

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



The Symbiotic Effectiveness and Diversity of Root Nodule Bacteria of Some *Acacia spp.* from Sodo Wereda, Guraghe Zone, Southern, Ethiopia.

A Thesis Submitted to the School of Graduate Studies Addis Ababa University
in Partial Fulfilment of the Required for the Degree of Master of Science in
Biology

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Addis Ababa, Ethiopia

August, 2017

Acknowledgements

First of all, I would like to thank my supervisor Dr. Fassil Assefa for his guidance, encouragement, constructive comments and useful suggestions since the onset till the completion of this work.

I would also like to thank those PhD students, Ato Yibeltal Merawi, Atsede Muleta, and Ato Belay Berza from the Department of Microbial cellular and Molecular Biology, at Addis Ababa University who technically helped me during the laboratory and greenhouse activities. I also acknowledge the members of the National Herbarium, Addis Ababa University, particularly Ato Weghe, for his help during the identification of the test plants.

I would like to extend my heartfelt thanks to my wife W/o Kuku Legesse, my little girl Betselot Abule, Ato Solomon Bekele, Elias Bekele, W/o Tadelech Dugida, W/o Lili Dugida, Ato Gashaw Bahiru, Ato Fikre Jida, Ato Endashaw Mulugeta, Ato Anmaw Jara Ato Mohamed Warga, Ato Lega Bereda and his son Solomon Lega, Ato Tariku Yemane, Ato Getu Karu and Ebrahim Mohamed, Secretary Saba Assefa for their support and love.

Last, but by no means the least, I thank colleagues and friends too numerous to mention by name to whose direct and indirect contributions this work owed its success

I am grateful to the Graduate School, Addis Ababa University for the financial help rendered to this work.

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Symbols and Abbreviations

BCP	Bromocresol purple
BCP-YEMA	Bromocresol purple-Yeast extract mannitol agar
BNF	Biological Nitrogen Fixation
BTB	Bromothymol blue
BTB-YEMA	Bromothymol blue- Yeast extract mannitol agar
CR	Congo red
CR-YEMA	Congo red- Yeast extract mannitol agar
E	effective
FG	Fast growing
HE	high effective
LM	Large mucoid
LW	Large watery
Mo-Fe protein	Molybdenum iron protein
N	Normality/The symbol of nitrogen
NDW	nodule dry weight
NN	nodule number
Rev/min	revolution/minute
SDW	shoot dry weigh

SE	symbiotic effectiveness
SG	Slow growing
YEMA	Yeast extract manitol agar medium

Abstract

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 for most of them fix nitrogen with rhizobia. However, nitrogen fixation by different species is not necessarily effective that needs compatibility of the rhizobia and the host varieties under different ecological conditions. Therefore, this was designed to isolate, phenotypically and symbiotically characterize acacia rhizobia from Sodo Woreda, Guraghe Zone, and Southern, Ethiopia under laboratory and greenhouse conditions. Thus, ten rhizobial isolates were collected from nodules of three different acacia species; *Acacia seyal*, *Acacia abyssinica* and *Acacia negrii*. They all induced nodulation upon re-inoculation on their homologous host plants. All isolates were fast growing, tolerant to high concentration of 6% NaCl concentration and grew over a wider range of temperature up to 43. Most of the isolates of *Acacia abyssinica* and one isolate of *Acacia negrii*, were capable of utilizing a various types of carbohydrates as carbon source. The symbiotic effectiveness of the isolates on the sand culture showed significant variations in nodule number, nodule dry weight, shoot dry weight, shoot length compared to their respective N- fertilized and N-fertilized -control groups. The maximum number of nodule and shoot dry weight of the inoculated plant were recorded by *Acacia negrii* with isolate AURAneg31. The minimum number of nodule was recorded by *Acacia abyssinica* with isolate AURAaby24. The highest shoot dry weight was observed N-fertilized (positive control) plant by *Acacia seyal*. However, shoot dry weight of N-unfertilized (negative control) plants less than both inoculated and uninoculated plants. Isolates of *Acacia negrii*, were very effective; AURAneg31 showed the highest value 91% of symbiotic effectiveness whereas both *Acacia abyssinica* and *Acacia seyal* were showed effective. Generally isolates of *A. negrii* were more competitive than other two species.

Key words: *Acacia abyssinica*, *Acacia negrii*, *Acacia seyal*, fast growing, Rhizobia, salt tolerance, symbioticeffectiveness

1. Introduction

Legumes are one of the dominant plant species that are widely distributed in different habitats as trees, shrubs, field crops and herbs, and classified into three subfamilies (Doyle and Luckow, 2003). They are multipurpose plants often selected for agro forestry production systems (Dommerugus, 1987; Young, 1988; Giller, 2001). Acacia is one of the leguminous plants that belongs to the Subfamily Mimosoideae and comprises over 1550 species that are widely distributed in Africa, Asia and Australia (Maslin et al., 2003). The selection of acacia trees and shrubs as important components in many agro forestry practices is attributed mainly to their capacity to nodulate and fix nitrogen in a symbiotic association with soil bacteria, collectively referred to rhizobia (Allen and Allen, 1981; Zehari et al., 2001). According to Dreyfus and Dommergus (1981), root nodule bacteria from acacia rhizobia that nodulate either by *Rhizobium*, or *Bradyrhizobium* or by both genera. Acacia species were traditionally classified as fast growing and slow growing rhizobia. Currently, the majority of the acacia microsymbionts are further classified within the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Leary et al., 2006).

Although acacia have the capacity for symbiotic N₂-fixation, there are variations depending upon the endosymbiont, type of provenance and environmental conditions with respect to the symbiotic effectiveness for N₂-fixation (Fassil Assefa and Kleiner, 1998; Burdon et al., 1999; Thrall et al., 2000). Fassil Assefa and Kleiner (1998), classified *A. abyssinica*, *A. negrii* and *A. etbaica* as high-nitrogen fixing legumes among nine Ethiopian acacia species;

Thrall et al. (2000) also showed significant variation on the performance of Australian acacias. Besides, they have demonstrated that there was a wide range in host specificity with respect to performance with different rhizobial isolates in that *A. melanoxyton* performed quite well with most isolates, whereas *A. mearnsii* was limited in its response different rhizobial species.

In Ethiopia, there are 49 indigenous and 9 exotic species recorded in the Ethiopian national Herbarium, which represent the fourth important genus in the country (Hunde and Tulin, (1989). They are distributed in different agro-ecosystems ranging from low altitude (1000-3000m asl), and persist as survivors in forest relics and scattered in farmlands in a heavily degraded and deforested landscape of the country. There has been limited information on the nodulation status and performance of indigenous acacia species in Ethiopia until the 1990's.

