ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Phenotypic and Symbiotic characterization of grass pea (*Lathyrus sativus*) rhizobial isolates from some major growing areas of South Wollo and West Shoa , Ethiopia

By

Musa Adal

A Thesis submitted to the school of graduate studies of Addis Ababa University inpartial fulfillment of the requirements for the degree of Master's of science in Biotechnology, Industrial Biotechnology

July 2009

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Approved by examining Board:

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Declaration

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

Name: Musa Adal
Place: Addis Ababa University
Signature: ____________________
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This thesis has been submitted for examination with my approval as university advisor

Name: Fassil Assefa (PhD)
Signature: __________________
Date: __________________

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List of symbols and Abbreviations

ATP	Adenosine triphosphate
AAUGR	Addis Ababa University Grass Pea rhizobia
BNF	Biological nitrogen fixation
BTB	Bromothymol blue
CR	Congo red
E	Effective
g / l	gram per liter
ha	hectare
HE	highly effective
I	ineffective
IAR	intrinsic antibiotic resistance
ICARDA	international central for agricultural research for in the dry areas
Kg	kilogram
LE	lowly effective
LM	large mucoid
LW	large watery
Mg	milligram
MGT	mean generation time
MM	millimole
Min	minute
MT	metric tone
N	nitrogen
N_2	nitrogen molecule
PGA	Peptone – Glucose – Agar

pH	hydrogen ion potential
PPM	parts per million
rpm	revolutions per minute
SW	South Wollo
SDM	Shoot dry matter
SDW	Shoot dry weight
µg /ml	microgram per milliliter
UPGMA	Unpaired Group Method Analysis
V/V	volume per volume
W/V	weight per volume
WS	West Shoa
YEMA	Yeast Extract Mannitol Agar
YEMA-BTB	Yeast Extract Mannitol Agar Bromothymol Blue
YEMA-CR	Yeast Extract Mannitol Agar Congred

Abstract

Grass pea (*Lathyrus sativus*) is the most widely distributed and the third most important pulse crop in Ethiopia. It grows in a wide range of altitudes and survives in scarce moist conditions and fixes nitrogen with rhizobia and performs well in less fertility soils. However, the effectiveness in nitrogen fixation varies depending upon the host varieties, type of strain and different environmental characters. It is, therefore, necessary to evaluate the efficiency of different isolates from different sampling areas. Thus, 68 grass pea rhizobial isolates (47 from South Wollo and 21 from West Shoa) were isolated and characterized on the basis of different characters. These isolates were reinoculated on pouch culture using "Wasse" variety of grass pea to authenticate nodulation and determine their symbiotic effectiveness. All but 5 isolates were authenticated as root nodule bacteria with their capacity to infect and nodulate their parent host. Most of the isolates displayed fast doubling time (1.2-4hrs) and large colonies (2-6.0 mm) and changed the BTB-YEMA media to yellow which showed that they are fast growing rhizobia and then categorized into *R. leguminosarum bv.* viceae. The preliminary screening of the isolates for symbiotic effectiveness on pouch culture showed that isolates AAUGR (31, 37, 41 and 47) of South Wollo and AAUGR (48, 49, 52, 59, 60, 61, 66 and 67) of West Shoa that comprised of 19% of the isolates were highly effective whereas, 43% were found to be effective. Three highly effective isolates from South Wollo (AAUGR 31, AAUGR 37, and AAUGR 41) and four highly effective isolates from West Shoa (AAUGR 48, AAUGR 59, AAUGR 61, and AAUGR 67) were reinoculated into soil culture to evaluate their performance in the natural environment (soil). All the selected isolates were found to accumulate 112-149 % of SDM that was higher than the controls. Isolate AAUGR 37 (SW) accumulated the highest SDM of all the inoculated treatments and the controls. All the selected isolates were found to perform better on pot culture than on pouch culture. Almost all of the isolates were able to grow at extremely low and high pH, low and high salt, low and high temperature, and utilized a wide range of carbohydrate and nitrogen sources and displayed high resistance to several antibiotics indicating that the isolates can compete in their natural environment. The result of the numerical analysis also showed the existence of diversity among the tested isolates.

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1. Introduction

Nitrogen is the fourth most abundant element in living organisms. It is part of the proteins, nucleic acids, adenosine triphosphate and other nitrogenous compounds. Although it makes up about 80% of the atmosphere mostly as dinitrogen (N_2), it is one of the most limiting factors that affect plant growth and crop production. It is often present in the atmosphere in an inert form and the oxidized form in the soil (NO_3) is available to plants. It should be necessarily reduced into biologically useful ammonia (NH_3, NH_4+) or nitrates (NO_3). This process is accomplished through industrial synthesis and biological nitrogen fixation (BNF).

BNF is the conversion of dinitrogen into ammonia (NH_3) which is carried out only by bacteria that are endowed with the enzyme, nitrogenase (Crews *et a*l., 2004; Postgate, 1998). The N_2- fixing microbes (many genera of bacteria, cyanobacteria, frankia) can exist as free-living organisms or in association with other microbes, plants or animals. It accounts to about 60% of the global nitrogen budget (Burdass, 2002) and is eco-friendly and cost effective (Zahran, 1999).

The most important of endosymbiotic association is between the plants belonging to the family Leguminasae and the root nodule bacteria, generally known as rhizobia. These plants include pulses (food legumes), shrubs and trees that account to the third largest group of flowering plants. The legumes are multi-purpose plants that serve as a source of food, feed, medicine, firewood, timber and other products. Food legumes (pulses) provide high quality protein food and feed, minerals and other nutrients. Consequently, 25% of the world’s major crop production, more than one-third of human protein needs and two-third of human livelihood is derived from pulses (Graham and Vance, 2003). The ability of many legumes to establish symbiosis with nitrogen fixing bacteria makes them a valuable source of soil nitrogen for other crops (Allen and Allen, 1981). Among the legumes, the pulses have played a great role as a source of food and feed and an integral part of the low-input agriculture. These plants fall into two principal groups based on climatic conditions and geographical distribution over the world, namely cool season and warm season legumes (Jayasundara *et a*l.,1998).The cool season legumes consist of the tribes viceae (Vicia, Lens, Lathyrus and Pisum), Cicereae (Cicer), and Genisteae (Lupinus) whereas, the warm season lowland tropical food legumes include cow pea (*Vagina unguiculata*), common bean (*Phaseolus vulgaris*), soy bean (*Glycine max*), ground nut (*Arachis hypogea*) and Pigeon pea (*Cajanus cajan*).

The genus *Lathyrus* is one of the cool season legumes belonging to the tribe viceae. It is widely distributed in Eurasia, America, Africa, India and Asia where its primary center of diversity is believed to be the Mediterranean region (Muehlbauer and Abebe, 1997). It is known by many common names, including chickling, vetch, Indian vetch (UK and USA), Khesari or Batura (Indian) and Guaya (Ethiopia) (Jennifer, 2003). However, the five different species that are distributed in Ethiopia are *Lathyrus sativus*, *L. pratensis*, *L. sphaericus*, *L. apaca*, *L adoratus* (Dawit, 1991). It is mostly grown in the highlands of Ethiopia at altitudes ranging from 1700-2220m above sea level with a maximum temperature of 15-20 ^{O}C in an operative range of 5-30 ^{O}C (Asmare *et al*., 2000).

The seed contains 18.2-34.6% protein, 0.6% fat, 58.2% carbohydrates (about 35% starch) and it also contains 1.5% glucose, 6.8% Pentosans, 3.6% Phytin, 1.5% lignin, 6.69% albumin, 1.5% prolamine 13.3% globulin, and 3.8% guttllin (Muehlbauer and Abebe, 1997). The same authors (Muehlbauer and Abebe, 1997) also reviewed that the essential amino acids found in grass pea seeds are (in grams per 16 grams of nitrogen): arginine 7.85, histidine 2.51, leucine 6.57, isoleucine 2.34, tryptophan 0.40, and valine 4.68 (like other cool season food legume, grass pea is deficient in methionine and tryptophan).

Grass pea (*Lathyrus sativus*) is the third most important pulse crop in Ethiopia after faba bean and chickpea with 142,170 ha of production area and 104,744 tones of production (Wuletaw and Endashaw 2001). It occupies 8.7% of the total area and 7.6% of the total production of food legumes in Ethiopia. It performs well on heavy black soils which are characterized by water logging. Grass pea production is mainly concentrated in the North

West Zone (58%), the central zone (16.3%), the North East (12%) and the Northern as well as the Southeast (12.9%) regions of Ethiopia (Wuletaw and Endashaw, 2001). However, the major grass pea producing administrative regions of Ethiopia are Shoa, Gojam, Gonder, Tigray, Wollo and Arsi (Adugna and Shelemew, 2001).

In the highlands of Ethiopia, major soils suitable for cool season food legumes are predominantly brown and chestnut vertisols (Adugna and Shelemew, 2001). Major soil related constraints to cropping are poor fertility, soil erosion on the slopes, acidity, and low phosphate availability (Angaw and Asnakew, 1994). Grass pea besides the nutritional benefits, has an important role as a legume crop in crop rotations, reportedly adding around 67 kgha^{-1}of nitrogen to the soil from symbiosis with *Rhizobium sp.* in a single season and conferring yield and protein benefits on the subsequent non-legume crop (Jennifer, 2003).

The development of grass pea inoculants can improve the fertility status of the soil and increase the crop yield. However, the development of such inoculants requires collection, isolation, identification and phenotypic characterization of large number of rhizobial isolates from different growing regions. Above all, testing the infectiveness and effectiveness of the isolates is also required.

Some research works have been done on grass pea with regard to its epidemiology, amount of neurotoxin, its production and its genetic resource conservation and utilization in some Agricultural Research Centers of Ethiopia (Berhanu *et al*., 2000). However; works on taxonomic status and symbiotic effectiveness of grass pea rhizobia is totally absent. Thus, this particular study is initiated with the aim of investigating the phenotypic diversity and symbolic effectiveness of rhizobia that nodulate grass pea from some major growing areas of South Wollo and West shoa.

2. Objectives

2.1. General Objective

To isolate and characterize the diversity and symbiotic effectiveness of root nodule bacterial isolates of grass pea (*Lathyrus sativus*) from major growing areas of South Wollo and West Shoa.

2.2. Specific Objectives

- To determine the different phenotypic characteristics of the isolates
- To evaluate the symbiotic effectiveness of the isolates on pouch culture
- To evaluate the performance of selected isolates on potted soil.

3. Literature Review

3.1. Legumes

Legumes are a family of plants Leguminosiae (Fabaceae) that have the ability to react in a symbiotic (mutually beneficial) relationship with root nodule bacteria to convert atmospheric nitrogen (N_2) from the soil atmosphere to ammonia nitrogen (NH_3) (Miller, 2008). Leguminosiae (Fabaceae) is composed of 18,000 species that are divided into three sub-families, Mimosoideae, Caesalpinoiodeae and Papilionoideae that are ranging from tiny annual herbaceous plants to a huge and perennial trees (Giller, 2001). They are used by mankind as a source of food and forage to mankind and animals (Howieson *et al*., 2000). According to De-Faria *et al*. (1989), about 23% of Caesalpinoiodeae, 90% Mimosoideae and 97% of Papillionoideae are known for their ability to form nitrogen (N_2) fixing root nodules with rhizobia.

The majority of legumes play a paramount role for agricultural purposes. Thus, some of the legumes that are frequently used in agriculture include *Vicia faba, Pisum sativum, Phaseolus vulgaris, Trifolium repens, Medicago sativa, Melilotus officinalis, Glycine max* and *Lathyrus sativa* (Alexander, 1997; Postgate, 1998). The symbiotic associations of legumes with rhizobia contribute a lot for the global nitrogen cycle in different ecosystems and also for ecological stability (Zahran, 1999). However; all legumes are not necessarily nodulated by root nodule bacteria due to genetic and several environmental factors. The association between Rhizobium and legume is a cheaper and usually more effective agronomic practice for ensuring an adequate supply of nitrogen for legume based crop and pasture production than application of chemical fertilizers.

It is estimated that Legume-rhizobia association fix 180 billion tones of atmospheric nitrogen (N_2) per year into ammonia (NH_3) (Freiberger *et al.*, 1997) which is equivalent to the expenditure of nitrogen fertilizer to about US 6.8 million dollar (Herridge and Rose, 2000). Generally, the inclusion of grain legumes in cropping sequences increases soil nitrate, grain yield, total nitrogen accumulation, food and feed (Badaruddin and

Meyer, 1994). Latta and carter (1998) also reviewed that their incorporation into the cropping system also reduces soil erosion losses, improve structure of the soil and nitrogen use efficiency of a subsequent cereal crop with inorganic fertilizer applied.

3.2. Grass pea

3.2.1. Ecology of Grass pea

The crop is widely cultivated in central, south and east Europe, the Mediterranean and Africa and India. The mean fluctuations during the growing season ranges from 10-30 ^{0}C with annual rainfall ranging from 600-1200mm and the crop is tolerant to extremely dry conditions in drought prone areas as in Ethiopia and also tolerant to excessive floods as in Bangladesh (Muehlbauer and Abebe, 1997). Asmare,*et al*.(2000) have also found that grass pea is grown in highlands of Ethiopia at altitude ranging from 1700-2220m with a maximum temperature of 15-20 ^{0}C in an operative range of 5-30^{0}C. It is a hardy crop suited to dry climates, producing good seed crops on poor soils. It is commonly cultivated on heavy clay soils. Black deep retentive soils are considered best for grass pea and it is sensitive to acidity and requires lime on acid soils (Muehlbauer and Abebe, 1997).

The growing conditions for grass pea have also been described at http://www.Hydra.Org.uk.(2001) as follows: i) annual rainfall: grass pea requires 300mm-1300mm.It is useful for very wet and very dry areas. ii) temperature: it grows at 4.5 ^{0}C to 27.5^{0}C and does well in colder areas and iii) soil type: it tolerates almost all types of soil from pH 4.5 to 8.3.It does best on clay soils.

3.2.2. Agronomic and economic importance of Grass pea

Grass pea seeds may be white, yellow-green, yellow, brown, gray or black. White seed is the most popular for human consumption. Common foods prepared from grass pea in Ethiopia includes roasted whole seeds (kolo), boiled whole seeds (nifro), pancake (kitta), leavened bread (injera) and the traditional sauce (shiro-wot). Grass pea performs well under adverse agricultural conditions, and its many cultivars possess different attributes including the ability to resist both drought and flooding, high climatic adaptability and the ability to grow in cool climates and at high altitudes (Jennifer, 2003).Grass pea cultures also have the ability to adapt to saline, alkaline, clay or otherwise poor soils, and are hardy and easy to cultivate (Sharma and Pandey, 2001).

One of the major drawbacks of grass pea is the fact that the seeds contain a major anti nutritional compound B-N-oxyalylamino-lalanine (BOAA) (also known as B-N-oxalyl-*L*-α, ß-diaminopropionic acid or ODAP (Jennifer, 2003). The neurotoxin causes a drastic and irreversible paralytic disease known as "Lathyrism or neuro Lathyrism" upon prolonged or excessive consumption of grass pea and the paralysis is manifested on leg muscle, muscular rigidity and weakness (Jennifer, 2003).

3. 3. Rhizobia

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants where they undertake symbiotic nitrogen fixation (Sahgal and Johri, 2003). They are gram negative rods, aerobic, motile and non-spore forming with one polar or sub-polar flagellum or 2-6 peritichous flagella and that are pleomorphic under adverse growth conditions (Jordan, 1984; Postgate, 1998). Rhizobia usually accumulate granules of poly-β-hydroxyl butyrate when carbon is in excess and possess respiratory type of metabolism with oxygen as the terminal electron acceptor (Jordan, 1984).

3. 3. 1. Taxonomy of rhizobia

The taxonomy of root nodule bacteria has changed considerably over the last 20 years. Beijerinck (1888) had for the first time isolated a bacterium from root nodule of legume and named it Bacillus radicicola. This was subsequently renamed Rhizobium (Frank, 1889). The earliest classification of rhibzobia was based on specificity of symbiotic plant range of bacterial species (Sahgal and Johri, 2003). Fred *et al.* (1932) recognized six species in the genus Rhizobium viz. *R. japonicum* (*Lathyrus*, *Lens*, *Pisum* and *Vicia*), *R. lupini* (Lupinus), *R. Meliloti* (*Melilotus*, *Medicago*, *Trigonella*) *R. phaseoli* (*Phaseolus*) and *R. Trfolii* (*Trifolium*) based on their host range and certain morphological and physiological properties .

Based on their growth rate bacteria were grouped as fast growers and slow growers, but were still placed in the genus Rhizobium till Jordan (1984) coined the new genus Bradyrhizobium for isolates from *Glycine max*. Norris (1965) observed that fast growers and slow growers differed in their symbiotic affinity. Accordingly, alkali producing slow growers was isolated with the tropical legumes whereas; fast growers from temperate legumes were acid producing (Sahgal and Johri, 2003).

