ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Symbiotic and Phenotypic Diversity of *Rhizobium leguminosarum* var *viceae* Isolates (*Vicia faba*) from Northern Gondar, Ethiopia.

By:

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A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Microbiology

May, 2006

ACKNOWLEDGMENTS

I would like to sincerely thank my advisor Dr. Fassil Assefa for his help in developing problem of study, guidance throughout the research work and useful comments, suggestions and critical feed back in preparation of this thesis.

I gratefully acknowledge the project “Microbial inputs in to Agroforstery” / SIDA/SAREC for funding this work and Educational Bureau of Oromia Regional States for sponsoring me in the MSc training.

I am highly indebted to Holeta Agricultural Research Institute for their supply of selected seed variety for my research work.

I am thankful to Dr. Tilahun Mamo who helped me in preparation of sample site map and encouraging and supporting me in one way or another.

My appreciation also goes to Ato Mamush Lelisa and Dr. Tsegaye Awano for providing me access to computer facilities, encouragement, and moral support during my postgraduate study.

I am very much thankful to my families, especially my sister Mulunesh Belay, friends, and brothers in Christ who remembered me in all circumstances during my work.

Finally, I extend my heart felt thanks to my wife Etagegnehu Mamo and my daughter Metsenanat Zerihun who, execute the burden of the family affairs quite confidently and give every time, need to do all my M.Sc training.

Above all, I would like to give glory to the only God through Jesus Christ forever, who alone is all-wise.

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LIST OF SYMBOLS AND ABBREVIATIONS

AUFR	Addis Ababa university faba bean rhizobia
CSFL	Cool season food legumes
IAR	Institute of agricultural research
IAR	Intrinsic antibiotic resistance
FAO	Food and agricultural organization
GS	Glutamine synthetase
ATP	Adinosine triphosphate
GOGAT	Glutamine-2-oxolglutamate-amino-transfrase
ICARDA	International center for agricultural research in the dry areas
EIAR	Ethiopian institute of agricultural research
MoA	Ministry of agriculture
YEMA	Yeast extract mannitol agar
CR	Congo red
BTB	Bromothymol blue
MGT	Mean generation time
CIAT	Centro internacional de agricultura tropical
DM	Dry matter
PCR	Polymrease chain reaction
REP	Repetitive extra genomic palindromic
RFLP	Restriction fragment length polymorphism
W/V	Weight/volume
m.a.s.l	meter above sea level
LW	Large watery
LM	Large mucoid

ABSTRACT

Twenty-one strains of *Rhizobium leguminosarum* var. *viceae* spp (*Vicia faba*) from 21 sites of North Gondar were isolated from a range of pH 5.8-7.5 to induce nodulation on “Degaga" cultivar of faba bean. Isolates were authenticated as root nodule bacteria by their ability to form nodules on their host up on re-inoculation, except AUFR13. All of the morphological features displayed by all isolates were characteristics of fast growing *Rhizobium leguminosarum* var *viceae,* except AUFR13. Almost all of the isolates grew between 15^0C and 35^0C and failed to grow at 40^0C, except AUFR22 and AUFR28. All isolates failed to grow at 1% NaCl, except AUFR12 and AUFR28. AUFR18 was the only osmotolerant strain capable of growing at a concentration of 5%. The isolates also grew on a wide range of moderate acidity and alkalinity but only 10% of the isolates grew at pH5.5. With the exception of gluconate, citrate, and tartarate, almost all isolates grew on 14 out of the tested carbohydrates. The Intrinsic Antibiotic Resistance (IAR) was found to be the best of all tested physiological characters to identify the heterogeneity of *Rhizobium leguminosarum* var. *viceae,* so that 95% of the tested isolate were resist erythromycin and chloromphenicol, and 14%, 24%, 38%, 38% and 43% of the isolates tolerated streptomycin, ampicillin, rifampcin, kanamycin and naldixic acid at highest concentration, respectively. The relative effectiveness expressed as percentage of shoot dry mass of inoculants over TN control, showed that 24%, 57%, 4.7% and 9.5% of the isolates were found to be highly effective, effective, lowly effective and ineffective, respectively. The highest scores of 88-100% effectiveness of symbiotic nitrogen fixation were displayed by AUFR14, AUFR15, AUFR24, AUFR28 and AUFR32. Selected strains on soil culture were found to accumulate 81-92% on Holeta soil and 90-103% on Ambagiorgis soil of their shoot dry mass compared to their respective N-fertilized controls. All isolates were also found to increase shoot dry weight by 0-43% higher than their respective sand cultures and response of the Holetta soil to inoculation by selected effective strains was much pronounced (47-67%) than Ambaghiorgis soil (8-25%) compared to the negative (N-) controls. The lower dry matter accumulation by Holeta soil indicates that the low pH and other soil-related factors severely affected survival, nodulation and nitrogen fixation of their indigenous rhizobia and the process can be rectified by inoculation of effective rhizobia.

Key words: Faba bean, Rhizobia, Phenotypic diversity, Symbiotic effectiveness.

1. INTRODUCTION

Grain legumes are protein rich source of food, which are essential diets in many parts of the tropics, particularly where meat is scarce. They play an important nutritional role in supplying essential amino acids (particularly lysine) that are not present in sufficient quantities in staple cereal corps (Giller, 2001). They are categorized in to two major groups, based on their geographical and climatic distribution in the world. These are cool season and warm season grain legume.

The cool- season food legumes (CSFL) belong to three tribes, the Vicieae (*Lens, Pisum*, *Vicia faba*, *Lathyrus*), the recently separated tribe Cicereae (*Cicer)* and Genisteae (*Lupinus*) (Summerfield and Bunting, 1980; Hawtin and Hebblethwaite, 1983). They are cultivated in temperate, Mediterranean, and subtropical regions of the world. These crops can also be found at higher elevation with in the tropics (Jayasundara *et al*., 1998).

The use of cool season food legumes, in agricultural production system dated as far back as 6000-8000 B.C. (Asfaw Telaye, 1988). The oldest seeds of faba bean were found in Jericho at 6250 B.C. (McVicar *et al.*, 2005). Faba bean was introduced to Ethiopia soon after domestication around the 5th millennium B.C. Hence the country together with Afghanistan, is considered as secondary center of diversity (Bond, 1976).

Faba bean is the most important pulse crops in Ethiopia and occupies about 3.9% of the total cultivated area. It is grown as a field crop mainly, in *weyna dega* or temperate zone between the altitudes of 1800 and 2400m a.s.l, and in the high land, *Dega*, within the limits of 3200m.a.s.l (Bond *et al*., 1985; CSA, 1996). The major production area includes the central high lands of Showa, all high lands of Gojam and Gondar, Northwest Wallo and Tigry. Arsi, Bale, and Wallega constitute the second major important production zones in the country (Asfaw Telaye *et al*., 1994).

Faba bean contain high protein and appreciable amount of minerals and vitamins (Senayit Yetneberk and Asrat Wondimu, 1994). It is an important source of protein supplement for the majority of the population, and incorporated in various popular Ethiopia dishes (Asfaw Telaye *et* *al*., 1994). Moreover, it provides large cash for producers and foreign exchange for the country (Desta Beyene, 1988).