However, a pioneering attempt was made to evaluate nodulation and nitrogen fixation, and the diversity of rhizobia from the dominant acacia species distributed in the different agro-ecological regions (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998). The acacia species were; *Acacia abyssinica*, *Acacia negrii* (highland); *A. senegal*, *A. prasinata*, *A. tortilis* (lowland); *A. seyal* and *A. etbaica* and *A. albida* (*Faidherbia albida*) distributed in a wider range of midland and lowland (Hunde and Thulin, 1989).

Based on acetylene reduction and agronomic studies under greenhouse conditions, the different acacia showed significant difference in nitrogen fixation (Fassil Assefa and Kleiner, 1998). Similarly Shishay Mesfin (2008) also studied the physiological and symbiotic diversity of acacia species from Tigray and he showed that *A. venosa* and *A. lahai* were nodulated by slow and fast growing rhizobia whereas *A. albida* were nodulate by slow growing rhizobia. However, the symbiotic effectiveness of significance difference one another; for example, *A. venosa* very effective whereas *A. lahai* were poor effective.

All taken together, studies on the diversity of rhizobia and their symbiotic nitrogen fixation are very limited to fewer species and regions. These necessitate further work to fully realize the potential of nitrogen fixation. In the present study, attempt has been made to evaluate the symbiotic effectiveness and the diversity of root nodule bacteria of some acacia species from Sodo Wereda, in the highland areas of southern, Ethiopia.

1. 1. Objectives

1. 1.1. General objective

- To study the symbiotic effectiveness and diversity of the root nodule bacteria of some acacia species grown from Sodo Wereda, Guraghe Zone, Southern Ethiopia.

1. 1.1. Specific objectives

- To isolate, characterize and authenticate the endo-symbionts from different acacia species.
- To evaluate the symbiotic effectiveness of the isolates on sand pot culture under greenhouse conditions

2. Literature review

2.1. Legumes

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; *Caesalpinioideae*, *Mimosoideae* and *Papilionoideae*. The legumes that are primarily tropical and subtropical are the subfamilies *Caesalpinioideae* and *Mimosoideae* trees and shrubs and mainly distributed in lowlands, midlands and arid ecosystems (Dommerugus, 1987). They are multipurpose plants; with a number of significant functions in the use systems (Dommerugus, 1987; Young, 1988).

Legumes are very important both ecologically and agriculturally. This is mainly attributed to their substantial contribution to the global flux of nitrogen from atmosphere N₂ 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 fix nitrogen; nevertheless some of them do not have this capacity.

2.2. Acacia

Acacia, with close to 1550 species, is the second largest genus with in both sub family and family of the *leguminosae* (Maslin *et al.*, 2003). Acacia consists of three sub genera, including *Acacia* (161), *Aculeiferum* (231) and *Phyllodineae* (960) (Maslin *et al.* 2003). And it is distributed throughout the world, particularly in Australia (>940 species), Africa (>140 species), Asia (>90 species) and the Americas (>180 species). They are rarely found in Europe. Acacia range from herbs to enormous trees but most are shrubs and small trees and they colonize different habitats which range from arid areas of low or seasonal rainfall to moist forest and riverbanks (Allen and Allen, 1981).

Acacias are valued as foliage, green manure, soil coverage to reduce erosion, and in gardening /land scapping (Wang *et al.*, 2006). Acacia forms symbiotic associations with strains of at least six genera of nodulating symbiotic bacteria (Leary *et al.*, 2006). Many of the associations fix nitrogen from the atmosphere; nevertheless there is variation in their nodulation pattern in that some acacias 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).

The results of several pot studied using a variety of methods have confirmed that acacias have the capacity for symbiotic N₂ fixation. Nevertheless, there are variations with respect to the symbiotic effectiveness for N₂ fixation (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998; Burdon *et al.*, 1990 and Thrall *et al.*, 2000

In most Ethiopian soils N is considered to be the limiting nutrient for plant growth (Girma Tadesse, 2001). Furthermore, the rising demand for increased food and fuel wood production by resource-poor farmers and concern for environmental degradation under intensive agriculture have increased interest in nitrogen-fixing trees in tropical regions (Danso *et al.*, 1992). Although nitrogen-fixing leguminous trees are contributing considerable amount for global biological nitrogen fixation, little has been done to describe their rhizobial specificity in terms of nodulation and nitrogen fixing effectiveness (Turk and Keyser, 1992).

Acacia seyal

Acacia seyal is a small to medium-sized tree, growing to 17 m tall and 60 cm in diameter at breast height; crown is umbrella shaped, resembling that of *A. tortilis*. A characteristic feature of the tree is its rust-coloured powdery bark; *A. seyal* var. *fistula* has whitish bark. Large, straight spines occur on the branches, and smaller, curved thorns are present near the tips of the branches. *Acacia seyal* which is more common on heavy soils some of the thorns are swollen and house symbiotic ants (Young, et al., 1996).

A seyal occurs from Senegal to the Red Sea and in Arabia. It is common in many other parts of Africa, especially north of the equator, from 10 to 12 degrees. It also occurs in east and southern Africa. In the southern and western Sudan, it is one of the most common trees in the savannah and often occurs as a pure forest over quite large areas of country.

Frequently, it grows in groups or patches, sometimes of considerable size, in areas inhabited by *A. senegal*. This species is characteristic of the Nile region. It is tolerant to high pH (6-8), salts and periodic flooding. *Acacia seyal* var. *fistula* is more tolerant to water logging than *A. seyal* var. *seyal*

Acacia abyssinica

Acacia abyssinica, *subsp. abyssinica* (*Fabaceae*, indigenous). A flat large topped tree to 20m when matured. Bark rough, grooved, dark brown and thorns are variable, short and long sometimes none. The common name is umbrella thorn, flat top acacia. In Ethiopia it occurs in wooded grassland, high land forests edges or Dry Moist and Wet Weyna Dega and Wet and Moist Dega agroforestry zone of Ethiopia. Altitude ranges from 1,500-2800m. Use for firewood, charcoal, poles, posts, tool handles, food (edible gum), medicine, fodder, bee forage, shade (for cattle), nitrogen fixation, soil conservation and fence (Azene Bekele Tesemma, 2007).

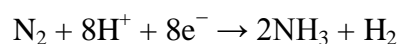
A. negrii

A. negrii is a shrub or tree up to 6 m with a flattened crown which grows in upland wooded grasslands, montane woodland, hilly grassland with scattered acacias, and rocky grazed area with Eucalyptus-stands, open dry hillside in low scrub and in disturbed habitats such as along roadsides. The seeds of *A. negrii* are very small in relation to pod width. The species is used as fuel wood and bee forage. *A. negrii* has been described as rather common in association with *Juniperus procera* forest, *Acacia negrii* is endemic to Ethiopia (Gondar, Welo, Gojam, Shewa, Harege regions) (Thulin, 1983).

