) values rated as ineffective (< 35%), effective (50-80 %) and high effective (> 80%)

**Table 2. N free media for legumes as indicated in Vincent (1970).**

| Reagents | Concentration (g/l) | vol. (ml) of stock solution |
|---|---|---|
| Per liter of medium | | |
| KCl | 29.8 | 2.5 |
| $K_2HP_{O4}$ | 69.6 | 2.5 |
| $MgSO_4\,7H_2O$ | 98.6 | 2.5 |
| Micronutrients | | 0.5 |
| $CuSO_4.5H_2O$ | 0.078 | |
| $Znso_4.7H_2o$ | 0.22 | |
| $Mns0_4.4H_20$ | 2.03 | |
| $(NH4)_6\ MO_70_{24}\ .4H_{20}$ | 0.01 | |
| $H_3BO_3$ | 1.43 | |
| Ferric citrate | 1.795 | 1.0 |
| $CaSO4.2H_20$ | 0.334g | |

**Harvesting test plants and Determination of Shoot dry weight**

Plants were grown for three months in green house (Addis Ababa University) under natural illumination with 12 h photoperiod. The shoots of the *Acacia* spp. were dehydrated in an oven at a temperature of $70^0$C for 48 hours and then weighed so as to determine the shoot dry matter of the respective *Acacia* species. With regard to nodule dry weight determination, nodules were first carefully collected from the roots of the host *Acacia* species, air dried and finally weighed.

## Data analysis

The data Analyzed using computer system by using SPSS 20 (Stastical Analytical method) Version. The significant difference of the isolates were separated by using
$P \leq 0.05$

# 5. Results

In this study, eleven isolates were collected from *A. abyssinica*, *A. albida (Faidherbia)*, *A. negrii* and *A. seyal.* All isolates were gram negative rod shaped bacteria (Table 3). They failed to absorb Congo red on CR-YEMA media and they did not grow on PGA.

**Table 3. Gram Stain, Size and Shape isolates**

| Rhizobial isolates | Size | Shape | Gram reaction |
|---|---|---|---|
| AURA al 11 | Small | Rod | Gm$^-$/Red Color/ |
| AURA al 12 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ab21 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ab22 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ab23 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ab24 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ne31 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ne32 | Small | Rod | Gm$^-$/Red Color/ |
| AURA ne33 | Small | Rod | Gm$^-$/Red Color/ |
| AURA sy41 | Small | Rod | Gm$^-$/Red Color/ |
| AURA sy42 | Small | Rod | Gm$^-$/Red Color/ |

Gm-, gram negative

## 5.1. Colony Characteristics

The isolates displayed different colony characteristics on YMA medium and were classified in to large mucoid (LM), large watery (LW) and small dry (SD) colonies (Table 4). Large mucoid (LM) colonies were mucoid range from translucent to opaque. Large watery (LW) colonies formed extensive areas of confluent watery growth (Table 4). Both of them grew more than 3mm in 5-7 days (Table 4). Small dry (SD) colonies were characterized by flat surface with little or no production of exopolysaccharides and attain a sizes of 1-2mm within 10 days (Table 4).

Isolates of AURAaby21, AURAaby22, and AURAaby23 (All from *A. abyssinica)* were found to have large watery (LW) colonies, while AURAaby24 *(A. abyssinica)* AURAneg31, AURAneg32, AURAneg33 (from *A. negrii)*, AURAsey41 and AURAsey42 (both from *A. seyal*) had large mucoid (LM) but AURAal 11 and AURAal 12 both from (*A. albida)* were characterized by small dry (SD) colonies types.

### 5.2. BTB reaction and Generation time

The growth of the isolates on BTB-YEMA revealed that 9 of the isolates from AURAaby21, AURAaby22, AURAaby23 and AURAaby24 (all from *A. abyssinica)* AURAneg31, AURAneg32 and AURAneg33 (all from *A. negrii),* AURAsey41 and AURAsey42 (both from *A. seyal)* were able to change the color of the media into yellow by the production of acid (Table 4). However; isolates, AURA al 11 and AURA al 12 (both from *A. albida*), were alkali producers that changed the color of the BTB-YEMA media into blue (Table 4).

