ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

Symbiotic Effectiveness and phenotypic Characterization of Rhizobia Nodulating Chickpea (*Cicer arietinum*) from Some growing areas of South and North Wollo zones

By:

Ahmed Seid

A thesis submitted to the school of graduate study of Addis Ababa University in partial fulfillment of the requirement for the degree of masters of Science in applied microbiology

June, 2010

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Declaration

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

Name Ahmed Seid
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Date_____________________________

This thesis has been submitted for examination with my approval as advisor
Fassil Assefa (PhD)
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Date________________________

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List of Symbols and abbreviations

AAUCR	Addis Ababa University Chickpea Rhizobia
ANOVA	analysis of variance
ATP	Adenine triphosphate
BNF	Biological Nitrogen Fixation
BCP	bromocresol purple
BTB	bromothymol blue
C. arietenium	*Cicer arietinum*
CFU	colony forming units
EPS	Extracellular polysaccharides
E	effective
HE	highly effective
IE	ineffective
gl^{-1}	gram per liter
GOGAT	Glutamine oxoglutarate aminotransferase
GS	glutamine synthetase
ha	hectares
IAR	Intrinsic antibiotic resistance
IGS	intergenic spacers
IS	insertion sequence
LM	large mucoid
LW	large watery
m.a.s.l	meter above sea level
Mll^{-1}	milliliter per liter
MGT	Mean generation time
mm	millimeter
μgml^{-1}	microgram per milliliter
mMl	milimole
mg	milligram
Mo-Fe	molybdenum iron cofactor
O.D.	Optical density
N	normality
N_2	dinitrogen
nm	nanometer
Nif	nitrogen fixing

Nod genes	nodulation genes
Nif genes	Nitrogen fixing genes
Kgha^{-1}	Kilogram per hectare
LE	lowly effective
PCR	polymerase chain reaction
PGA	peptone glucose agar
RAPDNA	random amplified polymorphic deoxyribonucleic acid (DNA)
spp.	Species
SE	symbiotic effectiveness
SIs	symbiosis islands
TRFLP	terminal restriction fragment length polymorphism
rRNA	ribonucleic acid (RNA)
UPGMA	Unweighted pair group method with the average
V/v	volume over volume
W/V	weight over volume
YEMA	yeast extract manitol agar (medium)
YEMB	Yeast Extract Manitol broth
YEMA-CR	yeast extract manitol agar congored
YEMA-BTB	yeast extract manitol agar bromothymol blue

ABSTRACT

Chickpea is one of the cool seasons legume cultivated in many part of the world. Ethiopia is the largest chickpea growing country in Africa. The major producing areas are concentrated in Amhara and Oromia Regional States. In areas where there is depletion of soil with nitrogen fertilizer, must be inoculated with the most symbiotically effective isolates to restore the fertility status of the soil. Therefore to develop inoculants, isolates must first be screened and characterized to know their status. The failure of some rhizobial isolates to re-inoculate their host in the previous work by Daniel Muleta (2009) needed further search for screening of isolates. So this study was aimed to characterize chickpea isolates from some major growing areas. Thirty eight chickpea root nodules were collected from farmers' plots in South and North Wollo zones. Ten of the isolates were rejected in the process of isolation and presumptive tests. The rest were reinoculated in to their host for authentication. From the reinoculated isolates, twenty seven formed nodules in their host but one isolate failed to form nodule upon re-inoculation. The authenticated rhizobia were characterized based on their, morphological and eco-physiological features. From the preliminary screening 96% of the isolates were fast growing rhizobia. Symbiotic effectiveness of isolates showed significant difference in percent effectiveness (34-92%) on the host plant. AAUCR 27 and AAUCR 9 were found to be the most effective isolates with percent effectiveness of 81% and 92% respectively. The majority of isolates were categorized under effective rate which posses percent of effectiveness 50-80%. Isolates were also tested for their tolerance to different eco-physiological features such as pH, temperature, salt concentration, intrinsic antibiotic resistance and utilization of different nitrogen sources. These tests showed the presence of wide physiological diversity between isolates. Plants inoculated with different isolates displayed differences in respect to nodule number, nodule dry weight and shoot dry weight. The numerical analysis of isolates based on forty six phenotypic features showed the presence of diversity among isolates and also statistical analysis indicates their diversity towards symbiotic effectiveness. Some isolates such as AAUCR 9 and AAUCR 27 that are highly effective and tolerant to a wide range of phenotypic features are promising in the development of inoculants.

Key words/ phrases: Biological nitrogen fixation, Nitrogenase, Inoculants, Phenotypic diversity

1. Introduction

The atmosphere contains about 4×10^{15} tones of inert N_2 gas (Hubbell and Kidder, 2003). It has to be transferred into utilizable form of NH_4 and NO_3 so as to be assimilated by plants and microorganisms. The conversion of N_2 into ammonia is facilitated through biological and chemical fixation of which 60% is fixed by biological nitrogen fixation (BNF) (Zahran, 1999). Biological Nitrogen fixation is undertaken by different groups of bacteria. A group of which the endosymbiotic root nodule bacteria fix the largest amount of fixed nitrogen in association with leguminous plants in nature. It is estimated that the various legume crops and pasture plants often fix about 200-300kg of N/ha/yr (peoples *et al*., 1995) and the global nitrogen fixation by BNF is estimated to be as much as 70×10^6 metric tons/yr (Brockwell *et al*., 1995). The nitrogen thus fixed is the major source of nitrogen to different biological production systems. Consequently, legumes are integrated into various cropping systems such as alley cropping, intercropping and crop rotations (Wani *et al*., 1995; Thomas, 1995).

The *Leguminosae* is one of the largest and diverse families of plants with approximately 730 genera and 19,400 species. It is divided into three closely related subfamilies, namely *Papilionoideae, Caesalpinioideae and Mimosoideae*. The subfamily *Papilionoideae* is the most dominant family containing 12,000 species, including peas, beans, peanuts, chick1peas, soybeans, clover, alfalfa, sweet pea, broom, and lupine (Menna *et al*., 2006).

The second subfamily contains the subfamily *Mimosoideae*, which includes acacias and mimosas and is represented by 3000 species or member of the family. The third subfamily is *Caesalpinioideae*, that include 3000 species of herbaceous and perennial plants such as Brazil wood, carob, honey locust, Judas tree, logwood, and tamarind (Giller, 2001).

The wide use of legumes as food crops, forages and green manure is mainly associated with their ability to establish symbiotic association with root nodulating rhizobia (Menna *et al*., 2006). This enabled legumes to grow in depleted and exhausted soils in nitrogen fertilizers. However, all legumes are not capable of nodule formation and nitrogen fixation. According to deFaria *et al*. (1989), about 23% of *Caesalpinioideae*, 90% *Mimosoideae* and 97% of *Papilionoideae* are known for their ability to form nitrogen (N_2) fixing root nodules. It is estimated that some legumes such as cowpeas, for example, can fix 45kg/ha of Nitrogen or more in the soil, equivalent to 112 kg/ha of urea or 225 kg/ha of ammonium sulphate (Winch, 2006).

Chickpea is one of the members of the subfamily *Papilionoideae* cultivated as food and fodder in different part of the world. It is grown in rotation with cereals and other crops in relatively well-drained black soils, in the cool semi-arid areas of the tropics, sub-tropics as well as the temperate areas. Chickpea was first produced in the Middle East about 7,000 years ago. At present, it is produced in over 40 countries represented in all continents. However, the most important chickpea producing countries in the world are India, Turkey, Pakistan, Iran, Mexico, Australia, Ethiopia, Myanmar, and Canada.

It is the third most important pulse gown in the world next to dry bean and pea, and constitutes about 20% of the world's pulse production. Chickpea is currently grown on about 10.7 million hectares worldwide with average annual production of 8.2 million tones. About 95% of chickpea cultivation and consumption is in the developing countries (Menale Kassie *et al*., 2009).

Chickpea is one of the cool season leguminous crops world-wide in more than 60 countries (Berger and Turner, 2007). There are five major center of diversity for chickpea that includes the Mediterranean basin, central and West Asia, the Indian sub-continent and Ethiopia. The average 2000 to 2005 global production data of chickpea indicated that more than 73% of chickpea products comes from South Asia, 13% from West Asia and North Africa, 6% from North America, 4% from East Africa, and 2% from Australia whereas Europe, East and central Asia contributed to less than 1% (Berger and Turner, 2007). Chick pea tolerates a wide range of soil types, except water logged ones. It is sensitive to acidity, and grows at optimum pH of 7-9 and is moderately tolerant to high salinity. It grows in places with annual rain fall of 600–750mm/year and is resistant to draught because of its ability to grow on residual moisture after rainy seasons (Winch, 2006).

Chickpea is rich in protein, complex carbohydrate, vitamins and minerals. It can be consumed raw and in roasted form. It can be used in mixture with cereals and root crops as a protein supplement in the country (Menale Kassie *et al*., 2009). It is a cash crop that contributes a significant portion of the total value of pulse export. In the year 2002, it contributed to about 48% of the pulse export volume and 27% of the total quantity of chickpea production (Shiferaw *et al*., 2007).

Chickpea can obtain a significant portion of its N_2 requirement through symbiotic N_2 fixation and is integrated with traditional agricultural system for it fixes nitrogen when grown in association with effective and compatible *Rhizobium* bacteria such as *Mesorhizobium ciceri* and *Mesorhizobium mediteraneum* and replenish soil fertility (Stephen, K. *et al*., 2002). It stores an estimated amount of $103\text{kgN}_2\text{ha}^{-1}\text{yr}^{-1}$ (FAO, 1984). Part of the nitrogen fixed thus can be transferred to subsequent crops such as wheat, barley and teff (Million, 1994).

In Ethiopia, chickpea grows in several regions with an altitude range of 1400-2300 meter above sea level (m.a.s.l.) mainly in Shoa, Gojam, Tigray, West Wollo, Gonder, East Bale and West Harargie (Geletu *et al*., 1996). There are two types of chickpea varieties in Ethiopia. The small desi type is widely cultivated in the country; whereas the large seeded Kabuli type is limited in distribution but preferred most in the market (Menale Kassie *et al.*, 2009).

Although chickpea is widely grown in Ethiopia, research on BNF has mainly focused on yield increase in field trials (Geletu *et al.*, 1996). Furthermore, most of the BNF works were limited to other highland pulse crops such as faba bean, field pea and other pasture legumes (Fikadu Shimekit, 2006; Zerihun Belay, 2006). Recently, Daniel Muleta (2009) screened root nodule bacteria from some important growing regions in Ethiopia. He reported that symbiotic effective endosymbionts are very limited to some regions and the failure of some isolates to re-inoculate their host requires further search for selection of inoculants. In the previous study the proportion of highly effective and effective isolates screened were very small in percent as compared to lowly effective and ineffective ones. In this study more symbiotically effective isolates will be screened that could help to develop inoculants.

This study is therefore focused on isolation and characterization of root nodule bacteria of chickpea from one of the best pulse growing regions of North and South Wollo zones. The search for symbiotic effective isolates could contribute to the development of rhizobial inoculants to fully realize the potential of BNF in low input agriculture in the country.

2. General Objective

The objective of this work is to identify and know the status of chickpea isolates that can be used to develop better inoculants from some growing areas of South and North Wollo zones.

2.1. Specific Objectives

To identify the different cultural and biochemical characteristics of chickpea rhizobia

To evaluate the symbiotic effectiveness of isolates on sand culture

3. Literature Review

3.1. Chickpea

Chickpea has been cultivated mainly in the Indian subcontinent, West Asia, and North Africa, but recently large acreages have been introduced in the Americas and Australia (Singh, *et al*., 2008; Cleyet-Marel, *et al*., 1990). Maâtallah, *et al*. (2002) reported that chickpea is an important legume food crop in many developing countries, and there are substantial research programmes to improve its yield, disease resistance and nutritional quality.

Ethiopia is one of the major centers of diversity for grain and legume crops, like field pea, grass pea, chickpea, lentil and wild species of cowpea. In Ethiopia, pulses are next to cereals in terms of production and are cheap sources of protein. They also play an increasingly important role in the export market. The total allotted area for the production of pulses is nearly 1.4 million ha, accounting for 13 percent of the total cultivated area and 11 percent of the total grain production (Institute of Biodiversity Conservation, 2008).

Chickpea covers over 160,000 ha with a total production of 160,000 tones which accounts for 12 percent of the total grain legume production in the country (Institute of Biodiversity Conservation, 2008). The related wild species of chickpea, (*C. cuneatum*) has been found in Northern Ethiopia. Although chickpea is widely grown in Ethiopia, the major producing areas are concentrated in the two regional states-Amhara and Oromia (Menale Kassie *et al.,* 2009). These two regions covered more than 90% of the entire chickpea area and constitute about 92% of the total chickpea production. The top 9 chickpea producing zones (North Gonder, South Gonder, North Shoa, East Gojam, South Wollo, North Wollo, West Gojam, and Gonder Zuria) belonging to the Amhara region and accounts for about 80% of the country's chickpea production (Geletu Bejiga, 1996). In Oromia region, the major producing zones are in West Shewa, East Shewa and North Shewa, which accounts for about 85% of the total area and production in the region (Menale Kassie *et al.*, 2009).

Chickpea is largely cultivated between 1400 and 2300 meter above sea level where annual rainfall ranges from 700 to 2000 mm/yr. It is usually planted on heavy black clay soils with pH ranging from 6.4 to 7.9. Such soils usually swell when wet and crack when dry. All chickpea in Ethiopia is grown under rain fed conditions (Geletu Bejiga, 1972). Ethiopia is the largest chickpea growing country in Africa, with a share of about 37% in area and 48% in production. During 2003/04, Ethiopia produced 195,800 tons of chickpea from an area of 176,554 ha (FAOstat, 2004). There has been an increase in about 10% in the area and 42% in the production of chickpea during the past decade (1994/95 to 2003/04). Most of the chickpea production is used for domestic consumption. However, there has been a substantial export of chickpea by Ethiopia during the past five years, with the highest of 48,549 tons accounting to value of US$14.7 million during 2002 (FAOstat, 2004).

The Debrezeit Agricultural Research Center (DZARC) is the leading institute for chickpea research in Ethiopia. It has collaborated with the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and the International Center for Agricultural Research in the Dry Areas (ICARDA), in chickpea improvement and released eight chickpea varieties in Ethiopia. Among these, three (DZ 10-4, DZ 10-11 and Dubie) were developed from its own breeding materials of this institute, four (*Mariye*, *Worku*, *Akaki* and *Shasho*) cultivare were developed from the breeding materials supplied by ICRISAT, and one cultivar; Ererti, from the breeding materials supplied by ICARDA. Three of these varieties (DZ 10-4, Shasho and Arerti) are *kabuli* type and the remaining are *desi* type. The Ethiopian chickpea production is dominated by *desi* chickpea (about 95%), however, in recent years there has been an increase in the interest of farmers in growing large-seeded *kabuli* varieties due to its higher price in the market (Ketema Daba *et al*., 2005).