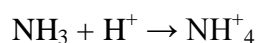
There are no known conservation measures specifically for *A. negrii*, and the species is not currently known to occur in the protected areas network. Samples of seed of *A. negrii* are currently stored in the Svalbard Global Seed Vault (Norway) as an *ex situ* conservation measure. The species has already been assessed as Vulnerable in the Red List of Endemic Trees and Shrubs of Ethiopia and Eritrea (Vivero *et al.* 2005).

2.3. Nitrogen fixation

Many legumes contain symbiotic bacteria called rhizobia within root nodule of their root system. These bacteria have the special ability of fixing nitrogen from atmospheric, molecular nitrogen (N₂) into ammonia (NH₃). The chemical reaction is (Abdel *et al.*, 1996) as follows:



Ammonia is then converted to another form, ammonium (NH₄⁺), usable by (some) plants by the following reaction:



This arrangement means that the root nodules are sources of nitrogen for legumes, making them relatively rich in plant proteins. All proteins contain nitrogenous amino acid. Nitrogen is therefore a necessary ingredient in the production of proteins. Hence, legumes are among the best sources of plant protein. When a legume plant dies in the field, for example following the harvest, all of its remaining nitrogen, incorporated into amino acid inside the remaining plant parts, is released back into the soil. In the soil, the amino acids are converted to nitrate (NO_3^-), making the nitrogen available to other plants, thereby serving as fertilizer for future crops (Abdel et al., 1991).

2.4. Significance of Biological Nitrogen Fixation to Soil Fertility

BNF is an efficient source of nitrogen (Peoples et al., 1995). The total annual terrestrial inputs of N from BNF as given by Burns and Hardy (Burns et al., 1975) and Paul (1988) range from 139 million to 175 million tonnes of N, with symbiotic associations growing in arable land accounting for 25 to 30% (35 million to 44 million tons of N) and permanent pasture accounting for another 30% (45 million tons of N). While the accuracy of these figures may be open to question Sprent (1990), they do help illustrate the relative importance of BNF in cropping and pasture systems and the magnitude of the task necessary if BNF is to be improved to replace a proportion of the 80 to 90 million tonnes of fertilizer-N expected to be applied annually to agricultural land by the end of the decade (Peoples et al., 1995).

Much land has been degraded worldwide, and it is time to stop the destructive uses of land and to institute a serious reversal of land degradation (Burris et al., 1994). BNF can play a key role in land remediation. *Rhizobium*-legume symbioses are the primary source of fixed nitrogen in land-based systems (Tat et al., 1995).

2.5. Environmental Conditions

Several environmental conditions are limiting factors to the growth and activity of the N₂-fixing plants. 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 and Bottomley, 1995), the *rhizobium*-legume symbiosis, which is a N₂-fixing system, the process of N₂ fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavourable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigour of the host legume (Peoples et al., 1995).

Currently, the subject of BNF is of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates in drinking water supplies) and the eutrophication of lakes and rivers (Al-sheriff, 1998). Further, while BNF may be tailored to the needs of the organism, fertilizer is usually applied in a few large doses, up to 50% of which may be leached (Sprent P, 1990). This not only wastes energy and money but also leads to serious pollution problems, particularly in water supplies.

2.6. Acacia in agroforestry

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 and Giller, 2001).

Agro forestry systems take advantage of trees for many uses; such as, for food, fodder for livestock, fuel, often fibres, to provide shade, construction materials, and protection of crops or animals by live fences, increase soil fertility through nitrogen fixation, or through bringing minerals from deep in the soil and depositing them leaf-fall (Young, 1998).

2.7. The nitrogen 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 N₂-fixation. Nevertheless, several workers have reported that there are variations with respect to the symbiotic effectiveness for N₂-fixation ((Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998; Burdon *et al.*, 1999; 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. melanoxyton* performed quite well with most isolates, whereas *A. mearnsii* was much more variable in its response to isolates from different species

3. Materials and Methods

3.1. Study sites

In this study, soil and seed sample were collected from field of rural areas around Buee Town Sodo Woreda, Guraghe Zone in Southern Regional State from September –January, 2016. The test plants were found around Buee town; *Acacia abyssinica* from (Guche Motebi, Amede Foshi), *Acacia negrii* (Buee hospital) and *Acacia seyal* (From Kessay, Wolasa kebele).

3.2. Seed and soil collection.

Soil samples from the rhizosphere of the different *Acacia spp* were collected randomly in the field. Seeds were collected from different acacia species and vouch specimens of the plants were also collected and brought to the National Herbarium of AAU for identification. They identified as *Acacia abyssinica*, *Acacia negrii* and *Acacia seyal*.

3.3. Induction of nodulation

Nodulation was induced by the ‘plant trap’ method of (Barnet *et al.*, 1985). Hard seeded acacia species of *A. abyssinica* and *A. negrii* were scarified mechanically whereas seeds of *A. seyal* were pre-treated with hot water to ensure rapid germination. They were surface sterilized with ethanol and sodium hypochlorate. Five germinated seeds then transferred into surface sterilized (70% alcohol), 3kg capacity plastic pots filled with soil samples collected from the rhizosphere of the parent *acacia spp* a 1% agar medium (Samosagren and Hoben (1994).

The seedlings were later thinned down to 3 per pot. Plants were watered three times a week and were grown for 12 weeks in a greenhouse under natural illumination with 12h Photoperiod.

3.4. Isolation of root nodule bacteria

Bacteria were isolated from root nodules as described by (Vincent, 1970). Three pink and healthy nodules were randomly picked from each acacia species and surface sterilized as before washed several times with distilled water. The nodules were then placed on Petri

dishes in a drop of sterile water, crushed and loop full of the specimens were transferred into YEMA medium (Table 1) and incubated at 28⁰c for ten days.

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 (Table 1).

Table 1. Yeast Mannitol Agar medium (YEMA) contains (Vincent, 1970)

Composition of Elements	Unit (g/ml)
Mg SO ₄ . 7H ₂ O	0.2
NaCl	0.2
K ₂ HPO ₄	0.5
Yeast extract	0.5
Mannitol	10
Agar	20
Congo red	0.025
Dist H ₂ O	1000 ml

3.5. Presumptive tests of the isolates

Even after surface sterilizing the nodules, bacterial contaminants such as the genus *Agrobacterium* can grow on YEMA. Therefore, all isolate that grew on the YEMA may not be rhizobia (Lupwayi and Hague, 1994). Consequently, the following tests were done to differentiate the contaminants from the true bacteria.

Gram reaction

All the isolates were gram stained; their shapes and gram staining reactions were observed under light microscopes.