**Table 4. Cultural characterization of the isolates**

| **Rhizobial isolates** | **Host** | **Colony size (mm)** | **Colony morphology** | **BTB reaction** | **groups** |
|---|---|---|---|---|---|
| AURA al 11 | *A. albdia* | 1 | SD | Blue | Slow growing |
| AURA al 12 | *A. albdia* | 2 | SD | Blue | Slow growing |
| AURA aby21 | *A. abyssinica* | 2.5 | LW | Yellow | Fast growing |
| AURAaby22 | *A. abyssinica* | 4 | LW | Yellow | Fast growing |
| AURAaby23 | *A. abyssinica* | 4 | LW | Yellow | Fast growing |
| AURAaby24 | *A. abyssinica* | 4 | LM | Yellow | Fast growing |
| AURAneg31 | *A. negrii* | 3 | LM | Yellow | Fast growing |
| AURAneg32 | *A. negrii* | 2.5 | LM | Yellow | Fast growing |
| AURA neg33 | *A. negrii* | 3.5 | LM | Yellow | Fast growing |
| AURA sey41 | *A. seyal* | 3 | LM | Yellow | Fast growing |
| AURA sey42 | *A. seyal* | 3 | LM | Yellow | Fast growing |

Legend: SD, Small Dry; LM, Large Mucoid; LW, Large Watery; SL, slow growing; FS, fast growing.

## *5.3. Physiological and Biochemical characteristics*

### 5.3.1. pH tolerance

All the isolates, with the exception of AURAal 11 (*A.* albida) and AURAal 12 (*A.* albida) were able to grow at pH 4. However, the fast growing isolates of AURAaby23, AURAaby24 (both from *A. abyssinica),* AURAneg31 (from *A. negrii),* AURAsey41 (from *A. seyal)* and slow growing isolates of AURAal 11 and AURAal 12 (both from *A. albida)* were capable of growing at acidic pH of 5, whereas all the isolates of both types tolerated pH of 8 and 9 (Table 5).

### 5.3.2. Salt tolerance

All the isolates except AURA al 11 and AURA al 12 (both from *A. albida*) grew at NaC1 concentration of 1% (Table 5). Most isolates also grew at 3 and 5% NaCl, except AURAal 11 (*A. albida*) and AURAal 12 (*A. albida*).

However, less than half of the isolates were resistant to NaCl concentration of 6%.

Thus, isolates from AURAaby21 and AURAab22 (both from *A. abyssinica*), AURAneg31, AURA neg32 and AURA neg33 (all from *A. negrii*) were able to grow at 6% NaCl concentration (Table 5).

### 5.3.3. Temperature Tolerance

All isolates from the four *Acacia* spp. grew on YEMA at 25 and $37^0$C (Table 5). The majority of the isolates grew at low temperature values i.e. $4^0$C, except AURAal 11, AURAal 12 (both from *A. albida*), AURAaby21 and AURAaby22 (both from *A. abyssinica*) (Table 5). Likewise, only isolates of AURAaby23, AURAaby24 (both from *A.abysinica),* AURAsey41 andAURAsey42 (both from *A. seyal*) were resistant to $45^0$C. Isolates from AURAneg31, AURAneg32 and AURAneg33 **(**all from *A. negrii)* were able to grow at 4 $^0$C but not at 45 $^0$C (Table 5).

**Table.5 pH, Salt and temperature tolerance of the Rhizobial isolates**

| Isolates | Growth at pH 4, 5 8 and 9 | | | | NaCl Concentration W/v (%) | | | | Temperature ($^0{}_C$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH 4 | pH5 | pH 8 | pH 9 | 1 | 3 | 5 | 6 | $4^0{}_C$ | $25^0$C | $37^0$C | $45^0{}_C$ |
| AURAal 11 | - | + | + | + | - | - | - | - | - | + | + | - |
| AURAal 12 | - | + | + | + | - | - | - | - | - | + | + | - |
| AURAaby21 | + | - | + | + | + | + | + | + | - | + | + | - |
| AURAaby22 | + | - | + | + | + | + | + | + | - | + | + | - |
| AURAaby23 | + | + | + | + | + | + | + | - | + | + | + | + |
| AURAaby24 | + | + | + | + | + | + | + | - | + | + | + | + |
| AURAneg31 | + | + | + | + | + | + | + | + | + | + | + | - |
| AURAneg32 | + | - | + | + | + | + | + | + | + | + | + | - |
| AURAneg33 | + | - | + | + | + | + | + | + | + | + | + | - |
| AURAsey41 | + | + | + | + | + | + | + | - | + | + | + | + |
| AURAsey42 | + | - | + | + | + | + | + | - | + | + | + | + |