3.1.1. Agronomic and economic importance of Chickpea

In Ethiopia chickpea serves as a multi-purpose legume. First, it fixes atmospheric nitrogen in soils and thus improves soil fertility and saves fertilizer costs in subsequent crops rotation. It is estimated that 103kg of nitrogen per year per hectare is fixed by chickpea (FAO, 1984). Second, it can be grown as a second crop using residual moisture. It is an excellent source of protein, fiber, complex carbohydrates, vitamins, and minerals, as a cash crop for smallholder producers. It also increases livestock productivity as the residue is rich in digestible crude protein content compared to cereals (Menale Kassie *et al*., 2009).

Chickpea is mainly used as human food in Ethiopia and seeds are consumed green, cooked, roasted, or germinated. Sometimes the dry seeds are mixed with wheat and/or barley and ground to powder to make "Kiyit Injera" (a type of local bread). Split seeds (kik) and powdered seeds (Shiro) are also used in making wot (type of sauce) or soup which is usually eaten with Injera (Geletu Bejiga, 1972).

Chickpea seed contains 29% protein, 59% carbohydrate, 3% fiber, 5% oil and 4% ash. The protein is rich in lysine and arginine but most deficient in sulphur-containing amino acids such as methionine and cysteine (Iqbal *et al*., 2006). It is also a good source of absorbable Ca, P, Mg, Fe and K (Chavan *et al*., 1986; Christodoulou, 2005).

3. 2. Rhizobia

The rhizobia are gram-negative soil bacteria that form nodules on the plant roots where the bacteria fix atmospheric nitrogen. They are bacterial symbionts with legume plants that are capable of fixing inert nitrogen in to the form plants can use to make protein and other organic materials. Nitrogen fixing microorganisms are roughly grouped in three categories: one group consists of free living microorganism such as *Klebsiella*, *Azotobacter* and *Rhodobacter* spp., the microorganisms of the second group are members of blue green algae that form symbiotic association with aquatic ferns such as *Azospirillum spp.*, *Anabaena*, and Nostoc (Boddey *et al*., 2000), and the third forms endosymbiosis with higher plants. In the latter group actinomycetes from the genus *Frankia* that are associated with wooden plants such as *Alnus (alder) or Casuarina* can be found as well as a group of rhizobia belonging to the α-proteobacteria subclass that form association with leguminous plants (Benson and Clawson, 2000).

Currently, 13 genera are widely recognized. The taxonomy of fast-growing rhizobia has been well developed, and has led to the proposal of about thirty new species. It is a group in continuous expansion. In contrast, slow-growing rhizobia (i.e. genera *Bradyrhizobium* and *Azorhizobium*) taxonomy remains unclear due to the inconsistency among the results obtained by different taxonomic methods (Jarabo-Lorenzo *et al*., 2003).

Microorganism belonging to the other rhizobial genera have also been found to nodulate legumes, i.e. genera *Metylobacterium* isolated from nodules of Crotalaria (Sy *et al*., 2001), *Blastobacter* from *Aeschynomene indica* (van Berkum and Eardly, 2002), *Devosia* isolated from an aquatic legume *Neptunia natans* (Vanparys *et al*., 2005), *Ochrobacterium* from *Lupinus honoratus* (Trujillo *et al*., 2005), and *Phyllobacterium* from *Trifolium pratense* (Valverde *et al*., 2005). Phylogenetic sequence analysis of nodulation genes which are shared by all different genera showed that they are closely related to rhizobial genes, suggesting that they were acquired by horizontal gene transfer (Sy *et al*., 2001; Trujillo *et al*., 2005 and Valverde *et al*., 2005).

The recent identification of ß-proteobacterial strains of the genus *Burkholderia* that are able to nodulate legumes changed the long-held dogma that only bacteria of the α-subdivision are symbionts of legumes (Moulin *et al*., 2001). These strains were subsequently described as *Burkholderia tuberum*, *Burkholderia phymatum* and *Burkholderia caribensis* which nodulate tropical legumes such as Aspalathus and Machaerium (Vandamme *et al*., 2003).

In addition, strains isolated from root nodules of Mimosa spp. were described as *Ralstonia taiwanensis* and were also classified as *ß-proteobacteria* (Chen *et al.*, 2001). The findings based on the sequencing of nodulation genes led to the hypothesis that ß-rhizobia evolved from diazotrophs through multiple lateral nod gene transfers and confirmed the phylogenetic diversity of nitrogen-fixing legume ß-rhizobial symbionts (Chen *et al*., 2003). Proteobacteria were also found associated with legume nodules including genera like *Enterobacter and Escherichia,* although their characteristics and role is yet to be defined (Benhizia *et al*., 2004).

3.2.1. Taxonomy of rhizobia

Early *Rhizobium* taxonomy has been mainly based on the nodulating host range (Fred *et al.*, 1932), although overlapping host ranges have already been reported more than fifty years ago (Wilson, 1944). The development of molecular techniques accelerated the taxonomic evaluation and led to the identification of many new rhizobial genera. Based on the sequence of the 16S rRNA gene; rhizobia could be grouped in the alpha subdivision of the *Proteobacteria* (Young and Haukka, 1996) and several genera have been defined including *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium*.

The first described *Rhizobium* species, *R. leguminosarum*, can be grouped in three biovars: *R. leguminosarum* bv. trifolii that nodulated clover, *R. leguminosarum* bv. Viciae that nodulate pea and faba bean, and *R. leguminosarum* bv. Phaseoli nodulating common bean (Jordan, 1984). Two species of *Bradyrhizobium* are well known to nodulate soybean, *B. japonicum* (Jordan, 1982) and *B. elkanii* (Kuykendall *et al*., 1992). Xu *et al*. (1995) described another soybean nodulating species, *B. liaoningiensis*; consisting of extremely slow-growing strains. In addition, yet unnamed species have been found that nodulate other legumes than soybean (Young and Haukka, 1996). The genus *Azorhizobium* includes strains that are very distinct from other rhizobia in many characteristics and *A. caulinodans* is the only species characterized up to now nodulating the roots and stems of *Sesbania rostrata* (Xu *et al*., 1995)). Recently, *Allorhizobium undicola* has been described as a new genus and species nodulating *Neptunia natans* (De Lajudie *et al*., 1998).

Sinorhizobium includes *S. fredii*, *S. meliloti*, *S. teranga, S. saheli* and *S. fredii* comprised of fast-growing strains nodulating soybean, although strains of this species are also able to nodulate and fix nitrogen on various legumes (Krishnan and Pueppke, 1994). *S. meliloti* was isolated from alfalfa, while *S. teranga* and *S. saheli* have been isolated from various tree legumes such as *Sesbania* and *Acacia* species (De Lajudie *et al*., 1994). *S. medicae* members are able to nodulate various alfalfa species but show a different host range than *S. meliloti* strains (Rome *et al*., 1996).

Recently, studies have aimed to disclose the nature of rhizobial symbionts in their native environments as it has been recognized that one of the major problems in the application of BNF technology is the establishment of introduced inoculants strain. In addition, molecular tools have become available to analyze diversity and population structure of bacteria. The 16S rRNA gene sequences are an indispensable parameter in *Rhizobium* taxonomy and methods based on differences in ribosomal RNA genes, which have been frequently applied to species identification (Laguerre *et al*., 1994).

The development of the polymerase chain reaction (PCR) led to the new fingerprinting methods. Arbitrary oligonucleotide PCR primers of random sequence (RAPD) have been used to generate strain-specific fingerprints of *Rhizobium* (Selenska-Pobell *et al.*, 1995; Paffetti *et al*., 1996). In addition, PCR primers based on short intergenic repeated sequences have been designed to fingerprint bacteria (De Bruijn *et al*., 1992; Versalovic *et al*., 1991) and this approach became a frequently employed technique for analysing bacterial communities (Laguerre *et al*., 1996; Sessitsch *et al*., 1997b).

Although the microsymbionts of plants other than crop species have been neglected for a long time, efforts have been undertaken to analyze rhizobia associated with economically less important leguminous plants such as nitrogen fixing trees (Mcinroy *et al*., 1999). Furthermore, the diversity of rhizobia occurring on native shrubby legumes in Southeastern Australia was recently investigated revealing a respectable diversity among the isolated strains (Lafay and Burdon, 1998). A high diversity has been found among bacteria establishing a symbiosis with common bean in European soils (Herrera-Cervera *et al*., 1999; Amarger *et al*., 1997; Sessitsch *et al*., 1997b); however, a molecular analysis revealed that the European strains are of American origin (Sessitsch *et al*., 1997a). *Rhizobium* nodulating alfalfa has demonstrated a tremendous diversity in various soils (Del Papa *et al*., 1999; Hartmann *et al*., 1998; Paffetti *et al*., 1996). In an Italian field a population of 96 *meliloti* isolates, which were phenotypically indistinguishable, proved to consist of 55 different strains when analyzed by RAPD-PCR method (Paffetti *et al*., 1996).

3.2.2. Rhizobia-legume interaction

The interaction between rhizobia and legumes results in a formation of highly specialized structures called nodules. On the basis of morphological, anatomical, and histological differences, legumes’ nodules are divided in to two main separate types: determinate and in-determinate nodules. In-determinate nodules retain their meristematic activity, while determinate nodules do not (Prell and Poole, 2006). The latter appears on legume plants of tropical climate like soybean, bean and cowpea plants, whereas in-determinate nodules are found in more temperate climate legumes like chick pea, pea, alfalfa, and clover.

Development of a *Rhizobium*-plant symbiosis involves a highly coordinated exchange of signals between the host plant and the bacterial symbiont which leads to a gradual and coordinated differentiation and adjustment of physiology and metabolism in both partners. The infection process is triggered by plant root exudates, flavonoids, to which the rhizobia respond by induction of nodulation genes (nod genes). The nod-ABC genes are present in almost all rhizobia and are required for the synthesis of the lipochitooligosaccharide backbone that can be modified by various chemical groups. Detection of nod factors by the host plant induces major developmental changes such as cortical cell division and root hair formation which are required for the entry of rhizobia into the host (Brencic and Winans, 2005).

The next step is the binding of *Rhizobia* to host root hairs. The weak Ca^{2+}-dependant binding is mediated by a bacterial protein called rhicadhesin and followed by a tight binding with cellulose fibrils also synthesized by the bacteria. Host lectins have also been shown to play roles in rhizobial adhesion. The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria and forming the infection thread (Gage, 2004).

Probably, a localized degradation of root hair wall occurs at the site of the infection. After bacteria entered a root hair, they begin to travel along an infection thread toward a developing nodule. The initiation and extension of the infection thread depends on the production of specific extracellular polysaccharides (EPS) by the bacteria. Afterwards, the bacteria are differentiated into "bacteroids" which differ from normal bacteria in size, form, and cell wall composition. Typically, cyclic glucanes, nitrogenase, and specific terminal oxidase are synthesized. Within the nodule, the plant supplies rhizobia with a carbon source in the form of dicarboxylic acids, which are then metabolized via the tricarboxylic acid cycle in the generation of ATP. In return, nitrogenase in the bacteroids catalyzes N_2 to ammonium that can be used as nitrogen source for the host plant (Lodwig *et al*., 2003).

The conditions in the nodules must be micro-aerofilic for nitrogen fixation to take place because of the sensitivity of the nitrogenase to oxygen. Oxygen concentration is the major signal controlling the expression of nif (coding nitrogenase) and fix genes (coding membrane-bound cytochrome oxidase). The central zone of nodules is protected by a layer of internal cortical cells bonded with glyco-proteins. Nodules also synthesize large amounts of leghemoglobin that binds oxygen and holds the oxygen concentration levels to approximately 25nm, and finally, the bacteroids produce the specific terminal oxidase cbb3 with high affinity to oxygen (Brencic and Winans, 2005).

3.3. Nitrogen fixation

Nitrogen is an essential element of biomolecules such as amino acids, proteins, vitamins, nucleic acids and many others required for growth and reproduction of organisms. The atmospheric nitrogen (N_2) represents approximately 78% of the Earth's atmosphere and is the biggest reservoir of nitrogen in the world. However, because of the strong triple bond between the two nitrogen atoms ($N \equiv N$), it cannot be used directly by animals or plants. The only living organisms capable of reducing N_2 to a form that is accessible to plants and animals belong to the domains of *Bacteria and Archaea.* The reduction process is known by the name biological nitrogen fixation.

Biological nitrogen fixation is performed by free living and endosymbiotic diazotrophs. It is estimated that endosymbiotic biological nitrogen fixation globally represents approximately 90% of all the fixed nitrogen in the terrestrial environment (Zaharan, 1999). Chemically fixed in the form of N-fertilizer contributes an estimated amount of 140 tons of additional fixed nitrogen each year which is mainly used for agricultural fertilizers (Gage, 2004).

Leguminous plants and strains of rhizobia show a degree of specificity; For instance, *R. leguminosarum biovar viciae* nodulate *V. itahisitalics*, Pisum, lens and lathyrus legume genera (Laguerre *et al.*, 2001). *Sinorhizobium ciceri* and *S. mediteraneum* are specific strain of *Rhizobia* that nodulate chickpea *(Cicer arietinum* L.) (Nour *et al.*, 1994). For rhizobia to fix nitrogen requires an enzyme collectively known by the name nitrogenase which is found located in the plasmid of *Rhizobium*.

3.3.1 Nitrogenase

The enzyme responsible for nitrogen reduction is the nitrogenase which can be irreversibly inactivated by oxygen. The reduction of N_2 to NH_3 is a highly endergonic and energy consuming reaction.

N_2 + 16Mg ATP + 8e^------------- Nitrogenase----------------2NH_3 + H_2 + 16MgADP + 16pi

Most nitrogenase are composed of two proteins: dintrogenase reductase and di-nitrogenase with iron molybdenum cofactors (FeMo-co). Two homologous alternative nitrogenase can be found in nature; V-containing nitrogenase and only Fe-containing nitrogenase (Rubio and Ludden, 2005).

The stoichiometry of the reaction showed above holds true only in laboratory conditions. In natural conditions up to 40 molecules of ATP can be hydrolyzed for the reduction of only one molecule of N_2 (Hill, 1992). Additionally for every reduced molecule of N_2 the nitrogenase complex produces another molecule of H_2. The hydrogen production has been described as one of the major factors that affect the efficiency of symbiotic nitrogen fixation; however, some rhizobial species have developed a system of hydrogenase that allows them to recycle the generated hydrogen (Baginsky *et al*., 2002).

3. 3.2. The biochemistry of biological nitrogen fixation (BNF)

Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$$\mathbf{N_2 + 8H+ 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

This reaction is performed exclusively by prokaryotes, using an enzyme complex; the nitrogenase. This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein, as shown in the diagram. The reaction occurs while N_2 is bound to the nitrogenase enzyme complex. The Fe-protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$.