Since the early nineties, sequence comparison of 16s r RNA genes and genetic finger printing methods based on PCR have been used extensively for characterizing rhizobia (Sahgal and Johri, 2003). These authors described that the use of sequencing of full 16s rRNA gene and different PCR tools are reliable to ascertain the phylogeny of rhizobial isolates. As Willems (2006) reviewed that the introduction of more genetic characteristics (DNA-DNA and DNA-rRNA hybridizaions, rRNA catalogues, rDNA sequencing) revealed more diversity among the rihizobia. This leads to a gradual increase in number of genera (Table I).

Table I. Rising number of species in the genera of the rhizobia.

Genus	Original Publication	Number of species					
		Before 1980	81-85	86-90	91-95	90-00	01-06
Rhizobium	Frank,(1889)	4	5	5	10	10	16
Brandy rhizobium	Jordan,(1982)		1	1	3	3	7
Sinorhizobium,	Chen et al.,(1988)			2	5	8	11
Azorhizobium	Dreeffus et al.,(1988)			1	1	1	2
Merorhizobium	Jarvis et al., (1997)					7	11
Allorhizobium	Delajudie et al.,(1998)					1	1
Total		4	6	9	19	30	48

Source: Willems (2006)

3. 3. 2. Rizobial diversity and characterization methods

The diversity of rhizobia in a particular soil may be influenced by the method used to isolate. Several arrays of techniques are used for detecting and describing rhizobial diversity. These are host range, comparative growth in culture, intrinsic antibiotic resistance, carbohydrate utilization, amino acid utilization and tolerance in pH, temperature and salt. These are among the most common methods that are considered as phenotypic characters and are used primarily to study rhizobial diversity (Ahmad *et al*., 1984; Maatallaah et al., 2002). The size, shape, color and texture of colonies and the ability to alter the pH of the media are generally stable characteristics useful in defining strains of isolates. Colonies could be discrete, round varying from flat to domed and even conical on agar surface. Moreover; they may be white, opaque, and milky and watery translucent (Ahmad *et al*., 1984).

Rhizobial colonies after incubation on YEMA-CR indicator remain white opaque or occasionally pink except some contaminants such as *Rhizobum melliloti* (Lupwayi and Haque, 1994). No or little growth of rhizobial isolates occur when streaked on Peptone Glucose Agar medium and incubated at 28 ± 2^0C for 3-5 days (Somasegaren and Hoben, 1994).

The fast growers form visible colony with in 2-3 days and their generation time is less than four hours. The fast growers are acid producers and the slow growers are base producers. This identification can be done by growing them on YEMA media in the presence of Bromthymol blue (BTB) indicator at pH 6.8 (Jordan, 1984; Lupwayi and Haque, 1994). Tolerance to acid or alkali, temperature sensitivity, resistance to antibiotics and heavy metals, tolerance to salinity, utilization of different carbon sources, utilization of different nitrogen sources and symbiotic characteristics were used by many researchers to determine a wide physiological diversity among tested isolates (Ahmad *et al*., 1984 and Maatallah *et al*., 2002). These phenotypic methods provide a valuable insight into rhizobial population structure and strain diversity. Thus, they are best regarded as preliminary screening methods for relative screening methods.

Modern molecular tools which are more preferred to phenotypic methods can also be used to evaluate the specificity of the stains (Thiess *et al*., 2001). Some of these molecular tools employed for rhizobial diversity identification includes: sequence comparison of 16S rRNA genes and genetic finger printing (Sahgal and Johri, 2003), plasmid profiling (Broughton *et al*, 1987), restriction fragment length polymorphism (RFLP) (Odee *et al.,* 2002), polymerase chain reaction based techniques (PCR) (Richardson *et al*., 1998), PCR-RFLP of 16S rRNA genes (Sahgal and Johri, 2003).

3. 4. Inoculation and their benefit

Soil contains millions of rhizobia but they may not be specific to the particular legume grown and in an active stage to induce infection and initiate N_2 fixation. This brings the necessity of inoculation of particular rhizobium inoculants into their specific legume partner. Lewis (1967) defined inoculation of legume as the introduction of legume bacteria into the soil or plant seedling to enable plants to fix or change in to usable form of atmospheric nitrogen. Mulongoy (2004) has also described inoculation as the introduction of rhizobia into the soil to ensure proper nodulation and nitrogen fixation, if specific and effective rhizobia are absent in the soil or if they are present in low numbers. The benefits of inoculation described by Miller (2008) include: i) increased yield -yields may increase 10-100% depending on specific soil conditions; soils with average fertility may have yield increase from 15-25%, ii) improved protein content, and iii) increase soil nitrogen for future crops – nitrogen fixation will vary depending on plant species, suitable bacterium population, soil nitrogen content, soil fertility level, soil pH, moisture, and temperature.

3.5. Nitrogen fixation

Nitrogen is an essential plant nutrient which is most commonly deficient in soils, contributing to reduced agricultural fields throughout the world (Montanez, 2000). The element nitrogen is an essential part of many of the chemical compounds, such as proteins, nucleic acids and other metabolites, which are the basis of all life forms. The atmospheric nitrogen in order to be incorporated in to amino acids and subsequently become an integral part of plant tissue, it has to be transformed in to ammonia (NH_3) through nitrogen fixation (Postgate, 1998). The different ways of N-transformation and fixation are (Smil, 2000):

1. The natural formation of NO from N_2 and O_2 due to lightening.
2. Combustion of fossil fuels: through automobile engines and thermal power plants that release different oxides of nitrogen.

3. Industrial nitrogen fixation: in the Haber-Bosch process, N_2 is converted together with hydrogen gas (H_2) into ammonia (NH_3) fertilizer.
4. Biological nitrogen fixation: bacteria are able to fix and assimilate it as organic nitrogen. Symbiotic nitrogen fixing bacteria such as rhizobium live in association with leguminous plants whereas, free-living nitrogen fixing bacteria such as azotobacter fix nitrogen without any association with plants.

Biological nitrogen fixation (BNF) is the process whereby atmospheric nitrogen (N=N) is reduced consequently in to ammonia and other reactive and usable forms in the presence of nitrogenase which is a biological catalyst found naturally only in prokaryotes. BNF is exclusively carried out by prokaryotes. The ability to reduce and siphon out an appreciable amount of nitrogen from the atmospheric reservoir and enrich the soil is confined to bacteria and archea (Tilak *et al*., 2005). These include a) symbiotic nitrogen fixing (N-fixing) forms, viz. rhizobium the obligate symbionts in leguminous plants and Frankia in non-leguminous plants and b) Non- symbiotic (free-living, associative or endophytic) N_2 – fixing forms such as *Cyanobacteria, Azosprillum, Azotobacter, Acetobacter, Diazotrophicus, Azoarcus*, etc.

BNF takes place in all ecological environments and conditions such as soils, fresh and salt water and sediments, on or within roots, stems, leaves of different type of leguminous plants (Postage, 1998; Zahran, 1999). Biological nitrogen fixation (BNF) is becoming more important for not only reducing energy costs but also in seeking more sustainable agricultural production and agricultural systems. Nitrogen is continually depleted by such processes as microbial denifrification, soil erosion, leaching, chemical volatilization and perhaps most important, removal of nitrogen-containing crop residues from the land. Since its deficiency is a problem in most soils of the tropics and subtropics (Hungria and Vargas, 2000), it's adequate supply, proper management and utilization efficiency are crucial to ensure improved agriculture in sustainable manner (Graham and Vance, 2003).

The atmosphere comprises of approximately 80% molecular nitrogen (N_2) that is about 4 X 10^{15} tones in the form of ditinrogen molecules, but only about 2 X 10^6 tones is found in compound form (Hubbell and Kidder, 2002). This molecular nitrogen is inert and unavailable for plants. It has to be transformed into utilizable form such as NH_4 through the process known as nitrogen fixation (Smith and Douglas, 1987; Postgate, 1998). The nitrogen reserve of agricultural soils must therefore be replenished periodically in order to maintain an adequate (non-growth limiting) level for crop production. This replacement of soil nitrogen is generally accomplished by the addition of chemically fixed nitrogen in the form of biological nitrogen fixation (BNF) systems (Hubble and Kidder, 2003).

The application of nitrogen fertilizer has increased approximately 10 fold to 90 million Mt between 1950 and 1995 with significant energy consumption for N-fertilizer synthesis and application (Frink *et al*., 1999 and FAO, 1994). However, in developing countries, several constraints such as the high cost of nitrogen fertilizer, the energy requirements for production and the suboptimal transportation capacities made the chemical fertilizers neither available nor affordable in food production on small farms (Bohlool *et al*., 1992; and Vance, 1997).

BNF is estimated to contribute 180 X 10^6 mt /year globally, of which 80% comes from symbiotic association and the rest 20% from free-living or associative systems (Tilak et al., 2005). This is by far greater than that of the industrially fixed nitrogen supply which is 65 X 10^6 tons per year. Roughly, half of the 23 million mt of nitrogen consumed as human food sources (grains and live stocks) come from BNF by prokaryotes (Frink *et al*., 1999). Out of this, rhizobia in root nodules are estimated to carry out about 80% of the world's BNF (Burris and Roberts, 1993).

Symbiotic nitrogen fixation (SNF) by rhizobium with legume crops fixes about 90 X 10^9 Kg of N_2 annually (Vance, 1998). In agricultural systems, symbiotically fixed nitrogen can: (i) be an immediate source of N to the fixing species for dry matter and seed production, (ii) be released from the species to comparison crops to complement their N needs and (iii) be useful as a green manure providing N to crops grown in relations (Peoples *et al*., 1995). SNF is dependent on several factors including the host plant genotype, the *Rhizobium* strain and the interaction of these symbionts with the pedoclimatic factors and the environmental conditions (Bordeleau and Prevost, 1994). Both the legume plant and the bacteria derive mutual benefit form the association. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium (Burdass, 2002).

3. 6. The biochemistry of biological nitrogen fixation (BBNF)

BNF, the process of reducing the triple bond of atmospheric nitrogen (N=N) in to ammonia and other usable forms take place in the presence of an oxygen sensitive enzyme, the nitrogenase. Nitrogenase is a complex – enzyme containing two oxygen sensitive metalloprotein components that are active independently but work synergetically (Dilworth and Glenn, 1991; Leigh, 2002).

Dinitrogenase (Component I), the larger heterotetramer component of nitrogenase with two different metalloclusters has two α- protein subunits and two β-protein subunits, 24 molecules of iron, two molecules of molybdenum and an iron molybdenum cofactor (FeMoCo) (Leigh, 2002). Component I, the dinitrogenase reductase, catalyses the actual conversion of N_2 to ammonia. Similarly, component II is composed of two protein subunits (different from that of component I) and a large number of iron molecules or iron-protein which plays a role in donating electrons to component I (Giller and Wilson, 1991; Dean and Jacobson, 1992; Maschner, 1995; Peters *et al*., 1995; Haward and Rees, 1996).

Each individual α, β dimmer component containing Fe-Mo-Co and a P-cluster is considered as a functional unit of nitrogen fixation and the Fe-protein is a homodimmer component containing two MgATP binding sites and a single (4Fe-45) cluster (Peters *et al.*, 1995). Dinitrogenase, a 240-KDa heterotertramer, binds N_2 and holds it while it is being reduced whereas; Dinitrogenase reductase a 64-KDa homodimer provides dinitrogenase with high energy electrons. Following binding, an electron is transferred from the Fe-protein to the Mg Fe-protein with concomitant hydrolysis of both bound ATP molecules to ADP; ultimately, the two components of nitrogenase dissociate (Giller, 2001). Thus, nitrogenase catalyses the conversion of N_2 to NH_3 which is represented as:

$$N_2 + 16\ ATP + 8e + 8\ H^+ \xrightarrow{Mg^{++}} 2NH^+_4 + H^+_2 + 16\ ADP + 16\ pi$$

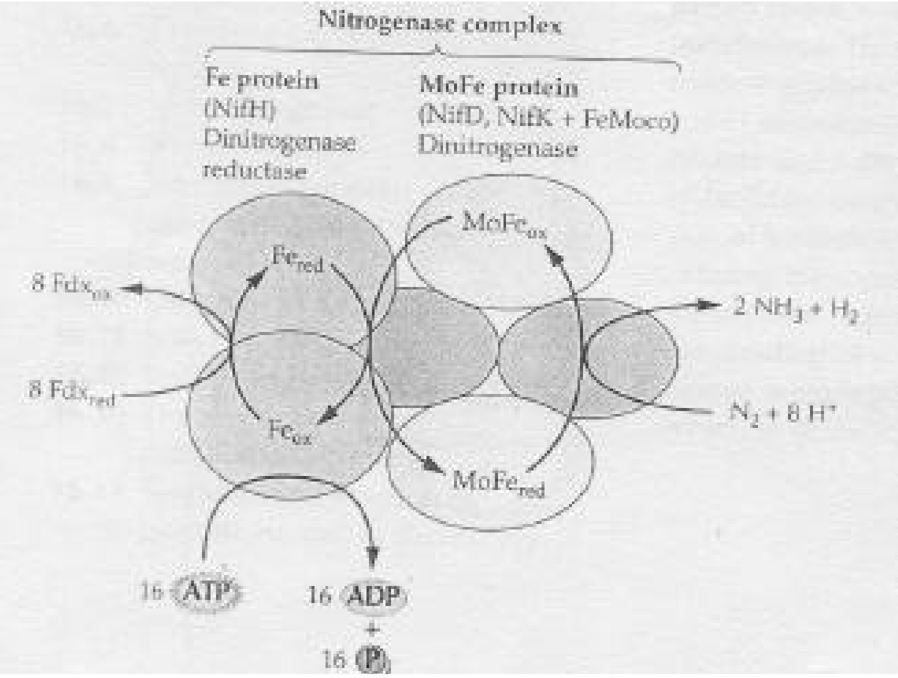

Fig. 1. The role of nitrogenase enzyme in nitrogen fixation

For the reaction to be catalyzed, nitrogenase requires anaerobic environment and a source of large amount of energy. BNF is energy intensive, efficient but requires much lower energy than Haber process (Zahran *et al.*, 1992). In symbiotic association, nitrogenase activity is protected form oxygen damage in two ways:one with strong and variable physical barrier to oxygen diffusion into nodule cortex and the second component of oxygen protection is with the presence of leg-hemoglobin within the infected cells. The role of leg-hemoglobin in the nodule is to facilitate oxygen supply for respiration, so that high oxygen tension support bacteroidal oxidative phosophorylation while keeping oxygen sequestered away from nitrogenous enzyme (Shaw, 1983).

3. 7. Ecological factor that affect BNF

BNF is a key to sustainable agricultural systems in tropical soils which are frequently deficient in nitrogen. This is achieved where there is establishment of effective symbiosis which requires the following factors (Bordeleau and Prevost, 1994).

A. Colonization and survival in soil by rhizobia as saprophytes in competition with other endogenous microbes which is also associated with soil infertility and extremes of temperature,
B. Rapid colonization of the rhizosphere prior to root infection and genetic compatibility between host and root nodule bacteria to establish an effective nodule, and
C. A favorable environment to allow maximum fixation.

In addition to the competitiveness of the rhizobia in forming nodule and the effectiveness of the rhizobium-host plant to fix N_2, a series of edaphic, chemical and biophysical factors affects N_2 fixation (Montanez, 2000). In the Rhizobium-legume symbiosis, N_2 fixation is strongly related to the physiological state of the host plant (Zahran, 1999). All environmental limitations that adversely affect plant growth and vigor also influence the symbiosis (Giller, 2001). Plants are highly dependent on physical and chemical conditions of the soil to give their natural potential for growth and nitrogen fixing activity (Negales *et al*., 2002; Zahran, 1999). As a result, an efficient rhizobial strain is not expected to express its full capacity for nitrogen fixation, if limiting factors imposed on the vigor of the host legume (Negales *et al*., 2002; Zahran, 1999).

Environmental factors can influence the ratio between rhizobia in the soil and rhizospehere microorganisms, and can also influence other steps in the nodulation process such as attachment, infection and nodule formation (Al-Falih, 2002). There are several environmental stresses (factors) that influence the legumes and the symbiotic partners, (Rhizobium) and the symbiosis which are soil acidity, drought, temperature, salinity, water stress, unfavorable soil pH, nutrient deficiency, insufficient or excess soil moisture, energy and carbon sources (Zahran, 1999).

3. 7.1. Soil PH

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity (Bordeleau and Prevost, 1994; Correa and Barneix, 1997). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic nitrogen fixation (Bordeleau and Prevost, 1994).

Acid soil conditions pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson, 1991). Legume and their rhizobia exhibit varied response to acidity. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within a species (Vargas and Graham, 1998). The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan, 1984) and a relatively few rhizobia grow well at pH less than 5.0 (Graham et al., 1994). Soil acidity constrains symbiotic N_2 fixation in tropical and temperature soils, limiting Rhzobium survival and persistence in soils and reducing nodulation (Graham et al., 1982).

Acidity affects early steps in the infection process including the exchange of molecular signals between symbiotic partners and attachment to the roots (Hungria and Vargas, 2000). Release of nod-gene inducers by soybean and common bean roots is less at pH 4.5 than at pH 5.8 (Hungria and Stancey, 1997), with some nodulation genes including nod A, switched off as the pH falls (Richardson *et al.,* 1988). Low pH can affect the production and exeretion of nodulation factors (Mcklay, 1993). Low pH is often associated with increase in Al and Mn toxicity and reduced Ca supply that affect the growth of rhizobia (Campo, 19995), of the host legume and symbiosis (Kim *et al*., 1985). The number of nodules, the nitrogenase activity, the nodule ultra-structure and the fresh and dry weights of nodules are affected to a greater extent at a low medium pH (< 4.5) (Vassileva *et al.,* 1997).