### 5.3.4. Carbon utilization

All the isolates failed to grow on starch, but utilized, xylose, glucose and fructose (Table 6). All the isolates, except AURAal 11 (*A. albida*) and AURAal 12 (*A. albida*) were capable of utilizing sodium citrate as their sole carbon source. However, all the isolates grew on all tested carbohydrate sources except, AURAal 11 and AURAal 12 (*A.albida)* that failed to grow on three of the tested disaccharides (maltose, lactose and sucrose) (Table 6).

### 5.3.5. Phosphate solubilization

Accordingly one isolate; AURAsy41 that was isolated from *A. seyal* and two isolates; AURAab21 and AURAab24 (both from *A.abyssinica*), were capable of forming clear zone around their colonies on the Pikovaskaya's medium (Table 6).

**Table. 6 Carbon utilization of the Rhizobia isolates**

| Isolates | Carbon utilization of the rhizobial isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Monosaccharides | | | disaccharides | | | | | | |
| | Xyl | Gluc. | Fruct. | Malt | Sucr | Lact | Na-citr | Star. | PS | SE |
| AURAal 11 | + | + | + | - | - | - | - | - | - | HE |
| AURAal 12 | + | + | + | - | - | - | - | - | - | LE |
| AURAaby21 | + | + | + | + | + | + | + | - | + | E |
| AURAaby22 | + | + | + | + | + | + | + | - | - | HE |
| AURAaby23 | + | + | + | + | + | + | + | - | - | LE |
| AURAaby24 | + | + | + | + | + | + | + | - | + | E |
| AURAneg31 | + | + | + | + | + | + | + | - | - | HE |
| AURAneg32 | + | + | + | + | + | + | + | - | - | E |
| AURAneg33 | + | + | + | + | + | + | + | - | - | LE |
| AURAsey41 | + | + | + | + | + | + | + | - | + | HE |
| AURAsey42 | + | + | + | + | + | + | + | - | - | E |

Legend: xyl, xylose; Gulc, Glucose; Fruct, fructose; Malt, maltose; Sucr, sucrose; Lact, lactose; Star, starch; Na-citr, Na-citrate; PS, phosphate solubilization; SE, symbiotic effectiveness

### Determination of symbiotic efficiency of rhizobia isolates

The symbiotic effectiveness of the isolates was calculated as the percentage of shoot dry weight of inoculated plant over N fertilized or positive control plants. Symbiotic effectiveness (SE) values rated as ineffective (`less than 35%), effective (50-80%) and highly effective (greater than 80%).