Ethiopia was a net exporter of faba bean. In the period of 1970-77, an average of 23000t were exported annually, to the Arabian Peninsula, Western Europe, and South-East Asia (Asfaw Telaye, 1979). Even though Ethiopia is the world's second largest producer of faba bean, next to the Peoples Republic of China, its share of production is 40.5% in Africa, and 6.96% of the world (Asfaw Telaye *et al*., 1994).

Faba bean is a legume capable of fixing nitrogen in an endosymbiotic association with root nodule bacteria: *Rhizobium leguminosarum* var. *viceae.* It is the most efficient nitrogen fixer of the pulse crops grown (McVicar *et al*., 2005). According to Somasegaran and Hoben (1994), the amounts of N_2-fixed (kg/ha) by faba bean have been 240-325.The dual contribution of faba bean as a source of protein for the majority of population, and its capability to fix nitrogen and improve soil fertility has been used in crop rotation and traditional mixed low-in put agricultural systems.

It is established that the symbiosis between *Rhizobium* and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume based crop and pasture production than the application of fertilizer N (Zahran, 1999). With increasing costs of chemical fertilizers and farmers inability to purchase them, the need to exploit inexpensive means of soil fertility maintains such as biological nitrogen fixation is becoming important (Tekalign Mamo and Asegelil Dibabe, 1994).

The amount of nitrogen fixed depends on many factors, such as the effectiveness of *Rhizobium* strain, the genetics of the host plant, physical factors of the environment and agronomic practice (Nutman, 1976). In order to realize an effective faba bean-*Rhizobium leguminosarum* bv. *viceae* for agricultural production, it is important to evaluate the compatible rhizobium and legume genotypes. The criteria for screening of the symbionts include the ability to compete and form effective N-fixing nodules, tolerance to environmental stress and persistence in the soil (Lupwayi and Haque, 1994).

To this end, for the last two decades attempts have been made to conduct research in the area of soil microbiology, specifically on rhizobiology of cool season food legumes in Ethiopia at the Institute of Agricultural Research (IAR). The major areas of research conducted so far include nodule collection and characterization, isolation and identification of *Rhizobium* strains and investigation of inoculation needs of pulse crops (Tekalegn Mamo and Asgelil Dibabe, 1994).

Most rhizobiology work conducted on faba bean was undertaken by Nile Valley Regional Program on cool season food legumes (Desta Beyene and Angaw Tsige, 1986; 1987; 1988: and Balesh Tulema and Asnakew Woldeab, 1993a,b, c; Tekalegn Mamo and Asgelil Dibabe, 1994). Recently, nodulation pattern, and effective nitrogen fixation of faba bean with and without application of NP fertilizers on different soil types have been documented (Asgelil Debabe, 2000; Amanuel Gorfu *et al.*, 2000; Ayneabeba Adamu *et al.*, 2001). However, there is a lack of sufficient information about phenotypic and symbiotic characteristics of rhizobial strains present in different agroecological zones of the country where faba bean are widely grown (Desta Beyene *et al.*, 2003).

2. OBJECTIVES

2.1 General objective

-To evaluate phenotypic and symbiotic diversity of *Rhizobium leguminosarum* var. *viceae* spp (*Vica faba*) from Northern Gondar, Ethiopia.

2.2 Specific objectives

-To examine the morphological and physiological characteristics of the isolates.

-To evaluate the symbiotic performance of the isolates under greenhouse experiment.

3. LITERATURE REVIEW

3.1. Legume

Legumes are dicotyledonous plants categorized into the third largest family of flowering plants, the family leguminosae. They are found in various habits of herbs, shrubs and trees. The family Leguminosae is estimated to contain 16,000- 19,000 species in about 750 different genera (Rendle, 1979; Allen & Allen, 1981), and divided into three subfamilies; the Papilionoideae (pea-like flowers), the Mimossoideae (compound inflorescences with reduced petals) and the Caesalpinioideae (flowers usually with five petals apparently radially symmetrical) (Polhill and Raven, 1981).

Sprent and Raven, (1992) reported that, the Mimosoideae and Caesalpinioideae are almost completely restricted to the tropics, where as Papilionoideae contains the majority of the most important grain legumes. The majority of the latter contains herbaceous plants that include the genera *Melilotus*, *Lotus*, *Lathyrus* and *Vicia* (Alexander, 1977; Rendle, 1979). Most of the genera in this subfamily are nodulated. The seeds, rich in starch and proteins, are a good source of food, as in the various beans, pea, vetch, groundnut, lentil, and others (Rendle, 1979).

3.1.1. Faba Bean

The faba bean (*Vicia faba*) is belonging to tribe Vicieae. Herbs with pinnate leaves and grows upright, ranging from 1-1.5 meters tall (Rendle, 1979). It is an annual legume with one or more strong, hollow and erect stems. Faba bean has a strong taproot, compound leaves, and large, white flowers with dark purple marking. A flower cluster may produce 1-4 pods. The pods are large (up to 10 cm long and 1-2 cm wide) and green, turning dark at maturity. Three to four oblong/oval seeds are contained with in each pod. Flowering occurs in 45-60 days and requires 110-130 days to mature (McVicar *et al.,* 2005)

According to Hawtin and Hebblethwaite (1983), faba bean (*Vicia faba*) is classified into two subspecies *paucijuga* and *eu-faba*. Three varieties are recognized within *eu-faba*, the large seeded var. major, the intermediate var.equina, and the small seeded var. minor. Although *Vicia faba* was originated in the Mediterranean-Near East region, it is a temperate crop in its adaptation and has similar requirement with other pulse crops in the tribe Vicieae.

In Mediterranean areas, it is fed by short rains or by irrigation in the winter. In strictly tropical areas, with higher elevation, and in the cooler monsoon tropics with medium elevation, the crop is grown under irrigation or on residual moisture in the dry season (Smartt, 1976). Faba bean should be grown with caution in the brown soil zones and light-textured soils unless irrigation is available. Planting faba bean on cold, poorly drained soils should be avoided, as it favors the development of seedling diseases and root rots.

Faba bean does not tolerate salt-affected soils, but more tolerant of temporary flooding compared to lentil, pea or dry bean. Faba bean is a legume capable of fixing nitrogen in association with root nodule bacteria and it is the most efficient nitrogen fixer of the pulse crops (McVicar ***et al.***, 2005).

3.2 Biological Nitrogen Fixation

Nitrogen is an essential element for plant growth and reproduction. Lack of mineral nitrogen in the soil often limits plant growth (Trevaskis ***et al.***, 2002). The atmosphere contains about 10^{15} tones of N_2 gas, and the nitrogen cycle involves the transformation of $3x10^9$ tones of N_2 per year on global bases (Posgate, 1982). However, N_2- fixation is not exclusively biological, lightning probably accounts for about 10% of the world's supply of fixed nitrogen (Sprent and Sprent, 1990). FAO, (1990) reported that, world production of fixed nitrogen in the form of chemical fertilizers accounts for about 25% of the earth's newly fixed nitrogen, and biological processes accounts for about 60%.

Biological fixation is the conversion of atmospheric N_2 gas, to ammonia (NH_3) that plants can use to make essential organic molecules such as amino acids and nucleotides (Moat and Foster, 1988). The capability of biological fixation of atmospheric nitrogen (N_2) is restricted to organisms with prokaryotic cell structure namely, bacteria and cyan bacteria (blue green algae). Some species in 11 of the 47 bacterial families are diazotrophs, i.e. capable of N_2 fixation (Peoples and Craswell, 1992).