Tolerance to high alkalinity has also been observed for rhizobial strain isolates with tolerance up to pH 9.5 for rhizobiaceae (Jordan, 1984). Nour *et al*. (1994) found the pH tolerance of rhizobial strains isolated from chickpea (*Cicer arietinum*) to be 10.0 and Shenbagarathi (1993) reported that rhizobial strain SBS- R100 isolated form *Sesbania procumbens* is capable of growing at pH 11. Rhizobia appear to be more tolerant to alkalinity than do their legume plants (Zahran, 1999). Rao and Sharama (1995) reported that while germination of pigeon pea was decreased at pH values of > 8.8, growth of rhizobia was unaffected up to pH 11.5. Rhizobial strains isolated form *Seseania formasa, Acacia farnesiana* and *Dulbergia sissoo* were well adapted to grow on pH 12.0 (Surange *et al.,* 1997).

Rhizobia with a higher tolerance to acidity have been identified (Graham *et al*., 1982). Aarons and Graham (1991) reported high cytoplasmic potassium and glutamate levels in acid-stressed cells of *Rhizobium leguminosarum* are linked with low pH tolerance. Furthermore, differences in LPS composition, proton exclusion and extrusion (Chen *et al*.; 1993), accumulation of cellular polyamines (Fujihara and Yoneyama, 1993), and synthesis of acid shock proteins (Hickey and Hirshfield, 1990) have been associated with the growth of cells at acid pH.

The composition and structure of outer membrane could also be a factor in pH tolerance (Graham *et al*., 1994). At least two loci of either megaplasmid or chromosomal location for pH genes are necessary for the growth of rhizobia at low PH (Chen *et al*., 1993). The expressions of one membrane proteins of 49.5 KDa and three soluble proteins of 66.0, 85.0, and 44.o KDa increased when the cells of acid-tolerant strains were grown at pH 4.0 (correa and Barneix, 1997). Soil acidity can be corrected by different practical measures. Two strategies that have been adopted to solve the problem of soil acidity are liming the acidic soil to ameliorate the effects of acidic conditions and selecting and isolating tolerant strains of rhizobia and plant cultivars to increase plant production on acidic soils (Graham, 1992; Bordeleau and prevost, 1994; Zahran, 1999).

3. 7.2. Salt stress

Salinity is a serious threat to agriculture in arid and semiarid regions (Zahran, 1999). Nearly 40% of the world's land surface can be categorized as having potential salinity problems (Cordovilla *et al.*, 1994). Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate, 1995). Salt tolerance in plants is a complex phenomena that involves morphological and developmental changes as well as physiological and biochemical processes. Salinity decreases plant growth and yield, depending up on the plant species, salinity levels and ionic composition of the salts (Delgado *et al*., 1994).

The response of legumes to salinity causes depends on such factors as climatic conditions, soil properties and the stage of growth and crop cultures (cordovilla *et al.*, 1995). Zahran (1999) and (Cordovilla *et al.*, 1995) reported variability in salt tolerance among legumes such as *Phaseolus vulgaris, Vicia faba, Glycine max*, alfalfa, clover and soybean. Legumes are generally more sensitive to osmotic stress than their microsymbionts are (Zahran, 1999). 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, 2002). According to Bordeleau and Prevost (2002) salt tolerant legumes include alfalfa and narrow- leafed lupins (*L. angustifolius*) that can grow in salinities equivalent to seawater.

Salt stress result in growth depression which can be attributed to the accumulation of toxic ions such as sodium and chlorine in plant tissue, where they can disturb enzyme activities (Bordeleau and Prevost, 2002). Increase in salt concentration affect microbial population, plant growth and yield as the result of inhibition of rhizobia-legume symbiosis, nodule initiation and nodule formation (Zahran, 1999). In addition, constraints of salt stress causes nutrient imbalance such as potassium, iron, boron and calcium when nodulated *pisum sativum* were analyzed (El-hamdaoui *et al.,* 2003). The reduction on nitrogen fixing activity by salt stress is usually the result of reduction in respiration of nodules and in cytosolic protein production particularly leg-hemoglobin by nodules.

Rhizobial cells subjected to high salt stress change their morphology (Zahran, 1999). The cell ultrastructure is severely affected and the cell envelop is distorted. These alterations affect the hosts and the symbiotic interactions that lead to changes in lipopolysaccharides (LPS) and surface antigens; which are the major determinants for adaptation and symbiosis (Zahran, 1999). These structures affect the capacity of rhizobium to infect and form effective nodules and hence the amount of nitrogen fixed per unit weight of nodules with salt stress (El-Shinnawi *et al*., 1989; Miller and Wood, 1996).

Rhizobial cells have various mechanisms for adapting high salt-stress conditions. Rhizobial adaptations to salt-stress are attributed to the accumulation of intracellular low molecular weight organic solvents (Osmolytes) such as glutamate, proline, glycine-betaine, glycine amino acids, ectoine, amide, polyoles called osmotically active solutes (Zahran, 1999). The accumulations of osmolytes (Osmotic adaptations) are thought to counteract the dehydration effect of low water activity in the medium but not to interfere with macromolecleular structure or function. They are involved in osmoregulation and prevention of cytoplasmic dehydration in saline environments (Bostford and Lewis, 1990; Miller and Wood, 1996). The disaccharide trehalose plays a role in osmoregulation when rizobia are growing under salt or osmotic stress (El-Sheikh and Wood, 1990). Trehalose accumulates to higher levels in cells of *R. leguminosarum* and peanut rhizobia under the increasing osmotic pressure of hyper salinity (Gittoni and Bueno, 1996).

3. 7.3. Temperature

Rhizobial species as other organisms do have their own range of optimum temperature for survival and growth. However; this optimum temperature differs from species to species. For most rhizobia, the optimum temperature range for growth in culture is 28 to 31^0C (Graham, 1992). Rhizobia isolated from nodules of *Acacia senegal* and *Proscopis chilensis,* growing in hot dry regions of Sudan, has had high maximum growth temperatures (44.2^0c) (Zahran *et al*., 1999; Zhang *et al.,* 1991). Hangar and Vargas (2000) have reviewed that the upper limits for rhizobial growth range between 32 and 47^0c although tolerance varies among species and strains. The upper limits for N_2 fixation in tropical legumes vary between 27 and 40^0c (Gibson, 1971). It is also reported that Strange *et al*. (1997) has isolated highly temperature (50^0c) tolerant strains of rhizobium nodulating trees from tropical soils.

High temperature affects the growth and survival of rhizobia (Zahran, 1999) and nodulation by influencing adherence, penetration of bacteria to form infection-thread (Vincent, 1980; Hungria and Vargas, 2000). It also influences nitrogen fixation by decreasing leg-hemoglobin synthesis, nitrogenase activity and allocation of fixed nitrate in nodules (Hungaria *et al*., 1989; Bordeleau and Prevost, 1994). Furthermore, high temperature accelerates nodule senescence (Hungria and Franco, 1993). The remedy is administration of inoculum in deeper soils and application of surface mulch to reduce soil temperature (Roughley, 1980). Likewise, it was also found that low temperature decrease nodulation and nitrogen fixation (Waughman, 1997).

The over all detrimental effects that come as a result of high temperature are due to physiological and genetic modifications in bacteria, including plasmid deletion and genomic rearrangement. Although, heat shock proteins have been found in rhizobium (Aarons and Graham, 1991), they cannot be produced during salt and osmotic stress (Zharan *et al*., 1999), which indicates that it is a specific response to heat stress.

3. 7.4. Water Stress

The survival and activity of micro-organisms may depend on their distribution among microhabitats and changes in soil moisture. Low water activity affects the legume cultivar, the micorsymbiont and the symbiosis. One of the immediate responses of rhizobia to water stress (low water potential) concerns the morphological changes. The modification of rhizobial cells by water stress will eventually lead to a reduction in infection and nodulation of legumes (Zahran, 1999). Water stress limits rhizobial survival, growth and their population structure in soil (Zahran, 1999; Hangria and Vargas, 2000).

Chemo tactic movement of rhizobia is influenced if there is no enough film of moisture in the medium (Bordeleau ad Prevost, 1994, Zahran, 1999) and they respond to low water activity by morphological changes (Zahran, 1999). This alteration together with low mobility results in low rhizosphere colonization and low infection of root hairs (Roughley, 1980). Furthermore, reduction in soil moisture affects formation and longevity of nodules and leghemoglobin synthesis (Hangria and Vargas, 2000). In general, nodule initiation, growth and activity are very sensitive to water (Zahran, 1999). Moreover, severe stress may lead to irreversible cessation of N_2 fixation (Vincent, 1980).

Water stress reduces both N_2 fixation and respiration of nodules by influencing legumes to produce short and stubby roots which are not suitable for infection by rhizobia. The effect of water stress on vegetative growth is more detrimental to nodulation and nitrogen fixation than during reproduction stage (Zahran, 1999). Sensitivity to moisture varies for a variety of rhizobial strains and different legume cultivars (Bordeleau and Prevost, 1994; Zahran, 1999). Water logging in the rhizospere exerts anaerobic condition which results in the build up of carbondioxide and ethylene reduction which thus restrict nodulation and nitrogen fixation (Bordeleau and Prevost, 2000).

Drought tolerant N_2 fixing legumes can be selected although the majority of legumes are sensitive to drought stress. Several mechanisms have been suggested to explain the various physiological responses of several legumes to water stress (Zahran, 1999). The legume with a high tolerance to water stress usually exhibit osmotic adjustment; this adjustment is partly accounted for by changing cell turgore and by accumulation of some osmotically active solutes (Ford, 1984). The accumulation of specific organic solutes (osmotica) such as proline, free amino acids and low molecular weight solutes (pinitol) and potassium is a characteristics response of plants subjected to prolonged severe water stress (Hungria and Vargas, 2000; Zahran, 1999).

3.7.5. Carbon and energy sources

Energy is required for the reaction of N_2 fixation from the host plant. The rhizobia use photon assimilated carbon via the carbon cycle and generate energy as ATP. The functioning of nodules depends directly on photosynthesis (Kouchi *et al*., 1986). As much as 25% of a legume's net photosynthate may be allocated below ground to support N_2 fixation (Atkins, 1984). Some of this carbon may be used directly to maintain nodule function while some is returned to the host plant in the form of carbon skeletons of the exported nitrogenous solutes (Crews, 2004). If the bacteroids face deprivation of their energy source (photosynthate), the proper functioning of the nodules becomes impaired.

3.7.6. Soil Nutrients

Soil salinity and acidity are usually accompanied by mineral toxicity (Specific ion toxicity), nutrient deficiency, and nutrient disorder (Zahran, 1999). In addition to their importance in the growth and survival of free-living rhizobia, some essential nutrients have been shown to have specific roles in the development and functioning of N-fixing symbioses. A group of essential nutrients Ca, P, Fe, and Mo are required at specific stages for the development of legume symbiosis and the symbiotic N fixation to the extent that their deficiencies limit the productivity of host legumes in some agricultural systems (Graham and Vance, 2000).

Phosphorous is one of several elements which affects N_2 fixation and along with N, it is a principal yield limiting nutrient in many regions of the world (Pereira and Bliss, 1989). Phosphorous appears essential for both nodulation and nitrogen fixation (Pereira and Bliss, 1989). Nodules are strong links for phosphorous (P) and range in P content from 0.72 to 1.2% (Hart, 1989); consequently, N_2 fixation dependent plants will require more of this element than those supplied with combined nitrogen (Cassman, 1981). Nodulation, N_2 fixation and specific nodule activity are directly related to the P supply (Singleton *et al.*, 1983). Phosphorous is required for signal transduction, membrane biosynthesis, nodule development and function (Israel, 1987) and nitrogenase activity (Al- Niemi *et al.*, 1997). Adaptive mechanisms of legumes are different to acquire P under phosphate limiting conditions in soil ranging from acidification of the rhizosphere, exudation of acid phosphase, changes in root architecture, enhance P transport and to mycorhizal symbiosis (Johnson *et al*., 1996).

Calcium deficiency has specific effects on the legume symbiosis during the pre-infection and nodule development stages (O. Hara *et al.*, 1988). Calcium has been suggested to have role in maintaining cell wall rigidity specifically, in lipopolysaccharides (LPS) structure (Ballen *et al.*, 1998) and expression of outer membrane proteins. Since the cell surface component of rhizobia are calcium dependent, attachment of rhizobia to root hair, appearance of nodule and nodulation can be affected by calcium deficiency (Caetano-Anollen *et al.*, 1989; Graham, 1992).

Molybdenum has a major role in symbiotic N fixation as a fundamental component for nitrogenase. Mo deficiency affects nodule development and nodule functioning by reducing bacteroid replication and delaying or preventing the onset of nitrogenase activity (Jongruaysup *et al.*, 1997).

Iron is required for legume nodulation, possibly for the proliferation of the infecting rhizobia in the host tissue (Tang *et al*., 1992). Lupines are sensitive to Fe deficiency at an early stage of nodule initiation (Tang *et al.*, 1992) whereas; peanut seem to be sensitive to Fe stresses at latter stages in nodule development (O'Hara *et al.*, 1988). The source of iron from seed reserves or supplied from soil, during nodule development may be an important factor in the efficiency of transfer of Fe from host tissues to the infecting rhizobia (O'Hara *et al*., 1993).

Regulation of dinitrogen fixation in response to available fixed nitrogen is mediated by the host legume rather than the bacterial symbiont. The soil nitrate will also inhibit or regulate the nitrogen fixation in three ways; low concentration of nitrate (1-2mm) actually promote nodulation by ensuring early rapid growth of the plant and development of a healthy root system that is able to nodulate, the intermediate concentration of nitrate partially inhibit nodulation and the effect may be manifested in the developing nodules being smaller, such that the nodule mass per plant is reduced while the total number of nodules remain almost unaltered and if the concentration of nitrate is excess, the effect will be actual inhibition of fixation and inactive nodules because nitrate could be restricted to the cytoplasm of nodule cells and the nitrogenase activity decreased due to an increase in the resistance of oxygen diffusion barrier (Arrese-Ignor *et al*., 1997).

4. Materials and methods

4. 1. Study sites

The sampling area covers 47 sampling sites from Kutaber, Legehida, Wereilu, and Jamma woredas (South Wollo) and 21 sampling sites from Gindeberet, Jeldu, and Dendi woredas (West Shoa). The nodule sample collection was done from these areas where grass pea has been growing for a long time with no earlier history (legend) of inoculation with rhizobia. The root nodules were collected at the end of October and at the beginning of November, 2008 during their flowering stage. The study sites are indicated in Figure 2 and Table 2.

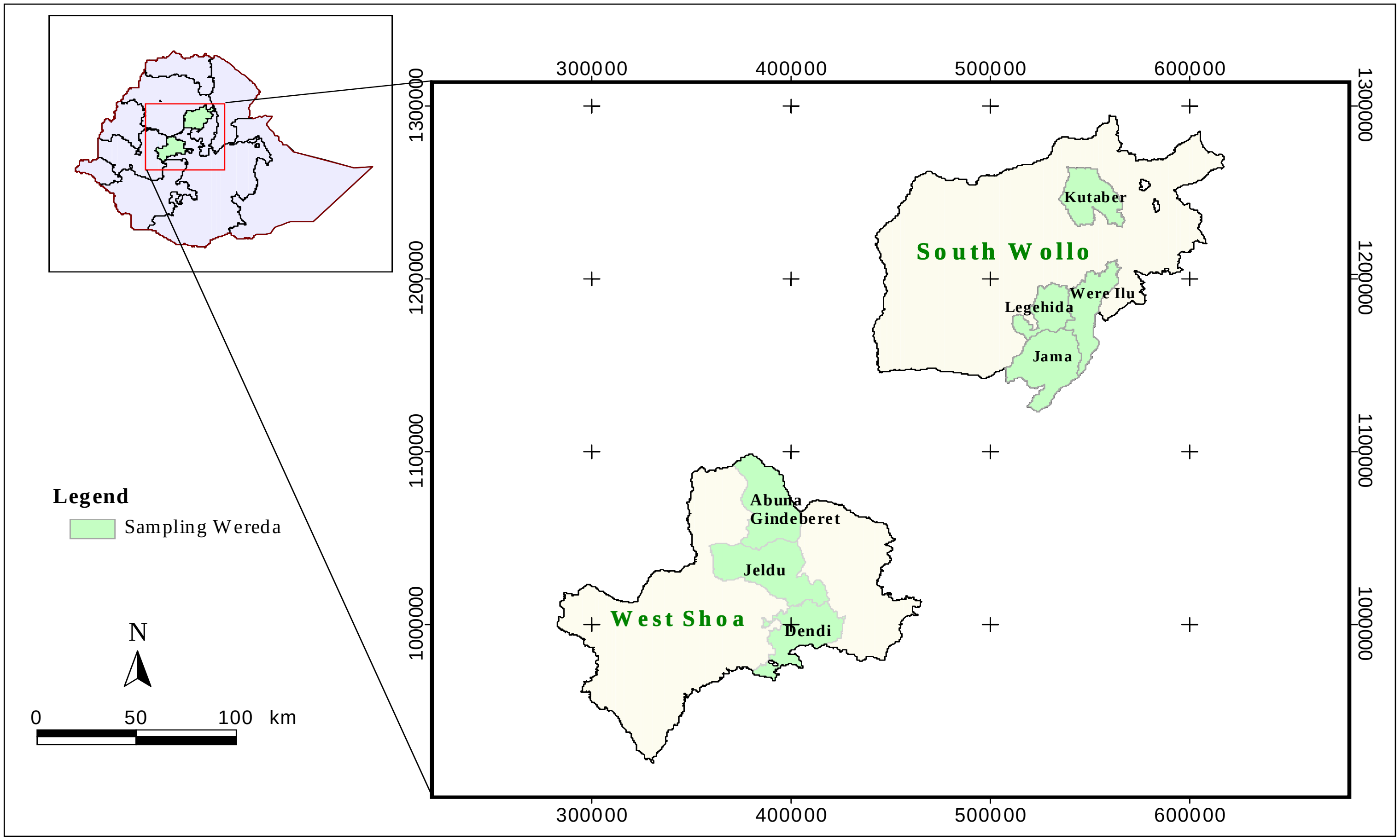

Fig.2. Location map of the study areas

4. 2. Nodule collection and isolation of rhizobia.