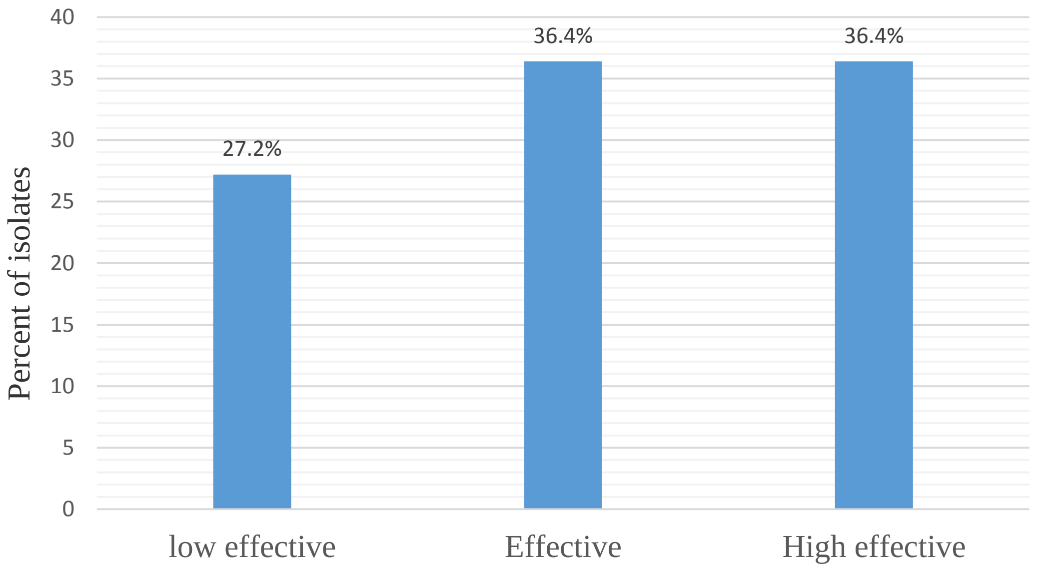

Fig.2 Effectiveness of isolate

## 5.4. Symbiotic effectiveness evaluation of isolates on sand

Plants were grown for three months in greenhouse of Addis Ababa University Under natural illumination with 12 h photoperiod. The plants were carefully uprooted to collected nodules to determine nodules number, nodule dry weight, shoot dry weight and shoot length. With regard to nodule dry weight determination, nodules were first carefully collected from the roots the host *Acacia* spp., air dried and finally weighted. However, the shoot of acacia species were dehydrated in an oven at a temperature of $70^0$C for 48 hours and then weighed so as to determine the shoot matter of the respective *Acacia* species.

The symbiotic effectiveness of the respective isolates were evaluated by comparing the data obtained from nodule number (NN),nodule dry weight (NDW),shoot dry weight (SDW), and shoot length (SL) of the different Acacia species grown on sand pots (Table7).

The data showed *Rhizobium* species induced nodules (NN/plant), *A. seyal* (44-59 NN/plant (mean =52), *A. abyssinica* 42-51 NN/plant (mean =47), *A. negrii*, 32-60 NN/plant (mean= 47) and *A. (Faidherbia) albida*, 21-47 NN/plant (mean=34) respectively (Table 7).

they also showed average nodule dry weight of 0.051 mg/plant (*A. negrii)*, 0.050 mg/plant (*A. seyal)* 0.044 mg/plant (*A. albdia*) and 0.041 mg/plant (*A. abyssinica)*, respectively (Table7). Likewise the inoculant plants accumulated shoot dry weight of mean=0.43 g/plant (*A. negrii)* mean=0.38 g/plant (*A. seyal*), mean=0.37 g/plant *(A. albdia*) and mean=0.30 g/p *(A.abyssinica)* respectively (Table 7). The maximum SDW of 0.69 g/plant was recorded from *A. negrii* inoculated with AURAneg31; followed by 0.51 g/plant was recorded from *A. albdia* inoculated with AURAal 11 (Table 7).

In terms of shoot length recorded by the inoculated plants range from 11 cm-8 cm (mean=10 cm) for *A. albdia* plant, from 13 cm-10 cm (mean=12 cm) for *A.abysinica*, from 14 cm-9 cm (mean=12 cm) for *A. negrii* from 12 cm-10 cm (mean=11 cm) for *A. seyal*. The maximum shoot length of 14 cm was recorded from *A .negrii* inoculated with AURAneg31, while the minimum shoot length of 8 cm was recorded from *A. albida* inoculated with AURAal 12. The maximum shoot length of 15cm was recorded from the positive control group *A.negrii* (Table 7).

The four isolates AURAal 11(*A. albdia*), AURAsey41 (*A. seyal*), AURAaby22 (*A. abyssinica*) and AURAneg31 (*A. negrii*), Showed the highest symbiotic effective with (HE) 93%, 90% 86% and 81% shoot dry weight accumulation respectively, (Table 7).

The lowest symbiotic effectiveness of 38% was recorded from the inoculation of AURIneg33 (*A. negrii*) (Table 7). In general, more than 70% of the isolates were effective/highly effective in nitrogen fixation on their respective hosts.