Depending on the type of microorganism, the reduced ferredoxin which supplies electrons for this process is generated by photosynthesis, respiration or fermentation. There is a remarkable degree of functional conservation between the nitrogenase proteins of all nitrogen-fixing bacteria. The Fe protein and the Mo-Fe proteins have been isolated from many of these bacteria, and nitrogen fixation can be shown to occur in cell free systems in a laboratory when the Fe protein of one species is mixed with the Mo-Fe protein of another bacterium, even if the species are distantly related (http://www.biology.ed.ac.uk/research/groups/jdeacon/microbes/nitrogen, 2009).

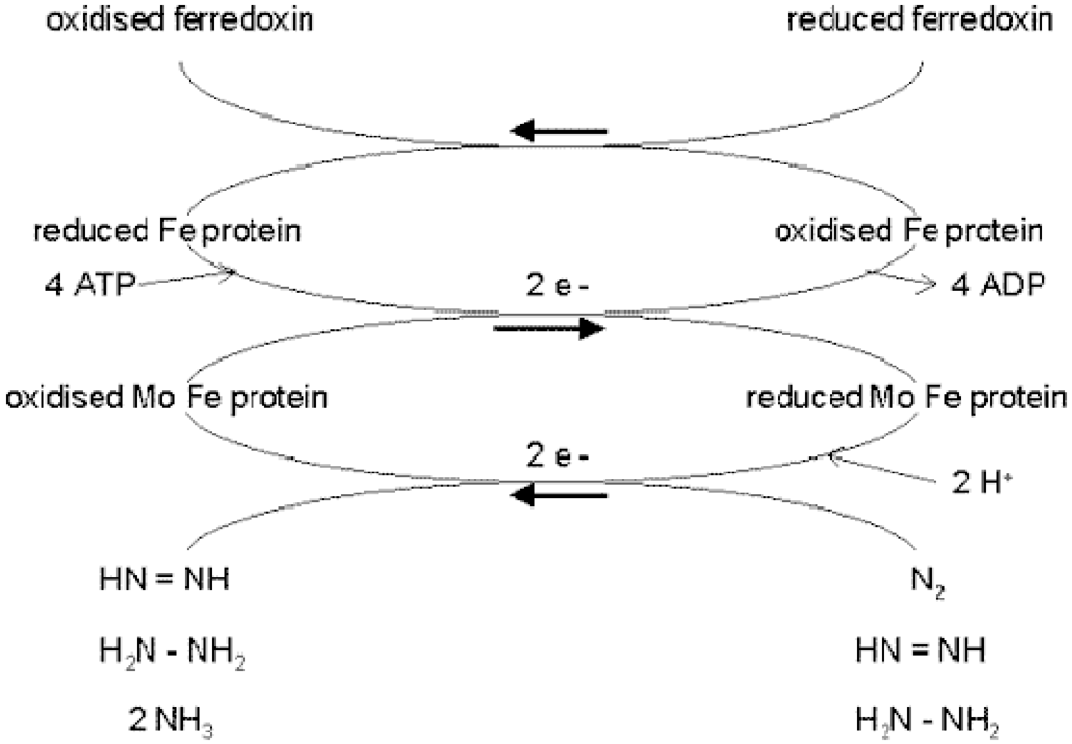


Fig.1. Biochemistry of Nitrogen reduction

3. 4. Ecological factors that affect BNF

The marked influence of environment on symbiotic nitrogen fixation has been known for a long time. The delicate balance between the host plant and the micro-symbiont is disturbed by adverse environmental conditions which have otherwise no effect on plant growth. Quite obviously, environmental factors affect the growth of both plants and bacteria. As plants must develop a rhizosphere to support rhizobial growth as well as build a morphologically developed nodule to house the invading rhizobia, we may assume that any factor that adversely affects plant growth will also profoundly affect competition for nodulation (Dowling and Broughton, 1986).

Some of the factors affecting biological nitrogen fixation are soil acidity, drought, salinity, high temperature and nutrient deficiencies (Giller, 2003; Hungria and Vergas, 2000). In the process of growth of legumes, nodulation is one of the most highly affected during the association between the Rhizobia and the host plant. Therefore, selection and breeding of legume crops tolerant to environmental stresses is very important to improve yield and productivity. In addition, selection of the micro-symbionts tolerant to the environmental stress and using them as inoculants will ensure the establishment of symbiosis in a positive way (Zahran, 1999).

High temperature, drought and soil acidity restrict legume root-nodule formation in the tropics (Hungria and Vergas, 2000). Photosyntate deprivation, salinity, soil nitrate, heavy metals, and biocides are also other typical environmental stresses faced by the growth of the host and their symbiotic partner (Walsh, 1995).

3.4.1. Soil Acidity

Legumes and their rhizobia exhibit varied responses to acidity. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within species (Brockwell *et al.,* 1995). The optimum pH for rhizobial growth is considered to be between 6 and 7 (Jordan, 1984), and relatively few rhizobia grow well at pH Less than 5. The fast growing strains of rhizobia have generally been considered less tolerant to acid pH than have slow growing strains of *Bradyrhizobium* (Graham *et al.,* 1994).

Although the basis for differences in pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is not clear (Correa and Barneix, 1997), differences in lipopolysaccharides composition, proton exclusion and extrusion accumulation of cellular polyamines, and synthesis of acid shock proteins (Zarhan, 1999), and composition and structure of outer membrane (Graham *et al*., 1994), have been implicated with pH tolerance of endosymbionts. Vlassak and Vanderleyden (1997) reported that, nodulation of legumes is reduced in acidic soil, mainly because of sensitivity of early nodulation events, such as attachment, root hair curling and initiation of infection thread formation. In addition, low pH can affect the production and excretion of nodulation factors in some strains of rhizobia. Lapinskas *et al*. (2005) showed that soil acidity was a decisive factor in the spread and symbiotic efficiency of *R. leguminosarum* bv.*viceae.*

Two strategies have been adopted to solve the problem of soil acidity. First selecting the optimal combination of rhizobial inoculums and the legume genotype for acidic soils where high H^+, Al, or Mn may limit the effectiveness of some rhizobia-host combination (Wood *et al.*, 1984; Rai, 1992) and, liming the acidic soil to improve the effects of acidic conditions (Rai, 1992). In this case liming has been considered the most efficient practice in overcoming soil acidity, with some of the benefits to legume crops not only due to increased soil pH, but also to increased availability of Ca^{2+} to plant, bacteria and the symbiosis (Hungria and Vargas, 2000).

3.4.2. Temperature stress

Rhizobia are mesophiles and most have a poor growth at temperature below 10°C or above 37 °C (Graham, 1992). Although responses to temperature is strain's dependent, rhizobia are found to tolerate between 4-42.4 °C. However, growth at 4°C is rare, and only *S. meliloti* can grow at 42.5 °C. *R. leguminosarum* isolates from lentil plants in Southern Nile Valley of Egypt were tolerant to 35 to 40°C inducing less effective symbiosis with their legume host (Moawad and Beck, 1991). For most rhizobia, the optimum temperature range for growth in culture is 28 °c to 31°C (Bordeleau and Prevost, 1994). Changes in temperature strongly affect bacterial infection and N_2 fixation in several legume species (Arayankoon *et al.*, 1990; Kishinevsky *et al.*, 1992). Nodulation and symbiotic nitrogen fixation depends on the nodulating strain in addition to plant cultivars (Arayankoon *et al.*, 1990). Elevated temperature may delay nodule initiation and development, and interfere with nodule structure and functioning in temperate legumes, whereas nitrogen fixation efficiency is mainly affected in tropical legumes. Furthermore, temperature changes affect the competitive ability of *Rhizobium* strains (Roughley, 1970; Bordeleau and Prevost, 1994). High soil temperature in tropical and subtropical areas is a major problem for biological nitrogen fixation of legume crops (Michiels *et al.*, 1994). Because high temperatures decrease rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rate of inoculation may frequently be required (Thies *et al.*, 1991).

3.4.3. Soil type in relation to Rhizobia

Soil is a reservoir of *Rhizobium* strains and the intrinsic makeup of the soil can affect the outcome of competition. The growth rate of *Rhizobium* in soil without plants is slow; a generation time of 200hr was estimated, as opposed to 12 hr in the rhizosphere of legumes. Similarly, no increase in size of *B. japonicum* inocula was observed in fallow soil, while a 100-fold increase was noted in the rhizosphere of soybeans (Dowling and Broughton, 1986).

3.4.4. Carbon and energy source

The reduction of N_2, whether accomplished chemically or biologically, requires a large amount of energy. The chemical process used to produce fertilizers utilizes vast amounts of fossil fuels as an energy source. These materials are non replaceable and, ultimately, exhaustible. BNF, however, obtains the required energy from the oxidation of carbohydrates which have been formed by the photosynthetic activity of green plants. The energy for photosynthesis comes from sunlight. The energy for BNF is therefore indirectly derived from a universally available and inexhaustible source. The direct source of energy (carbohydrate) for BNF is therefore potentially available wherever conditions permit the growth of photosynthetic organisms (http://edis.ifas.ufl.edu/SS180, 2009).

The carbon cycle generates energy in the form of ATP. The life process of nodulation is directly dependent upon photosynthesis (Kouchi et al., 1986). In leguminous plants as much as 25-40% of Photosyntate is exuded in the form of root exudates (Atkins, 2004). Part of the carbon is directly used to maintain nodule function while part of it is returned to the host plant in the form of carbon skeleton (Crews, 2004). If the bacteroids face lack of their carbon source, proper functioning of nodules becomes impaired.

3.4.5. Salt stress

Salt stress is one of the major environmental stresses adversely affecting legume production in arid and semi-arid regions (Bernstein and Ogata, 1966). High soil salinity can deleteriously affect symbiotic association between legume and *Rhizobium* by osmotic stress and ionic toxicity and imbalance (Sprent, 1972; Aparicio-Tejo and Sanchez-Diaz**,** 1982). Increased salt concentration affect the soil micro-biota by causing osmotic stress on them. Additionally saline soils are generally deficient in nutrients and microbial activities and population is low (Hussain *et al.,* 2002).

The legume-*Rhizobium* symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are rhizobia (Zahran, 1991; Graham, 1992). In contrast to their host legumes, some rhizobia can survive in the presence of extremely high levels of salt both in culture and in soil (Bordeleau and Prevost, 1994). Thus, organisms such as *S. meliloti* tolerate 200-300mM (milimole) NaCl, while nodulation and nitrogen fixation in their host can be inhibited at 50-100mM salt concentration (Serraj and Sinclair, 1998). Hussain *et al*. (2002) also indicated that, nodulation and nodule dry weight of *Trifolium* Spp. *alexandrium* inoculated by *R. trifolii* was depressed significantly with consistent increase in salinity.

The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of nodules and a reduction in cytosolic protein production specifically legheamoglobin (Delgado *et al*., 1994). The depressive effect of salt stress on N_2-fixation by legumes is directly related to the salt induced decline in dry weight and N_2 content in the shoot (Cordovilla *et al*., 1995).

Many species of bacteria adapted to saline conditions by intracellular accumulation of low molecular weight organic solutes; the osmolytes (Csonka and Hanson, 1991). The accumulation of osmolytes is thought to counteract the dehydration effect of low water activity in the medium but not to interfere with macromolecular structure or function. Rhizobia utilize this mechanism of osmotic stress adaptation (Smith *et al.*, 1994). Zahran *et al.* (1997) examined that the rhizobial cells responded to high salt stress by changing their cellular morphology.

Generally salinity is a serious threat to agriculture in arid and semi-arid regions (Rao and Sharma, 1995). Nearly 40% of the world's land surface can be categorized as having potential of salinity problems (Cordovilla *et al*., 1994). Most of these areas are confined to the tropics and Mediterranean regions. Successful *Rhizobium*-legume symbiosis under salt stress requires the isolation and development of salt tolerant rhizobial inoculant (Zahran, 1991). The selection of salt tolerant plant gene also plays an important role in confronting the salt stress when it is matched with the salt tolerant gene of *Rhizobium* (Cordovilla *et al*., 1995).

3.4.6. Water deficiency Stress

Shortage of water compromises plant and rhizobial growth, and is a major cause of nodulation failure and low N_2-fixation. The modification of rhizobial cells by water stress will eventually leads to a reduction in infection and nodulation of legumes (Zahran, 1999). Symbiotic nitrogen fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes and shrub legumes exhibited a reduction in nitrogen fixation when subjected to soil moisture deficit. This is due to the fact that water stress affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function (Guerin *et al.*, 1991).

In general, the wide range of moisture level characteristic of ecosystems where legumes have been shown to fix nitrogen suggested that rhizobial strains with different sensitivity to soil moisture can be selected. Studies have shown that sensitivity to moisture stress varies for a variety of rhizobial strains (Fuhrmann *et al.*, 1986). Thus, it can be assumed that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host. Optimization of soil moisture for growth of the host plant, which is generally more sensitive to moisture stress than bacteria, results in maximal development of fixed-nitrogen inputs into the soil system by the *Rhizobium*-legume symbiosis (Tate, 1995).

3. 5. Inoculation and their benefit

The symbiotic relationship between rhizobia and legumes is agriculturally important as the input of nitrogen through biological nitrogen fixation increases soil fertility, whilst decreasing the need for synthetic nitrogen fertilizers. Where effective strains of rhizobia are not present in the soil, they can be supplied by inoculation of the seed with selected and most effective strains. Inoculation of legume seed with root-nodule bacteria can result in a large benefit-cost ratio as the cost of inoculant is only about 1% of the total cost of input (Michelle Irene Lindeque, 2007).

It is necessary to apply commercial inoculants to nitrogen deficient fields especially when the indigenous rhizobial population is limited or has a poor nitrogen-fixing ability. Availability of suitable inoculant is often a limiting factor to successful cropping in many regions of the world.

The number of rhizobia added to the legume seed and the number of indigenous rhizobia capable of nodulating the host influences the inoculant success, as does the specificity of the host plant and environmental conditions (a dissertation by Michelle Irene Lindeque, 2007).

4. Materials and methods

The rhizobial isolation, identifications and sand pot experiments were carried out in the applied Microbiology Laboratory at the Department of Biology, Addis Ababa University.

4.1. Sampling sites

The selected sample sites of this study covered were in the major chickpea growing areas of South Wollo and North Wollo zones of the Amhara Region. In these areas chickpea has been growing for a long time without any history of inoculation with rhizobia. The root nodules were collected between October and November, 2002. Fig. 2 shows the sample sites of the study areas and the pH of the soil sample.