4. 2. 1. Nodule collection

After the study sites were selected, nodule samples were collected from the farmer’s field randomly. The nodules were immediately kept in sealed vials containing a desiccant (Silica gel) covered with 1cm of cotton wool (Somasegaren and Hoben, 1994) till isolation of the rhizobia was undertaken.

4. 2. 2. Isolation of Rhizobia

Dehydrated or desiccated root nodules were immersed in sterile distilled water for overnight in labeled sets of peri-dishes (Vincent, 1970). The imbibed nodules were surface sterilized according to Somasegaren and Hoben (1994). They were first subjected to 70% ethanol for 10 seconds and then to 5 % (v/v) solution of sodium hypochlorite (NaoCl) for 4 minutes. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals.

The surface sterilized nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85%NaCl) inside a laminar air flow hood. A loopful of suspensions (crushed nodule saps) were streaked across the surface of Yeast Extract Mannitol Agar (YEMA) plates containing 25 ppm of congored (YEMA-CR) and incubated at 28 ± 2^{0}C fro 3-5 days.

The YEMA-CR medium comprises of the following components (g / l):

Mannitol ---------------------------------10g
K_2 $HP0_4$ ---0.5g
$Mg\ So_4.\ 7H_2O$----------------------------0.2g
NaCl ---------------------------------------0.1g
Yeast extracts ------------------------------1g

Agar --------------------------------------15g

Congored ----------------------------------10ml

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

They were autoclaved for 121^0C for 15 minutes.

Source: Somasegaren and Hoben (1994).

Table 2. The study area of nodule collection

Isolates	Zone	Woreda	Kebele	Soil PH
AAUGR1	South Wollo	Kutaber	Chistiane	6.3
AAUGR2	South Wollo	Kutaber	Gono	6.1
AAUGR3	South Wollo	Kutaber	Jamlako	6.5
AAUGR4	South Wollo	Kutaber	Gono	6.4
AAUGR5	South Wollo	Kutaber	Ruga	6.6
AAUGR6	South Wollo	Kutaber	Dihamatebia	6.8
AAUGR7	South Wollo	Kutaber	Boru	6.7
AAUGR8	South Wollo	Kutaber	Kemmeda	6.5
AAUGR9	South Wollo	Legehida	Teftef	6.6
AAUGR10	South Wollo	Legehida	Aley	6.2
AAUGR11	South Wollo	Legehida	Sirgut	6.1
AAUGR12	South Wollo	Legehida	Adalo	6.4
AAUGR13	South Wollo	Legehida	Mendel	6.3
AAUGR14	South Wollo	Legehida	Godo	6.7
AAUGR15	South Wollo	Legehida	Nifas Amba	6.8
AAUGR16	South Wollo	Legehida	Guramba	6.9
AAUGR17	South Wollo	Legehida	Tatity	7.1
AAUGR18	South Wollo	Legehida	Ablko	6.5
AAUGR19	South Wollo	Legehida	Shefait	6.2
AAUGR20	South Wollo	Legehida	Zerkamy	7.2
AAUGR21	South Wollo	Legehida	Liguama	6.2
AAUGR22	South Wollo	Legehida	Shikif	6.7
AAUGR23	South Wollo	Woreilu	Amayo	6.5
AAUGR24	South Wollo	Woreilu	Abewold	6.6
AAUGR25	South Wollo	Woreilu	Gurachalie	6.9
AAUGR26	South Wollo	Woreilu	Tulumojo	6.8
AAUGR27	South Wollo	Woreilu	Gerbo	6.5
AAUGR28	South Wollo	Woreilu	Mechela	6.8
AAUGR29	South Wollo	Woreilu	Dereba	6.7

AAUGR31	South Wollo	Woreilu	Geshober	6.6
AAUGR32	South Wollo	Woreilu	Kabie	6.5
AAUGR33	South Wollo	Woreilu	Gebrelu	6.9
AAUGR34	South Wollo	Woreilu	Korkie	7.1
AAUGR35	South Wollo	Woreilu	Dolu	6.9
AAUGR36	South Wollo	Woreilu	Tulu	6.7
AAUGR37	South Wollo	Woreilu	Chifa	6.8
AAUGR38	South Wollo	Jamma	Soramicha	7.0
AAUGR39	South Wollo	Jamma	Ketari	7.1
AAUGR40	South Wollo	Jamma	Faji	6.8
AAUGR41	South Wollo	Jamma	Kubi	7.1
AAUGR42	South Wollo	Jamma	Jarso	7.2
AAUGR43	South Wollo	Jamma	Boren	7.1
AAUGR44	South Wollo	Jamma	Yedo	7.0
AAUGR45	South Wollo	Jamma	Amuma	7.2
AAUGR46	South Wollo	Jamma	Shilafaf	7.1
AAUGR47	South Wollo	Jamma	Aejerti	6.9
AAUGR48	West shoa	A/Gindeberet	Berbabo	6.8
AAUGR49	West shoa	A/Gindeberet	Mendida	6.6
AAUGR50	West shoa	A/Gindeberet	Korkie	6.9
AAUGR51	West shoa	A/Gindeberet	Kersa	6.7
AAUGR52	West shoa	A/Gindeberet	Gitre	6.5
AAUGR53	West shoa	A/Gindeberet	Bekekelate	6.6
AAUGR54	West shoa	A/Gindeberet	Erjajo	6.7
AAUGR55	West shoa	Jeldu	Gojo	7.2
AAUGR56	West shoa	Jeldu	Tulubultuma	6.7
AAUGR57	West shoa	Jeldu	Sunko	6.8
AAUGR58	West shoa	Jeldu	Felo	6.7
AAUGR59	West shoa	Jeldu	Chilankie	6.8
AAUGR60	West shoa	Dendi	Golelo Bolo	6.7
AAUGR61	West shoa	Dendi	Awash Bolto	6.8
AAUGR62	West shoa	Dendi	Denbi	6.7
AAUGR63	West shoa	Dendi	Ubdo legabatu	6.8
AAUGR64	West shoa	Dendi	Jemiem legabaut	6.9
AAUGR65	West shoa	Dendi	Ababor	6.7
AAUGR66	West shoa	Dendi	Awaro kolo	6.8
AAUGR67	West shoa	Dendi	Tulu meda	6.9
AAUGR68	West shoa	Dendi	Chonkie	6.9

4. 3. Purification of isolates

Single and seemingly different colonies were picked from plates with sterile inoculating loop and transferred into 6ml of sterilized YEM broth in test tubes. The test tubes were vortexed and placed on rotary shaker for 48 hrs at room temperature. After two days, a loop-full of culture suspensions from each test tube was taken and streaked on sterile YEMA plates and incubated at 28 ± 2^0C for 3-5 days. The purity and uniformity of the colony type was exhaustively tested through repeated re-streaking.

4. 4. Preservation of isolates

A single well isolated colony which was obtained after repeated purification was picked and streaked to YEMA slant containing 0.3% (w / v) $CaCO_3$ in a culture test tube and incubated at 28 ± 2^0C for 3 days. After sufficient growth was observed, the culture slants were then transferred to and preserved inside refrigerator adjusted at 4^0C (Vincent, 1970).

4. 5. Designation of the isolates

All the isolates were designated as AAUGR (Addis Ababa University Grass pea Rhizobia) with different numbers representing each strain.

4. 6. Seed samples

The seeds of variety “Wasse” with gray seeds were provided by Debrezeit Agricultural Research centre

4. 7. Authentication of the isolates and preliminary screening of their symbiotic effectiveness on pouch experiment

To identify the definitive test of all the rhizobial isolates, they were then screened for infectivity and effectivity using pouch. The pouches were washed, surfaced sterilized and autoclaved as indicated in Somasegaren and Hoben (1994).

The grass pea variety called “Wasse” was used for the greenhouse experiment. Gray undamaged seeds of uniform size were selected and surface sterilized and were rinsed in five changes of sterile water and placed to 0.75 (w/v) water agar plates. They were incubated at 28 ± 2^0C for 3 days. After three days, two germinated seeds were transferred into each of pouch for germination.

After 3 days, each seedling was inoculated with 1ml of 72 hr YEMA grown culture. The experiment was statistically laid out with three replications using a complete random design in a greenhouse with a 12hr. photoperiod and an average of 28^0C and 15^0C day and night temperature. Each block contained three pouches for a negative control devoid of both the chemical N-fertilizer (KNO_3) and the inoculum whereas, the other three contained positive control treated with 70mg/l nitrogen(0.05 KNO_3 (w/v))solution every week. All the pouches were fertilized with quarter strength of Broughton and Dilworth N-Free medium per week as described in Somasegaren and Hoben (1994) Table 3.

Table 3. N-Free nutrient solution (Brouthton and Dilworth, 1970)

Stock solution	Chemical	G / liter
1	$Ca\ Cl_2.2H_2O$	294.1
2	$K\ H_2\ PO_4$	136.1
3	$Fe\ C_6\ H_5O_7.\ 3H_2O$	6.7
	$Mg\ SO4.\ 7H_2O$	123.3
	$K_2\ SO_4$	87.0
	$Mn\ SO_4.\ H_2O$	0.33
4	H_3BO_3	0.247
	$ZnSO_4.\ 7H_2O$	0.288
	$CU\ SO4.\ 5H_2O$	0.100
	$CoSO_4.\ 7H_2O$	0.056
	$Na_2MoO_2.\ 2H_2O$	0.048

Source: Adapted form Somasegaren and Hoben (1994)

4. 8. Relative effectiveness of the isolates

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

$$\%SE = \frac{\text{Inoculated plant DM}}{\text{N – Fertilized plant DM}} \text{ X } 100$$

Where, DM = dry matter, N= nitrogen, SE= symbiotic effectiveness

The rate of nitrogen fixing effectiveness is evaluated as: Highly effective > 80%, Effective 50-80%, Lowly effective 35-49% and Infective <35%.

4. 9. Characterization of isolates

4.9.1. Colony morphology

The morphological characteristics of the isolates were determined according to Lupwayi and Haque (1994). A loopful of rhizobial isolates from 48 hrs old broth culture was inoculated by streak plating onto YEMA and incubated at 28 ± 2^0C for 3-5days. After 5days, colony diameter and morphology, colony texture were recorded as large mucoid (LM), large watery(LW), Elastic and buttery respectively as indicated in Martinez-Romero *et al.*, (1991).

4. 9.2. Acid-base production

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

4. 9.3. Determination of Growth rate

Each isolate was inoculated into a 10ml YEM broth (YEMB), vortex-dispersed and shaken on orbital shaker at 120 rev. min^{-1} for 48 hrs at room temperature. Then, half ml of each broth culture (cell suspensions) was inoculated into 50ml sterilized YEM broth in 125ml Erlenmeyer flask and kept on orbital shaker at a rev. of 120min^{-1}. Turbidity was measured by taking an optical density (OD540nm) reading of the YEM broth cultures just at the time of inoculation (0hr) and every 6hrs interval by using spectrophotometry (Jenway, 6405 UV/ VIS 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 dispensed on to sterilized YEMA plates and spread by using alcohol flamed hockey-like glass rod spreader to determine the colony forming units (CFU) (Somasegaren and Hoben, 1994). Mean generation (doubling) time was calculated from the logarithmic phase (White, 1995).

4. 10. Biochemical and physiological tests

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

4. 10.1. 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, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11.0, and 12.0 as described by Bernal and Graham (2001).

4. 10.2. 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 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12% and 13% of NaCl as indicated in Bernal and Graham (2001).

4. 10. 3. 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, 10^0C, 15^0C, 20^0C, 25^0C, 30^0C, 35^0C, 40^0C, 45^0C and 50^0C as indicated in (Lupwayi and Haque, 1994).

4. 10.4. Intrinsic antibiotic resistance

The resistance of the isolates to intrinsic antibiotics 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 Hagqe (1994). The antibiotics were Streptomycin, Erythromycin, Ampicillin, Chloroamphinicol, and Nalidixic acid. Each antibiotic was used at a concentration of 2.5 µg/ml, 5 µg/ml, 10 µg/ml. Streptomycin and erythromycin were dissolved in ethanol whereas, the other three were dissolved in sterile water. The stock solution of each antibiotic was prepared by dissolving 2 g of it in 100ml of water. The required concentrations were then added to the media aseptically with single pipette for each antibiotic. If the required concentration is 10 µgml,$^{-1}$ 0.05ml stock was added to 100ml YEMA.

4.10.5. Carbohydrate utilization

The carbohydrate requirement of the rhizobial isolates was evaluated on 16 carbon sources: fructose, mannitol, sucrose, lactose and glucose that are heat resistant carbohydrates whereas, sorbitol, arabinose, galactose, xylose, maltose, mannose, cellulobiose, cellulose, starch, Na-citrate and Trehalose that are of heat liable carbon sources (Somasegaren and Hoben, 1994).

One gram (1g) carbohydrate from each heat stable and heat liable was dissolved in 10ml of distilled water and was kept in refrigerator. Basal media (carbohydrate free media containing the following ingredients (g/l) was also prepared :

- K_2 HPO_4---------------------------1 g
- K H_2PO_4---------------------------1 g
- Fe $Cl_3.6H_2O$------------------------0.01 g
- $MgSO_4.7H_2O$----------------------0.2 g
- $CaCl_2$-------------------------------0.1 g
- $(NH_4)_2SO_4$-------------------------1 g
- Agar --------------------------------15 g

The heat stable carbohydrate sources were autoclaved together with the basal media. The heat liable carbon sources were first filter sterilized using sterile 0.22µm disposable membrane filters. They were then added to the autoclaved and cooled basal media. 48 hrs old rhizobial broth cultures were inoculated on to the basal media containing the different carbon sources.

4. 10. 6. Amino acid utilization

Different types of amino acids which include L-alanine, L-arginine, L-asparagine, L-glutamate, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-tryptophan and L-tyrosine were used to determine the ability of the isolates to utilize amino acids. These amino acids were added at a concentration of 0.5 g / l to a similar basal media used for carbohydrate sources 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). 48hrs old rhizobial suspensions were inoculated in to these basal media.

4. 11. Soil analysis

Soil sample for pot experiment was brought from Awash Bolto from where the nodules were collected. Then, the soil was analyzed to determine its pH, total nitrogen, organic carbon following the manual produced by Sahlemedhin Sertsu and Taye Bekele, (2002).

4. 12. Screening for symbiotic effectiveness of potted field soil

The effectiveness of seven (7) selected isolates (3 isolates from South Wollo and 4 isolates from West Shoa) was determined through pot experiment in a greenhouse. The soil was properly mixed, sieved and air–dried. 3 Kg of this soil was distributed to plastic pots. Grass pea “Wasse variety” was surface sterilized as before and rinsed in five changes of distilled sterilized water. Five ungerminated seeds were sown in each pot and later thinned down to three after germination for one week. After a week, each seedling was inoculated with 1 ml of each isolate grown for 72 hrs YEM broth. The experiment was set up in replicates under a greenhouse temperature of 19 ± 1^0C mean minimum and $30+/-1^0$C mean maxmum. The pots were arranged in complete random design with each block consisting of negative control (without N and uninoculated) and positive control (uninoculated but with N). The nitrogen fertilizer (KNO_3) was given at a concentration of 0.5 g / l per week until the plants were harvested. All the pots were watered every two days.