**Table.7 Symbiotic properties of isolates from *A. abyssinica, A. albdia, A. negrii* and *A. seyal* on sand culture under greenhouse condition.**

| Isolates | Groups | Host | NN/ Plant | NDWmg/ plant) | SDW g/plant | SLcm /plant | SE % | effectiveness |
|---|---|---|---|---|---|---|---|---|
| AURAal 11 | *Bradyrhizobium* | *A.(Faidherbia)* | 47 | 0.045 | 0.51 | 11 | 93 | HE |
| AURAal 12 | *Bradyrhizobium* | *A.(Faidherbia)* | 21 | 0.043 | 0.23 | 8 | 42 | LE |
| Average | | | 34 | 0.44 | 0.37 | 10 | | |
| N+(control) | - | - | - | - | 0.55 | 13 | | |
| N-(control) | - | - | - | - | 0.20 | 5.5 | | |
| AURAaby21 | *Rhizobium* | *A. abysinica* | 42 | 0.042 | 0.23 | 10 | 50 | E |
| AURAaby22 | *Rhizobium* | *A. abysinica* | 51 | 0.047 | 0.41 | 13 | 86 | HE |
| AURAaby23 | *Rhizobium* | *A.abysinica* | 43 | 0.034 | 0.21 | 10 | 45 | LE |
| AURAaby24 | *Rhizobium* | *A.abysinica* | 49 | 0.042 | 0.35 | 12 | 74 | E |
| Average | | | 47 | 0.041 | 0.3 | 11 | | |
| N+ (control) | - | - | - | - | 0.47 | 13 | | |
| N- (control) | - | - | - | - | 0.20 | 7 | | |
| AURAneg31 | *Rhizobium* | *A. negrii* | 60 | 0.069 | 0.60 | 14 | 81 | HE |
| AURAneg32 | *Rhizobium* | *A. negrii* | 49 | 0.039 | 0.40 | 11 | 51 | E |
| AURAneg33 | *Rhizobium* | *A. negrii* | 32 | 0.044 | 0.30 | 9 | 38 | LE |
| Average | | | 47 | 0.051 | 0.43 | 11 | | |
| N+ (control) | - | - | - | - | 0.78 | 13 | | |
| N- (control) | - | - | - | - | 0.27 | 7 | | |
| AURAsey41 | *Rhizobium* | *A. .seyal* | 59 | 0.063 | 0.46 | 12 | 90 | HE |
| AURAsey42 | *Rhizobium* | *A. seyal* | 44 | 0.036 | 0.29 | 10 | 57 | E |
| Average | | | 52 | 0.050 | 0.38 | 11 | | |
| N+ (control) | - | - | - | - | 0.51 | 14 | | |
| N- (control) | - | - | - | - | 0.20 | 8 | | |

Legend: NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; SL, shoot length; SE, symbiotic effectiveness; HE, high effectiveness; LE, low effectiveness; E, effectiveness

## 6. Discussion:

In this work, all the isolates were gram negative rod shaped bacteria, failed to absorb Congo red on CR-YEMA medium and did not grow on PGA and tentatively identified as root nodule bacteria (Vincent, 1970). They were induced nodules in their respective hosts during authentication experiment.

With regard to the cultural characteristics of the isolates, 6 of them displayed LM colonies, with relatively larger colony diameters (2.5-4.5 mm) and were associated with excessive expolysaccharide production on YEMA in 3-5 days. About, 3 of the isolates showed LW colonies that were characterized by watery growth. However, AURAal 11 (*A. albida*) and AURAal 12 (*A. albida*), displayed SD colony types with colony diameters of 1-2mm with little or no production of expolysaccharides (Table 4). These findings are consistent with Jordan (1984) and Elkan 1987) who reported that fast growing *Rhizobial* isolates grow more than 1 mm with excessive production of exopolysaccharide in 5-7 days, whereas slow growing (SG) *Bradyrhizobial* (SD) isolates produced less gum and did not grow more than 1mm in 10 days (Table 4). The different isolates changed the color of the BTB-YEMA media into yellow or blue. Accordingly, all isolates, except AURAal 11 and AURAal 12 (both from *A. albdia*) changed the BTB-YEMA medium in to yellow that were accompanied by the production of acid (Table 4).

Accordingly isolates from AURAal 11 (*A. albida*) and AURAal 12 (*A. albida*) were slow growing and designated as Bradyrhizobium, whereas isolates from *A. abyssinica*, *A. negrii* and *A. seyal* were all *fast* growing *Rhizobium* (Table 4). Although this finding was consistent only to the character of *A. albdia* that it is nodulated by slow growing *Rhizobium*, Fassil Assefa and Kleiner, 1998 showed that *A. abyssinica*, *A. negrii* and *A. seyal* were nodulated by both *Rhizobium* and *Brady rhizobium*.