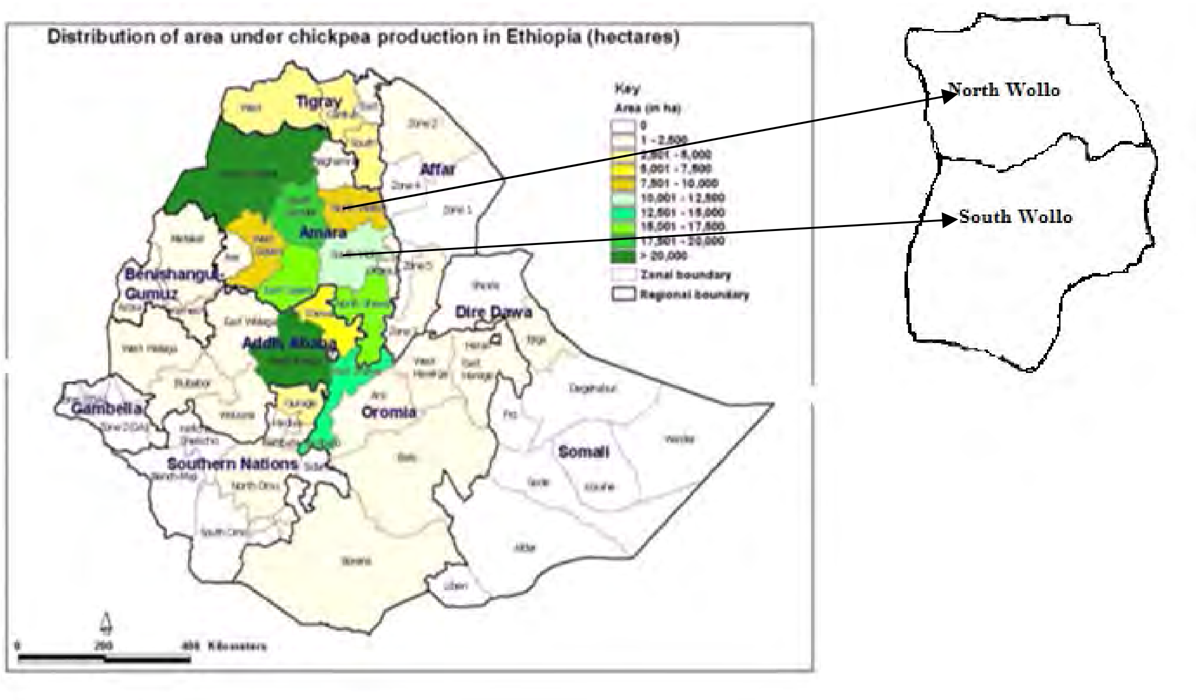


Fig.2. Map of Ethiopia showing sample sites of North and South Wollo zones

4.2. Sample Collection and isolation of rhizobia

4.2.1. Collection of Nodules

Nodule samples were randomly collected from the farmer's field and immediately kept in sealed vials containing a desiccant (Silica gel) covered with 1cm of cotton wool for isolation of rhizobia (Somasegaren and Hoben, 1994).

4.2.2. Isolation of root nodule bacteria

Dehydrated or desiccated root nodules were immersed in sterile distilled water overnight in labeled sets of flasks to imbibe water. The imbibed nodules were surface-sterilized with 70% ethanol for 10 seconds and then to 3% (v/v) solution of sodium hypochlorite ($NaHClO_3$) for 3 minutes according to Somasegaren and Hoben (1994). The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse off and remove the sterilizing chemicals.

The surface-sterilized nodules were then transferred to sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar flow hood. Stock solution of Congo red (CR) was prepared by dissolving 0. 25g of CR in 100ml sterile distilled water (Vincent, 1970) and then 10ml of the CR stock solution was added to one liter of YEMA before autoclaving. Finally, Loopful of crushed nodule suspensions were streaked on YEMA plates with CR and incubated at 28 ± 2^{0}C wrapped with aluminium foil to keep it in a dark condition for 3-7 days.

The YEMA-CR medium of volume 1000ml consists of the following components (g/l):

$MgSo_4.7H_2O$----------------------------------0.2g
NaCl --0.2g
K_2HP04--0.5g
KH_2po_4---0.5g
Yeast extracts ----------------------------------0.5g
Mannitol ---10g
Agar ---15g
Congored -------------------------------------10ml/l

Distilled water --------------------------------1000ml

(Somasegaren and Hoben, 1994).

4.3. Purification and preservation of isolates

Single dome-shaped colonies were picked with sterile inoculating loop and streaked on sterile YEMA plates and incubated at $28\underline{+}2^0$C. The purity and uniformity of colony types were carefully examined through repeated re-streaking and a single well isolated colony was picked and transferred to YEMA slant containing 0.3% (W/V) $CaCO_3$ in a culture tube and incubated at $28\underline{+}2^0$c. When sufficient growth was observed, the culture was transferred to be preserved at 4^0c for future use (Vincent, 1970).

4.4. Presumptive test of the isolates

Each isolate was examined for presumptive purity using Peptone Glucose Test (PGT), gram staining and growth response to YEMA-CR medium (Somasegaren and Hoben, 1994).

4.4.1. Congored absorption

Stock solution of Congo red was prepared by dissolving 0.25g of Congo red in 100ml of sterile distilled water. From stock solution, 10ml was added to a liter of YEMA and autoclaved. Loop full of test isolates were streaked on the medium and covered with aluminium foil to dark-incubate at $28\underline{+}2^0$C for 3 to 7 days to detect Congo red absorption by the colonies (Vicent, 1970).

4.4.2. Peptone glucose test

Peptone Glucose Test was prepared according to the procedure of Lupwayi and Haque (1994) by dissolving 5g of glucose, 10g of peptone, 15g of agar and 10ml of bromocresol purple (BCP) in a liter of distilled water and the pH was adjusted to 6.8 with 1N NaoH and HCl. Stock solution of BCP was prepared by dissolving 1g of BCP in 100ml of ethanol. Three days old yeast extract manitol broth culture containing approximate number of cells (10^4 cells ml^{-1}) was streaked on to the Peptone Glucose Medium to observe the growth after having incubated at 28±2^0C for 3 to 7 days (Lupway and Haque, 1994).

4.5. Authentication of the isolates and preliminary screening of Symbiotic effectiveness of isolates on sand pot experiment

In order to test the definitive purity of all rhizobial isolates, nodulation test was carried out for each of the purified isolates. They were inoculated into the host plant potted in to 3Kg capacity plastic pots containing sterilized and nitrogen free sand (Somasegeren and Hoben, 1994). The sand was thoroughly washed with 1N sulfuric acid whereas the pots were surface sterilized with 95% ethanol. Six seeds were sown in each pot and thinned down to three after germination. Each isolate was inoculated into Erlenmier flask for 3 days and 1ml of the culture suspension was inoculated into each seedling. The pots were arranged in a randomized design in a green house with 12h/12h photo periods at a maximum and minimum temperature of 20-25°C and 12-18°C respectively. The control plants were fertilized with 100ml of 0.05% KNO_2 once every week. The plants were irrigated with N-free medium nutrients four times in their life (Broughton and Dilworth, 1970).

4.6. Cultural characterization of isolates

4.6.1. Colony morphology

The morphological characteristics of the isolates were determined according to Lupwayi and Haque (1994). A loopful of 48 hrs old grown broth culture from each isolate was inoculated onto YEMA and incubated at 28±2^0C for 3-7days. After 7days, colony diameter, morphology and colony texture were recorded as indicated in Martinez-Romero *et al*., (1991).

4.6.2. Acid-base production

To determine the ability of the rhizobial isolates to produce acid or alkaline in the medium, YEMA containing bromothymol blue (BTB) (0.025 w/v) was used. A loop full of the isolates from a 48 hrs old culture broth was streaked on to the YEMA–BTB medium and incubated for 3-7 days so as to record the color changes of the medium (Jordan, 1984).

4.6.3. Determination of growth rate

Each isolate was inoculated into a 10ml YEM broth (YEMB), vortex-dispersed and shaken on orbital shaker at 125 rev. min^{-1} for 3 days. Then, 1ml of each broth culture (cell suspensions) was inoculated into 100ml sterilized YEM broth in 250ml erlenmeyer flask and kept on orbital shaker at 125 rev min^{-1}. Turbidity was measured by taking optical density (OD540nm) reading of the YEM broth cultures just at the time of inoculation (0hr) and every 6hrs interval by using spectrophotometer (UV-7804C, Ultraviolet Visible spectrophotometer) after calibrating it to zero with sterile uninoculated YEM broth as a blank. Isolates were immediately taken, serially diluted (10^{-1}–10^{-10}) with sterile distilled water). 0.1 ml sample from each solution was dispersed on to the sterilized YEMA plates and spreaded by using alcohol flamed spreader made out a glass rod to determine the colony forming units (CFU) (Somasegaren and Hoben, 1994). Mean generation (doubling) time was calculated from the logarithmic phase of either the optical density (OD) reading of spectrophotometer or viable count of colony forming units (c.f.u) (White, 1995).

4.7. Designation of isolates

All the isolates were designated as AAUCR (Addis Ababa University Chickpea Rhizobia) with different numbers representing each isolate.

4.8. Seed samples

The seed variety “D-Z-10-4” which is categorized under grey Kabuli type seed were provided from Ethiopian Institution of Agricultural Research (EIAR), Debrezeit Agricultural Research Centre.

4.9. Biochemical and physiological tests

For each biochemical and physiological test, inoculation of a loopful of 48hrs old broth culture was streaked on to the YEMA medium. The inoculated YEMA plates were incubated at 28 ± 2^0C for 3-5 days (Somasegaren and Hoben, 1994). For each experiment, three replicates and controls were used per test as indicated in Maatallah *et al*., (2002). Ultimately, the growth of each rhizobial isolate was determined as (+) for positive growth, (++) for abundant growth and (-) for no growth.

4.9.1. Phosphate solubilization test

All isolates were tested for their ability to solubilize tri-calcium phosphate according to Lupway and Haque (1994). The Basal Sperber agar medium that is used to test the ability of isolates to solubilize tri-calcium phosphate was prepared using the following ingredients.

Yeast extract---------------------------10g/l
$CaCl_2$ ----------------------------------0.1g/l
$MgSo_4.7H_2O$--------------------------0.25g/l
$Ca_3(Po_4)_2$ -----------------------------2.5g/l
Glucose--------------------------------10g/l
Agar------------------------------------10g/l

After mixing these components, the Basal Sperber agar medium was autoclaved at 121°C for 15 minutes and 48hr old culture broth was streaked on to the Basal Sperber agar medium and incubated at 28± 2°C to measure the diameter of halo zone on each culture medium.

4.9.2. Amino acid utilization

Different types of amino acids including L-arginine, L-glutamate, L-leucine, L-phenylalanine, L-tryptophane, urea and L-tyrosine were used in this experiment in order to determine the ability of the isolates to utilize the amino acids as a nitrogen source. These amino acids were added at a concentration of 0.5g/l to a basal media source that lack ammonium sulfate and supplemented with 1g/l of mannitol. The membrane filter sterilized amino acids were added to the autoclaved and cooled (approximately 55^0C) basal media as indicated in Amargar *et al.* (1997). Finally 48hr old rhizobial suspensions were inoculated in to these basal media and incubated at 28±2 °C for 3-5 days.

4.9.3. pH tolerance

The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating each isolate on YEMA adjusted at a pH of 4.0, 4.5, 5.0, 8.0, 8.5, 9.0, 9.5 and 10.0, using 1N NaOH and HCl as described by Bernal and Graham (2001).

4.9.4. Salt tolerance

The ability of the isolates to grow at different level of salt concentrations was determined by inoculating each isolate on the YEMA media containing 1%, 4%, 5%, 6%, 7%, 8%, 9% and 10% of NaCl as indicated in Lupwayi and Haque, (1994).

4.9.5. Temperature tolerance

The growth of each isolate at different incubation temperatures was evaluated by inoculating each isolate on YEMA plates. The inoculated plates were incubated at a temperature of 4^0C, 10 ^{0}C, 15^0C, 20^0C, 25^0C, 30^0C, 35^0C, 40^0C, 45^0C and 48^0C as indicated in Lupwayi and Haque, (1994).

4.9.6. Intrinsic antibiotic resistance

The resistance of isolates to different antibiotics at different concentration was evaluated by streaking each isolate on YEMA containing freshly prepared filter sterilized antibiotics using 0.22μm sized membrane filters. The stock solution of each antibiotic was first prepared as described in Lupwayi and Haque (1994) and was kept in refrigerator until they were used in the test. The antibiotics were Tetracycline, Erythromycin, Ampicillin, Chloroamphinicol, and penicillin. Each antibiotic was tested at the following concentrations. Ampicilin at 10μg/ml, Chloroamphinicol at 2.5μg/ml and 5μg/ml, Tetracyclin at 2.5μg/ml, Erythromycin at (2.5 μg/ml, 5μg/ml and 10μg/ml) and penicillin at (2.5 μg/ml, 5μg/ml and10μg/ml). Erythromycin was dissolved in ethanol, whereas the other four were dissolved in sterilized water. The stock solution of each antibiotic was prepared by dissolving 2g of each antibiotic in 100ml of water. The required concentration was aseptically added to the media using a single pipette for each antibiotic. The stock solution of each antibiotic was filter sterilized using a milipore filter (0.22μm) and aseptically added to autoclaved YEMA (kept at 50^0C in water bath) at the final concentrations of 2.5, 5 and 10 μg/ml, which is 12.5, 25 and 50μl of antibiotic solution per 100 ml medium, respectively, and finally poured separately in to plates.

4.10. Relative effectiveness of the isolates

After sixty days of planting upon re-inoculation, the plants were uprooted to measure nodule number, nodule dry weight and shoot dry weight. The effectiveness of isolates in accumulating plant shoot dry matter was calculated as described in Somasegaren and Hoben (1984) and Molungoy (2004) as follows:

$$SE = \frac{\text{Inoculated plant D.M. X 100}}{\text{N-Fertilized plant D.M.}},$$

Where, D.M. = dry matter, S.E. = symbiotic effectiveness

The rate of nitrogen fixing effectiveness is evaluated as: Highly effective > 80%, Effective 50-80%, Lowly effective 35-50% and Ineffective <35%.

4.11. Data Analysis

Symbiotic effectiveness of the strains was measured in terms of the number of nodules, shoot dry weight and nodule dry weight from greenhouse trial. Phenotypic variability was analyzed using a computer cluster analysis applying the unweighed pair group method with the average (UPGMA) by PCORD statistical software ver. 5.0 of hierarchical clustering method (Maatallah *et al.*, 2002). One-way analysis of variance of data was also undertaken using the SPSS statistical program ver.15.0. Mean separation was calculated using the Turkey's values when the F-test was significant at P=0.05.