4. 13. Plant total nitrogen content analysis

The nitrogen content of the plant samples was evaluated using the modified Kjeldhal method (Bremer, 1965). The plant samples were grounded. At first 0.3 g of the ground sample was measured in 100 ml digestion tube for the analysis. A 0.5 g of mixture (10 g K_2 SO_4, 2 g $CuSO_4$. $5H_2O$ and 0.2 g Selenium) was added to the ground sample to be used as a catalyst. Sulfuric and salicylic acid mixtures at an amount of 7 ml were mixed to the ground sample and permitted to react for 30 minutes. 0.5 g of $NO_2S_2O_3.5\ H_2O$ was also added and shaken to react for five minutes. A blank consisting of 0.5 g salt mixture and 7 ml of sulfuric-salicylic mixture was also prepared. The digestion of the ground sample was undertaken at a temperature of 300^0C for 2hrs until the content turned to colorless. Then after, the sample was distilled by adding 75 ml of 40% NaoH and its nitrogen was collected with a flask containing 20 ml boric acid until the volume reached 110 ml. ultimately, the distillates become titrated using 0.1 N H_2SO_4 and the reading of the burette was recorded and the percentage nitrogen was calculated by:

$$\text{Nitrogen (\%)} = \frac{(V_1 - V_2) \text{ X N X } 0.014 \text{ X } 100}{S}$$

Where, V_1 = ml of titrant used for the sample
V_2 = ml of titrant used for the blank
N = Normality for the acid
S = weight of the plant material

4. 14. Numerical analysis

The isolates's phenotypic variability was analyzed with a computer cluster analysis using similarity coefficient. A denodrogram was constructed by the Unweighed Pair Group Method with the Average (UPGMA) clustering method using NTSYS-Pc version 2.1. The analysis was carried out on the basis of the parameters done during the physiological tests (Tolerance of Temperature, pH, salt, antibiotic resistance and utilization of amino acids and carbohydrates).

4. 15. Data analysis

Comparison between the treatments was done by one way analysis of variance (ANOVA) (Turkey's HSD test) using statistical program.

5. Result

5.1. Isolation and Authentication of rhizobia

A total of 68 Grass pea rhizobial bacteria were isolated from South Wollo (47 isolates) and West Shoa (21 isolates). In order to determine the capacity of all isolates to form nodules, they were re-inoculated on the host plant on sterile pouch. As a result, 63 out of 68 isolates formed nodules and were authenticated as root nodule bacteria by forming nodules. The remaining five isolates that failed to nodulate and authenticate were AAUGR (1, 5, 7, and 18) from South Wollo and AAUGR 65 from West Shoa .

5. 2. Characteristics of the isolates

5. 2. 1. Morphological and cultural characteristics

The different isolates showed variations in colony texture and diameter (Table 4). Accordingly, 22% of the isolates showed large watery colonies (LW) with extracellular exopolysaccharide production whereas, 78% of the isolates displayed large mucoid (LM) texture on YEMA media. The colony diameter of all the isolates was between 2.0mm and 6.0mm. The largest colony diameter of 6.0 was displayed by isolates AAUGR (35, 41and 47) from South Wollo and AAUGR (49 and 55) from West Shoa whereas, the smallest diameter of 2.0mm was exhibited by isolates AAUGR (21, 22, 29, 31, 34, and 37) from South Wollo and AAUGR(48, 54 & 66) from West Shoa. The colony texture was distributed evenly among the sampling sites. The number of isolates that showed large water (LW) was 5 and 3 from South Wollo and West Shoa, respectively. Whereas, those that displayed large mucoid (LM) were 14 isolates from each sampling region.

Table 4 – Growth characterization of the isolates

Isolates	**Collection sites**	**Colony (mm) diameter**	**Colony morphology**	Mean generation **time (MGT)**
AAUGR-4	SW	2.5	LM	1.34
AAUGR-9	SW	3.0	LM	1.36
AAUGR-19	SW	4.0	LM	2.03
AUGR-21	SW	2.0	LW	1.24
AAUGR-22	SW	2.0	LW	1.34
AAUGR-29	SW	2.0	LM	2.34
AAUGR-31	SW	2.0	LW	1.99
AAUGR-32	SW	5.0	LM	2.39
AAUGR-34	SW	2.0	LM	3.98
AAUGR-35	SW	6.0	LW	3.74
AAUGR-37	SW	2.0	LM	3.97
AAUGR-38	SW	3.0	LM	3.89
AAUGR-39	SW	4.0	LM	3.31
AAUGR-40	SW	5.0	LM	2.13
AAUGR-41	SW	6.0	LM	2.21
AAUGR-42	SW	4.5	LM	1.96
AAUGR-43	SW	3.0	LM	5.82
AAUGR-46	SW	4.0	LW	4.71
AAUGR-47	SW	6.0	LM	3.69
AAUGR-48	WS	2.0	LM	3.99
AAUGR-49	WS	6.0	LM	3.48
AAUGR-52	WS	5.5	LM	2.31
AAUGR-53	WS	4.0	LM	3.99
AUGR-54	WS	2.0	LM	3.85
AUGR-55	WS	6.0	LM	3.85
AUGR-56	WS	4.0	LM	3.82
AAUGR-57	WS	4.0	LM	3.41
AAUGR-58	WS	4.5	LM	3.11
AAUGR-59	WS	4.5	LM	2.33
AAUGR-60	WS	5.0	LM	2.57
AAUGR-61	WS	4.0	LM	3.97
AAUGR-63	WS	3.0	LW	2.99
AAUGR-64	WS	3.0	LW	6.12
AAUGR-66	WS	2.0	LW	3.91
AAUGR-67	WS	4.5	LM	3.49
AAUGR-68	WS	5.0	LM	3.41

WS= West Shoa, SW= South Wollo, LM= Large mucoid, LW= Large watery

5. 2. 2. Growth on YEMA-BTB and Mean generation time of the isolates

All the isolates changed the YEMA-BTB medium into yellow colour (data not shown). With regard to their growth rate, the isolates displayed different doubling times that ranges from 1.24 – 6.12 hrs (Table 4). Six isolates (17% of the isolates) that were all from South Wollo showed the fastest growth with doubling times less than 2 hrs. These isolates were AAUGR 4, AAUGR 9, AAUGR21, AAUGR 22, AAUGR 31 and AAUGR 42 with doubling time 1.34, 1.36, 1.24, 1.34, 1.99 and 1.96, respectively. The doubling time of 75% of the isolates (27 isolates) was between 2.0 - 4.0 hrs. The remaining 8% (three) of the isolates exhibited slow growth with doubling time of 4.71 – 6.12 hrs. Isolates AAUGR 43 and AAUGR 46 of South Wollo and isolate AAUGR 64 of West Shoa showed slow growth with doubling time of 5.82, 4.71 and 6.12 hrs, respectively.

5. 3. Eco-physiological characters

The isolates showed diversity in physiological tolerance to pH, salt, temperature, IAR, and utilization of carbohydrate and amino acid as substrates.

5. 3. 1. pH tolerance

All the isolates (100%) were found to tolerate pH 5.0 to pH 12 with abundant growth at pH 6-10 (Table 5). Likewise, all but five isolates from South Wollo and four isolates from West Shoa managed to grow on all the tested pH indicating that there are variations amongst the isolates based on the site (soil) of isolation. Isolates AAUGR (31, 37, and 41) from South Wollo and isolates AAUGR (48,49, 52, 58, 59, 61, and 67) from West Shoa showed abundant growth on all the tested pH values whereas, the isolates that were found to be sensitive at pH 4 and pH 4.5 were isolates AAUGR (34, 35, 38, 43, 46, 54, 57, 64, and 66). In general, 75% of the isolates managed to grow on all pH media.

Table 5 Tolerance of rhizobial isolates to different pH

Isolates	Site	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	11	12
AUGR-4	SW	+	+	+	+	++	++	++	++	++	++	++	++	++	++	++
AUGR-9	SW	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-19	SW	+	+	+	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-21	SW	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-22	SW	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-29	SW	+	+	+	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-31	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-32	SW	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-34	SW	_	_	++	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-35	SW	_	_	++	+	++	++	++	++	++	++	++	++	++	++	++
AUGR-37	SW	++	++	++	+	++	++	++	++	++	++	++	++	++	++	++
AUGR-38	SW	_	_	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-39	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-40	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-41	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-42	SW	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-43	SW	-	-	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-46	SW	-	-	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-47	SW	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-48	WS	++	++	++	+	++	++	++	++	++	++	++	++	++	++	++
AUGR-49	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-52	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-53	WS	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-54	WS	_	_	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-55	WS	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-56	WS	+	+	+	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-57	WS	_	_	+	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-58	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-59	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-60	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
AUGR-61	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-63	WS	+	+	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-64	WS	_	_	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-66	WS	-	-	+	+	++	++	++	++	++	++	++	++	++	+	+
AUGR-67	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AUGR-68	WS	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++
TOTAL		**27**	**27**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**	**36**
%		**75**	**75**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

- = No growth, + = Slight growth, ++= abundant growth

SW = South Wollo , WS = West Shoa

5. 3. 2. Salt tolerance

The isolates displayed differences in tolerance to grow on YEMA adjusted at different NaCl concentrations (Table 6). All the isolates (100%) grew at a salt concentration of 0.5%. There is a progressive decrease in tolerance ranging from 1%-13%. However, the number of isolates that managed to grow at NaCl concentration of 11, 12 and 13% displayed up to 25%, 17% and 11% of tolerance, respectively. The most tolerant isolates that were found to grow on all the tested salt media were AAUGR 37 from South Wollo and AAUGR 48, AAUGR 59, and AAUGR 61 from West Shoa. On the other hand, isolates AAUGR 9, AAUGR 22, AAUGR 32, AAUGR 43, AAUGR 46 and AAUGR 47 (South Wollo) and AAUGR 63, AAUGR 64, AAUGR 66, and AAUGR 68 from West Shoa were found to be within a narrow range of salt tolerance.

Table 6. Tolerance of rhizobia nodulating grass pea at different level of salt concentrations

Isolates	Site	0.5%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	13%
AAUGR-4	SW	+	++	++	+	+	+	+	-	-	-	-	-	-	-
AAUGR-9	SW	+	-	-	-	-	-	-	-	-	-	-	-	-	-
AAUGR-19	SW	+	+	+	+	+	+	+	+	+	+	+	+	-	-
AAUGR-21	SW	+	+	+	+	+	+	+	+	-	-	-		-	-
AAUGR-22	SW	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AAUGR-29	SW	++	++	++	+	+	+	+	+	-	-	-	-	-	-
AAUGR-31	SW	+	+	+	+	+	+	+	+	+	+	-	-	-	-
AAUGR-32	SW	++	++	++	+	+	+	+	+	-	-	-	-	-	-
AAUGR-34	SW	++	++	++	++	++	++	++	+	+	+	+	+	+	-
AAUGR-35	SW	+	+	+	+	+	+	+	-	-	-	-	-	-	-
AAUGR-37	SW	++	++	++	+	+	+	+	+	+	+	+	+	+	+
AAUGR-38	SW	++	++	++	++	++	+	+	+	+	+	+	-	-	-
AAUGR-39	SW	+	+	+	+	+	+	+	-	-	-	-	-	-	-
AAUGR-40	SW	++	++	++	+	+	+	+	+	+	+	-	-	-	-
AAUGR-41	SW	++	++	++	++	++	++	++	+	+	-	-	-	-	-
AAUGR-42	SW	+	+	+	+	+	+	+	-	-	-	-	-	-	-
AAUGR-43	SW	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AAUGR-46	SW	+	-	-	-	-	-	-	-	-	-	-	-	-	-
AAUGR-47	SW	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AAUGR-48	WS	++	++	++	+	+	+	+	+	+	+	+	+	+	+
AAUGR-49	WS	++	++	++	++	++	++	++	++	-	-	-	-	-	-
AAUGR-52	WS	++	++	++	++	++	+	+	+		-	-	-	-	-
AAUGR-53	WS	+	+	+	+	+	+	+	+	+	+	+	-	-	-
AAUGR-54	WS	+	+	+	+	+	+	+	+	+	+	+	+	-	-
AAUGR-55	WS	++	++	++	+	+	+	+	+	+	+	-	-	-	-
AAUGR-56	WS	+	+	+	+	+	+	+	+	+	+	-	-	-	-
AAUGR-57	WS	++	++	++	++	++	+	+	+	+	+	+	-	-	-
AAUGR-58	WS	++	++	++	++	++	++	++	+	+	+	+	+	-	-
AAUGR-59	WS	++	++	++	++	++	++	++	+	+	+	+	+	+	+
AAUGR-60	WS	+	+	+	+	+	+	+	+	+	+	+	-	-	-
AAUGR-61	WS	++	++	++	+	+	+	+	+	+	+	+	+	+	+
AAUGR-63	WS	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AAUGR-64	WS	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AAUGR-66	WS	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AAUGR67	WS	++	++	++	++	++	++	++	+	+	+	+	+	+	-
AAUGR-68	WS	++	+	+	+	+	+	+	+	-	-	-	-	-	-
TOTAL		**36**	**33**	**31**	**26**	**26**	**26**	**25**	**22**	**18**	**15**	**13**	**9**	**6**	**4**
%		**100**	**92**	**86**	**72**	**72**	**72**	**69**	**61**	**50**	**42**	**36**	**25**	**17**	**11**

- = No growth, + = Slight growth, ++= abundant growth , SW = South Wollo , WS = West Shoa

5. 3. 3. Temperature tolerance

The ability of the isolates to tolerate different incubation temperatures is shown in Table 7. All the tested isolates (100%) grew at temperatures between 15^{O}C and 35^{O}C. Likewise, 72%, 53% and 28% of the isolates were found to tolerate 40^{O}C, 45 ^{O}C and 50 ^{O}C, respectively. Whereas, 53% and 61% of the isolates were found to grow at 5 ^{O}C and 10 ^{O}C, respectively. The isolates that tolerated all the tested range of temperature (5-50 ^{O}C) include AAUGR19, 31, 35, 37 and 41 (South Wollo) and AAUGR 48, 55, 59, 61, and 67 from West Shoa. On the other hand, isolates that grew within narrow range of temperature (15-45) were AAUGR 9, 43, 46, and 47 (South Wollo) and AAUGR 53, 63, 64, and 66 (West Shoa).

Table 7. Tolerance of grass pea rhizobia to different levels of temperature

Isolates	Site	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50°C
AUGR – 4	SW	+	+	+	+	+	+	+	+	-	-
AUGR – 9	SW	-	-	+	+	+	+	+	+	-	-
AUGR –19	SW	+	+	+	+	+	+	+	+	+	+
AUGR –21	SW	+	+	+	+	+	+	+	-	-	-
AUGR –22	SW	+	+	+	+	+	+	+	-	-	-
AUGR –29	SW	+	+	+	+	+	+	+	-	-	-
AUGR –31	SW	+	+	+	+	+	+	+	+	+	+
AUGR –32	SW	+	+	+	+	+	+	+	-	-	-
AUGR –34	SW	-	-	++	++	++	++	++	++	+	-
AUGR –35	SW	+	+	+	+	+	+	+	+	+	+
AUGR -37	SW	+	+	++	++	++	++	++	++	+	+
AUGR –38	SW	+	++	++	++	++	++	++	++	+	-
AUGR –39	SW	-	+	+	+	+	+	+	+	+	-
AUGR –40	SW	-	+	++	++	++	++	++	-	-	-
AUGR -41	SW	+	+	++	++	++	++	++	+	+	+
AUGR –42	SW	+	++	++	++	++	++	++	+	+	-
AUGR –43	SW	-	-	+	+	+	+	+	+	-	-
AUGR –46	SW	-	-	+	+	+	+	+	+	-	-
AUGR –47	SW	-	-	+	+	+	+	+	+	-	-
AUGR –48	WS	+	+	+	+	+	+	+	+	+	+
AUGR –49	WS	++	++	++	++	++	++	++	-	-	-
AUGR –52	WS	++	++	++	++	++	++	++	++	+	-
AUGR –53	WS	-	-	+	+	+	+	+	+	-	-
AUGR –54	WS	+	+	+	+	+	+	+	+	+	-
AUGR –55	WS	++	++	++	++	++	++	++	+	+	+
AUGR –56	WS	+	+	+	+	+	+	+	+	+	-
AUGR –57	WS	-	-	+	+	+	+	+	+	+	-
AUGR –58	WS	-	++	++	++	++	++	++	+	+	-
AUGR –59	WS	+	++	++	++	++	++	++	++	+	+
AUGR –60	WS	-	+	+	+	+	+	+	+	+	-
AUGR –61	WS	+	+	+	+	+	+	+	+	+	+
AUGR –63	WS	-	-	+	+	+	+	+	-	-	-
AUGR –64	WS	-	-	+	+	+	+	+	-	-	-
AUGR –66	WS	-	-	+	+	+	+	+	-	-	-
AUGR –67	WS	++	++	++	++	++	++	++	++	++	+
AUGR –68	WS	-	-	+	+	+	+	+	+	+	-
Total		19	22	36	36	36	36	36	26	19	10
%		53	61	100	100	100	100	100	72	53	28

- = No growth, + = Slight growth, ++= abundant, SW = South Wollo, WS = West Shoa

5. 3. 4. Intrinsic antibiotic resistance

The isolates exhibited a wide range of resistance to different concentrations of different antibiotics (Table 8). All the isolates tolerated and grew at the lowest concentrations (2.5 μg ml^{-1}) in ampicillin, chloroamphenicol and Nalidixic acid. Three isolates from South Wollo (AAUGR 31, AAUGR 37 and AAUGR 42) and ten isolates form West Shoa AAUGR (48, 49, 55, 56, 57, 58, 59, 60, 61 and 67) were found to tolerate all the tested antibiotics at different concentrations. The most sensitive isolates that grew within a narrow range of antibiotic resistance include AAUGR (9, 21, 53, 63, 64 and 66). In general, 36 % of the isolates tolerated all the tested antibiotics at different concentrations whereas, 64 % showed fluctuation in range of tolerance. The antibiotic that showed an inhibitory effect against the rhizobial isolates was erythromycin at 10.0 μg ml^{-1} .