Congo red absorption

The bacterial isolates were streaked on YEMA media contains 0.25% Congo red (CR-YEMA) and incubated at 28⁰c (Vincent, 1970) Here the plates were wrapped with aluminium foil to provide darkness.

Peptone glucose test

A 72 hours old YEMA rhizobia cultures were streaked on to the peptone glucose media (Table2) and incubated at 28⁰c for ten days to check rhizobia growth.

Table 2 .The peptone glucose medium contains (Vincent, 1970)

Composition of Elements	Unit (g/ml)
Glucose	1.5
Peptone	3.0
Agar	6.0
Bromocreesol purple (% in ethanol)	3ml
Dist. H ₂ O	300 ml

3.6. Bacterial designations of the *rhizobial* isolates

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

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

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

3.7. Characterization of the *rhizobial* isolates

The following tests were undertaken on YEMA medium; adjusted to pH 6.8 and inoculated with 10⁶ cells/ml and incubated at 28⁰C for 5-10 days, unless stated otherwise.

3.7.1. Acid or alkali production (colours on BTB-YEMA)

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

3.7.2. Colony characteristics

Colony characteristics were described based on colony parameters such as transparency, colony size and the production of a watery or mucilaginous gum as indicated in Ahmed *et al.*, (1984).

3.7.3. Physiological characteristics

3.7.3.1. Sodium chloride tolerance

The ability of the isolates to grow at different NaCl concentrations was tested by streaking a loop full of YEM broth at YEMA plates containing 2%, 3%, 5%, and 6% NaCl% (w/v) as described by Samosagren and Hoben (1994)

3.7.3.2. Temperature tolerance

The ability of the isolates to grow at different temperature ranges was tested by incubating the YMA cultures at temperatures; 4⁰c, 25⁰c, 28⁰c, 37⁰c), and 43⁰c (Samosagren and Hoben, 1994).

3.7.3.3. pH tolerance

PH tolerance of the isolates was tested by incubating them into YMA plates adjusted to pH values 5, 7 and 9(Samosagren and Hoben (1994).

3.7.3.4. Utilization of carbohydrates

Carbon utilization of the isolates was determined by streaking them on plates on carbon sources were (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). Final concentration of 1g/L to a basal medium containing (g/L): K_2HPO_4 , 1; KH_2PO_4 , 1; $FeCl_3 \cdot 6H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.1; $(NH_4)_2SO_4$, 1; and 15g of agar (Amarger *et al.*, 1997). The carbon sources after filter sterilizing the heat sensitive carbohydrates; (D-arabinose, D-mannose, sorbitol, D-galactose, Maltose, Na-citrate, Xylose, trehalose, cellobiose and glycerol) were using a Millipore filter (0.22Nm) and by using autoclaving to avoid the risk of decomposing of these heat sensitive carbohydrates.

3.7.3.5. Phosphate solubilisation

The ability of the isolates as Phosphate solubilises was examined by streaking a loop full of 72 hours old YEM broth cultures of the rhizobial isolates on Pikovaskaya's medium as indicated by Tawari *et al.*(2004). Then the plates were incubated at 28°C for 5-7 days. Clear zones formation around their colonies indicated phosphate solubilisation.

Table3. The Pikovaskaya's medium contains Tawari *et al.*(2004):

Composition of Elements	Unit (g/ml)
Glucose	10.0
Mg SO ₄ . 7H ₂ O	0.1
NaCl	0.2
(NH ₄) ₂ SO ₄	0.5
Yeast extract	0.5
MnSO ₄ .H ₂ O	0.002
Agar	15.0
KCl	0.2
Ca(PO ₄)	5.0
Distilled Water	1000ml

3.8. Authentication and symbiotic effectiveness isolation on sand culture

Experimental design:

The ability of rhizobial isolates to form root nodules on their respective host (authentication) and their symbiotic effectiveness on the acacia hosts was determined under greenhouse condition, according to Samosagren and Hoben (1994). Seeds were surface sterilized and germinated as before and seedlings transferred into 3kg sterile capacity plastic pots filled with acid washed 95% H₂SO₄). They were thinned down to 3 plants/pots in the greenhouse at (AAU). For this experiment, after a week each isolates was inoculated with active rhizobial culture (10⁹/ml) into pots in triplicates with KNO₃ (0.05%) fertilized as positive control pots and unfertilized pots as negative control.

Seed germination and sowing

Viable and healthy acacia seeds were selected and hard seeded acacia species including *A. negrii* and *Acacia abyssinica* was scarified mechanically and that of *A. seyal* and were pre-treated with hot water while seeds. The seeds were surface sterilized as before and germinated by the same procedure mentioned under the section 'Induction of nodulation'. Five geminated seeds had been were then planted in each pot that were thinned down to three seedlings per pots.

Preparation of sterile sand

River sand was thoroughly washed many times with tap water, drained and autoclaved at 121⁰c/15mmHg for 1 hour. Then 2kg of this sterile sand was added in to the surface sterilized plastic pots.

Inoculation

Isolates were grown on YEM broth 3 days from which 1 ml YEMB culture of each isolate (10^9 cells) was transferred into 100 ml sterilized YEMB in 250 ml Erlenmeyer flask and put on rotary shaker shaking at 150 rev/min. for four days. Then each seedling was inoculated with 1 ml inoculants (approximately 10^9 cell/ ml) immediately after sowing and were also re-inoculated after 10 days later.

Watering plants with water and N free media

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

Table 4. N free media for legumes as indicated in Vincent (1970).

Reagents per litter of medium	Concentration (g/l)	vol.(ml) of stock solution
KCl	29.8	2.5
$\text{K}_2\text{HP}_{\text{O}_4}$	69.6	2.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	98.6	2.5
Micronutrients		0.5
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.078	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22	
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	2.03	
$(\text{NH}_4)_6 \text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.01	
H_3BO_3	1.43	
Ferric citrate	1.795	1.0
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$		0.334g

Harvesting and determination of shoot dry weight

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 collect nodules to determine nodule dry weight (NDW) and shoot dry weight (SDW) and shoot length (SL).

Determination of symbiotic efficiency of rhizobia isolates

Symbiotic effectiveness (SE) of the each isolate can be calculated as follows:

$$(\%SE) = \frac{\text{Mass of the shoot dry matter of inoculated plants}}{\text{Mass of the shoot dry matter of N}^+ \text{ fertilized plant}} \times 100(\%)$$

Symbiotic effectiveness (SE) values rated as ineffective (< 35%), effective (50-80%) and highly effective (> 80%) as described in Beck *et al.*, (1993).