5. Result

5.1. Isolation and authentication of rhizobia

A total of 38 chickpea nodule samples were collected from different sites of South and North Wollo, from which 21 isolates from South Wollo and 6 isolates from North Wollo were isolated and characterized. Ten of them were rejected in the presumptive test and one is discarded upon authentication. All isolates did not grow on PGA and absorb congored on YEMA-CR media. All of them changed YEMA-BTB medium into yellow color (Table-1). All but one isolate, formed nodules and authenticated as root nodule bacteria after they were re-inoculated into the host plant.

5. 2. Characterization of the isolates

5. 2. 1. Morphological and growth characteristics of isolates

Isolates were grown on YEMA medium to determine colony type, colony diameter and colony texture. With regard to colony texture 52% showed large watery colonies (LW) with exo-polysaccharide production and 48% of the isolates were characterized as large mucoid (LM) texture on YEMA media (Table-1). The colony diameter of all the isolates ranged between 2 and 6mm. The largest colony diameter of 6mm was observed on isolates AAUCR 4 (North Wollo) and AAUCR 28 (South Wollo), whereas the smallest diameter of 2mm was recorded for isolates AAUCR 12, AAUCR 17, AAUCR 22 (south Wollo) and AAUCR 30 (North Wollo zone).

All isolates displayed generation times between 2-4hrs except, AAUCR 15 that took 6.2hr to double its population (Table-1). Isolates AAUCR 2, AAUCR 7 from South Wollo and AAUCR 33 from North Wollo showed the fastest doubling time of 1.8hr (Table-1).

Table-1. Colony morphology and mean generation time of isolates

No̲	**Isolates**	**Sample sites**	**Collection Zones**	**Colony diameter (mm)**	**Colony Morphology**	**Mean generation time (MGT)**	**Growth on BTB**
1	AAUCR 1	Sulula	South Wollo	4	L.W.	2.0	Yellow
2	AAUCR 2	Hitecha	South Wollo	3.5	L.W.	1.8	»
3	AAUCR 3	Langa	North Wollo	5	L. M.	1.9	»
4	AAUCR 4	Diobulko	North Wollo	6	L. M.	2.0	»
5	AAUCR 6	Debreselam	South Wollo	5.5	L.W.	2.1	»
6	AAUCR 7	Tita	South Wollo	5	L.W.	1.8	»
7	AAUCR 8	Buke	South Wollo	2.5	L. M.	2.6	»
8	AAUCR 9	Haratehuledere	South Wollo	2.5	L. M.	3.2	»
9	AAUCR 10	Woldelulo	South Wollo	2.5	L.W.	3.5	»
10	AAUCR 12	Qulqualo	South Wollo	2	L.W.	2.5	»
11	AAUCR 13	Wulawula	South Wollo	5	L.W.	3.6	»
12	AAUCR 14	Haraworebabo	South Wollo	2.5	L. M.	3.0	»
13	AAUCR 15	Gobeya	South Wollo	2.5	L.W.	6.2	»
14	AAUCR 16	Amumo	South Wollo	3.5	L. M.	3.5	»
15	AAUCR 17	Goha	South Wollo	2	L.W.	3.4	»
16	AAUCR 18	Wowa	South Wollo	3	L. M.	2.9	»
17	AAUCR 20	Golbo	South Wollo	2.5	L. M.	4.0	»
18	AAUCR 21	Enchini	South Wollo	3	L.W.	3.4	»
19	AAUCR 22	Serdem	South Wollo	2	L.W.	3.6	»
20	AAUCR 23	Korkie	South Wollo	5	L. M.	2.3	»
21	AAUCR 24	Wurgessa	South Wollo	3.5	L. M.	3.7	»
22	AAUCR 25	Lalibela	North Wollo	3	L. M.	4.0	»
23	AAUCR 26	Merssa	North Wollo	3	L.W.	3.5	»
24	AAUCR 27	Harbu	South Wollo	4	L.W.	2.4	»
25	AAUCR 30	Sirinka	North Wollo	2	L. M.	3.6	»
26	AAUCR 31	Chefa	South Wollo	5	L.W.	3.8	»
27	AAUCR 33	Tekeze	North Wollo	3.5	L. M.	1.8	»

LM, large watery; LW, large watery

5. 3. Physiological tests

All isolates were characterized with the following physiological and biochemical tests; phosphate solubilization and amino acid utilization, eco-physiological characters (pH, salt and temperature tolerance) and intrinsic antibiotic resistance. All isolates did not solubilize tri-calcium phosphate on basal Sperber agar medium. Isolates were found to utilize different amino acid substrates as sources of nitrogen (fig. 3). Most of the isolates (93%) were able to utilize phenylalanine except, AAUCR 18 and AAUCR 21 from South Wollo followed by 78%

of the isolates that were capable of utilizing tryptophan as a nitrogen source. There was no isolate that showed growth on urea. Isolates AAUCR 2, AAUCR 13, AAUCR 27 and AAUCR 15 from South Wollo, and AAUCR 3 from North Wollo utilized large number of nitrogen sources (86% of the tested nitrogen source), whereas isolates AAUCR 1, AAUCR 18, AAUCR 21 and AAUCR 22 from South Wollo, and AAUCR 30 and AAUCR 33 from North Wollo were found to utilize the least number of amino acid (Glutamic acid, phenylalanine, glycine and tryptophan) (Annex-4).

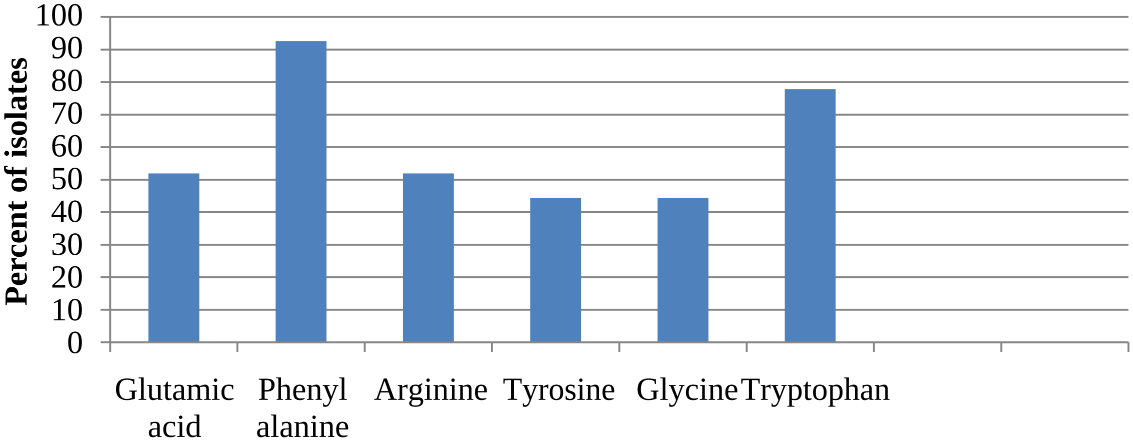


Fig.3. Pattern of amino acid utilization by different chickpea rhizobial isolates

5.4. pH tolerance

All isolates tolerated pH levels of 6-8.5 (Fig. 4), and 59% of the isolates were found to be tolerant to pH 4.5. The other 41% of the isolates grew at a pH 10. Isolates AAUCR 1, AAUCR 10 and AAUCR 14 from South Wollo, and AAUCR 3 and AAUCR 4 from North Wollo, showed growth on all tested pH levels 4.5-10 (Annex-1).

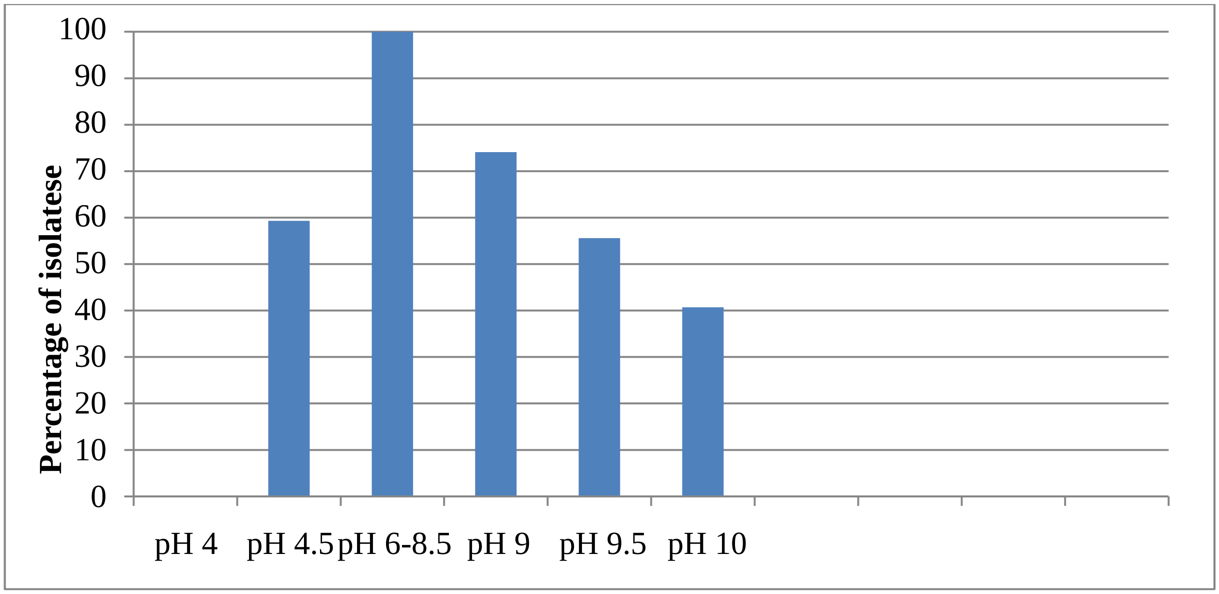


Fig.4. pH tolerance of rhizobial isolates from North and South Wollo

5. 5. Salt tolerance

Isolates displayed differences in growth on YEMA medium adjusted at different NaCl concentrations (fig. 5). All isolates were tolerant to salt concentration of 2%, but showed a steady decrease in growth when they were inoculated into the medium containing 4 to 8% salt concentration. Consequently, 85% of the isolates grew at 4% NaCl, whereas 33% of isolates were resistant to salt concentration of 7%. Isolates AAUCR 9 and AAUCR 14 (South Wollo) were found to be the most tolerant strains that grew at Salt concentration of 8%. The most sensitive isolates were AAUCR 6 and AAUCR 7 (South Wollo), and AAUCR 3 and AAUCR 25 (North Wollo); that were able to grow at salt concentration of only 2% (Annex-2).

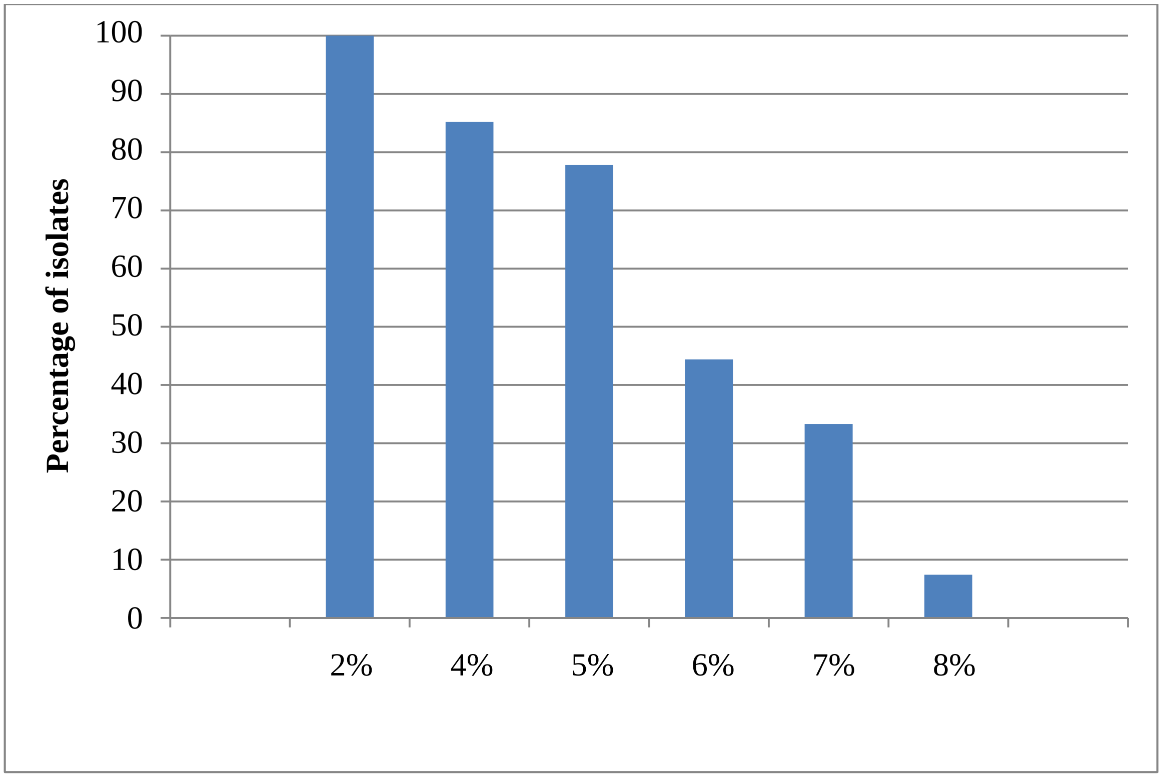


Fig.5. Tolerance of rhizobial isolates to different salt concentrations

5.6. Temperature tolerance

The ability of isolates to grow at different temperature levels is shown in (Fig. 6). All isolates were able to grow within temperature range of 15 to 30°c. Isolates AAUCR 1, AAUCR 9, AAUCR 10, AAUCR 17, AAUCR 21 and AAUCR 22 (south Wollo) and AAUCR 25 and AAUCR 30 (North Wollo) were grown at the lowest temperature of 4°C. There was a progressive decrease in growth of isolates from a temperature range of 35°C to 40°C. Only isolates AAUCR 9 and AAUCR 10 (South Wollo) were grown at all tested incubation temperature (4-40 °C) (Annex-3).