All the isolates, with the exception of AURAal 11 and AURAal 12 (both from *A. albida*) Were able to grow at pH4 (Table 5). The fast growing (FG) isolates of AURAaby23, AURAaby24 (both from *A.abyssinica)* AURAneg31 (*A. negrii*) AURAsey41 (*A. seyal*) (*Rhizobium*) and slow growing isolates (SG) of AURAal 11 and AURAal 12 (both from *A. aldida*) capable of growing acidic pH of 5 (Table 5), whereas all the isolates of both types tolerated pH of 8 and 9 (Table 5).

Previous reports (Jordan, 1984; Elkan, 1987) however, showed that fast growing isolates tolerate high pH, while slow growing isolates grow at lower pH supporting Norris' hypothesis that fast growing and slow growing isolates produce acid and alkali to counter the soil pH of their habitat of basic soil in the temperate region and acidic soil in the tropics, respectively. But according to Broughton 1990 it is difficult to prove the authenticity of the relationship between tolerance of acid or base production by fast growing and slow growing isolates respectively.

All the isolates, except AURAal 11 and AURAal 12 that were isolated from *A. albida* grew well at NaCl concentration of 1% (Table 5). The majority of the (FG) isolates were tolerate to 3% and 5% NaC1 concentration. However, all isolates failed to grow at 6% NaC1 concentration except, AURAaby23 (*A. abyssinica*), AURAaby24 (*A. abyssinica*) AURAsey41 *(A. seyal*) and AURAsey42 (*A. seyal*) (Table 5). *Rhizobium* strains isolated from woody legumes such as *Acacia*, *Prosopis* and *Leucaena* can tolerate NaCl concentration up to 5% (Surange *et al*., 1997). From the data, it appears that FG isolates were more tolerant (3-6% NaCl) than the SG isolate(less than 2%NaCl). Zang *et al.* (1991) isolated a large number of fast growing tree rhizobia from the Sudanese soil that grew well at 3% NaC1. This finding agree with the report of Odee *et al*, (1997) who observed fast growing rhizobia more tolerant to higher NaCl concentration compared to slow-growing isolates collected from woody legumes growing in diverse Kenyan condition.

All the isolates grew on YEMA at $25^0$C and $37^0$C (Table 5). Shishay Mesfin (2008) showed a 100% growth of root nodulating bacteria from *Acacia* species at the same incubation temperature of 15-$35^0$C. The majority of the isolates grew at low temperature i.e. 4°C, except AURAal 11 and AURAal 12 (both from *A. albida*), AURAaby21 and AURAaby22 (both from *A. abysinica*) (Table 5). Likewise, the majority of the isolates did not grow at temperature of $45^0$C, except AURAaby23 *and* AURAaby24 *(both from A. abysinica),* AURAsey41 and AURAsey42) (both from *A. seyal*) (Table 5). Zehari *et al*. (2000), Fassil Assefa and Kleiner (1998) isolated rhizobial isolates that could grow at temperature values of $40^0$C and $43^0$C, respectively.

Zahran (1999) had reviewed that rhizobial isolates isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis*, growing in hot dry regions of Sudan, had high maximum growth temperatures of (44.2$^0$C). In general, the isolates of rhizobia, collected in this study seemed to be slightly more tolerant to high salt concentration and temperature values than the rhizobial isolated from temperate legumes (Zhang *et al*., 19991; Fassil Assefa, 1993, Fassil Assefa and Kleiner, 1998, Odee *et al*., 1997; Zehari *et al*., 2000).

All the isolates utilized many of the tested carbohydrates as carbon sources. The majority of the isolates utilized Na-citrate except, AURAal11 *(A. albdia*) andAURAal12 *(A. albdia*). Likewise, none of the isolate could utilize starch as their sole carbon source (Table 6). This result is similar to some other findings of Bala *et al,* (2004) who showed the failure of most of their isolates to utilize starch. Moreover, the majority of slow growing rhizbia failed to utilized maltose, sucrose and lactose as a sole carbon source (Table 6). Fassil Assefa (1993) isolated slow growing rhizobia from legume trees that were more specific in their requirements with some carbohydrates, especially disaccharides and polysaccharides. Generally, the range of carbon compounds utilized by slow growers is narrow compared to the fast growing strains (Zhang *et al*., 1999; Zehari *et al*., 2000).