Three major types of N_2 fixation can be differentiated in terrestrial ecosystems symbiotic, associative and free living nitrogen fixing organisms, differing in both, energy source and fixation capability. On average the relative contribution of symbiotic, associative and free living N-fixing systems is in the order of 70% and 30% non-symbiotic (Peoples and Craswell, 1992).

The predominant groups of N-fixers are Rhizobia (bacteria) and the others are cynobacteria (blue green algae), which are found both as free-living species and in association with the variety of plants, most notably the aquatic fern *Azolla.* The third groups of important N_2 fixers are the actinomycets ***Frankia*** species, which form symbiotic association with flowering plants from a number of different families. Almost all of their host plants are woody perennials. Forth group of N_2- fixer is more loosely associated with plants. This includes *Azospirillum,* which colonize the root epidermis of host species including wheat, maize and rice (Vande Brock *et al.*, 1993). The last group of N-fixing organisms that contribute to the N-balance in tropical cropping system is the free-living fixers. Organisms such as ***Klebsillea*** and *Azotobacter* live in the soil and fix N_2 where the forms N are unavailable.

3.3 Biochemistry of Nitrogen Fixation

In all N_2 fixing microorganisms the principle steps of this reaction are the same. The key enzyme complex, referred to as *Nitrogenase*, is unique to N_2-fixing microorganisms (Thornely, 1992). Nitrogenase consists of two proteins in the ratio of 2:1 azoferredoxin and molydoferredoxin. A common property of all nitrogenase preparation is their sensitivity towards oxygen. The enzyme is irreversibly inactivated by oxygen so that nitrogen fixation can be regarded as a strictly anaerobic process (Smith, 1999).

Biological reduction of N_2 to NH_3 is also a highly endergonic process with a minimum energy requirement of Ca.960 KJ mol^{-1} N- fixed (Sprent and Raven, 1985). For the nitrogenase reaction, energy in the form of ATP and reducing equivalents (electrons) are required, supplied by respiration (ATP) and electron carriers, usually ferrodoxin. Nitrogenase catalyzes the reduction of several substrates, including H^+, N_2, and C_2H_2. The principal reaction for dinitrogen reaction is as follows:

$$N_2+8H^++8E^-+16Mg.ATP \longrightarrow 2NH_3+H_2+16\ Mg.ADP+16Pi$$

Concomitant reduction of at least two protons to hydrogen is an inevitable part of the reaction (Simpson and Burris, 1984). Hydrogen gas evolved during nitrogen fixation in legume root nodules can be recycled through the activity of an H_2-uptake (Hup) system synthesized by certain strains of rhizobia (Evans *et al.,* 1985). This H_2 recycling systems, whose first component is a membrane-bound, dimeric, and nickel- and iron-containing hydrogenase, has a potential to increase legume productivity (Evans *et al.,* 1985; Evans *et al.,* 1988). *Rhizobium leguminosarum* bv. *viciae* expresses an uptake hydrogenase in symbiosis with peas (*Pisum sativum)* but, unlike all other characterized hydrogen-oxidizing bacteria, cannot express it in free-living conditions (Evans *et al.,* 1988; Brito *et al.,* 1997).

All N derived from N_2-fixation is obtained in the form of ammonia. The predominant assimilation pathway for ammonia is a two-step process. Glutamine synthetase (GS) adds ammonium to glutamic acid to form glutamine with concomitant hydrolysis of ATP. This glutamine is then used by glutamine-2-oxoglutamate-amino-transfrase (glutamate synthetase or GOGAT) to aminate two molecules of 2-oxaloglutaric acids, with the production of two molecules of glutamate (Lea *et al.,* 1990).

3.5 Rhizobia, Taxonomy and Host Specificity

Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria that were originally classified together with their nodulating members of leguminosae (Somasegaran and Hoben, 1994).

Morphologically, they are medium-sized, rod-shaped cells, 0.5-0.9 µm in width and 1.2-3.0 µm in length. They are gram-negative, motile by a single polar filagellum or six peritrichous flagella. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of 0_2 and utilize relatively simple carbohydrates and amino compounds. Optimal growth of most strains occurs at a temperature range of 25^0 C-30^0C and pH of 6.0-7.0(Somasegaran and Hoben, 1994).

Until the early 1980s, all symbiotic nitrogen fixing bacteria from leguminous plants were classified in the single genus *Rhizobium.* Six species were identified in to *R.leguminosarum, R.*

meleloti, R. trifolii, R. phaseoli, R. lupine and *R. japonicum* based on their cross-inoculation groups with pea, alfalfa, clover, bean, lotus, and soybean, respectively.

Taxonomy based on the concept of cross-inoculation groups failed because of the many exceptions to this rule. It was also widely recognized that rhizobium classification should adjust to general bacterial taxonomy and include a panel of genomic, phenotypic and phylogentic features instead of the sole nodulation properties (Zakhia and de Lajudie, 2001).

Differences in rates of growth allowed early separation of rhizobia into two basic groups, fast growers and slow growers. Fast growers have generation times of less than 6 hours and generally forms visible colonies (2-4 mm in diameter) on agar media within 2-5 days; whereas slow growers have generation times exceeding 6 hours and give detectable growth after more than 5 days under standardized conditions. Most of the slow growing rhizobia produce alkali while fast growers produce acid (Jordan, 1984).

According to current classification rhizobia belong to the alpha subdivision of protobacteria, that were first classified into two genera, the genus *Rhizobium* including the fast growing strains and the new genus *Bradyrhizabium,* created for the slow growing ones (Jordan, 1984). Since then, isolation of rhizobia from an increasing number of plant species around the world and their characterization by modern polyphasic taxonomy has necessitated the description of additional new genera and species. A total of 6 genera; *Rhizobium, Bradoyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium, Allorhizobium,* and 28 species have been recognized (Zakhia and de Lajudie, 2001)

The species name of the microsymbionts reflects in most cases the corresponding host plant nodulated and suggests that symbiosis is a species-specific process. It is clear that the degree of host specificity varies tremendously among the rhizobia (Young and Johnston, 1989). Some strains have a very narrow host range, for example *R.leguminosarium* bv. *trifoli*, have only clover while others, like *R. leguminosarum* bv. *viciae* have a very broad host range such as peas, vetches;(*Vicia* and *Lathyrus*) and lentils (Spaink *et al.,* 1989).

Specificity of *Rhizobium*-Legume symbiosis is also governed by specific genes on the bacterial chromosomes that codes for proteins involved in recognition and uptake of specific signal molecules present in root exudates. These root exudates contain chemical attractants such as flavonoids and betains, secreted by the roots (Barbour *et al*., 1991; Krishanan and Pueppke, 1993). Each species of *Rhizobium* possesses a system for sensing the flavonoids produced by the plants species with which it interacts (Matthysse, 1996).

These compounds stimulate the expression of a set of *Rhizobium* genes; the nod genes (Heidstra and Bisseling, 1996). The nod genes encode approximately 25 proteins required for bacterial synthesis and export of nod factor. Nod factor is a lipooligosaccharide signal consisting of a chitin backbone, four to five N-acetyl-glucosamine units in length, with a lipid attached to nonreducing end and host specific modification on the back bone (Gage, 2004).