Table 8. Antibiotic resistance of isolates after 5-7 days of incubation

		Ampicillin			Chloro-amphenicol			Erythro-mycin			Nali-dixic acid			Strepto-mycin		
Isolates	Site	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
AAUGR–4	SW	+	+	-	++	++	-	+	+	+	+	+	-	++	++	++
AAUGR-9	SW	++	++	+	+	+	+	-	-	-	+	-	-	+	+	-
AAUGR-19	SW	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-
AAUGR 21	SW	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-
AAUGR 22	SW	++	+	-	+	-	-	+	+	+	+	-	-	++	++	++
AAUGR 29	SW	++	++	+	+	+	+	+	+	+	+	+	-	+	+	-
AAUGR 31	SW	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR 32	SW	+	+	+	++	++	+	+	-	-	++	++	+	+	-	-
AAUGR–34	SW	+	-	-	+	+	+	-	-	-	+	+	+	++	++	+
AAUGR–35	SW	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
AAUGR-37	SW	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–38	SW	++	++	+	+	+	+	+	-	-	++	++	+	+	+	+
AAUGR–39	SW	++	++	+	+	+	+	+	-	-	+	+	+	+	+	+
AAUGR–40	SW	++	++	+	+	+	+	+	+	+	++	+	-	++	++	+
AAUGR-41	SW	++	+	+	++	++	+	+	++	++	+	-	-	+	+	+
AAUGR–42	SW	++	+	+	++	++	+	+	++	++	++	++	+	+	+	+
AAUGR–43	SW	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-
AAUGR–46	SW	+	+	+	+	-	-	+	+	-	+	+	+	-	-	-
AAUGR–47	SW	++	+	+	+	+	+	+	-	-	+	+	-	+	+	+
AAUGR–48	WS	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–49	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–52	WS	++	++	++	++	++	++	++	+	-	+	+	+	+	+	+
AAUGR–53	WS	+	-	-	++	+	-	-	-	-	+	+	+	++	+	-
AAUGR–54	WS	++	+	+	++	++	++	++	++	-	+	+	+	+	+	+
AAUGR–55	WS	+	+	+	++	++	++	++	++	++	+	+	+	+	+	+
AAUGR–56	WS	+	+	+	+	+	+	++	++	++	+	+	+	+	+	+
AAUGR–57	WS	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–58	WS	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–59	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–60	WS	++	++	++	++	++	++	++	+	+	+	+	+	+	+	+
AAUGR–61	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–63	WS	+	+	++	+	-	-	-	-	-	+	+	-	+	-	-
AAUGR–64	WS	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-
AAUGR–66	WS	+	+	-	+	-	-	-	-	-	+	-	-	+	+	-
AAUGR–67	WS	+	+	+	++	++	++	++	++	+	+	+	+	+	+	+
AAUGR–68	WS	++	+	+	++	++	++	++	+	-	+	+	+	+	+	+
Total		36	32	28	36	30	26	30	26	21	36	32	25	30	26	22
%		100	89	78	100	83	72	83	72	58	100	89	69	83	72	61

- = No growth, + = Slight growth, ++= abundant growth, SW= South Wollo, WS= West Shoa

5. 3. 5. Utilization of carbon sources

All of the isolates were able to catabolize a large variety of carbon sources (Table 9). All the isolates (100%) were found to catabolize glucose, fructose, maltose, mannose, galactose, xylose, sucrose and mannitol. However, lactose, sorbitol, cellulobiose, arabinose, Na-citrate, trehalose, starch and cellulose were utilized by 94, 92, 89, 78, 69, 69, 69, and 67%, respectively. Isolates AAUGR 4, AAUGR 21, AAUGR 29, of South Wollo failed to grow on sorbitol whereas; all the rest grew very well. Fifteen isolates (42% of the isolates) were found to catabolize and grew on the basal medium containing all the 16 tested carbon sources while isolates AAUGR37, AAUGR 39, and AAUGR 40 of South Wollo and isolates AAUGR48, AAUGR 59, AAUGR 61 and AAUGR 67 of West Shoa showed abundant growth on all tested carbon sources. On the other hand, the isolates AAUGR 4 of South Wollo and AAUGR 49, AAUGR 52 and AAUGR 56 of West Shoa utilized less number of carbon sources (69%) out of the tested carbon sources.

Table 9. Grass pea rhizobial utilization to different carbohydrate sources

Isolates	Site	Glucose	Arabinose	Ffuctose	Sorbitol	Maltose	Mannose	Galactose	Lactose	Xylose	Sucrose	Cellulobiose	Manitol	starch	Na-citrate	cellulose	Trehalose
AAUGR–4	SW	++	+	+	-	+	+	+	-	+	+	+	+	+	-	-	-
AAUGR-9	SW	+	+	+	++	++	++	++	++	+	+	-	+	++	+	+	+
AAUGR-19	SW	+	++	++	++	+	++	+	++	+	+	+	++	-	-	-	+
AAUGR 21	SW	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR 22	SW	++	-	+	+	+	+	+	+	+	+	++	+	+	++	++	+
AAUGR 29	SW	+	+	+	-	+	+	+	+	+	+	++	++	++	-	-	-
AAUGR 31	SW	+	++	++	++	+	++	+	++	+	+	++	++	+	+	+	+
AAUGR 32	SW	+	+	++	++	+	+	+	+	+	+	-	+	+	++	-	-
AAUGR–34	SW	++	++	++	+	+	++	+	+	+	+	++	++	++	-	+	+
AAUGR–35	SW	++	++	++	++	+	+	+	+	+	+	+	+	-	+	+	+
AAUGR-37	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–38	SW	++	+	+	++	+	+	+	++	+	+	++	++	-	-	+	-
AAUGR–39	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–40	SW	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR-41	SW	++	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–42	SW	++	-	++	+	+	+	+	+	+	++	++	++	-	-	+	-
AAUGR–43	SW	+	++	++	+	+	+	+	++	+	+	+	+	-	+	+	+
AAUGR–46	SW	+	++	+	++	++	++	++	++	+	+	++	++	+	++	+	++
AAUGR–47	SW	+	++	+	+	+	+	++	++	+	+	++	++	++	++	++	++
AAUGR–48	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++
AAUGR–49	WS	++	-	+	++	+	+	+	+	+	+	++	++	-	-	-	-
AAUGR–52	WS	++	-	+	++	+	+	+	+	+	+	++	++	-	-	-	-
AAUGR–53	WS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUGR–54	WS	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
AAUGR–55	WS	++	-	++	+	+	++	+	++	++	+	++	+	-	-	-	+
AAUGR–56	WS	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
AAUGR–57	WS	+	+	++	+	+	++	+	++	+	+	++	+	+	++	+	+
AAUGR–58	WS	++	++	++	++	++	++	++	++	++	++	++	++	-	+	-	+
AAUGR–59	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–60	WS	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+
AAUGR–61	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–63	WS	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
AAUGR–64	WS	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
AAUGR–66	WS	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
AAUGR–67	WS	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AAUGR–68	WS	++	+	++	++	++	++	+	++	++	++	+	++	++	++	++	++
Total		36	28	36	33	36	36	36	34	36	36	32	36	25	25	24	25
%		100	78	100	92	100	100	100	94	100	100	89	100	69	69	67	69

- = No growth, + = Slight growth, ++= abundant growth, SW= South Wollo, WS= West Shoa

5. 3 6. Amino acid utilization

Almost all of the isolates were able to catabolize a large variety of nitrogen sources (Table 10). All the isolates (100%) utilized tyrosine, methionine, arginine and asparagine likewise, 92, 94, 94, 89, and 83% of the rhizobial isolates catabolized alanine, lysine, glutamate, leucine, tryptophan, respectively. Only 78% of the isolates utilized phenylalanine which was found to be the most resistant amino acids.

Fifty percent (50 %) of the isolates that include AAUGR (9, 29, 31, 32, 34, 37, 39, 40 and 41) of South Wollo and isolates AAUGR (48, 49, 57, 58, 59, 61, 64, 67 and 68) from West Shoa utilized 100% of the tested amino acids whereas, isolates AAUGR 42 of South Wollo and AAUG 66 of West Shoa were found to be much fastidious with the ability of catabolizing 70% of the tested amino acids.

Table 10. Utilization of grass pea rhizobia on different nitrogen sources

Isolates	Site	Alanine	Arginine	Asparagine	Gluta mate	Leucine	Lysine	Methionine	Phenylalanine	Tryptophane	Tyrosine
AAUGR–4	SW	+	+	+	+	+	+	+	-	+	+
AAUGR-9	SW	+	++	++	++	++	++	++	++	++	++
AAUGR-19	SW	+	+	+	+	+	+	+	-	+	+
AAUGR 21	SW	-	+	+	+	+	+	+	+	+	+
AAUGR 22	SW	++	++	++	++	++	-	++	++	++	++
AAUGR 29	SW	+	+	+	+	+	+	+	+	+	+
AAUGR 31	SW	++	++	++	+	++	++	++	++	++	++
AAUGR 32	SW	+	+	+	+	+	+	+	+	+	+
AAUGR–34	SW	++	++	++	+	+	+	+	++	++	++
AAUGR–35	SW	+	+	+	+	+	++	+	-	+	+
AAUGR-37	SW	+	++	++	+	+	+	++	+	++	++
AAUGR–38	SW	+	+	+	+	-	+	+	-	+	+
AAUGR–39	SW	++	++	++	++	++	++	++	+	++	++
AAUGR–40	SW	++	++	++	++	++	++	++	+	++	++
AAUGR-41	SW	+	+	+	++	+	+	+	+	+	+
AAUGR–42	SW	-	+	+	++	-	-	+	+	+	+
AAUGR–43	SW	+	+	+	-	+	+	+	+	+	+
AAUGR–46	SW	++	++	++	-	++	++	++	++	++	++
AAUGR–47	SW	++	++	++	+	++	+	+	+	-	+
AAUGR–48	WS	++	++	++	+	++	+	+	+	++	++
AAUGR–49	WS	+	+	+	+	+	+	+	+	+	+
AAUGR–52	WS	+	+	+	+	+	+	+	-	-	+
AAUGR–53	WS	++	++	++	+	-	+	+	-	+	+
AAUGR–54	WS	+	+	+	++	+	+	+	+	-	+
AAUGR–55	WS	+	+	+	+	+	+	+	+	-	+
AAUGR–56	WS	+	+	+	+	+	+	+	+	-	+
AAUGR–57	WS	++	++	++	+	++	+	+	+	+	++
AAUGR–58	WS	++	++	++	+	++	+	+	+	+	++
AAUGR–59	WS	++	++	++	+	++	+	+	+	+	++
AAUGR–60	WS	++	+	+	+	+	+	+	-	+	++
AAUGR–61	WS	++	++	++	++	++	++	++	++	++	++
AAUGR–63	WS	-	+	+	+	+	+	+	+	++	++
AAUGR–64	WS	++	++	++	+	++	++	+	+	++	++
AAUGR–66	WS	+	+	+	+	-	+	+	-	-	+
AAUGR–67	WS	+	+	+	++	+	+	+	+	+	+
AAUGR–68	WS	+	+	+	+	+	++	+	+	+	+
Total		33	36	36	34	32	34	36	28	30	36
%		92	100	100	94	89	94	100	78	83	100

- = No growth, + = Slight growth, ++= abundant growth, SW= South Wollo, West Shoa

In all the eco-physiological tests undertaken isolates AAUGR 37, 48, 59, 61 and 67 were found to be the most tolerant strains whereas, isolates AAUGR 43, 63, 64, and 66 were also found to be the most sensitive. Among the most tolerant and sensitive isolates, isolates AAUGR 37 and 43 were from the South Wollo whereas, all the rest were from West Shoa (Table 11).

Table 11. Summary of the most resistant and sensitive isolates to physiological characters

	Site	pH	NaCl% (0.5-13)	Temp. 5-50 ^{0}C	IAR utilization	Carbohydr-ate Utilization.	Amino acid utilization
Most tolerant isolates	SW	4,9,19,21, 22,29,31, 32,36,37, 38,39,40, 41,42,47	34,37,	19,31,37,	37,42	37,39,40	9,29,32,34, 37,39,40
	WS	48,49,52, 53,55,56, 58,59,60, 61 ,67,68	48,55,58,59, 61,67	48,55,59, 61,67	48,49,55,56,57, 58,59,60,61,67	48,59, 61,67	48,49,57, 58,59,61, 64,67,68
Most sensitive isolates	SW	34, 35, 38,43,46	9,22,31, 43,46	43,46,47	9,21	43	42
	WS	54,57, 64,66	63,64,66,68	63,64,66	53,63,64,66	49, 52, 52, 56	66

5. 4. Numerical analysis

The numerical analysis based on 76 phenotypic features allowed the grouping of all isolates in to four similarity groups plus one ungrouped at 50% relative similarity coefficient (Fig.3). Cluster I include 7 isolates AAUGR (4, 19, 29, 32, 35, 54, and 56) with two separated sub- clusters in which they were separated at 80% level of relative similarity. Cluster II again consist of 7 isolates AAUGR (38, 41, 42, 49, 52, 55 and 67) that displayed two sub–clusters at 70% level of relative similarity. Cluster III which is the biggest cluster encompassed 13 isolates AAUGR (9, 22, 31, 37, 39, 40, 47, 48, 57, 58, 60, and 61) that were separated further in to two sub-clusters at 60 % level of similarity. Cluster IV comprised of 6 isolates AAUGR (21, 43, 53, 63, 64, and 66) that were further divided in to two sub-clusters at 64% level of similarity. Isolate AAUGR 68 had been left out of the clusters at 50% level of similarity (Fig.3).

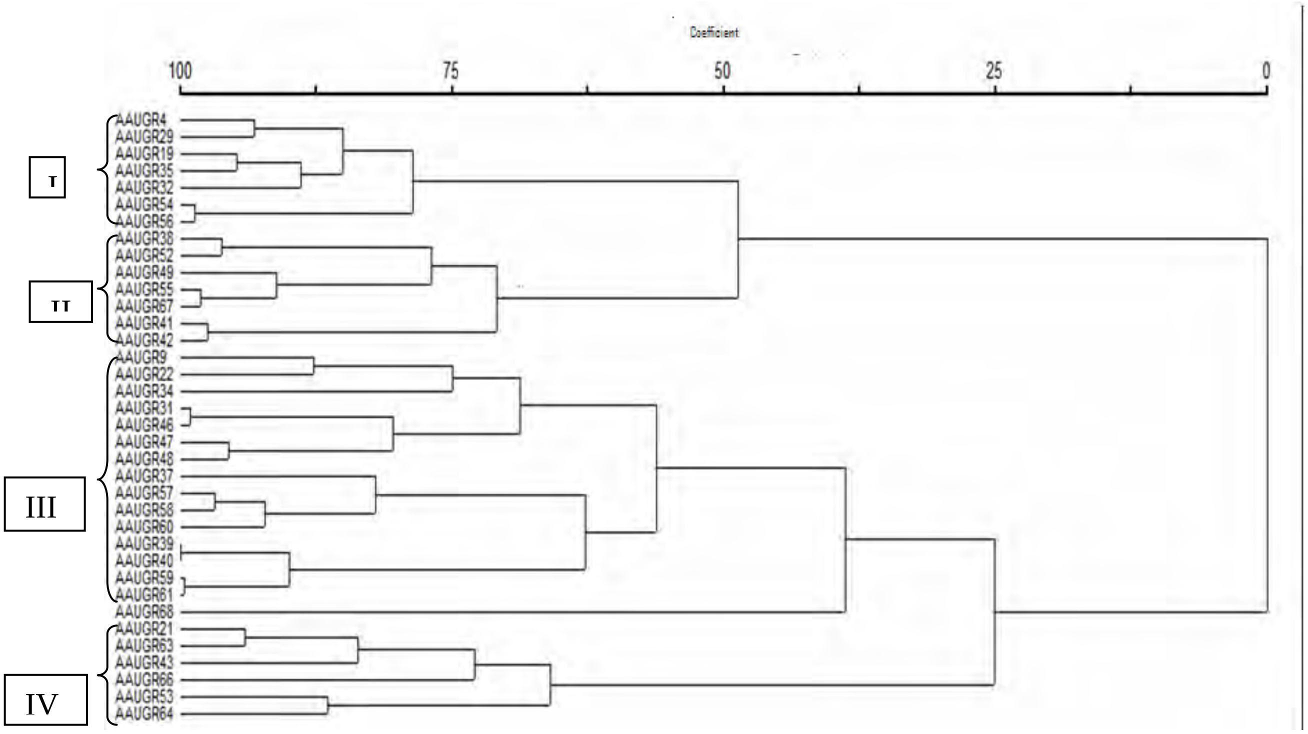

Fig.3. Dendrogram highlighting the phenotypic diversity of grass pea rhizobial isolates

5. 5. Soil analysis

The soil brought from Awash Bolto was analyzed for its pH, available phosphorous, total nitrogen and organic carbon (Table 12). The soil was almost neutral with lowest N (0.09) and relatively high P (12.88).