Only three isolates (30%) of AURAaby21 and AURAaby24 (both from *A.abysinica*) and AURAsey41 (from *A.seyal*) (*Rhizobium*) were able to solubilize phosphate. In this study some of the isolates that solubilized inorganic phosphate produce acid on YEMA-BTB media (Table 3). There are other related reports that suggested the positive correlation of acid production with inorganic phosphate (Halder and Chakrabartry, 1993). This may strengthen the fact that some rhizobia could be some phosphate solubilizers (Tewari *et al.,* 2004*).*

From the result of sand culture study on nodule induction and symbiotic effectiveness of the isolates of the four *Acacia* species ($p \leq 0.05$ ) variation showed in nodule number, nodule dry weight, shoot dry weight and shoot length . Accordingly, AURAneg31 from *A. negrii* and AURAal 11 from *A. albida* induced the maximum number of 60 nodule/plant and a minimum number of 21 nodule/plant respectively (Table 7).

Shishay Mesfin (2008) reported that nodule from *Acacia* spp. were as high as 107 nodules/plant and as low as 25 nodule/plant. In general, the tested *Acacia* spp. of in this study produced slightly less number of nodule per plant compared to Shishay Mesfin (2008).

The nodule dry weight of the plant ranged from 0.034 mg/p to 0.069 mg/p for AURAaby23 (*A. abysinica*) and AURAneg31 (*A. negrii*) respectively (Table 7). The difference between the performances of different rhizobia isolates can be indicated by variation in infection of effective nodulation (Karuppasamy *et al.*, 2011). four isolates of AURAal 11(*A. albdia*), AURAsey41 (*A.* seyal), AURAaby22(*A. abysinica*) and AURAneg31 (*A. negrii*) and showed high symbiotic effectiveness with 93%, 90%, 86% and 81% of shoot dry weight accumulation compared to the N-fertilized control respectively. Shishay Mesfin (2008) also reported some rhizobial species isolated from *A.venosa* higher symbiotic effectiveness reaching to 115% shoot dry weight accumulation compared to the N-fertilized control. In this study 73% of the isolates showed effective/high effective compared to Shishay Mesfin, (2008) showed 81% of them were effective/high effective. This study has built up a basis for more comprehensive examination and screening of high effective/effective isolates that tested in the greenhouse condition.

# 7. Conclusion and recommendation

## 7.1 Conclusion

From the results of phenotypic characterization of the rhizobial isolates of the test *Acacia* species were diverse with regard to their morphological and physiological characteristics. Most of the isolates were Fast growing rhizobia that were able to utilize a wide range of carbon sources which can give them a competitive advantage to utilize a wide range of carbon sources. The rhizobial isolates from the *Acacia* species were generally tolerant to high NaCl concentration, pH and temperature ranges. All isolates from the four different *Acacia* species were able to re-infect and induce nodule formation on their homologous host plants. The nodulation pattern of the four acacia species showed that three of them (*A. abysinica, A. negrii* and *A. seyal*) can be nodulated by fast growing isolates. Likewise, *A. albida* spp. were nodulated by slowing growing isolates only.

The symbiotic effectiveness of the rhizobial isolates showed significant $p \leq 0.05$ variation in mean shoot dry weight compared to their respective N+ and N- control groups.

## 7.2 Recommendations

Based on the results of this study, the following points are recommended:

- The highly competent isolates of AURAal 11(*A. albdia*), AURAaby21, AURAaby22, AURAaby24 (all from *A. abysinica*), AURAneg31, AURAneg32 (both from *A. negrii*), AURAsey41 and AURAsey42 (both from *A. seyal*) that were tested in greenhouse conditions need to be tested at field to ensure their competitiveness under the actual environmental conditions. Among the above listed isolates, AURAal 11 (A. albdia) is the best candidate inoculant for plantation of Acacia Species
- Further isolation and characterization from a wide area in the region can bring more rhizobia with novel characters to fully realize the benefits of biological nitrogen fixation.
- Cross inoculation test of the isolates on their various acacia host may reveal their compatibility as inoculants for their heterologous hosts.