In response to nod factors, many of the developmental changes seen in the host plant early in the nodulation process including root hair deformation to establish a meristem and nodule prmordeium. Rhizobia continue to differentiate inside the nodule and synthesize proteins required for nitrogen fixation and for the maintenance of the mutalistic partnership (Gage, 2004).

3.6 Environmental Factors Affecting BNF and Legume Production

Biological nitrogen (N) fixation is key to sustainable agricultural system in tropical soils, which are frequently deficient in nitrogen. Environmental factors influence all aspects of nodulation and symbiotic N_2-fixation. According to Hungria and Vargas (2000), high temperature, drought and soil acidity constrain legume root-nodule formation in the tropics. Photosyntate deprivation, salinity, soil nitrate, heavy metals, and biocides are also typical environmental stresses faced by the growth of the host and their symbiotic partner (*Rhizobium*) (Walsh, 1995).

3.6.1 Extreme Temperature

Rhizobia are mesophiles and most have a poor growth at temperature below 10^0C or above 37^0C(Graham, 1992). Although response to temperature is strains dependent, rhizobia are found to tolerate between 4-42.4 ^{0}C. However, growth at 4^0C is rare, and only *R. meliloti* can grow at 42.5^0C. *R.leguminosarum* isolates from lentil plants in Southern Nile Valley of Egypt were tolerant to 35 to 40^0C inducing less effective symbiosis with their legume host (Moawad and Beck, 1991). For most rhizobia, the optimum temperature range for growth in culture is 28 to 31^0C. (Bordeleau and Prevost, 1994).

Changes in temperature strongly affect bacterial infection and N_2 fixation in several legume species (Arayankoon *et al.*, 1990; Kishinevsky *et al.,* 1992). Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to plant cultivars (Arayankoon *et al.*, 1990). Elevated temperature may delay nodule initiation and development, and interfere with nodule structure and functioning in temperate legumes, whereas nitrogen fixation efficiency is mainly affected in tropical legumes. Further more, temperature changes affect the competitive ability of *Rhizobium* strains (Roughley, 1970; Bordeleau and Prevost, 1994).

High soil temperature in tropical and subtropical areas is a major problem for biological nitrogen fixation of legume crops (Michiels *et al.,* 1994). Maximum soil temperatures in the tropics regularly exceed 40^0C at 5 cm and 50^0C at 1cm depth (Eaglesham and Ayanaba, 1984). Because high temperatures decrease rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rate of inoculation may frequently be needed (Thies *et al.,* 1991).

3.6.2 Soil Moisture Deficiency

Shortage of water compromises plant and rhizobial growth, and is a major cause of nodulation failure and low N_2 fixation. Water stress affects rhizobial morphology, survival and growth and population structure in soil (Guerin *et al.*, 1991). The modification of rhizobial cells by water stress will eventually lead to a reduction in infection and nodulation of legumes (Zahran, 1999)

Symbiotic N_2 fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes and shrub legumes exhibit a reduction in nitrogen fixation when subject to soil moisture deficit. This is due to the fact that water stress affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function (Guerin *et al.*, 1991).

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

3.6.3 Soil Acidity

Legume and their rhizobia exhibit varied responses to acidity. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within species (Brockwell et *al.,* 1995). The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan 1984), and relatively few rhizobia grow well at pH Less than 5.0. The fast growing strains of rhizobia have generally been considered less tolerant to acid pH than have slowly growing strains of *Bradyrhizobium* (Graham *et al.,* 1994).

Although the basis for differences in pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is not clear (Correa and Barneix, 1997), differences in lipopolysacchardes composition, proton exclusion and extrusion accumulation of cellular polyamines, and synthesis of acid shock proteins (Zarhan, 1999), and composition and structure of outermebrane (Graham *et al*., 1994), have been implicated with pH tolerance of endosymbioants.

Vlassak and Vanderleyden (1997) reported that, nodulation of legumes is reduced in acid soil, mainly because of sensitivity of early nodulation events, such as attachment, root hair curling and initiation of infection thread formation. In addition, low pH can affect the production and excretion of nodulation factors in some strains of rhizobia (Mckay and Diordjevic, 1993). Lapinskas *et al*. (2005) showed that soil acidity was a decisive factor in spread and symbiotic efficiency of *R. leguminosarum* bv.*viceae.*

In general, low soil pH is often associated with increased Al and Mn toxicity, reduced calcium (Ca) supply phosphorus (P) and molybdenum (Mo) deficiencies (Hungria and Vargas, 2000). These additional stresses affect the growth of rhizobia, of the host legume and symbiosis. The effect on symbiosis is evident from the fact that, nodulated legumes are more sensitive to Al and Mn toxicity than plants receiving mineral N (Hungaria and Vargas, 2000).

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 Barnex, 1997). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depended on symbiotic N_2 fixation (Bordeleau and Prevost, 1994).

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

3.6.4 Salt Stresses

Increasing salt concentrations may have detrimental effect on soil microbial populations as a result of direct toxicity as well as osmotic stress (Tate, 1995). Additionally saline soils are generally deficient in nutrients and microbial activities and population is low (Hussain ***et al.,*** 2002).

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

The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of nodules and a reduction in cytosolic protein production specifically legemoglobin (Delgado *et al*., 1994). The depressive effect of salt stress on N_{2-} fixation by legumes is directly related to the salt induced decline in dry weight and N content in the shoot (Cordovilla *et al*., 1995).

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

Salinity is a serious threat to agriculture in arid and semi-arid regions (Rao and Sharma, 1995). Nearly 40% of the world's land surface can be categorized as having potential of salinity problems (Cordovilla *et al*., 1994). Most of these areas are confined to the tropics and Mediterranean regions.

Successful *Rhizobium*-legume symbiosis under salt stress requires the selection of salt tolerant rhizobia from the indigenous population (Zahran, 1991). It is also suggested that a need to select plant genotype that is tolerant to salt stress and then match them with the salt tolerant and effective strain of rhizobia (Cordovilla *et al*., 1995).

3.7 Inoculant Production

Legume inoculation is the process of introducing commercially prepared source of rhizobia to promote nitrogen fixation. Commercial inoculants are composed of rhizobial strains selected for maximum fixation potential. Inoculation has the potential of increasing dry matter yield, N yield, and residual N levels. Legume crops are inoculated to increase yields and decrease the input costs of nitrogen fertilizer in agricultural production.

The production of rhizobial inoculants is simple and requires only modest technology. Pure cultures of the required strains are grown in broth culture in a fermentor. The culture then inoculated in to a solid carrier, which can be used to apply the inoculants to seeds. Lime usually added to adjust to neutral pH for good survival of rhizobial (Somasegaran, 1985).

The most widely used form of inocula is pure culture of rhizobial strains impregnated in to a solid substrate such as finely ground peat (Williams, 1984). The peat serves as a carrier to allow the inoculants to be coated with the surface of seeds, and also serves as to protect the bacteria against desiccation. The main criteria for selection of a suitable solid carrier for inoculants are high water holding capacity, ability to support the growth of rhizobia and an ability to favor survival of rhizobia (Hynes et al., 1995).