Table 12. Physical and chemical analysis of selected soils

parameters	Value
pH	7.1
Total nitrogen (%)	0.09
Organic carbon (%)	0.87
Available phosphorous (ppm)	12.88

5. 6. Symbiotic effectiveness test on pouch culture for preliminary screening

The pouch culture study on nodulation and symbiotic effectiveness of isolates showed marked variations among the isolates in nodule number, nodule dry weight and shoot dry weight at P=0.05(Table 13).

Table 13. Symbiotic effectiveness of isolates of grass pea rhizobia from different sampling sites

Isolates	Nodule number per plant	Nodule dry Wt. per plant(g)	Shoot dry Wt. per plant (g)	% SE	Effectiveness
AAUGR 4	35 ± 22ce	0.054 ± 0.005a-g	0.173 ± 0.047 a-d	68	E
AAUGR 9	24 ± 5c-e	0.047 ± 0.009 a-g	0.131 ± 0.089 b-d	52	E
AAUGR 19	36 ± 10c-e	0.040 ± 0.014b-g	0.144 ± 0.144b-d	57	E
AAUGR 21	38 ± 27c-e	0.035 ± 0.022c-g	0.133 ± 0.112b-d	52	E
AAUGR 22	33 ± 13c-e	0.027 ± 0.002 d-g	0.128 ± 0.047b-d	50	E
AAUGR 29	28 ± 6c-e	0.029 ± 0.016 d-g	0.145 ± 0.026b-d	57	E
AAUGR 31	90±22a-e	0.062 ±0.007a-f	0.266 ± 0.055 a-d	108	HE
AAUGR 32	59 ± 7a-e	0.047 ± 0.010 a-g	0.160 ± 0.006b-d	63	E
AAUGR 34	49 ± 10a-e	0.046± 0.007a-g	0.181± 0.095a-d	71	E
AAUGR 35	44 ± 8b-e	0.054 ±0.018a-g	0.152± 0.024b-d	60	E
AAUGR 37	135±57a	0.086±0.012a	0.296± 0.034a	120	HE
AAUGR 38	67±6a-e	0.036±0.003c-g	0.164± 0.042b-d	65	E
AAUGR 39	42 ± 25b-e	0.038±0.012b-g	0.143±0.126b-d	56	E
AAUGR 40	75 ± 18a-e	0.061 ± 0.012 a-g	0.169 ± 0.047a-d	67	E
AAUGR 41	83±27a-e	0.066 ± 0.018 a-f	0.264 ± 0.018a-d	104	HE
AAUGR 42	73±6a-e	0.059 ± 0.006 a-g	0.151 ± 0.077b-d	59	E
AAUGR 43	66 ± 6a-e	0.043 ± 0.016 a-g	0.160 ± 0.066b-d	63	E
AAUGR 46	62 ± 23a-e	0.065 ± 0.005 a-g	0.127 ± 0.064b-d	50	E
AAUGR 47	78 ± 23a-e	0.070 ± 0.017 a-d	0.216 ± 0.040a-d	85	HE
AAUGR 48	130±55ab	0.082±0.013ab	0.287 ± 0.062ab	116	HE
AAUGR 49	53 ± 6a-e	0.043 ± 0.005 a-g	0.215 ± 0.105a-d	85	HE
AAUGR 52	73 ± 8a-e	0.058 ± 0.005 a-g	0.220 ± 0.066a-d	87	HE
AAUGR 53	55 ± 37a-e	0.036 ± 0.012 c-g	0.198 ± 0.116a-d	78	E
AAUGR 54	26 ± 20c-e	0.025 ± 0.011 e-g	0.135 ± 0.074b-d	53	E
AAUGR 55	65 ± 28a-e	0.062 ± 0.012 a-f	0.167 ± 0.075a-d	66	E
AAUGR 56	48 ± 11a-e	0.041 ± 0.010 a-g	0.149 ± 0.073b-d	59	E
AAUGR 57	48 ± 4a-e	0.048 ± 0.008 a-g	0.168 ± 0.055a-d	66	E
AAUGR 58	68 ± 25a-e	0.035±0.028a-g	0.158 ± 0.033b-d	62	E
AAUGR 59	116±59a-c	0.075±0.013a-c	0.275 ± 0.047a-c	112	HE
AAUGR 60	57±16a-e	0.051 ± 0.001 a-g	0.249 ± 0.027a-d	98	HE
AAUGR 61	111±82a-d	0.073 ±0.011a-c	0.267 ± 0.048a-d	108	HE
AAUGR 63	73 ± 11a-e	0.059 ± 0.013 a-g	0.153 ± 0.067b-d	60	E
AAUGR 64	45 ± 12a-e	0.055 ± 0.003 a-g	0.178 ± 0.031a-d	70	E
AAUGR 66	48 ± 32a-e	0.069 ± 0.005 a-e	0.259 ± 0.176a-d	104	HE
AAUGR 67	108±46a-e	0.064 ± 0.005a-f	0.269 ± 0.023a-c	108	HE
AAUGR 68	65 ± 40a-e	0.053 ± 0.001 a-g	0.152 ± 0.111b-d	60	E
- ve control	-	-	0.082 ± 0.010d	32	
+ ve control	-	-	0.254 ± 0.013a-d	100	

Note: Levels not followed by the same letter/letters are significant at p=0.05 (Tukey's HSD test)

The physical appearances of inoculated plants were clearly differentiated from the negative control. The control plants appeared shorter whereas, the inoculated plants showed deeper green leaves and long shoots with many branches. The inoculated plants also had pink colored nodules (data not shown).

With regard to the number of nodule of the tested plants, the highest nodule number of 135/plant was recorded from plants inoculated with isolate AAAUGR 37 followed by 130 nodules/plant displayed by isolate AAUGR 48 (Table 13). Likewise, the smallest nodule number was displayed by AAUGR 8 with 9 nodules/ plant followed by AAUGR 15 with16 nodules/plant. Regarding nodule dry weight, the highest nodule dry weight (86mg/plant) was recorded from plants inoculated with isolate AAUGR 37 whereas, the least record (11mg/plant) was from isolate AAUGR 6.

The data also showed variations amongst the isolates with regard to shoot dry matter accumulation. The highest shoot dry matter accumulation of 296mg/plant was recorded from the plants inoculated with isolates AAUGR 37 followed by isolates AAUGR (41, 31, 61, 67, 59 and 48) that induced nodulation on the host with shoot dry weight ranging from 264mg/plant to 287mg/plant. The least effective isolates were isolates AAUGR (6, 8, 16, 25, 26 and 45) that were found to accumulate 66-76mg/plant in shoot dry weight that were comparable to the uninoculated negative control (82mg/plant). Based on the relative plant dry matter accumulation of the inoculated plants with uninoculated and nitrogen fertilized control (positive control), 18% (12 isolates) were highly effective (85 – 120 effectiveness), 43 % (27 isolates) were effective (50-78% effectiveness) and 29% (18 isolates) were lowly effective (35-49% effectiveness) whereas, 6 isolates (10%) were ineffective (26-33 effectiveness). In general, 61% of the isolates were found to be effective compared to the 39% of the isolates that were lowly effective and ineffective.

In this study, five isolates (8%) that include AAUGR (6, 8, 16, 25, 26 and 45) that were all from South Wollo were much more ineffective than the negative control. On the other hand, 8 isolates (13%) that include AAUGR(31, 37, and 41) (South Wollo) and isolates

AAUGR (48, 59, 61, 66, and 67) from West Shoa displayed much more effectiveness as compared to uninoculated and nitrogen fertilized control. The data also showed a pattern of effectiveness on the basis of site of collection. Accordingly, 95% of the isolates from West Shoa and 47% of the isolates from South Wollo induced effective nodulation and nitrogen fixation and shoot dry weight accumulation (Table 14). Most of the effective isolates were recorded from West Shoa.

Table 14. Rate of effectiveness of isolates by sampling regions

Sampling regions	%HE	%E	Total	%LE	%IE	Total	HE isolates	E isolates
SW	10	37	47	17	6	23	31,37,41,47	2,4,9,19,21,22,29,32,34, 35,38,39,40,42,43,46
WS	40	55	95	5	_	5	48,49,52,59, 60,61,66,67	51,53,54,55,56,57,58, 62,63,64,,68

5. 7. Symbiotic effectiveness of selected isolates on potted soil

After pouch culture experiment, based on the shoot dry matter, the top four isolates from West Shoa and three isolates form South Wollo were selected based on their performance among the isolates. These include AAUGR 31, AAUGR 37, and AAUGR 41 of South Wollo and AAUGR 48, AAUGR 59, AAUGR 61 and AAUGR 67 from West Shoa. Grass pea, “Wasse” variety grew on potted neutral (7.1) Awash Bolto soil.

The *in vivo* symbiotic test of isolates on soil culture showed differences in nodule number, and weight, shoot dry weight and nitrogen fixation (Table 15). The highest nodule number was recorded by plants inoculated with isolate AAUGR 37 (165 nodule/plant) followed by isolate AAUGR 48 (147 nodules/plant) whereas, the least number of nodule (93 nodule/plant and 97 nodule/plant) was recorded from isolates AAUGR 41 and 61, respectively. There was a significant difference in nodule number amongst the isolates. Regarding nodule dry weight, the isolates AAUGR 37 and AAUGR 48 showed the highest nodule mass of 1.0g/plant and 0.98g/plant that were significantly different from the nodule dry weight recorded by the other isolates. The least nodule mass of 0.70g/plant was displayed by isolate AAUGR 61.

The highest shoot dry matter accumulation of the rhizobia treated plants was recorded from the inoculation of plants with isolate AAUGR 37 followed by isolates 48, 59 and 67 with shoot dry mass of 1.95g/plant, 1.79g/plant, 1.67g/plant, and 1.67g/plant, respectively. The least shoot dry weight of 1.47g/plant was recorded from isolate 41 that was significantly different from the uninoculated negative control (0.93g/plant). The shoot dry matter accumulation by the selected isolates on pot trial was found to be much higher than pouch culture (Table 16). The nitrogen content of all the tested isolates was also within the range of 2.6-3.9 with one another (Table 15). The highest nitrogen content of 3.9% was recorded by isolate AAUGR 37 whereas, the least (2.6%) was displayed by isolate AAUGR 41. The nitrogen content of the inoculated plants was found to be twice more than the nitrogen content of the uninoculated negative control (1.2%).

Table.15. Symbiotic effectiveness of selected isolates of grass pea rhizobia on potted soil

Isolate	Nodule number per plant	Nodule dry weight per plant(g)	Shoot dry weight per plant(g)	Total nitrogen per plant (%)
AAUGR 31	117± 1d	0.073 ± 0.3d	1.51 ± 0.2e	2.8 ± 0.1f
AAUGR 37	165± 1a	1.011 ± 0.9a	1.95± 0.6a	3.9 ± 0.3a
AAUGR 41	93± 1f	0.071 ± 0.4de	1.47 ± 0.3g	2.6 ± 0.8g
AAUGR 48	147± 1b	0.098 ± 0.8b	1.79 ± 0.5b	3.7 ± 0.5b
AAUGR 59	135± 1c	0.081± 0.7c	1.67 ± 0.4 c	3.3 ± 0.2d
AAUGR 61	97± 1e	0.070 ± 0.4e	1.50 ± 0.7f	3.2 ± 0.6d
AAUGR 67	133± 1c	0.079 ± 0.6c	1.67 ± 0.7d	3.5 ± 0.7c
- ve control	19 ± 3g	0.007 ± 0.1f	0.93 ± 0.8i	1.2 ± 0.9h
+ ve control	17 ± 1g	0.004 ± 0.2g	1.31 ± 0.1h	3.1 ± 0.4e

Note: Levels not followed by the same letter/letters are significant at p=0.05 (Tukey's HSD test)

The *in vivo* test that was undertaken in the greenhouse both on pouch and soil culture showed significant differences in nodule number, nodule dry weight and shoot dry weight. The comparative percentage of effectiveness between soil and pouch was 82-85%. Accordingly, the isolates accumulated much shoot dry weight on soil culture than on pouch culture (Table 16).

Table 16. Shoot dry weight and %SE of selected isolates on both pouch and pot cultures

	On pouch culture		On potted soil		Comparative % effectiveness between soil /pouch
Isolates	Shoot dry wt./p	SE (%)	Shoot dry wt./plant	SE (%)	
AAUGR31	0.27	108	1.51	115	82
AAUGR37	0.3	120	1.95	149	85
AAUGR41	0.26	104	1.47	112	82
AAUGR48	0.29	116	1.79	137	84
AAUGR59	0.28	112	1.67	127	83
AAUGR61	0.27	108	1.51	115	82
AAUGR67	0.27	108	1.67	127	84
-VE CONTROL	0.08	32	0.93	71	91
+VE CONTROL	0.25	100	1.31	100	81

The result of the physiological and symbiotic tests of this study showed that grass pea rhizobial isolates were more tolerant and effective than their cross inoculation group of *R. leguminosarum var viceae*. Consequently, the isolates of this study were found to tolerate high salt, high temperature and high pH and performed better symbiotic effectiveness on sand culture as compared to other rhizobial isolates from faba bean and field pea (Table 17).

Table 17. Comparative analysis of maximum tolerance and effectiveness of different *R.leguminosarum var viceae* isolates from faba bean and field pea

Researcher	Maximum pH	% of isolates Grown.	Maximum Temp. (^{0}C)	% of isolates grown	Maximum NaCl (%)	% of isolates grown	% SE on sand	% TN
Aregu Amsalu (2007)	10	82	40	10	6	33	133	2.93
Gebremeskel Gebremariam (2007)	9.5	85	45	20	6	35	85	2.80
Zerihun Belay (2006)	9.0	100	40	10	5	5	96	3.9
Getaneh Tesfaye (2008)	10	23	40	13	7	5	91.4	3.19
This work	12	100	50	25	13	11	120	3.9

6. Discussion

In this study, root nodule rhizobia from grass pea (*Lathyrus sativus*) were collected from four districts of South Wollo and three districts of West Shoa. They were characterized on the basis of their different phenotypic and symbiotic characteristics. Out of the 68 isolates, 63 of them were authenticated as root nodule bacteria according to Vincent (1970). According to Brockwell (1998), the ability to form nodules along with the subsequent capacity of fixing nitrogen are widely used means to evaluate the inherent links between the rhizobia and their host plant. Although failure to nodulate could emanate from loss of symbiotic plasmids (Sym-plasmids) that govern the symbiotic interaction between the two partners (Zhange *et a.l*, 2001), the failure of these five isolates to re-nodulate their parent host may be due to other intruding bacteria that penetrated the nodule (Johnston and Beringer, 1976) or they may not originally be a rhizobial isolate.

The majority of the colonies (78%) showed large mucoid (LM) whereas, 22% of them displayed large watery colonies (LW). Fifty eight percent (58%) of the isolates displayed large colony diameters of (2-4 mm) while 42% exhibited very large ones (4-6 mm) after five days of inoculation. The largest colony diameter (6.0mm) was displayed by AAUGR 35, AAUGR 41, AAUGR 47 from South Wollo and AAUGR 49 and AAUGR 55 from West Shoa. Likewise, 25% of the isolates (9 isolates) showed the smallest colony diameter of 2.0mm. Almost all isolates were fast growers with excessive production of exopolysaccharides as described by Jordan (1994).

All the authenticated isolates changed the BTB –YEMA media to yellow which showed that they were acid producers and fast growers (Jordan, 1984; Lupwayi and Haque, 1994). With the exception of isolates AAUGR43, 46, and 64, all the isolates showed doubling times of less than 4hrs characteristic of fast growing root nodule bacteria (Jordan, 1984). The fact that most of the isolates displayed fast doubling times (1.24-4hrs), had larger colonies (2-6mm) and changed the BTB-YEMA media to yellow showed that they were fast growing rhizobia (Jordan,1984). Since the isolates nodulated grass pea (*Lathyrus sativus),* they can safely be categorized into *R. leguminosarum var viceae*.

The result revealed that the different rhizobial isolates grew at a different range of pH (pH 4-12) (Table 5). The majority of the isolates were able to tolerate higher pH and nine of the isolates (25% of the isolates) failed to grow at pH 4.0 and 4.5. All of the isolates (100%) were found to grow at pH 5- pH 12 which is consistent with (Graham *et al.*, 1982; Surange *et al.*, 1997; Merih *et al.*, 2006) that reviewed the tolerance of rhizobia that nodulate alfalfa and narrow leafed lupins (*L. angustifolius*) to higher pH (pH 8-12). In this study, the rhizobial isolates that nodulated grass pea *(Lathyrus sativus*) were found to be highly resistant to higher and lower acidity and alkalinity than *R. leguminosarum* that nodulated other cool season legumes (Zerihun, 2006; Aregu, 2007; Gebremeskel, 2007 and Getaneh, 2008) (Table 17).