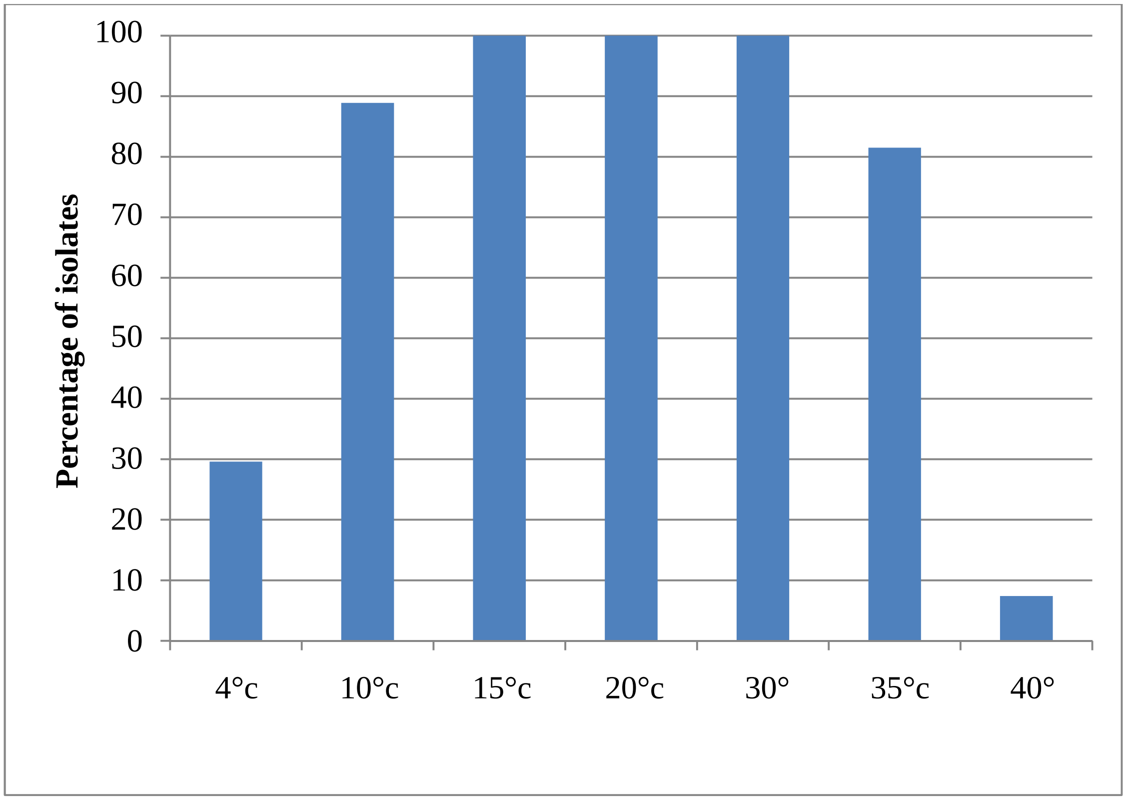


Fig.6. Temperature tolerance of isolates from North and South Wollo

5.7. Intrinsic antibiotic resistance

Most of the isolates (89%) were found to be resistant to erythromycin at a concentration of 2.5mg/ml (Table-2). The isolates, in general, were sensitive to tetracycline, except isolate AAUCR 2, AAUCR 8 and AAUCR 21 that were able to grow on the media containing 2.5mg/ml of the antibiotics. Isolate AAUCR 3 (North Wollo) was found to be the most resistant of the isolates to different antibiotics followed by isolate AAUCR 18, AAUCR 20, AAUCR 8, AAUCR 22 and AAUCR 24 (South Wollo) (Table-2). The most sensitive isolate was AAUCR 6; did not grow on YEMA medium containing any antibiotic, followed by isolates AAUCR 1, AAUCR 15 and AAUCR 31 (South Wollo) and AAUCR 33 (North Wollo) that were able to grow on the media containing one or two antibiotics (Table-2).

Table.2. Tolerance of isolates to various antibiotics in different concentrations (µg/ml)

Isolate	Ampicilin	Chloroam phinicol		Tetracycline	Erythromycin			Penicillin		
	10	2.5	5	2.5	2.5	5	10	2.5	5	10
AAUCR 1	-	-	-	-	+	+	+	-	-	-
AAUCR 2	-	-	-	+	+	+	+	+	+	-
AAUCR 3	+	+	+	-	+	+	+	+	+	+
AAUCR 4	+	+	-	-	+	+	+	-	-	-
AAUCR 6	-	-	-	-	-	-	-	-	-	-
AAUCR 7	-	_	-	-	+	-	-	+	+	+
AAUCR 8	-	+	+	+	+	+	-	+	+	-
AAUCR 9	+	+	+	-	+	+	+	-	-	-
AAUCR10	-	-	-	-	+	+	-	+	+	-
AAUCR12	+	-	-	-	+	+	-	+	-	-
AAUCR13	+	+	-	-	+	+	+	-	-	-
AAUCR14	+	+	+	-	+	+	-	-	-	-
AAUCR15	+	+	+	-	-	-	-	-	-	-
AAUCR16	+	-	-	-	+	+	+	-	-	-
AAUCR17	+	-	-	-	+	-	-	+	+	-
AAUCR18	+	+	+	-	+	+	-	+	+	+
AAUCR20	+	+	+	-	+	+	+	+	+	-
AAUCR21	+	-	-	+	+	+	-	+	+	-
AAUCR22	+	+	-	-	+	+	+	+	+	-
AAUCR23	+	-	-	-	+	+	+	-	-	-
AAUCR24	+	+	+	-	+	+	+	+	-	-
AAUCR25	+	-	-	-	+	+	+	-	-	-
AAUCR26	+	+	+	-	+	+	-	-	-	-
AAUCR27	-	-	-	-	+	+	+	+	+	-
AAUCR 30	+	+	+	-	+	+	-	+	-	-
AAUCR 31	+	-	-	-	+	+	-	-	-	-
AAUCR33	-	+	-	-	-	-	-	+	+	-
No of isolates	19	14	10	3	24	22	13	15	12	3
%	70	52	37	11	89	81	48	56	44	11

(+) growth and (-) no growth

5.8. Numerical analysis

The twenty seven isolates were clustered in to 4 groups based on 46 phenotypic features used in physiological test. Cluster I consists of 4 isolates that includes AAUCR 1, AAUCR 9, AAUCR 30, and AAUCR 14 which forms two sub-clusters at around 80% similarity coefficient. Cluster II comprised of 6 isolates which included AAUCR 6, AAUCR 7, AAUCR 33, AAUCR 12, AAUCR 23 and AAUCR 27. Group three contained the largest cluster of 13 isolates which were grouped into two sub-clusters at 40% similarity coefficient. Cluster four comprised of four isolates (Fig. 7).

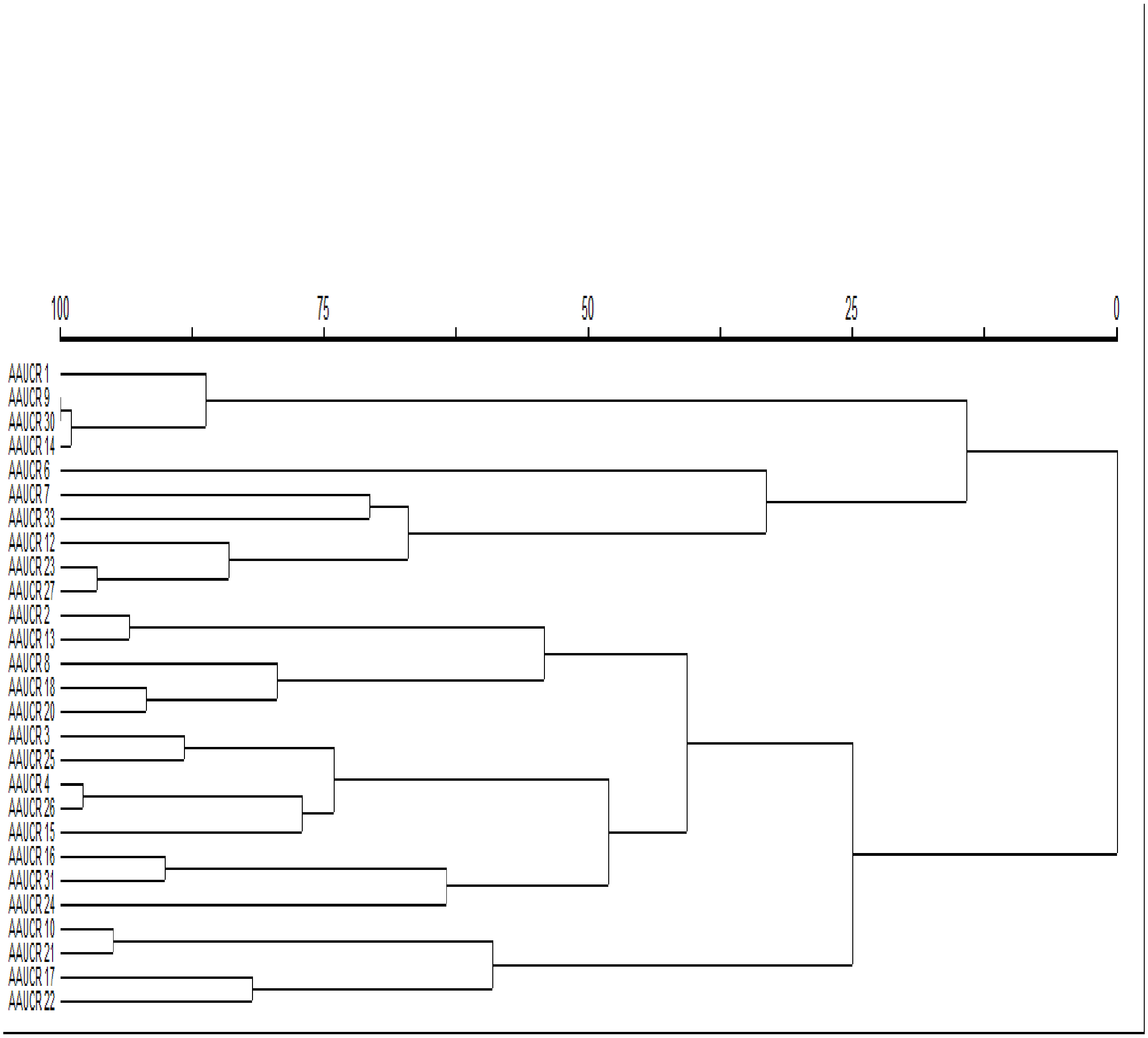


Fig.7. Dendrogram highlighting the phenotypic diversity of chickpea rhizobial isolates

5.9. Symbiotic effectiveness test on sand culture

The sand pot culture study on the symbiotic effectiveness showed variations among the inoculated plants. Plants that were inoculated with effective rhizobia showed green colored leaves, long and branched shoots and pink nodules as compared to the plant inoculated with the least effective isolates and the uninoculated control.

The inoculated plants showed differences in nodule number (Table-3). Plants inoculated with isolate AAUCR 10 showed the highest nodule number (61 nodules per plant), whereas the least number of nodules (2 nodules per plant) was recorded from the plants treated with isolates AAUCR 3 (North Wollo), AAUCR 6 and AAUCR 7 (South Wollo). Similarly, the highest nodule dry weight of 76mg/plant was recorded from plants inoculated with isolate AAUCR 10 (South Wollo), whereas the lowest nodule dry weight of 10mg/plant was shown by the host inoculated with isolate AAUCR 3, AAUCR 4 and AAUCR 6 (Table-3).

Likewise, the highest shoot dry matter accumulation was recorded from plants inoculated with isolate AAUCR 9 (South Wollo) followed by plants treated with isolate AAUCR 27 and AAUCR 21 with shoot dry matter accumulation of 526mg/plant, 460mg/plant and 410mg/plant respectively (Table-3). The least dry mass was recorded from plants inoculated with isolate AAUCR 6 (South Wollo) that yielded 196mg/plant (Table-3).

The relative dry matter accumulation of plants in relation to the nitrogen fertilized positive control, 7% of isolates were found to be highly effective (80 - 100% effectiveness), 67% of the isolates were effective (50-80% effectiveness), while 22% and 4% of the isolates were also found to be lowly effective and ineffective with effectiveness rate of 35-50% and less than 35% effectiveness respectively in relative to the dry mass accumulated by the positive control. In general, 74% of the isolates were effective and very effective whereas 26% were lowly effective and ineffective strains.

Table-3. Nodulation data of collected isolates of chick pea rhizobia

Isolates	**Nodule number per plant**	**Nodule dry Wt. per plant(Mg)**	**Shoot dry Wt. per plant (Mg)**	**% SE**	**Rate**
AAUCR 1	4± 0.58^{f-g}	26± 0.01^{d-g}	285± 0.01^{d-g}	50	E
AAUCR 2	17± 3.18^{c-g}	46± 0.01^{a-g}	256± 0.01^{d-g}	45	LE
AAUCR 3	2± 0.00^{g}	10± 0.00^{g}	250± 0.01^{d-g}	44	LE
AAUCR 4	4± 1.73^{f-g}	10± 0.00^{g}	297± 0.02^{d-g}	52	E
AAUCR 6	2± 0.33^{g}	10± 0.00^{g}	196± 0.01^{fg}	34	IE
AAUCR 7	2± 0.58^{g}	16± 0.03^{fg}	240± 0.01^{d-g}	42	LE
AAUCR 8	48± 4.33^{a-c}	60± 0.01^{a-d}	306± 0.02^{c-g}	54	E
AAUCR 9	56± 5.49^{ab}	70± 0.01^{ab}	526± 0.02^{ab}	92	HE
AAUCR 10	61± 1.45^{a}	76± 0.03^{a}	386± 0.04^{b-e}	68	E
AAUCR 12	38± 1.45^{a-e}	66± 0.33^{a-c}	380± 0.04^{b-e}	67	E
AAUCR 13	4± 1.73^{f-g}	36± 0.01^{b-g}	346± 0.08^{c-g}	61	E
AAUCR 14	32± 2.89^{a-g}	56± 0.03^{a-e}	350± 0.02^{b-f}	61	E
AAUCR 15	55± 4.04^{ab}	30± 0.01^{c-g}	356± 0.05^{b-f}	62	E
AAUCR 16	9± 2.03^{e-g}	20± 0.01^{e-g}	290± 0.01^{d-g}	51	E
AAUCR 17	35± 14.72^{a-f}	46± 0.01^{a-g}	310± 0.08^{c-g}	54	E
AAUCR 18	25± 6.35^{b-g}	30± 0.01^{c-g}	297± 0.02^{d-g}	52	E
AAUCR 20	42± 1.45^{a-d}	56± 0.01^{a-e}	266± 0.03^{d-g}	46	LE
AAUCR 21	39± 4.91^{a-e}	60± 0.01^{a-d}	410± 0.05^{a-d}	72	E
AAUCR 22	53± 7.50^{ab}	66± 0.01^{a-c}	360± 0.03^{b-f}	63	E
AAUCR 23	8± 0.58^{e-g}	36± 0.01^{b-g}	230± 0.03^{e-g}	40	LE
AAUCR 24	21± 6.35^{c-g}	50± 0.00^{a-f}	326± 0.02^{c-g}	57	E
AAUCR 25	32± 7.32^{a-g}	60± 0.02^{a-d}	350± 0.02^{b-f}	61	E
AAUCR 26	39± 2.03^{a-e}	56± 0.02^{a-e}	296± 0.02^{c-g}	52	E
AAUCR 27	31± 5.49^{a-g}	40± 0.01^{a-g}	460± 0.01^{a-c}	81	HE
AAUCR 30	6± 0.58^{fg}	66± 0.01^{a-c}	295± 0.01^{d-g}	52	E
AAUCR 31	16± 0.35^{d-g}	40 ± 0.01^{a-g}	295± 0.01^{d-g}	52	E
AAUCR 33	19± 2.31^{c-g}	40± 0.01^{a-g}	250± 0.04^{d-g}	44	LE
+ve cont.	0	0	570± 0.01^{a}	100	
-ve cont.	0	0	170± 0.05^{g}	30	