Production of inoculants of consistently high quality requires checking of cultures for contamination by other microbes at all stage of production. It is also essential that the parent cultures be checked frequently for effectiveness in N-fixation on the target legume as inadvertent substitution of cultures, contamination, or genetic instability in the inoculant strain can lead to loss of inoculum effectiveness (Vincent, 1977). The success of inoculum often is limited by several factors, including environmental conditions, the number of effective cells applied, the number of competing indigenous rhizobia, and the inoculation method (Brock well *et al*., 1995).

3.8 Selection of Elite Strains of Root Nodule Bacteria

In order to maximize productivity in legume-based agriculture, there is an essential requirement for matching of the root nodule bacteria to both host legumes and soil conditions (Beck and Duc, 1991; O'Hara *et al*., 2002). The root nodule bacteria are a diverse group of ubiquitous soil inhibiting gram-negative bacteria (O'Hara, 2001) and it is very likely that all agricultural soils contain some bacteria capable of nodulating some legumes (O'Hara *et al*., 2002).

Beck and Duc, (1991) reported that, most soils where faba bean is grown contain (10^2 -10 ^{4}/g) number of rhizobia, which can vary in their symbiotic capability. However, the root nodule bacteria present in any given soil may not be able to nodulate legumes being grown as crops or pastures or, may not undertake effective symbiosis that contribute nitrogen to the host legume and to the farming system (O'Hara *et al*., 2002). This is quite common for the situation where new legumes are being introduced to new lands.

The abundant diversity in soil populations of root nodule bacteria provides a large resource of natural germplasm to screen for desired characteristics (Sadowsky and Graham, 1998; Dilworth *et a*l., 2001). The essential characteristics for inoculum strains of root nodule bacteria is effective nitrogen fixation on a wide range of host legume species and other beneficial characteristics such as stress tolerance, competitive ability to nodulate the hosts, genetic stability and satisfactory growth and survival (Brockwell *et al.,* 1995; O’Hara *et al.,* 2002).

According to Howieson *et al.* (2000), the current technique used to select appropriate strains of root nodule bacteria require a combination of glasshouse and fieldwork. The strain selection program has four phases. These are; germplasm acquisition, (isolation and storage of strains), authentication of isolates as root nodule bacteria and screening for effective nitrogen fixation, and taken to the field for their performance on the fields. The final phase involves validation of strain performance using a smaller number of strains in large-scale rotation trails in farming systems on different locations.

A current challenge for selection of inoculants strains is the need for single inoculants strains to effectively nodulate a broad range of legumes. This can be exemplified by isolating a strain of *R.legminosarum* bv. *viciae* that nodulate species of *Lens*, *Lathyrus*, *Vicia* and *Pisum* (Howieson *et al*., 2000).

3.9 Ethiopian Background

Faba bean, chickpea, field pea, lentil and grass pea are the major cool-season food legumes in Ethiopia. The crops have various benefits to Ethiopian agriculture and diet; however, they were not given the proper research and development emphasis (Asfaw Telaye and Mahmoud Solh, 1994). Through ICARDA's Nile Valley regional program, the Ethiopian scientists have undertaken laboratory research and field trail demonstration to improve the productivity of cool-season food legumes.

Of the cool-season food legumes grown in Ethiopia, only faba bean and chickpea have received some attention while few studies on rhizobiology have been made (Tekalign Mamo &Asegelil Dibabe, 1994). Desta Beyene &Angaw Tsigie (1986; 1987; 1988a,b) studied nodulation on faba bean under Ethiopian conditions, nodulation and rhizobial strains in faba bean, selection of effective *Rhizobium* strains in faba bean and evaluation of root nodules collected from faba bean respectively.

Similarly, evaluation of faba bean rhizobium strains on vertisols (Balesh Tulema & Asnakaw Wolde Ab, 1993a), effect of inoculation on nodulation and yield of faba bean (Balesh Tulema & Asnakaw Wolde Ab, 1993b), and isolation and selection of *Rhizobium* from collected nodules field pea, chickpea, and lentil (Balesh Tulema & Asnakaw Wolde Ab, 1993c) have been undertaken. Further more Ayneabeba Adamu *et al*. (2001), and Asefa Keneni (2002) studied the importance of Rhizobium inoculation and fertilizer treatment on growth and production of faba bean (*Vicia faba)* in some yield depleted and yield sustained regions of Semien Shewa.

From such background information, it is evident that the knowledge of the diversity and relative symbiotic effectiveness of natural population of *Rhizobium leguminosarum* on faba bean in different regions is limited. Therefore, it is necessary to bridge the information gap of symbiotic effectiveness of rhizobia from different faba bean growing regions of the country.

4. MATERIALS AND METHODS

The rhizobium isolation, identifications, pot experiments, and total nitrogen analysis were carried out at the Department of Biology, Addis Ababa University. Soil chemical analysis was done at the National Soil laboratory, Ethiopian institute of Agricultural Research (EIAR).

4.1. Study Site

The study sites are located in Northern Gondar Zone, Amhara Regional State, 650km away from the capital, Addis Ababa (Fig.1). According to MoA (2000) the agroecological classification of the zone is between 1500m.a.s.l (*erteb kola*) sub-moist and 3500m.a.s.l cold (*dega*) regions. The study sites are characterized by mean annual temperature of 10-20^{0}C and average annual rainfall of 900mm. Soil pH of the study sites are generally neutral and near neutral, except Aba Matabo with a pH of 5.8 (Table 1).