The symbiotic effect (SE) of the respective isolates were evaluated by comparing the data obtained from nodule number (NN), nodule dry weight (NDW), and shoot dry weight (SDW) of *Acacia seyal*, *Acacia abyssinica* and *Acacia negrii* plants grown on sand pots.

According to Date *et al.*, (1990) in Phrcino *et al.*, (2002), variation in symbiotic effectiveness among legume rhizobia symbiosis as very effective (>80%), effective (50-80%), low effective (35-50%) and ineffective (<35%) is indicated.

3.9. Data analysis

The data Analyzed using computer system by using SPSS20 (Statically Analytical method) Version. The significant difference of the isolates were separated by using $P \leq 0.05$.

4. Results

In this study, ten isolates were authenticated as root nodule bacteria of which 4 isolates belonged to *A. abyssica* whereas 3 isolates were recorded each for *A. seyal* and *A. negrii*. All isolates were found to be gram negative and rod shaped bacteria. They all failed to absorb Congo red on CR-YEMA media and to grow on peptone glucose media (data not shown)

4.1. Colony characteristics

The isolates displayed different colony characteristics. Most of the isolates (7isolates) showed large mucoid (LM) Isolates like AURasey11, AURasey12, AURasey13, AURAneg31, AURAneg32, AURAneg33 and AURAaby23and 3 isolates were characterised by large watery (LW) colonies as mostly *A. abyssica* (AURAaby21, AURAaby22 and AURAaby24) (Table5). The LW the isolates showed large watery (LW) colonies with excessive areas of watery growth on YEMA.

4.2. BTB reaction and Generation time

Most of the growth of the isolates on BTB-YEMA medium showed that the isolates were able to change the colours of the media into yellow due to the production of acid. The result displayed mean generation time ranging from 1.0h-2.5h indicated that they all isolates were fast growing rhizobia (Table5).

Table5 Cultural characterization and mean generation time (MGT) of the isolates

Isolates	Host Acacia	Colony	Colony (diameter in mm)	MGT in(hrs)	BTB reaction	Fast Growing	Rhizobium
AURAsy11	<i>A.seyal</i>	LM	1.4	2.5	Yellow	FG	Rhizobium
AURAsy12	<i>A.seyal</i>	LM	2.0	1.5	”	”	”
AURAsy13	<i>A.seyal</i>	LM	1.0	1.5	”	”	”
AURAaby21	<i>A.abysynica</i>	LW	2.2	1.0	”	”	”
AURAaby22	<i>A.abysynica</i>	LW	0.9	1.5	”	”	”
AURAaby23	<i>A.abysynica</i>	LM	0.7	1.0	”	”	”
AURAaby24	<i>A.abysynica</i>	LW	0.8	2.0	”	”	”
AURAneg31	<i>A.negrii</i>	LM	2.3	2.0	”	”	”
AURAneg32	<i>A.negrii</i>	LM	2.1	2.5	”	”	”
AURAneg33	<i>A.negrii</i>	LM	1.5	2.0	”	”	”

LW, large watery; LM, large mucoid; Y,yellow (acid reaction)

4.3. Physiological and Biochemical characteristics of the Rhizobial

Isolates:

4.3.1. Salt tolerance

The isolates grew at the different NaCl concentration in YEMA medium shown in (Table6).More than half of the isolates were grew at NaCl concentration of 0.5% except isolate of *A. negrii* and one isolate from *A. abysynica*. All most all isolates grew 6% NaCl concentration except isolates from *A. abysynica*.

4.3.2. Temperature tolerance

Isolates from the three acacia species grew on YEMA at 25°C- 43°C.However, more than half of the isolate were grew at low (4°C) and fewer, isolates were grew at the temperature 43°C except AURAsy11,AURAsy12 and AURAsy13 from *A. seyal* and one isolate from

AURAneg33 from *A.negrii*. Almost all isolates from *A. negrii* and *A. seyal* were tolerant to 43°C (Table6).

4.3.3. pH tolerance

Almost all isolates grew at pH value 5.5 except isolates AUARneg32 and AUARneg33 from (*A. negrii*).Others failed to grow at pH 9.0 except isolates AUARaby21, AUARaby22, AURAaby23, AUARaby24 from (*A.abysinica*), and AUARneg31, AUARneg32 both from *A.negrii*(Table6)

Table 6. Temperature, Salt and pH tolerance of the rhizobial isolates

Rhizobial isolates	Temperature (°C)		NaCl concentration		PH		Phosphate solubilisation(PKM)
	4	43	0.5%	6%	5.5	9	
AURA-sey 11	+	-	+	+	+	-	-
AURA-sey 12	+	-	+	-	+	-	-
AURA-sey13	+	-	+	+	+	-	-
AURA-aby21	-	+	+	-	+	+	+
AURA-aby22	-	+	+	-	+	+	+
AURA-aby23	-	+	+	-	+	+	+
AURA-aby24	-	+	-	+	+	+	+
AURA-neg31	+	+	-	+	+	+	-
AURA-neg32	+	-	-	+	-	+	-
AURA-neg33	+	+	-	+	-	+	-

+, Isolates grown; - Isolates not grown

4.3.4. Carbon utilization

All isolates were grown on a given simple (hexoses) but AUARaby21, AUARaby22, AUARaby23 and AUARaby24 failed to grow on maltose as carbon source. However, all isolates of *A.seyal*(AURAsey11,AURAsey12, AURAsey13) and that of *A.negrii*

(AURAneg31, AURAneg32 and AURAneg33) were grown on sucrose. The most revealed isolate that grows on 6 carbon with AURAb21. But, all the isolates failed to grow on starch.

Table 7. Carbohydrate utilization of isolates

Isolates	Carbohydrates							Total grown
	Hexoses		Acid	Disaccharides			Polysaccharide	
	Glucose	Fructose	Citrate	Maltose	Sucrose	Lactose	Starch	
AURasey11	+	+	-	+	+	-	-	4
AURasey12	+	+	-	+	+	-	-	4
AURasey13	+	+	-	+	+	-	-	4
AURAb21	+	+	+	-	+	+	-	5
AURAb22	+	+	+	-	+	+	-	5
AURAb23	+	+	+	-	+	+	-	5
AURAb24	+	+	-	-	+	+	-	4
AURAneg31	+	+	-	+	+	+	-	5
AURAneg32	+	+	-	+	-	+	-	4
AURAneg33	+	+	-	+	-	+	-	4
Total grown	10	10	3	6	8	7	-	

+, growth; -, no growth

4.3.4. Phosphate solubilization

Out of the isolates AURAb21, AURAb22, AURAb23, AURAb24 from *A. absinica* were able to solubilize inorganic phosphate (Table 6). They formed clear zones around their colonies on the Pikovskaya's media.