The inhibitory effect of different salt concentrations varied among isolates (Table 6). The isolates showed tolerance to 0.5%-13% NaCl. The result indicated that the percentage of tolerance continued to decrease as the concentrations of NaCl increases. Fifty percent of the isolates grow at 8% concentrations whereas, 4 (11%) of the isolates were found to tolerate 13% concentration of NaCl. Likewise, some isolates which included AAUGR 37 (SW) and AAUGR 48, AAUGR 59 and AAUGR 61 (WS) were found to grow at all tested salt levels of concentrations as described in Boredleau and Prevost (2002) that reported that some rhizobia can survive in the presence of extremely high levels (12%) of salt concentrations both in culture and in soil while some others can tolerate salinities that are equivalent to sea water. Almost all the isolates of this study were found to be highly tolerant to higher levels of NaCl concentrations in contrast to the *R. leguminosarum* of other cool season food legumes (Zerihun, 2006; Aregu, 2007; Gebremeskel, 2007 and Getaneh, 2008) (Table 17).

The isolates grew within varied range of temperature 5-50 ^{O}C (Table 7). All the isolates (100%) grew well between 15^{O}C and 35^{O}C as reported in (Getaneh, 2008; Zerihun, 2006

Aregu, 2007). 72%, 56% and 28% of the isolates were also found to grow at a temperature of 40^{O}C, 45^{O}C and 50^{O}C, respectively. This result is still in harmony with (Hungria and Vargas, 2000; Zahran , 1999; Al-Falih, 2002) that reviewed the tolerance of some strains at 40 ^{O}C, 47 ^{O}C and 50 ^{O}C. The high temperature (50^{O}C) tolerant strains of this work were AAUGR (19, 31, 35, 37 and 41) of South Wollo and AAUGR (48, 55, 59, 61, and 67) of West Shoa. The result of this work is again unique in that several isolates tolerated a temperature of 45 ^{O}C and 50 ^{O}C in contrast to earlier works on other cool season food legumes (Zerihun, 2006; Aregu, 2007; Gebremeskel, 2007 and Getaneh, 2008) (Table 17).

The isolates showed diversity in intrinsic antibiotic resistance on the tested antibiotics (Table 8). All the isolates (100%) were able to resist ampicillin, chloroamphenicol and nalidixic acid that were adjusted at a concentration of 2.5 µg ml $^{-1}$. However, no isolate was found to display such resistance (Aregu, 2007; Zerihun, 2007; Getaneh, 2008). Although some isolates were sensitive to erythromycin and streptomycin particularly at a concentration of 5µg ml $^{-1}$ and 10µg ml $^{-1}$, the percent of tolerance was higher as compared to the results of (Aregu, 2007; Zerihun, 2007; Getaneh, 2008). 72 and 61% of grass pea rhizobial isolates were able to tolerate streptomycin at concentrations of 5µg ml $^{-1}$ and 10µg ml,$^{-1}$ compared to 52 and 32 % of the isolates from field pea in Aregu (2007). Thus, grass pea rhizobial isolates were found to develop better resistance to different antibiotics at different levels of concentrations and thereby could have better coexistence with antibiotic producing soil microorganisms as reviewed in Muller *et al*., (1988).

Isolates AAUGR 31, AAUGR 37, AAUGR 42, of South Wollo and AAUGR 48, AAUGR 49, AAUGR 55, AAUGR 56, AAUGR 57, AAUGR 59, AAUGR 60, AAUGR 61 and AAUGR 67 from West Shoa exhibited the broadest antibiotic resistance on all the tested antibiotics at all concentrations. On the other hand, isolates AAUGR 21 of South Wollo and AAUGR 53, AAUGR 63, AAUGR 64 and AAUGR 66 from West Shoa displayed the narrowest range of antibiotic resistance in all tested antibiotics (Table XI).

The tested isolates showed little variation in their range of carbohydrate utilization (Table 9). All isolates (100%) grew on D-glucose, D-fructose, maltose, D-mannitol, galactose, xylose, sucrose and mannose and greater than 75% of the isolates were found to grow on arabinose, sorbitol, lactose and cellobiose. This result is consistent with (Stowers, 1985; Amargar *et a*l, 1997; Hungria *and Vargas,* 2000) which described that the greater proportion of carbon sources were utilized by most of the isolates and rhizobia has the ability to utilize a wide variety of carbon sources for growth and energy with several pathways for carbon metabolism. In this study, 69%, 69%, 69%, and 67% of the isolates utilized starch, Na-citrate, and trehalose and cellulose, respectively. Upon utilization of Na-citrate only two isolates (Gebremeskel, 2007), eleven isolates (Aregu, 2007) and no isolates (Getaneh) whereas, 25 (69%) of grass pea rhizobial isolates were found to utilize Na-citrate. Isolates AAUGR 4 from South Wollo and isolates AAUGR 49, AAUGR 52 and AAUGR 56 from West Shoa showed a narrow range of carbohydrate utilization. On the other hand, isolates AAUGR (31, 37, 39, 40, 41, 46 and 47) from South Wollo and AAUGR (48, 53, 57, 59, 60, 61, 67 and 68) from West Shoa displayed wide range of utilization of all the tested carbohydrate sources (Table 11) whereas, 15% of the isolates from faba bean exhibited such broad range of utilization (Getaneh, 2008). The results of grass pea rhizobia was in harmony with Sukrita *et al.* (1981) that concluded that rhizobia catabolize a wide range of substrates and have ecological advantage over those with specific carbon sources. Similarly, Jordan (1984) reported that fast growing rhizobia utilize wide range of carbohydrates as sole sources of carbon.

All the isolates (100%) were able to utilize L-Arginine, L-Asparagine, L-Methionine and L-Tyrosine and greater than 80% of the tested isolates were also found to catabolise L-tryptophan, L-lysine, L-glutamate and L-alanine. This result agreed with (Sessisch *et al*., 1997) that reported most of the nitrogen sources were utilized by rhizobia nodulating faba bean. This result is inconsistent with other cool season legumes nodulated by *R. leguminosarum var viceae* (Zerihun, 2007 and Getaneh, 2008). On the other hand, 78% of the tested isolates showed less capacity of utilization to phenylalanine as described in Jordan (1984). Eighteen isolates (50% of the isolates) displayed wide range of utilization of nitrogen sources. Whereas, two isolates AAUGR 42 (South Wollo) and AAUGR 66 (West Shoa) showed less range of nitrogen utilization relatively (Table 10).

A cluster analysis on grass pea rhizobia was carried out to evaluate the phenotypic relationships among the isolates of the different districts of South Wollo and West Shoa. The dendrogram displayed the occurrence of a high level of diversity among the isolates. At 50% similarity coefficient, the thirty – six isolates grouped in to four clusters and one unclustered isolate. Each cluster and sub-cluster encompasses isolates of different sampling areas. The dendrogram indicated that relatedness of the isolates decrease as similarity coefficient level increase. As similarity coefficient increase, the number of clusters increased. This shows that similarity analysis of 76 phenotypic characteristics could be helpful to determine the genetic diversity among the isolates.

The preliminary screening for infectiveness (ability to form nodules) and effectiveness (ability to form enough nitrogen) of the isolates were evaluated on the basis of nodule number, nodule dry weight and shoot dry weight. Although there was a discrepancy in nodule number and weight among the treated plants, a significant difference existed with respect to their shoot dry matter at P=0.05. The result of the symbiotic effectiveness of the isolates revealed that 61% (39 isolates) rated highly effective and effective by accumulating 50 - 120% symbiotic effectiveness whereas, with respect to sites of the samples, 95% and 47% of the isolates that were found to record both highly effective and effective were from West Shoa and South Wollo, respectively. Although the isolates that were able to display high effectiveness were isolates AAUGR (31, 37, and 41) from South Wollo and AAUGR (48, 49, 52, 59, 60, 61, 66 and 67) from West Shoa, the isolates of West Shoa performed better than isolates of South Wollo without any ineffective isolate (Table 14). The shoot dry matter accumulated by these highly effective isolates was also too much greater than that accumulated by both the inoculated treatments and the uninoculated controls.

The shoot dry matter accumulated in the infected host plant was used to evaluate the relative symbiotic effectiveness of the isolate. According to Lupwayi and Haque (1994); peoples *et al.* (2002) and Mulongoy (2008), shoot dry matter is a good indicator of relative isolate effectiveness and there exists a sound correlation between the nitrogen fixing capacity of legumes and their shoot dry matter accumulation. In general, a significant difference (P=0.05) in shoot dry matter was recorded among inoculated grass pea and the controls and also between the positive control (0.25g/p) and the negative control (0.08g/p).

The soil experiment which was done using Awash Bolto soil with pH 7.1 for ascertaining infectivity and effectivity displayed marked differences among the isolates in nodule number, shoot dry matter and plant total nitrogen. Most of the selected isolates (5 of 7 isolates) showed slight increase in nodule number on soil experiment (93-165 nodules/plant) than on pouch culture (83-135 nodules/plant). The controls, both positive and negative were also able to form a few nodules that were small in size and white in colors from the indigenous rhizobia. The inoculated isolates formed nodule ranging from 93-165 while the positive and negative control formed nodules 17 and 19 respectively. This result can lead us to a conclusion that inoculation increased the amount of nodules formed.

Shoot dry matter accumulation of the rhizobial-inoculated plants revealed differences on the performance of the isolates in soil. The isolates were found to increase shoot dry matter by 82-85% in soil than on pouch cultures (Table 16). As Ayeneabeba *et al.* (2002) reported a 45-50% increase in shoot dry matter between sand culture and soil culture experiments on faba bean production in the Northern Shoa. Kang and Mills (2004) also reviewed that the high nitrogen content of the soil, additional nodulation by the background indigenous strains of the tested soil and other rhizosphere effects on plant growth might have contributed to the increased shoot dry matter on the soil culture as compared to the sand culture.

In this experiment, a significant difference in total nitrogen among the effective isolates was also recoded at P = 0.05. The isolates were able to accumulate 2.6-3.9% total nitrogen. On the other hand, the negative control was able to accumulate 1.2% total nitrogen. The total nitrogen accumulated by grass pea rhizobial isolates was found to be higher than that accumulated by other cool season legumes (Aregu, 2007; Gebremeskel, 2007 and Getaneh, 2008) (Table 17). Thus, grass pea associated microsymbionts are to some extent more effective in nitrogen fixing efficiency.

7. Conclusion and Recommendations

7.1. Conclusion

This particular study that focused on the phenotypic and symbiotic characterization of rhizobia nodulating grass pea in major growing areas of South Wollo and West Shoa has been done for the first time in Ethiopia. The result of plant shoot dry matter, total nitrogen and literatures supported that grass pea has greater intrinsic potential to enrich the nitrogen deficient soil. Apart from this, grass pea grows in poor soils, in water logging and drought conditions and in general, in soils at extreme conditions. Thus, its cultivation best suits the agricultural conditions of the country.

In this study, all isolates changed the YEMA-BTB medium to yellow colour. With the exception of few isolates, all isolates showed a mean doubling time of less than four (4) hrs, and induced nodulation on grass pea. All these and other characteristics can qualify these isolates of the cross-inoculation group of *Rhizobium leguminosarum var viceae*.

Most of the isolates were tolerant for most of the environmental factors like high pH and low pH, high and low temperature, low and high salt concentrations. The majorities of the isolates were able to utilize a wide variety of carbon and nitrogen sources and also displayed a considerable resistance to many of the tested antibiotics. When the physiological test result of grass pea rhizobia is compared with the result of other cool reason legumes that were carried out in the past few consecutive years, the tolerance of grass pea rhizobia to these extreme abiotic factors was found to be slightly better than other isolates from other cool season legumes.

This capacity may favor the establishment of the rhzobia in the soil and may represent an advantage to be used as inoculants for grass pea. This gives an ecological advantage in colonizing the soil or rhizosphere as compared to strains having limited preference and competitive advantage for the isolates when they live as saprophytes in the soil. This is an essential component in strain selection and for production of inoculants. The fact that 61% of the isolates were effective in screening trials indicates that some of the grass pea growing areas evaluated mostly in South Wollo are in need of inoculants that could enhance the symbiotic performance of the crop.

7.2. Recommendations

On the basis of the above conclusion, the following recommendations are suggested:

- The isolates were taken from two sampling sites of South Wollo and West Shoa. More should be studied from different grass pea growing areas of the country in order to reveal the true character of grass pea rhizobia.

- In order to reveal the true diversity of grass pea rhizobia, phenotypic characterization should be supplemented with polyphasic approach that includes genetic characterization.

- The highly effective isolates that were tested in soil culture in the greenhouse should be re-tested under field conditions to ascertain their performance *in vivo*.

- Among the highly effective isolates, isolate AAUGR 37 (SW) and isolates AAUGR 48, 59, 61 and 67 from West Shoa are strongly suggested to be used as inoculums in field conditions.

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9. Appendices

Appendix 1. Inoculated and control plants after 45 days of growth on pouch culture in greenhouse


N - VE
N + VE
AUGR_61
9 14:26


N - VE
N + VE
AUGR_62
9 14:49

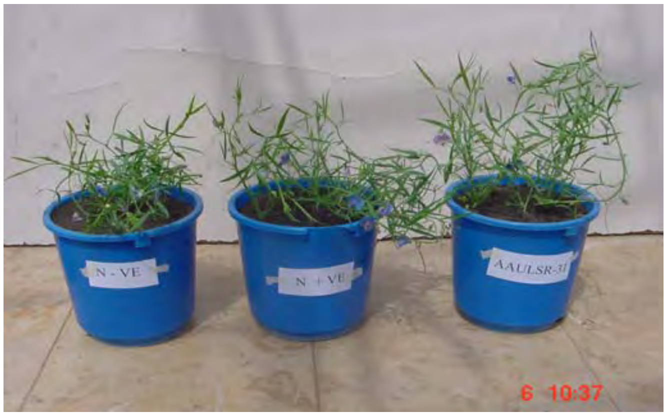

N - VE
N + VE
AUGR_64
9 14:11


N - VE
N + VE
AUGR_66
9 14:50


N - VE
N + VE
AUGR_67
9 14:09


N - VE
N + VE
AUGR_68
9 14:21

Appendix 2. Inoculated and control plants after 45 days of growth on potted soil in greenhouse



Appendix 3. Authenticated and non-authenticated isolates that that are not characterized.

Isolates	Nodule number per plant	Nodule dry Wt. per plant(g)	Shoot dry Wt. per plant (g)	% SE	Effectiveness
AAUGR1	-	-	-	-	Non-authenticated
AAUGR2	46±21a-e	0.039±0.012b-g	0.135±0.049b-d	53	E
AAUGR3	22±10de	0.024±0.016fg	0.116±0.095cd	46	LE
AAUGR5	-	-	-	-	Non-authenticated
AAUGR6	23±15de	0.011±0.008g	0.066±0.057d	26	IE
AAUGR7	-	-	-	-	Non-authenticated
AAUGR8	9±3e	0.028±0.008fg	0.075±0.057d	30	IE
AAUGR10	35±10c-e	0.035±0.022c-g	0.125±0.051b-d	49	LE
AAUGR11	44±26b-e	0.050±0.005a-g	0.117±0.056cd	46	LE
AAUGR12	22±11de	0.021±0.013fg	0.096±0.079d	37	LE
AAUGR13	31±6c-e	0.017±0.007g	0.104±0.041cd	41	LE
AAUGR14	16±9e	0.043±0.010a-g	0.111±0.028cd	41	LE
AAUGR15	27±27c-e	0.043±0.016a-g	0.122±0.097cd	44	LE
AAUGR16	25±13c-e	0.023±0.004fg	0.085±0.033d	33	IE
AAUGR17	19±10de	0.020±0.004fg	0.119±0.061cd	47	LE
AAUGR18	-	-	-	-	Non-authenticated
AAUGR20	31±24c-e	0.016±0.008g	0.099±0.078d	39	LE
AAUGR23	30±13c-e	0.025±0.011fg	0.121±0.040cd	48	LE
AAUGR24	27±18c-e	0.025±0.002fg	0.117±0.047cd	46	LE
AAUGR25	20±6de	0.022±0.008fg	0.076±0.014d	30	IE
AAUGR26	26±20c-e	0.010±0.002g	0.069±0.068d	27	IE
AAUGR27	34±21c-e	0.038±0.005c-g	0.122±0.044cd	48	LE
AAUGR28	31±13c-e	0.023±0.010fg	0.099±0.014d	39	LE
AAUGR30	51±8a-e	0.059±0.015a-g	0.124±0.050cd	49	LE
AAUGR33	30±23c-e	0.024±0.019fg	0.089±0.013d	35	LE
AAUGR44	28±7c-e	0.023±0.010fg	0.093±0.043d	37	LE
AAUGR45	29±12c-e	0.022±0.005fg	0.067±0.025d	26	IE
AAUGR50	74±16a-e	0.060±0.003a-g	0.114±0.050cd	45	LE
AAUGR51	44±49b-e	0.035±0.024c-g	0.138±0.037b-d	54	E
AAUGR62	60±39a-e	0.061±0.003a-g	0.158±0.030b-d	62	E
AAUGR65	-	-	-	-	Non-authenticated

Note: Levels not followed by the same letter/letters are significant at p=0.05 (Tukey's HSD test)