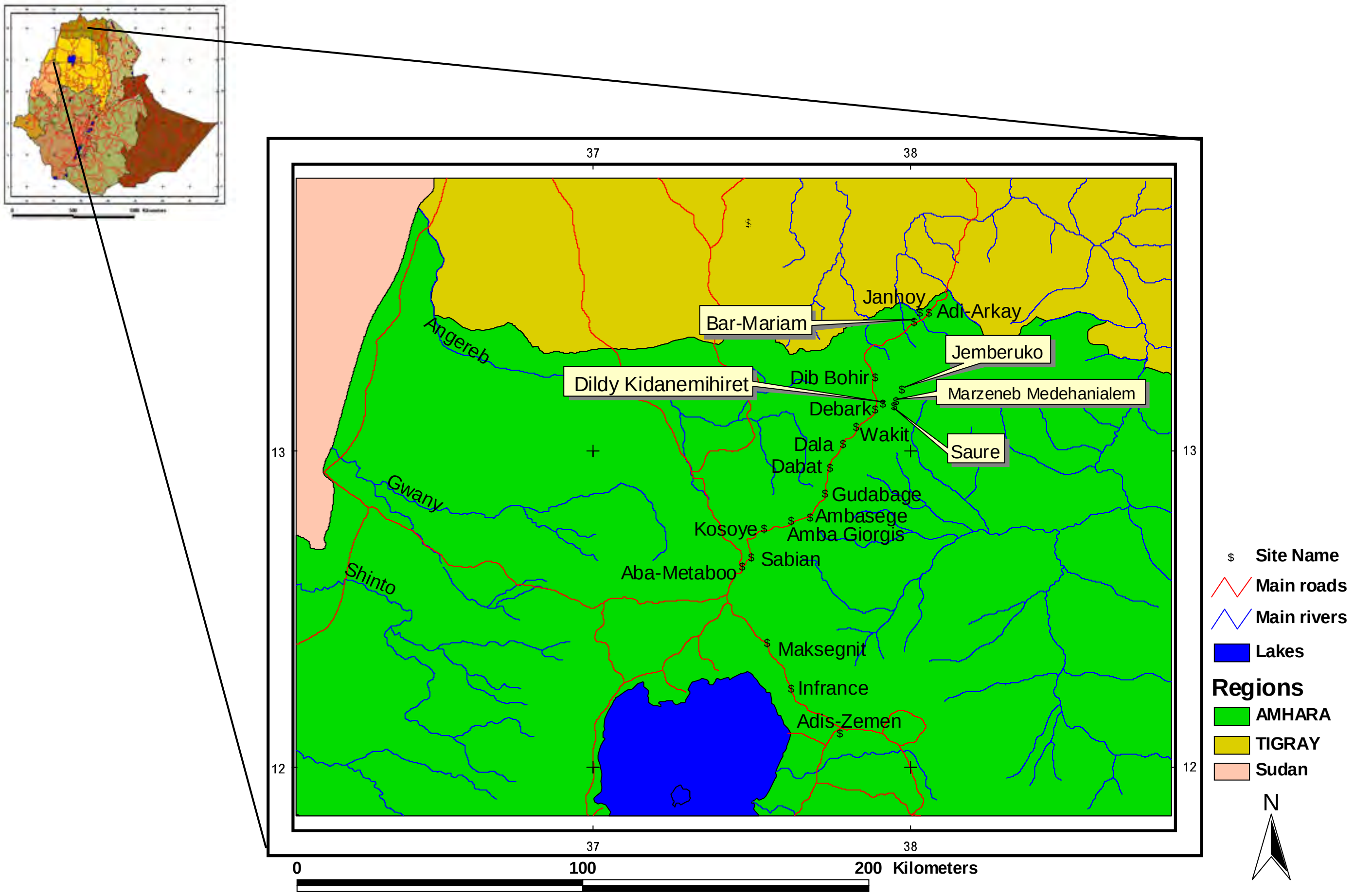

Figure 1. Location Map of the Sampling Sites

4.2. Soil Sampling

Samples were collected from twenty-one soil sites in Oct. 2004 (Table 1). From each site five farmer fields were selected, and samples from10-15 cm depth were pooled, composited and collected in alcohol sterilized plastic bags. The samples were taken to the laboratory of the Department of Biology, AAU, for further work.

Table 1. Sample location, altitude, soil pH and types of legumes on the soil field

Sample number	Name of the sites	Altitude (m.a.s.l)	Location		Type of legume on the soil field	Soil pH H_2O (1:2.5)
			Latitude (N)	Longitude (E)		
12	Infranz	ND	12^{0}15.	037^{0}37.529’	Faba bean	6.7
13	Maksegnit	1929	12^{0}39.856’	037^{0}33.122’	Faba bean	7.2
14	Aba Matabo	2264	13^{0}43.151’	037^{0}28.355’	Faba bean	5.8
15	Sabian	2419	12^{0}45.233’	037^{0}30.083’	Faba bean	6.7
16	Janhoy	2693	12^{0}47.433’	037^{0}29.511’	Faba bean	6.6
17	Kosaye	2898	12^{0}51.82’	037^{0}32.41’	Faba bean	6.3
18	Ambasege	2694	12^{0}56.831’	037^{0}41.104’	Faba bean	6.8
19	Godabage	2767	13^{0}01.257’	037^{0}43.988’	Faba bean	7.4
20	Dabat	2634	13^{0}04.463’	037^{0}44.839’	Faba bean	6.7
21	Dala	2672	13^{0}01.257’	037^{0}47.351’	Faba bean	6.7
22	Wokin	2758	13^{0}04.463’	037^{0}49.790’	Faba bean	6.5
23	Debark	2795	13^{0}07.912	037^{0}53.484’	Faba bean	6.3
24	Jemberko	3196	13^{0}11.704’	037^{0}58.440’	Faba bean	6.9
25	Merzeneb medhanlem	3074	13^{0}09.462’	037^{0}57.382’	Faba bean	6.1
26	Saurae	3042	13^{0}08.467’	037^{0}57.042’	Faba bean	7.3
27	Dildykidanemihret	2923	13^{0}09.029’	037^{0}54.939’	Faba bean	6.6
28	Dib-bahir	2163	13^{0}13.982’	037^{0}53.376’	Faba bean	6.1
29	Bar-Mariyam	1586	13^{0}24.574;	038^{0}00.773’	Chick pea	7.5
30	Adi-Arkay	1529	13^{0}206.224’	038^{0}03.498’	Soya bean	6.8
32	Amba-Georgis	2837	12^{0}46.750’	037^{0}38.909’	Faba bean	7.3
34	Adis-Zemen	1923	12^{0}06.364’	-	Chick pea	7.5

ND- not determined

4.3 *Rhizobium* Isolation

Rhizobia were isolated from the soil samples by using 'plant induction' method (Vincent, 1970). Each representative soil sample was thoroughly mixed and sieved using 2mm sieve. The soil from each sample was filled into 3 kg capacity plastic pots, which had been surface sterilized by swabbing with 70% alcohol. Undamaged and selected seeds of faba bean were surface sterilized briefly with 95% ethyl alcohol for 10seconds and 0.2% acidified mercuric chloride solutions for 3 minutes (Vincent, 1970). After rinsing five times with sterile water, five seeds were planted on each pot, which were later thinned down to three after germination. The pots were watered twice a week at full field capacity, and arranged in a complete random design to allow plant growth in a green house with 12/12h light/dark cycle. After 45 days of planting, plants were uprooted and nodules were randomly picked, surface sterilized as before and crushed. Loop full of crushed nodules sap were transferred on Yeast Extract Mannitol Agar (YEMA) and incubated at 28^0C for 3-5 days.

Yeast Extract Mannitol Agar (YEMA) (Vincent, 1970) composition contains: -

Mannitol	10 g/l
K_2HP0_4	0.5 g/l
$MgS0_4 .7H_20$	0.2 g/l
NaCl	0.1 g/l
Yeast Extract	0.5 g/l
Agar	15 g/l
Distilled Water	1000 ml
pH	7±0.1

Autoclaved at 121^0C for 15 minutes.

Plates were examined for growth and single colonies were picked up and periodically purified by re-streaking on new YEMA plates. Pure isolates were then preserved on YEMA slants containing 0.3% (w/v) $CaCO_3$ and stored at 4^0C (Vincent, 1970).

4.4 Characterization of the Isolates

All colonies were checked on YEMA containing 25 μgml^{-1} Congo red to evaluate their ability to absorb the dye. Isolates were also inoculated on YMA containing 25 μg ml^{-1} bromothymol blue (BTB) to determine their ability to produce acid or base and change the medium (Lupwayi and Haque, 1994). The isolates were also characterized by colony appearance, colour, extracellular polysaccharide (EPS) production and the size of the colony, on YEMA plates incubated at 28^{0}C for 3-5 days (Ahmed *et al*., 1984).