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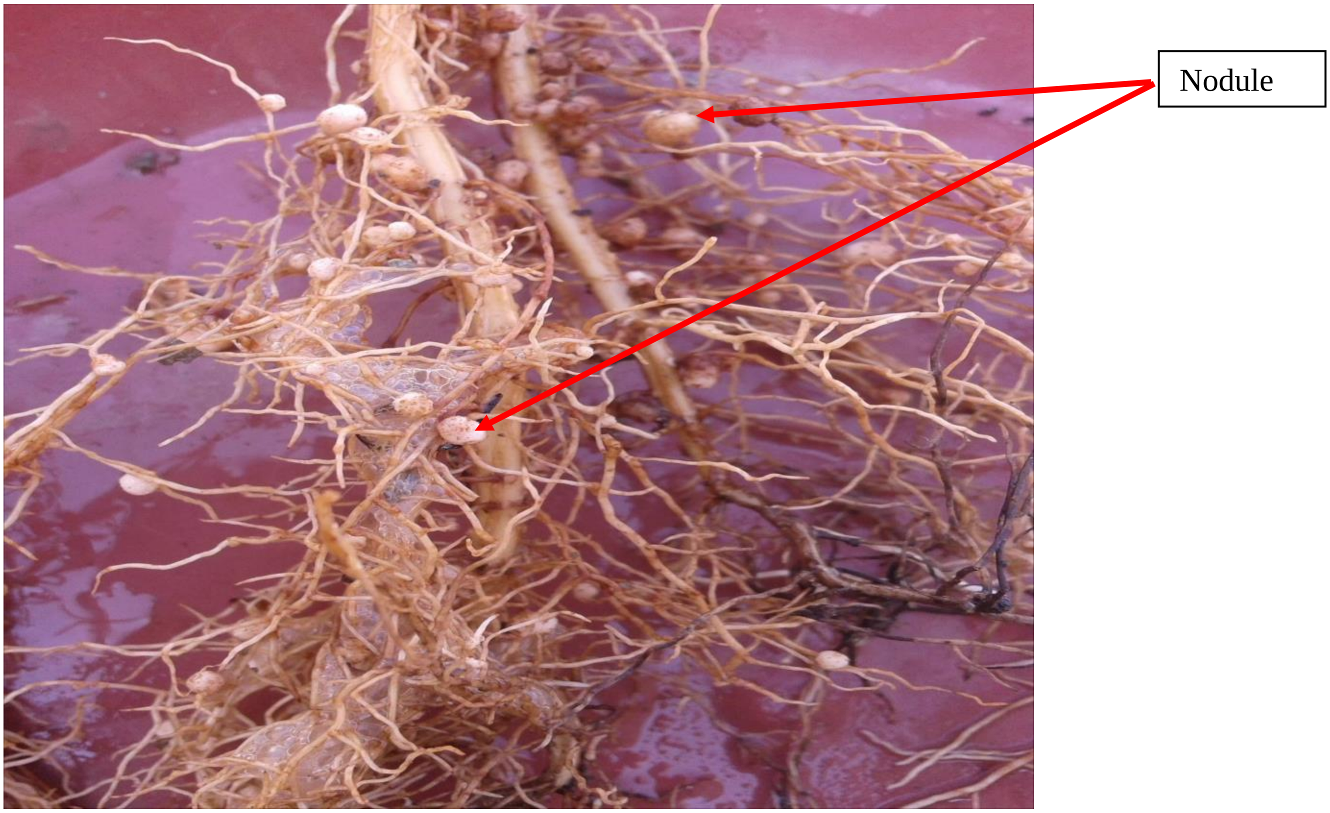
**Appendix I A:** seedling of *Acacia albida* and *Acacia abyssinica* growing In the Greenhouse

**Appendix I B:** seedling of *Acacia negrii* and *Acacia seyal* growing In the greenhouse



**Appendix II: Root nodules from *Acacia negrii***

## Declaration

I declare that the dissertation hereby submitted by me for the Degree of Masters in Biology to the School of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else at another University. The materials obtained from other sources have been duly acknowledged in the dissertation.

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