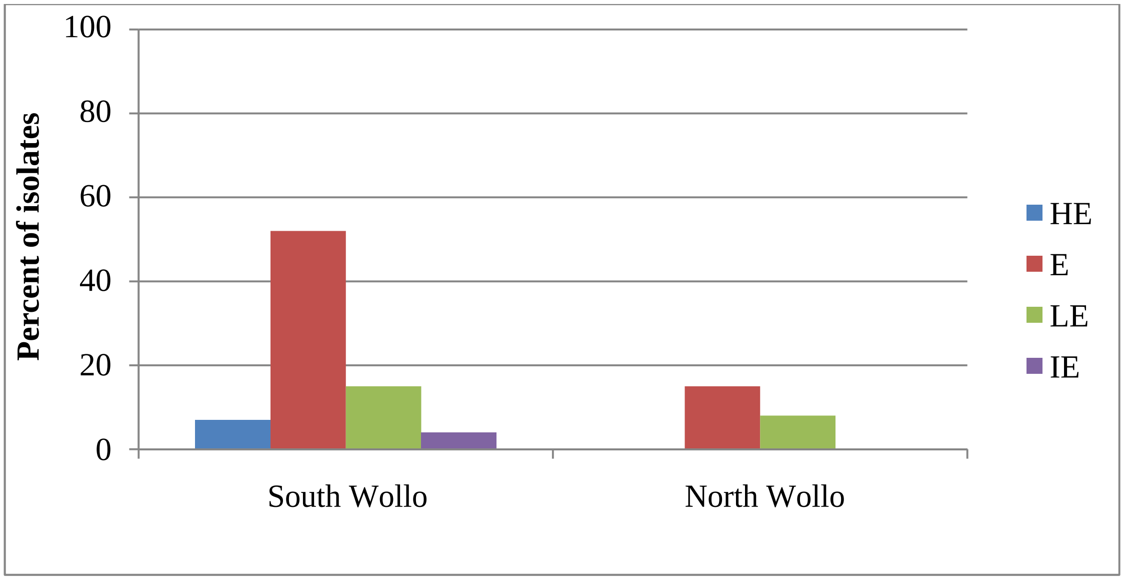
∗Means in the same column followed by the same letters are not significantly different at $p<0.05$ (Tukey's HSD test).

HE, highly Effective, E, Effective, LE, Lowly Effective, IE, Ineffective

There was a correlation between nodule number, nodule dry weight and shoot dry weight of inoculated plants. Plants with the lowest nodule number and nodule dry weight displayed lowest shoot dry weight. Plants inoculated with isolate AAUCR 3, AAUCR 6 and AAUCR 7 with 2 nodules/plant each, had nodule dry weight of 10, 10 and 16mg/plant respectively. These plants had shoot dry weight of 250, 196 and 240mg/plant respectively (Table-3). The highest nodule number recorded was 61 and 56 nodules/plant for isolate AAUCR 10 and AAUCR 9 and their nodule dry mass was 76 and 70 mg/plant respectively. These plants had also shoot dry mass of 386 and 526mg/plant respectively (Table-3).

With respect to the site of collection, 8% from South Wollo and 67% of the isolates from South and North Wollo were found to be highly effective and effective, respectively (fig. 8). Likewise, 22% of the isolates from North and South Wollo were also found to be lowly effective and one isolate from South Wollo was ineffective (Fig. 8).

Fig.8. Comparison of rate of effectiveness of isolates by sample site



HE, highly effective E, effective   LE, lowly effective IE, ineffective

In order to screen for a very competitive future inoculants against the backdrop of severe competitions for niche and habitat in the soil ecosystem, the effective and highly effective isolates were compared with another with regard to their tolerance to different environmental factors (Table-4)

Table-4. Summary of Eco-physiological and symbiotic properties of highly effective and effective chickpea isolates.

Isolates	Sites	Colony texture	Amino acid	T (°C)	Salt (%)	pH	IAR	RE	Rate
AAUCR1	Sulula	LM	2	4-35	6	8	3	50	E
AAUCR4	Diobulko	LW	4	10-35	5	8	5	52	E
AAUCR8	Bukie	LM	3	20-30	7	7	9	54	E
AAUCR9	Woldelulo	LM	4	4-40	6	6	8	92	HE
AAUCR10	Qulqualo	LW	2	4-40	4	8	6	68	E
AAUCR12	Haraworebabo	LW	4	10-35	4	6	6	67	E
AAUCR13	Gobeya	LW	6	10-35	6	4	7	61	E
AAUCR14	Amumo	LM	3	10-35	7	8	7	61	E
AAUCR15	Goha	LW	6	10-35	4	7	5	62	E
AAUCR16	Wowa	LM	4	10-35	4	6	4	51	E
AAUCR17	Golbo	LW	4	4-35	5	4	6	54	E
AAUCR18	Enchini	LM	2	10-35	6	5	10	52	E
AAUCR21	Enchini	LW	1	4-30	4	6	8	72	E
AAUCR22	Serdem	LW	1	4-35	4	5	9	63	E
AAUCR24	Wurgessa	LM	3	10-30	3	5	9	57	E
AAUCR25	Lalibela	LM	3	4-35	2	7	6	61	E
AAUCR26	Mersa	LW	3	10-35	6	5	7	52	E
AAUCR27	Harbu	LW	6	20-35	4	4	7	81	HE
AAUCR30	Sirinka	LM	2	4-35	6	6	6	52	E
AAUCR31	Chefa	LW	4	4-30	4	4	3	52	E

The isolates, in general showed differences to different parameters. In most of the tests, the highly effective AAURC 9 (South Wollo) showed a wide range of tolerance in almost all phenotypic characters range (intrinsic antibiotic resistance, salt tolerance, temperature tolerance). The other highly effective isolate, AAUCR 27 also showed a strong resistance to antibiotics, wide range of amino acid utilization, with mild tolerance to temperature and pH compared to the other isolates. Isolates AAUCR14, AAUCR10, AAUCR8, AAUCR13, AAUCR15 and AAUCR18 were among the effective isolates that displayed a wide tolerance to different environmental variables. These isolates were also among the rhizobia that induced the highest shoot dry matter accumulation (60-70%) compared to the N-fertilized control.

6. Discussion

A total of 27 isolates were identified as root nodule bacteria of chickpea based on several presumptive and authentication tests (Vicent, 1970; Lupway and Haque, 1994). One isolate failed to form nodules on the host upon re-inoculation. However, failure in nodulation in this work was not as pronounced as that of the chickpea rhizobia isolated from other parts of the country where 47% of the isolates did not form nodule upon re-inoculation on the host plant (Daniel Muleta, 2009).

Based on colony size, texture and confluent growth rate with gum production, the isolates were categorized as fast growing rhizobia. They also changed the YEMA-BTB medium to yellow color indicating that the isolates were acid producing root nodule bacteria. These are general characteristics of fast growing rhizobia according to Jordan (1984). This result is also similar to the cultural characteristics of chickpea rhizobia isolated from Morocco by Matallah *et al* (2002). Several works on cool season legumes in Ethiopia (Zerihun Belay, 2006; Fekadu Shimekit, 2006; Aregu Amsalu, 2007) and chickpea (Daniel Muletta, 2009) also showed that fast growing root nodule bacteria are the dominant endosymbionts of important agricultural legumes of the highlands of Ethiopia.

Although the majority of the cool season agricultural legumes are categorized into the cross-inoculation group of *Rhizobium leguminosarum* var. *viceae* (Vincent, 1970; Jordan, 1984), chick pea rhizobia are separately grouped into distinct groups of fast growing bacteria known as *Mesorhizobium ciceri*, and *Mesorhizobium mediteraneum* (Stephen *et al*., 2002)

Most of the isolates in the current work utilized phenylalanine and tryptophan. None of the isolates catabolized urea as a nitrogen source (Annex-4). Isolates AAUCR 3, AAUCR 2, AAUCR 13, AAUCR 15 and AAUCR 27 (South Wollo) utilized wide range of amino acids (Annex-4). This is similar to the findings of Daniel Muleta (2009) on chickpea rhizobia isolated from different parts of Ethiopia. However, the pattern of amino acid utilization was different from that of Kucuk and Kivank (2008) who showed that glycine was the most readily utilizable nitrogen source by all isolates of chickpea rhizobia.

Isolates showed great diversity of growth at different pH (Annex-1). All isolates grew at pH level of 6-8.5 and 41%-74% of the isolates grew within pH of 9-10. The most tolerant isolates grew at all tested pH (4.5-10) were; AAUCR 1, AAUCR 10 and AAUCR 14 (South Wollo), AAUCR 3, AAUCR 4 (North Wollo). This is in contrary to the reports of Kucuk and Kivanc (2008) that showed chickpea rhizobia grew on YEMA medium with pH levels of 5 and 8. It was also interesting to note that 59% of the isolates from South and North Wollo tolerated wide range of pH 4.5-9.5 as opposed to the report of Nour *et al.* (1984) who reported that chickpea rhizobia showed a neutral and baso-tolerant tendency; and Maatallah *et al*. (2002) reported that 90% to 100% of the isolates from Morocco grew on medium with lightly acidic (pH 4.5-6) and neutral pH.

All isolates tolerated salt concentration of 1-2% NaCl whereas some isolates grew on YEMA medium containing up to 8% (Annex-2). Isolates like AAUCR 8 and AAUCR 14 (South Wollo) were the most tolerant to all salt concentrations. The pattern is similar to salt tolerance by chickpea rhizobia from Morocco (Maatallah *et al*., 2002) and higher than the range (0.5-1% NaCl tolerance) shown by chickpea rhizobia from Ethiopia (Daniel Muleta, 2009).

Isolates showed great diversity in temperature tolerance. In this study, all isolates were tolerant to temperature ranging from 20°C to 30°C. This is similar to the report of Maatallah *et al.,* (2002) from Morocco and Kucuk and Kivanc (2008) from Turkey. In this study, however, many isolates (more than 80%) were tolerant to 35^{o}C, and a few isolates (8%) were grown at the incubation temperature of 40°C. Isolate AAUCR 6 and AAUCR7 (South Wollo) grew within a wider range of temperature (4-40°C).

The evaluation of antibiotic resistance of chickpea rhizobia showed that all isolates were tolerant to ampicilin at a concentration of $5\mu gml^{-1}$ (Table-2). Most isolates (89 and 82%) were resistant to erythromycin at a concentration of $2.5\mu gml^{-1}$ and $5\mu gml^{-1}$ and Chloroamphinicol ($2.5\ \mu gml^{-1}$ and $5\mu gml^{-1}$), respectively. Many of the isolates were most sensitive to tetracycline at all concentrations ($2.5\mu gml^{-1}$) and penicillin at a concentration of $10\mu gml^{-1}$. The most tolerant isolate to all the antibiotics tested at all concentrations were AAUCR 3 (North Wollo) followed by AAUCR 18 and AAUCR 20 (South Wollo) so these isolates could have better ability to adapt the rhizosphere environment than the most sensitive isolates such as AAUCR 6 (South Wollo) followed by AAUCR 15 (South Wollo) (Table-2)

With regard to pattern of resistance to different antibiotics, Kucuk and Kivanc (2008) from Turkey reported that chickpea rhizobia showed a high level of resistance against erythromycin, penicillin and Chloroamphinicol. However, Daniel Muleta (2009) reported that most chickpea rhizobia were resistant to penicillin (100%) at a concentration of 5 and 10μgml^{-1}, and Chloroamphinicol (70%) at a concentration of 10 μgml^{-1}.

On the basis of numerical analysis the twenty seven isolates based on their biochemical and physiological test record were grouped into four clusters below the average similarity coefficient of 85% (Fig.7). The first and fourth clusters consisted of four isolates each and cluster III contained the largest number of 13 isolates. The clustering of isolates did not show any affinity with isolation sites. This result is more or less similar to the report of Maatallah *et al* (2002) where 56 root nodule bacteria from chickpea were grouped into 5 groups at 82% average similarity. The work of Daniel Muleta (2009) also showed that isolates were clustered in to two major clusters and four minor ones at 0.71 level of relative similarity. The data in general, showed that isolates were dominated by one major group of chickpea rhizobia without excluding the presence of others. However, there is a need for more collections and undertaking many tests on large number of phenotypic and genetic characters to see the true picture of rhizobial diversity on chickpea.

Symbiotic effectiveness of isolates on the host plant was evaluated after 60 days of planting by measuring nodule number, nodule dry weight and shoot dry weight. The number of nodules ranged between 2-61nodules per plant. The highest number of nodule per plant (61) was recorded from plants inoculated with isolate AAUCR 10 followed by isolate AAUCR 9 and the least number of nodules (2) was recorded from plants inoculated with isolate AAUCR 3, AAUCR 6 and AAUCR 7 (Table-3). Plants inoculated with isolates with the highest number of nodules as they are found in highly effective and effective category, they can be selected as good isolates to develop inoculants. In contrast those plants inoculated with isolates that are with the lowest number of nodules are found in lowly effective and ineffective category. Daniel Muleta (2009) also reported that chickpea rhizobia induced various numbers of nodules ranging from 4 and 45 nodules/plant. In the current work significant variation in nodule dry weight was also observed in plants inoculated with different rhizobial isolates. The nodule dry weight was ranged between 10mg for plants inoculated with isolates AAUCR 3, AAUCR 4 and AAUCR 6 to 76mg for plants inoculated with isolate AAUCR 10 per plant (Table-3). Similarly the shoot dry mass was ranged between 196Mg/plant for isolate AAUCR 6 to 526Mg/plant for isolate AAUCR 9. This result supported previous findings of Talukdar *et al*. (2008) in such a way that dry weight of shoot was influenced by rhizobium inoculation. The highest dry weight of shoot per plant was recorded for inoculated plant 70% higher than that of uninoculated control. The lowest dry weight of shoot was recorded for uninoculated control.

Correlation between nodule number, nodule dry weight and shoot dry weight was also observed (Table-5). Plants with the lowest nodule number and nodule dry weight were with the lowest shoot dry weight. In the same way plants with the highest nodule number and nodule dry weight were with the highest shoot dry weight (Table-3). Correlation between nodule number, nodule dry mass and shoot dry weight was observed. The correlation between nodule number and nodule dry weight was 0.732 at 0.001 probability value. The correlation between nodule number and shoot dry weight also was 0.645 at 0.001 probability value; whereas the correlation between nodule dry weight and shoot dry weight was 0.573 at 0.01 probability value.