4.4.1 Determination of Mean Generation Time (MGT)

Growth of the isolates was determined as described by Somasegaran and Hoben (1994). Bacterial growth was assessed on YEMB broth in Erlenmeyer flask incubated in a Gyratory shaker at 100 rev. min^{-1}, by measuring the optical density using Spectrophotometer (Jenway 6405 UV/vis.) at 540nm every 6hrs. Viable counts were also taken every 6 hrs on YEMA by Colony Spread Plate method. Mean generation time or doubling time was calculated from the logarithmic phase (White, 1995).

4.4.2 Physiological Characteristics

All tests were carried out on triplicate YEM agar plates except pH that tested on Kesyer defined medium and spot inoculated using 3.3μl calibrated inoculation loop containing (10^5 cells ml^{-1}), against control plates and incubated at 28^{0}C (Lupwayi and Haque, 1994). Growth was determined qualitatively and recorded as (+) for growth or (-) for no growth.

4.4.2.1 Salt Tolerance

The strains were tested for their tolerance to the salinity in YEM agar medium supplemented with NaCl at concentrations of 0.1, 0.3, 0.5,0.8,1,2,3,4, 5 and 6% (w/v) (Lupwayi and Haque, 1994).

4.4.2.2 Temperature Tolerance

The ability of bacterial strains to grow at high and low temperatures was monitored using YEMA medium incubated at 5^{0}C, 10^{0}C, 15^{0}C, 35^{0}C, 40^{0}C and 45^{0}C(Lupwayi and Haque, 1994).

4.4.2.3 pH Tolerance

The ability of the strains to grow in acid and alkaline media was tested by inoculating them into Kesyer defined medium, to which the pH was adjusted to 4,4.5,5,5.5,8 and 9 with sterile HCl or NaOH. Kesyer defined medium modified by (CIAT, 1988) contains

A. Micronutrients stock solution 0.5ml

$MnCl_2.4H_2O$	0.504g/l
$ZnSO_4.7H_2O$	0.277g/l
$CuCl_2.2H_2O$	0.034g/l
$NaMO_2.2H_2O$	0.008g/l
B. Phosphate stock solute	1.0ml
KH_2PO_4	1.36g/l
C. Vitamin stock solution	1.0ml
Thiamine HCl	0.4g/l
d-pantothonic acid (Ca)	0.4g/l
Biotin	0.0001g/l

Medium	**Per liter**
Glycerol	5 ml
K_2 $S0_4$	0.131g
Na-glutamate	0.220g
$MgSO_4.7H_2O$	0.074g
$CalCl_2.2H_20$	0.007g
Fe-EDTA	0.035g
Solution A	0.5ml
Solution B	1.0ml
Solution C	1.0ml
Agar	20g

4.4.2.4 Utilization of Carbohydrates

Carbon utilization of strains was determined following the method of Somasegaran and Hoben (1994) on seventeen carbohydrates. Carbohydrates were prepared as 10% (w/v) solution in water. The carbohydrates free medium, which was essentially similar to YEMA medium, was modified by reducing the yeast extract to 0.05g/ liter. Heat labile carbohydrates already were sterilized by membrane filtration using millipore with pore size of 0.22µm and added to the autoclaved carbohydrate free basal medium. The heat-stable carbohydrates were autoclaved together with the medium. YEMA medium with out carbon source and with mannitol were used as a negative and positive controls, respectively.

4.4.2.5 Intrinsic Antibiotic Resistance (IAR)

The intrinsic antibiotic resistance of isolates was determined on solid YEM medium containing filter sterilized antibiotics using 0.22 µm size membrane filters, at concentration of 2.5,5 and 10µg/ml according to Beynon and Josey, (1980). The antibiotics were Ampcillin, Streptomycin, Rifampcin, Kanamycin, Erythromycin, Nalidixic acid, and Chloramphenicol.

4.4.2.6 Numerical Analysis

A computer cluster analysis of phenotypic variables of the isolates was carried out using a similarity coefficient and phenogram was constructed by the un weighted pair group method with the average (UPGMA) clustering method, using (NTSYS Version 2:1) software

4.5 Authentications and Evaluation of Nitrogen –fixing Effectiveness

The effectiveness of rhizobial strains was tested in pot experiment in controlled glass house using sterile sand and unsterilized field soil culture systems.

4.5.1 Screening under sand pot experiment

About 3kg of carefully washed, sieved, and acid sterilized river sand was filled into alcohol sterilized plastic pots. The faba bean cultivar called “Degaga” obtained from Holeta Agricultural Research Institute was used in this study. Seeds of uniform size and colour were surface sterilized as described before (Vincent, 1970) and transferred to 0.75% (w/v) water agar plates for germination and incubated at 25^0C for 3 days. Four seedlings were transferred to each pot, which were latter thinned dawn to three. Each strain of rhizobia grown on YEM liquid medium to logarithmic phase (10^9 cells ml^{-1}) and (1ml/seedling) of the culture was inoculated. The experiment was set up as a randomized complete block design with three blocks or replications. There were 21 treatments, a plus –N control with no inoculation, and a noninoculated control with no N. The plus control contains 70mg/liter of N applied as a 0.05% KNO_3 (w/v) solution every week (Somasegaran and Hoben, 1994). Plants were supplied with distilled water every two days, and fertilized once a week with quarter strength of N-free nutrient solution (Broughton and Dilworth, 1970 cited in Lupwayi and Haque, 1994).

Broughton and Dilworth (1970) medium contain;

Nutrient	**Form**	**g/l**
Ca	$CaCl_2.2H_2O$	294.1
P	KH_2PO_4	136.1
Fe	Fe-Citrate	6.7
Mg	$MgSO_4.7H_2O$	123.3
K	K_2SO_4	87.0
Mn	$MnSO_4.H_2O$	0338
B	H_3BO_3	0.247
Zn	$ZnSO_4.7H_2O$	0.288
Cu	$CuSO_4.5H_2O$	0.100
Co	$CoSO_4.7H_2O$	0.056
Mo	Na Mo O_3H_2O	0.048

Plants were grown in a green house with a 12/12h light/dark cycle and $29/18^0$C day/ night temperature. All plants were harvested forty-five days after sowing, the roots scored for nodulation, and the top plants and nodules oven dried at 70^0C for 48hrs to determine the dry weight.

4.5.1.1 Relative effectiveness of isolates

Strain effectiveness was calculated according to the equation proposed by Date *et al.* (1993) cited in Purcino *et al.* (2000) [100× inoculated plant DM/ N-fertilized plant DM) with Nitrogen fixing effectiveness classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

4.5.2 Screening under soil pot experiment

In order to determine the effectiveness of the strains at two different non-sterile soil conditions, finely sieved soil samples (as before) from Holeta (Shoa) and Ambagirogis (Northern Gondar) were used for the experiment. Table 2 shows the chemical analyses of the two soils.