4.4. Symbiotic effectiveness evaluation of isolates on sand

The symbiotic performance of the isolates of the three nodulating acacia species (*A. seyale*, *A. abssinica* and *A. negrii*) showed significant variations at $p < 0.05$ (Turkey's test) in mean nodule number, nodule dry weight and shoot dry weight inoculation to their respective positive (N^+) and negative (N^-) control groups.

AURAneg31 and AURAneg32 both from *Acacia negrii* induced the maximum number of 71 nodules/plant and the minimum number of 50 nodules/plant, respectively. The nodule dry weight of the isolate found between (0.26-0.39gm/plant) the lower and the higher, respectively. With regard to shoot dry weight of *Acacia negrii* plants, the minimum shoot dry weight of 0.36 gm/plant was recorded by isolate AURAneg32, whereas the maximum shoot dry weight of 0.41 gm/plant recorded from the isolate AUR Aneg 31 (Table 8). The shoot length found between (10-10.9cm/plant), was recorded by isolate AURAneg32 (lower) and AURAneg31 (higher), respectively. However, the shoot length of the N^+ controlled plant recorded the highest value (11.6cm/plant) and the N^- controlled plant shown the least value recorded i.e.8.2cm/plants. Shoots dry weight among the plants inoculated with uninoculated were show variation. The N^+ control plants displayed the highest shoot dry weight i.e. 0.45gm/plant compared with all the other groups.

The symbiotic effectiveness of the isolates ranged between (84%-91%). Similarly, AURAaby21 and AURAaby24 (*A. abyssinica*) induced 49 and 30 nodules; maximum and minimum nodules, respectively. The nodule dry weights between (0.04-0.09gm/plants) were high for AURAaby24 and low that of (AURAaby22) isolates. Likewise, shoot dry weights were high for isolate AURAaby21 and low AURAaby22 compared with the four isolates. The minimum shoot dry weight of *Acacia abyssinica* plant was 0.22gm/plant (inoculated by isolate AURA24) and the maximum shoot dry weight was 0.35 gm/plant (isolate AURAaby21). The symbiotic effectiveness of the isolates was between (50% _ 76 %). Shoot length found between (9.0-10.8 cm/plants), the lowest and the highest values recorded by

isolates AURAaby24 and AURAaby21, respectively. The N⁻ controlled plant was recorded the least value (8cm/plant). The symbiotic effectiveness of the isolates was (50% – 76%).

As shown in (Table 8), the *A. seyal* plants showed nodule number (38-48 nn/plants) between high nodulation from isolate AURAsay12 and low nodulation AURAsay13 (Table 8). Whereas, the nodule dry weight of *Acacia seyal* ranged from 0.07-0.09(gm/plant) that were induced by isolates AURAsay12 and AURAsay13, respectively. Shoot length of the inoculated plants no significant differences with one another but less N⁻ plants. However, the plants did not show significant differences in nodule dry weight (0.07-0.09). Shoot length of the inoculated plants no significant differences with one another but less N⁻ plants. The symbiotic effectiveness of the isolates was (67%-75%).

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. Two isolates AURAneg31 and URAneg33 from *A. negrii* showed the highest and lowest symbiotic effectiveness with 91% and 84%, respectively (Table). The lowest symbiotic effectiveness of 50% was recorded from the inoculation of AURAaby24 and the highest symbiotic effectiveness of 76% was recorded from isolate AURAaby21 on *Acacia abyssinica*, respectively. The highest symbiotic effectiveness of 75% was recorded from isolate AURAsay12 whereas the lowest symbiotic effectiveness of 67% was recorded from the inoculation of AURAsay13, on *A. seyal* hosts (Table 8). *A. negrii*, *Acacia seyal* and *Acacia abyssinica*, respectively.

Table (8). The effect of rhizobia nodulating of *A. negrii*, *A. seyal* and *Acacia abyssinica* based on the nodule number (NN), nodule dry weight (NDW); shoot dry weight (SDW) from plants grown on sand pots, respectively.

**Table 8. Evaluation of symbiotic performance of the nodulated isolates
With *Acacia negrii*, *Acacia seyal* and *Acacia abyssinica*, respectively.**

Isolate	NN/plant	NDW(gm/plant)	SDW(g/plant)	SL(cm/plant)	%SE	Effectiveness
AURAneg31	71a	0.39a	0.41a	11.4a	91	HE
AURAneg32	50b	0.26a	0.36a	10.4a	80	HE
AURAneg33	58b	0.34a	0.38a	10.8a	84	HE
Average	60	0.34	0.38	10.9	85	HE
AURAsey11	40a	0.08b	0.36b	10.2b	69	E
AURAsey12	48a	0.07b	0.39b	10.9b	75	E
AURAsey13	38a	0.09b	0.39b	10.8b	67	E
Average	42	0.29	0.37	10.6	70	E
AURAaby21	49a	0.09b	0.35b	10.8a	76	E
AURAaby22	35b	0.07c	0.32b	10.3a	70	E
AURAaby23	33b	0.04a	0.24c	9.8a	52	E
AURAaby24	30a	0.07a	0.22c	9.0a	50	E
Average	37	0.07	0.28	10.0	62	E
N ⁺	-	-	0.48a	11.7a	-	-
N ⁻	-	-	0.26c	7.9d	-	-

SE, Symbiotic Effectiveness; HE, High Effective; E, Effective

NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, shoot length=SL
Numbers are the means of variables of three replicates of four plants per pot

Letters in vertical columns (a, b, c and d) are ranks of the mean nodule number, nodule dry weight, shoot dry weight and shoot length respectively.

4.5. Comparative evaluation of the pattern of symbiotic effectiveness among highly effective and effective isolates

Based on the physiological, biochemical and symbiotic effectiveness performance of isolates, grades were assigned for the following mentioned tests and comparisons were made among isolates (Table 9). Isolates were given grades 2.5 up to 5, 2.5 being poor for having the lowest performance and 5 being excellent for having the highest performance on different physiological, biochemical and symbiotic situations.