The highest symbiotic effectiveness was recorded from isolates AAUCR 9 and AAUCR 27 (South Wollo); with symbiotic effectiveness greater than 80%. Eighteen isolates were found to be effective with the range of effectiveness of 50-80%. The other six isolates were rated as lowly effective with their effectiveness ranged between 35-50% whereas one isolate was found to be ineffective with effectiveness value of 34%. This effectiveness rate showed that most of the isolates were found in the symbiotic effectiveness range of effective; i.e.50-80% effectiveness rate. The number of isolates that were included in highly effective and effective categories was 7% and 67%, respectively (Table-3). Daniel Muleta (2009) reported that out of 55 isolates reinoculated onto the host plant, 59% were lowly effective and 27% were ineffective. The proportion of highly effective (14.4%) and effective isolates (27%) was very small but in the current work the proportion of highly effective (7%) and effective isolates (67%) is higher which showed that more effective isolates were screened in the current work. The trend in the low proportion of highly effective and effective isolates of chickpea rhizobia is an indication that there is a requirement for taking large number of samples from different sites to select isolates for future inoculants production.

In this study, attempts were also made to correlate symbiotic effectiveness of isolates with their tolerance to different environmental factors. This is essential to screen best isolates for future screening and production of inoculants. In most of the tests, the highly effective AAURC9 (South Wollo) showed a wide range of tolerance in almost all phenotypic characters. The other highly effective isolate, AAUCR27 also showed a strong resistance to antibiotics, wide range of amino acid utilization, with mild tolerance to temperature and pH compared to the other isolates. Isolates AAUCR14, AAUCR10, AAUCR8, AAUCR13, AAUCR15, and AAUCR18 were among the effective isolates that displayed a wide tolerance to different environmental variables. These isolates were also among the rhizobia that induced the highest shoot dry matter accumulation (52-92%) compared to the N-fertilized positive controls. It may well be that isolates that were screened under laboratory conditions that may thrive and prove effective in ecological competitiveness to survive in the soil and outcompete the indigenous one in nodule occupancy and nitrogen fixation. This, however, needs further tests under greenhouse and field conditions to realize the full benefits of BNF in the production of chickpea in low-input agriculture.

7. Conclusion and Recommendation

7.1. Conclusions

The result of this work is an indicative of the presence of wide diversity in rhizobial isolates of chickpea collected from different growing areas of South and North Wollo zones of the Amhara Regional State. Diversity of the isolates was observed based on their morphological, physiological, host infection and symbiotic effectiveness.

This study showed that rhizobial isolates showed diversity with respect to symbiotic infectiveness and effectiveness with their host. Similarly, the numerical analysis also confirmed that these isolates were phenotypically diverse which indicates that their real diversity must also be confirmed by genetic analysis using molecular techniques.

The tolerance of isolates to different pH levels, temperature, salinity, and antibiotics is an important quality of rhizobial strains to screen and develop inoculants that are endowed with ecological competitiveness. In the presence of different environmental stresses, the tolerant isolates would survive, occupy nodules, and fix nitrogen and provide the host to boost plant production.

Accordingly, the following isolates were found to be effective in nitrogen fixation and resistant to different environmental stresses. In most of the tests, the highly effective AAURC9, AAUCR27 was found to be the best of all the isolates followed by isolates AAUCR14, AAUCR 10, AAUCR 8, AAUCR 13, AAUCR 15, and AAUCR 18.

7.2. Recommendations

Based on the result of this study, the following is recommended;

- To get infective, symbiotically effective and ecologically adaptive chickpea rhizobia, collection of large samples from many chickpea growing areas is mandatory
- Isolation and characterization of highly effective isolates *in vitro* condition may not give the same result at *in vivo* condition so this must be supported by trial at field condition by researchers and different agricultural experts to optimize the benefits of inoculant development for large scale production
- Isolates that are tolerant to different environmental factors must be screened to develop inoculants as they are ecologically competitive
- Isolates like AAUCR 9, AAUCR and AAUCR 27 that are categorized under highly effective isolates showed wide range of tolerance to different phenotypic features must be used to develop inoculants as they are ecologically competitive, they can outcompete the indigenous *Rhizobia* to nodule occupancy and nitrogen fixation
- Isolates like AAUCR 8, AAUCR 10, AAUCR 13, AAUCR 15 and AAUCR 18 are among effective isolates that showed a wide tolerance to different environmental variables, are promising to develop inoculants

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9. Annexes

Annex-1. Tolerance of rhizobial isolates to different pH levels

Isolates	**pH 4**	**pH 4.5**	**pH 5**	**pH 6.5**	**pH 8**	**pH 8.5**	**pH 9**	**pH 9.5**	**pH 10**	**pH4-10**
AAUCR 1	_	+	+	+	++	+	+	+	+	8
AAUCR 2	_	+	+	+	+	+	_	_	_	5
AAUCR 3	_	+	+	+	+	+	+	+	+	8
AAUCR 4	_	+	+	+	+	+	+	+	+	8
AAUCR 6	_	+	+	+	+	+	_	_	_	5
AAUCR 7	_	_	_	+	+	+	+	_	_	4
AAUCR 8	_	+	+	+	+	+	+	+	_	7
AAUCR 9	_	_	_	+	+	+	+	+	+	6
AAUCR 10	_	+	+	+	+	+	+	+	+	8
AAUCR 12	_	_	_	+	+	+	+	+	+	6
AAUCR 13	_	_	+	+	+	+	_	_	_	4
AAUCR 14	_	+	+	+	+	+	+	+	+	8
AAUCR 15	_	+	+	+	+	+	+	+	_	7
AAUCR 16	_	+	+	+	+	+	+	_	_	6
AAUCR 17	_	_	+	+	+	+	_	_	_	4
AAUCR 18	_	_	+	+	+	+	+	_	_	5
AAUCR 20	_	_	_	+	+	+	+	+	_	5
AAUCR 21	_	_	+	+	+	+	+	+	_	6
AAUCR 22	_	+	+	+	+	+	+	_	_	6
AAUCR 23	_	_	_	+	+	+	+	+	+	6
AAUCR 24	_	_	+	+	+	+	+	_	_	5
AAUCR 25	_	_	+	+	+	+	+	+	+	7
AAUCR 26	_	_	+	+	+	+	+	+	_	6
AAUCR 27	_	_	_	+	+	+	+	-	_	4
AAUCR 30	_	_	_	+	+	+	+	+	+	6
AAUCR 31	_	_	+	+	+	+	_	_	_	4
AAUCR 33	_	_	_	+	+	+	_	_	_	3
Total	0	11	19	27	27	27	21	15	10	
%	0	41	70.4	100	100	100	78	56	37.03	

(+) is for growth and (-) is for no growth

Annex-2. Tolerance of the isolates to different salt concentrations

Isolates	**1%**	**2%**	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**1-9%**
AAUCR 1	+	+	+	+	+	+	_	_	6
AAUCR 2	+	+	+	+	+	+	_	_	6
AAUCR 3	+	+	_	_	_	_	_	_	2
AAUCR 4	+	+	+	+	+	_	_	_	5
AAUCR 6	+	+	_	_	_	_	_	_	2
AAUCR 7	+	+	_	_	_	_	_	_	2
AAUCR 8	+	+	+	+	+	+	+	_	7
AAUCR 9	+	+	+	+	+	+	_	_	6
AAUCR 10	+	+	+	+	_	_	_	_	4
AAUCR 12	+	+	+	+	_	_	_	_	4
AAUCR 13	+	+	+	+	+	+	_	_	6
AAUCR 14	+	+	+	+	+	+	+	_	7
AAUCR 15	+	+	+	+	_	_	_	_	4
AAUCR 16	+	+	+	+	_	_	_	_	4
AAUCR 17	+	+	+	+	+	_	_	_	5
AAUCR 18	+	+	+	+	+	+	_	_	6
AAUCR 20	+	+	+	+	+	_	_	_	5
AAUCR 21	+	+	+	+	_	_	_	_	4
AAUCR 22	+	+	+	+	_	_	_	_	4
AAUCR 23	+	+	+	_	_	_	_	_	3
AAUCR 24	+	+	+	_	_	_	_	_	3
AAUCR 25	+	+	_	_	_	_	_	_	2
AAUCR 26	+	+	+	+	+	+	_	_	6
AAUCR 27	+	+	+	+	_	_	_	_	4
AAUCR 30	+	+	+	+	+	+	_	_	6
AAUCR 31	+	+	+	+	_	_	_	_	4
AAUCR 33	+	+	+	+	_	_	_	_	4
Total	27	27	23	21	12	9	2	0	
%	100	100	85.2	77.8	44.4	33.3	7.4	0	

Annex -3. Tolerance of isolates to different temperature

Isolate	**4°c**	**10°c**	**20°c**	**30°c**	**35°c**	**40°c**	**45°c**	**4-45°c**
AAUCR 1	+	+	+	+	+	_	_	5
AAUCR 2	_	+	+	+	-	_	_	3
AAUCR 3	_	+	+	+	+	_	_	4
AAUCR 4	_	+	+	+	+	_	_	4
AAUCR 6	_	_	+	+	+	_	_	3
AAUCR 7	_	+	+	+	+	_	_	4
AAUCR 8	_	_	+	+	+	_	_	3
AAUCR 9	+	+	+	+	+	+	_	6
AAUCR 10	+	+	+	+	_	_	_	4
AAUCR 12	_	+	+	+	+	_	_	4
AAUCR 13	_	+	+	+	+	_	_	4
AAUCR 14	_	+	+	+	+	_	_	3
AAUCR 15	_	+	+	+	+	_	_	4
AAUCR 16	_	+	+	+	+	_	_	4
AAUCR 17	+	+	+	+	+	_	_	5
AAUCR 18	_	+	+	+	+	_	_	4
AAUCR 20	_	+	+	+	+	+	_	5
AAUCR 21	+	+	+	+	_	_	_	4
AAUCR 22	+	+	+	+	+	_	_	5
AAUCR 23	_	+	+	+	+	_	_	4
AAUCR 24	_	+	+	+	_	_	_	3
AAUCR 25	+	+	+	+	+	_	_	5
AAUCR 26	_	+	+	+	+	_	_	4
AAUCR 27	_	_	+	+	+	_	_	3
AAUCR 30	+	+	+	+	+	_	_	5
AAUCR 31	_	+	+	+	_	_	_	3
AAUCR 33	_	+	+	+	+	_	_	4
Total	8	24	27	27	22	2	0	
%	29.6	88.9	100	100	81.5	7.4	0	

Annex-4. Amino acid utilization of isolates

Isolates	**Glutamic acid**	**Phenylalanin**	**arginin**	**Tyrosin**	**Glycin**	**Tryptophan**	**urea**	
AAUCR 1	+	+	_	_	_	_	_	2
AAUCR 2	+	+	+	+	+	+	_	6
AAUCR 3	+	+	+	+	+	+	_	6
AAUCR 4	+	+	+	_	_	+	_	4
AAUCR 6	_	+	+	+	+	+	_	5
AAUCR 7	_	+	+	+	_	+	_	4
AAUCR 8	_	+	_	+	_	+	_	3
AAUCR 9	+	+	+	+	_	_	_	4
AAUCR 10	_	+	_	_	_	+	_	2
AAUCR 12	_	+	+	_	+	+	_	4
AAUCR 13	+	+	+	+	+	+	_	6
AAUCR 14	+	+	_	_	+	_	_	3
AAUCR 15	+	+	+	+	+	+	_	6
AAUCR 16	_	+	+	+	_	+	_	4
AAUCR 17	_	+	+	+	_	+	_	4
AAUCR 18	+	_	_	_	+	_	_	2
AAUCR 20	+	+	_	_	_	+	_	3
AAUCR 21	_	_	_	_	_	+	_	1
AAUCR 22	_	+	_	_	_	_	_	1
AAUCR 23	+	+	+	_	_	+	_	4
AAUCR 24	+	+	_	_	+	_	_	3
AAUCR 25	_	+	_	+	_	+	_	3
AAUCR 26	+	+	_	_	_	+	_	3
AAUCR 27	+	+	+	+	+	+	_	6
AAUCR 30	_	+	_	_	_	+	_	2
AAUCR 31	_	+	+	_	+	+	_	4
AAUCR 33	_	+	_	_	+	+	_	3
Total	14	25	14	12	12	21	0	
%	51.9	92.6	51.9	44.4	44.4	77.8	0	

Annex-5. N-free nutrient composition (Broughton and Dilworth, 1970)

Solutions	**Chemical**	**g/liter**
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
4	H_3BO_3	0.247
	$MnSO_4.H_2O$	0.338
	$FeC_6H_5O7.3H_2O$	6.7
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

Adopted from Somasegaren and Hoben (1994)

Annex-6. Sample sites and pH of sample soil

N<u>o</u>	**Kebele**	**Zone**	**Soil pH**
1	Sulula/Haik	South Wollo	6.05
2	Hitecha	South Wollo	5.58
3	Langa	North Wollo	5.58
4	Diobulko	North Wollo	6.54
6	Debreselam	South Wollo	6.68
7	Tita	South Wollo	6.38
8	Buke	South Wollo	6.48
9	Haratehuledere	South Wollo	6.62
10	Woldelulo	South Wollo	6.53
12	Qulqualo	South Wollo	6.68
13	Wulawula	South Wollo	7.08
14	Haraworebabo	South Wollo	6.68
15	Gobeya	South Wollo	7.58
16	Amumo	South Wollo	6.16
17	Goha	South Wollo	6.78
18	Wowa	South Wollo	7.08
20	Golbo	South Wollo	6.48
21	Enchini	South Wollo	6.33
22	Serdem/Kombolcha	South Wollo	6.60
23	Korkie	South Wollo	7.18
24	Wurgessa	South Wollo	6.61
25	Lalibela	North Wollo	6.63
26	Merssa	North Wollo	6.48
27	Harbu	South Wollo	6.58
30	Sirinka	North Wollo	6.88
31	Chefa	South Wollo	6,84
33	Tekeze	North Wollo	6.00

Annex.7. Comparison of inoculated symbiotically effective and control plants after 60 days of growth on pot culture in the greenhouse


AAUCR 9
POSITIVE CONTROL
NEGATIVE CONT


AAUCR 33
POSITIVE CONTROL
NEGATIVE CONTROL


AAUCR 2
POSITIVE CONTROL
NEGATIVE CONTROL


AAUCR 12
POSITIVE CONTROL
NEGATIVE CONTROL


AAUCR 9
POSITIVE CONTROL
NEGATIVE CONTROL


AAUCR 22
POSITIVE CONTROL
NEGATIVE CONTROL

Annex. 8. Nodules of symbiotically effective isolates