From table 9 isolates like AURAneg31 and AURAneg32 both from *A. negrii* were found to be highly competent among all the isolates with highest total grade (26 out of 30) and high symbiotic effectiveness. On the contrary, three other isolates from *A. seyal* (AURA11, AURA12 and AURA13) were found to be very low competent among all the isolates with lowest total grade of 19 out of 30. Isolates from *Acacia abyssinica* were ranked second with a total score of 25 out of 30

Table 9 Comparisons of the results of the physiological and biochemical with SE among isolates

Isolate	Grades given to isolates based on different competence potentials						
	Temperature	Salt	pH	Carbon sources	Phosphate solubilisation	SE (%)	Total
AURasey11	2.5	5	2.5	4	2.5	2.5	19
AURasey12	2.5	2.5	2.5	4	2.5	2.5	19
AURasey13	2.5	5	2.5	4	2.5	2.5	19
Average	-	-	-	-	-	-	19
AURaaby21	2.5	2.5	5	5	5	5	25
AURaaby22	2.5	2.5	5	5	5	5	25
AURaaby23	2.5	2.5	5	5	5	5	25
AURaaby24	2.5	2.5	5	5	5	5	25
Average	-	-	-	-	-	-	25
AURAneg31	5	3	5	5	3	5	26
AURAneg32	3	5	5	5	3	5	26
AURAneg33	5	3	5	5	3	5	26
Average	-	-	-	-	-	-	26

5. Discussion

In this work, all isolates were found to be gram negative rod shaped bacteria, and all of them failed to absorb Congo red and did not grow on PGA. Thus, the isolates were presumptively tested as rhizobia (Vincent, 1970).

Colony morphology

Fast growing isolates grown on YMA were classified into large mucoid (LM) and large watery (LW) colony types. Most of the isolates (about 70%) showed large mucoid (LM) colonies, associated with excessive production of exo-plysaccharides on YMA whereas the isolates (about 30%) showed large watery (LW) colonies. Isolates like AURasey11, AURasey12, AURasey13, AURAneg31, AURAneg32, AURAneg33 and one isolate from *A. abyssinica* (AURAaby23) displayed LM colony types whereas the other (AURAaby21, AURAaby22 and AURAaby24) showed LW. Thus, based on the colony morphology of the isolates shown LM and LW. Other reports shown, FG strains isolated from wild legumes displayed LM and LW colony types typical of *Rhizobium leguminosarum* Ahmed *et al.* (1984).

Acid and base reaction

All isolates produced yellow coloration on YEMA-BTB (Norris medium) that confers the production of acid (pH 5.3-6.8), (Table 1). The isolates shown their generation time range between 1h-2.5h. According to Jordan (1984) and Elkan (1987), isolates that show fast growth with 2-4h, with large colony types and release acid on YMA medium are categorized as Fast growing (FG) (*Rhizobium*). Thus, all the rhizobia isolates were belong to Fast growing rhizobia.

According to Odee *et al.*, 1997 and Endalkachew Wolde-Meskel *et al.*, 2004, isolates from woody which are fast-grower acid-producing and intermediate-grower acid-producing are classified as legume *Rhizobium*. However, Dreyfus and Dommergues (1981), Fassil and Kleiner (1998), showed that *A. abyssinica* and *A. seyal* could also be nodulated by both fast and slow growing rhizobia.

According to Fassil and Kleiner(1998) also reported that *A. abyssinica* , *A. seyal* and *A. negrii* were nodulated and pattern of infection both by *Rhizobium* and *Bradyrhizobium*. This included both FG and SG nodulating bacteria, respectively. However, this work showed that all the nodulating isolates were FG rhizobia.

Salt tolerance

Most of the isolates from these acacia species tolerated lower salt up to 0.5% NaCl concentration (Table6). However, more than half of the isolates were tolerant to 6% NaCl concentration except AURAneg31, AURAneg32, AURAneg33 from (*A. negrii*) and one isolate from AURAaby24 (*A. abyssinica*) (Table 6). This result is similar to some other findings that showed tolerance of up to 3% NaCl concentrations by a number of tree (*Acacia* and *Prosopis spp.*) rhizobia from the Sudan and Saudi Arabia (Zhang *et al.*, 1991). *Rhizobium* strains isolated from woody legumes such as *Acacia*, *Prosopis* and *Leucaena* can tolerate NaCl concentration up to 5% were FG *Rhizobium*.

However, Graham and Parker (1964) reported that FG *rhizobia* are more salt tolerant than SG brandyrhizobia which was later disputed by Singleton *et al.* (1982). Elkan (1987) suggested that the NaCl tolerance be used as a diagnostic feature to separate the salt-sensitive FG from the salt-tolerant SG isolates respectively. Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate, 1995).

Temperature tolerance

All FG isolates were grown at 4°C except AURAaby21, AURAaby22 AURAaby23and AURAaby24. However, most of the isolates failed to grow at a high temperature of 43°C except AURasey11, AURasey12, and AURasey13 and one isolate fro *A. negrii* (AURAneg32. From this study, the isolate were grow at lower temperature (4°C) and others grew at higher temperature (43°C). The isolates were more tolerant to high salt concentration and temperature values than the rhizobial strains isolated from temperate legumes (Zhang *et al.*, 19991; Fassil Assefa, 1993; Odee *et al.*, 1997; Zehari *et al.*, 2000). But most of the isolates grew at temperature range of 15°C-35°C.

Shishay Mesfin, (2008), also reported that a 100% growth of root nodulating bacteria, at the same incubation temperature between 15°C -35°C, for example, isolated from *Acacia spp.* growing in northern part of Ethiopia. Therefore, screening of tropical root nodule bacteria on the basis of their temperature tolerance in laboratory culture may be useful tool to select strains better suited to soil environments where high temperature is a limiting in the symbiosis. This, together with the selection of the corresponding legume species and provenances (Craig *et al.*, (1991), are important for the application of BNF technology in agro forestry in the arid and semiarid region.

pH tolerance

All isolates grew at pH value 5.5 except isolates AUARneg32 and AUARneg33 from (*A. negrii*). Others failed to grow at pH 9.0 except isolates AUARaby21, AUARaby22, AURAaby23, AUARaby24 (*A. abyssinica*), and AUARneg31, AUARneg32 both from (*A. negrii*). Surange *et al.*, (1997) also showed that rhizobial isolates from tree legumes (*Acacia farnesiana*, *Dalbergia sissoo* and *Sesbania formosa*) can grow well at pH 9.0.

According to Shishy Mesfin(2008) showed that all of FG rhizobial isolates except (*A. lahai*), were able grow at pH4.0. However, according to Fassil Assefa (1993) found it difficult to make generalization with regard the relationship between pH tolerance and acid or alkali production indicating that several factors; such as composition and structures of outer membrane, changes in protein expression and cytoplasmic potassium and glutamate levels are supposed to contribute to the pH tolerance ability of rhizobial isolates(Aaron and Graham,1991; Graham et al.,1994).

Phosphate solubilisation

Majority of the isolates did not exhibit the ability to solubilize inorganic phosphate AURAaby21, except isolates of *A. abyssinica* (AURAaby21, AURAaby22, AURAaby23, and AURAaby24) and were able to form clear zone around their colonies. According, Shishay Mesfin (2008) reported that a few isolates of *A. asak*(less than 50 %) were able to done this process. In this study, similar results obtained (4 out of 10 isolates) or 40% of the isolates have a potential to done this process.

Carbon Utilization

All isolates utilized glucose and fructose. Most isolates were capable of metabolizing on hexoses (monosaccharide) followed by rigorous grow. But there were variation among the isolates in using acids and disaccharides. Most of the isolates grew on maltose except AURAaby21, AURAaby22 and AURAaby23 and AURAaby24. Majority the isolates grown on sucrose except AURAneg32 and AURAneg33. All *A. seyal* isolates did not grow on lactose. Likewise, none of the tested isolates could utilize starch. Out of the isolates, except (AUAaby21, AURAaby22, and AURAaby23) from *A. abyssinica* failed grow on citrate their carbon source. Fassil and Kleiner(1998), also shown that 50% of the isolates from acacia utilizes less disaccharides but isolates from *A seyal* and *A.abysinica*, were more resources utilizing disaccharides than other isolates. Fassil Assefa (1993) showed no growth on starch but sucrose (lactose) grew about 90%.

Pattern of nodulation and Relative effectiveness of the isolates on sand

The nodulation pattern of the isolates showed from the three acacias (*Acacia abyssinica*, *A seyal* and *A negrii* are nodulated by fast growing (*Rhizobium*) a root nodule bacteria. Similar results showed the promiscuity of these species (Fassil Assefa and Kleiner, 1998; Endalkachew Wolde-Meskel *et al.*, 2004) which is very important for the potential identification of novel 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 plants grown on sand pots.

From the data, when the result compared with one another as follows: from *Acacia negrii* isolate like AURAaby31 and AURAneg32 showed the maximum number of 71 nodules/plant and the minimum number of 50 nodules/plant, respectively. Similarly, AURAaby21 and AURAaby24 (*A. abyssinica*) induced 49 and 30 nodules; maximum and minimum nodules, respectively. According to Shishay Mesfin (2008), acacia plants inoculated with rhizobia ranging from 25NN/plants- 107 NN/plants,

The nodule dry weights of *A. abyssinica* between (0.04-0.09mg/plants) were high for AURAaby24 and low that of (AURAaby22) isolates. Whereas, the nodule dry weight of *Acacia seyal* ranged from (0.04 - 0.08gm/plant) that were induced by isolates AURasey13 (low) and AURasey12 (high), respectively. There were no significant differences between *Acacia seyal* and *A. abyssinica*, the nodule dry weights. However, *Acacia negrii* showed the nodule dry weight ranged between (0.36 -0.39mg/plant) from isolates of AURAneg31 and AURAneg32, respectively.

With regard to shoot dry weight, two isolates (AURAneg31 and AURAneg32,) from *Acacia negrii* accumulated higher shoot dry weight than the negative controls; this result showed that the potential of some rhizobial species accumulating higher shoot dry weight on their host plant when compared to the same but uninoculated nitrogen provided plants (Shishay Mesfin, 2008). Similarly, Shishay Mesfin, (2008) reported that some rhizobial species isolated from *Acacia venosa* have higher symbiotic effectiveness reaching up to 115% shoot dry weight accumulation compared to the N-fertilized control.

From the results of the sand culture study on the symbiotic performance of the isolates of the three nodulating acacia species (*A. seyal*, *A. abyssinica* and *A. negrii*), there was significant variations at $p < 0.05$ (using turkey's test) in mean shoot dry weight, nodule dry weight, nodule number and within their respective N^+ and N^- control groups. According to Date *et al.*, (1990) in Phrcino *et al.*, (2002), variation in symbiotic effectiveness among the rhizobia with

symbiosis as very effective (>80%), effective (50-80%), low effective (35-50%) and ineffective (<35%) is indicated.

Accordingly, AURAneg31 and AURAneg33 from *A. negrii* showed the highest symbiotic effectiveness (SE) isolates with 91% and 84% shoot dry weight accumulation compared to the N⁻ fertilized (negative control), respectively (Table 8). All the remaining isolates were effective with SE 50% from AURAaby24 and SE of 76% (*A. abyssinica*). The SE range between (67-75%) by *A. seyal*.

Hence, most isolates of *A. negrii* were very effective; whereas AURAneg31 assuming the highest (SE) value i.e. 91%. Isolates of *A. abyssinica* had better performance next to isolates of *A. negrii*. However, *A. seyal* less effective even if SE of *A. seyal* shown better symbiotic effectiveness than *A. abyssinica*. According to Fassil Assefa and Kleine(1998) also reported that among nine Ethiopian acacia species, *A. abyssinica*, *A. negrii* and *A. etabaica*. were effective.

Based on the criteria given from table (8 and 9) result, among the isolates of the three acacia species were *A. negrii* shown highly component isolates. However, *Acacia seyal* showed less competent than the others.

6. Conclusion and recommendations

6.1. Conclusion

All isolates from the three different acacia species (*A. seyal*, *A. abyssinica*, and *A. negrii*) were able to re-infect and induce nodule formation on their homologous host plants under greenhouse conditions. The nodulation pattern of the three acacia species showed that all of them were nodulated by *rhizobial* strains i.e., fast growing. They also utilized a wide range of carbon sources which can give them a competitive advantage to utilize a wide range of carbon sources, for example, *A. abyssinica*. The *rhizobial* isolates from the acacia species were generally tolerant up to 6%NaCl concentration, extreme pH ranges and temperature up to 43°C ranges.

The symbiotic effectiveness of the *rhizobial* isolates on their homologous host plants showed significant difference at $p < 0.05$ (Turkey's test) in nodule numbers, nodule dry weight, mean shoot dry weight, and within their respective N^+ and N^- control groups. Generally, isolates of *A. negrii* (84%-91%) were very effective (HE), followed by *A. abyssinica*. isolates that showed effective (50%-76%) fixation. and the third one was *A. seyal* indicating symbiotic effectiveness of nitrogen fixation differed from one endosymbionts to another.

6.2. Recommendations

The data showed prospects to find effective nitrogen fixing rhizobia for acacia species. Since the work was not exhaustive, more isolates could be screened from more acacia species from wider sampling sites in the Wereda and elsewhere to fully exploit nitrogen fixation for afforestation and rehabilitation of degrade lands using acacia species.

The highly competent isolates which have been tested in greenhouse conditions need to be tested in the field to ensure their competitiveness ability of the isolates in the natural environment (soil).

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From AAU greenhouse (nodulation) results as follows:

Appendix-A



Fig.1. Acacia seedlings in AAU under greenhouse

Appendix-B



Fig.2. Nodules induced by isolates; AURAneg31

Appendix-C



Fig.3. The inoculated, the N⁺ and N⁻ control groups under the greenhouse

Declaration

I declare that the dissertation here by me for the Degree of Master in Biology to 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 university. The materials obtained from other sources have been dually acknowledged in the dissertation.

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