Table 2. Soil chemical analyses

Parameter	**Holeta**	**Ambagiorgis**
Depth (cm)	0-30	0-30
pH H_2O 1:2.5	4.8	7.3
EC (ds/m) [1]	0.059	0.172
Na (Meq/100gm soil) [2]	2.60	2.78
K (Meq/100gm soil) [3]	1.81	2.18
Ca (Meq/100gm soil) [4]	6.24	28.84
Mg (Meq/100gm soil) [5]	8.72	10.78
CEC (Meq/100gm soil) [6]	30.00	56.20
T.N. (%)[7]	1.66	2.09
O.C. (%)[8]	1.549	2.071
C: N [9]	9	10
Av.P.ol. (Ppm) [10]	6.44	47.08
HCO_3 (Me/100g) [11]	0.11	0.23

1,Electron conductivity; 2,Total sodium; 3, Total potassium; 4, Total calcium; 5, Total magnesium; 6, Cation exchange capacity; 7, Total nitrogen; 8, Organic carbon; 9,Ratio of carbon to nitrogen content; 10, Available phosphorous; 11, Total bicarbonate

Soil samples were filled in 3kg capacity of surface sterilized pots and once treated with fertilizer recommended in Somasegaran and Hoben (1994). The composition of the fertilizers in kg/ha: 500 KH_2PO_4, 382 KCl, 50 $MgS0_4.7H_2O$, 46.8 $ZnS0_4.7H_2O$, 1.76 (NH_4) 6 $Mo_7O_{24}.H_2O$. 222kg ha^{-1} urea Co $(NH_2)_2$ was added as nitrogen source for N-Control pots. Before planting the soils were watered to approximately 75% field capacity. Healthy selected seeds of Degaga variety faba bean were surface sterilized as described before (Vincent, 1970) and allowed to germinate on a water agar for three days at 25^0C. Based on their symbiotic efficiency selected rhizobial isolates were prepared by growing in YEM nutrients broth media and adjusted their number approximately to be 10^9 cells ml^{-1}. One ml of each culture suspension was flooded on each seedling for one hour. The inoculated seedlings were planted on each pot, which were later thinned down to three. A N+ control with no inoculation and a non-inoculated control with no N, were also included. The experiment was set up as a randomized complete block design with three blocks or replications. The pots were watered every two days to field capacity of 75%. After 45 days of growth, the whole plants were carefully up rooted to determine nodulation score, nodule dry weight, shoot dry mass and total nitrogen.

4.5.3 Plant total nitrogen analysis

Total nitrogen is quantitatively determined by the modified "Wet" Kjeldahl method. The Kjeldahl procedure is based on the principle that by treating plant material with concentrated sulpheric acid to oxidize the nitrogen in to ammonium sulphate. The ammonia liberated in the distillation process with NaOH is trapped by acid. The ammonia is adsorbed in the form of NH_4^+ ion in boric acid and back titrated with standard H_2SO_4 (Sahlemedhin Sertsu and Taye Bekele, 2000). Then the percentage of total nitrogen was calculated as follows.

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

Where,

a = ml of H_2S04 required for titration of sample

b = ml of H_2S04 required for titration of blank

S = Sample weight in mg

N = Normality of H_2So_4

0.014$_=$ meq weight of nitrogen in g *Mcf*=moisture correction factor

4.6 Data Analysis

Comparison between treatments was analyzed using by one-way ANOVA (Tukey's HSD tests) (SPSS.10).

5. Results

5.1 Morphological and growth characteristics of the isolates

Twenty-one isolates were recovered from the nodules of the trap host grown in soils collected from various sites of Northern Gondar Zone. All colonies of the isolates were similar in appearance with large mucoid (LM) and large watery (LW) with diameter of 2-4.5mm except colony of isolate AUFR13, which was, less translucent, dry, smaller, and less than lmm in diameter after 3-5 days growth. All isolates showed no color absorption on congored except AUFR12 that completely absorbed congored under dark incubation. All isolates showed deep to moderate yellow colour on BTB medium. On the basis of their generation time, isolates displayed different doubling times ranging from 1.9 to 4.3hrs. Among the isolates, AUFR20 showed the fastest growth with 1.9hrs and isolate AUFR32, with slow growth of 4.3hrs. The doubling times of 90% of the isolates were between 2.5 and 4.3hrs (Table 3).

Table 3. Colony Morphology, Colony Diameter, growth on CR and BTB, and Growth rate of the isolates

Isolates	Colony characteristics after 4-5 days	Colony diameter	Growth on YEMA-CR	Growth on BTB	MGT
AUFR 12	Circular, less translucent, raised and mucoid	2.5mm	Red color	Yellow	2.1hrs
AUFR 13	Poor growth of colony, smaller, dry, and less shine	<1mm	Colour less	Yellow	2.6hrs
AUFR 14	Circular less translucent raised and mucoid	3.5m	Colour less	Yellow	3.1hrs
AUFR 15	Circular, less translucent, raised and mucoid	3.5mm	Color less	Yellow	3.2hrs
AUFR 16	White, raised and mucoid colony	3mm	Color less	Moderate yellow	4.2hrs
AUFR 17	Less translucent, raised and mucoid colony	4mm	Color less	Yellow	4.1hrs
AUFR 18	Irregular, less translucent mucoid, raised and larger colony	5mm	Color less	Yellow	2.9hrs
AUFR 19	Less translucent, raised, mucoid and larger colonies	4.5mm	Color less	Deep yellow	3.0hrs
AUFR 20	Less translucent, raised and mucoid colony	4.5mm	Color less	Deep yellow	1.9hrs
AUFR 21	Less translucent, raised and mucoid colony	3.5mm	Color less	Deep yellow	2.9hrs
AUFR 22	Less translucent, raised and mucoid colony	3mm	Color less	Deep yellow	4.1hrs
AUFR 23	Less translucent, raised and mucoid colony	3mm	Color less	Deep yellow	2.9hrs
AUFR 24	Less translucent, raised and mucoid colony	3.5mm	Color less	Deep yellow	3.2hrs
AUFR 25	Less translucent, raised and mucoid colony	3.mm	Color less	Deep yellow	3.3hrs
AUFR 26	Less translucent, raised and mucoid colony	4mm	Color less	Deep yellow	3.8hrs
AUFR 27	Highly mucoid, very less translucent and elastic	4mm	Color less	Deep yellow	3.0hrs
AUFR 28	Less translucent, mucoid and raised colony	2.5mm	Color less	Deep yellow	3.3hrs
AUFR 29	Shiny (translucent), raised and mucoid colony	2.5mm	Color less	Deep yellow	3.1hrs
AUFR 30	Less translucent, raised and mucoid colony	2mm	Color less	Deep yellow	3.2hrs
AUFR 32	Less translucent, raised and mucoid colony	2.5mm	Color less	Deep yellow	4.3hrs
AUFR 34	Less translucent, raised and mucoid colony	2.5mm	Color less	Deep yellow	2.5hrs

5.2 Physiological Characterization

5.2.1 Temperature tolerance

As indicated in Table 4, all rhizobial isolates grew at 15^0C and 35^0C except isolate AUFR13 that showed no growth at temperature of 15^0C.Isolates AUFR22 and AUFR28 were found to grow at a temperature of 40^0C whereas two isolates AUFR18 and AUFR26 showed growth at temperatures of 5^0C and10^0C.

5.2.2 Salt tolerance

Tolerance of rhizobial isolates to NaCl concentration showed variation among strains. All isolates showed growth at YEMA containing 0.1% of NaCl. However, only 57%, 33% and 19% of the isolates were found to grow on YEMA supplied with 0.3%, 0.5% and 0.8% NaCl, respectively. At higher concentrations, number of tolerant strains decreased rapidly so that only isolate AUFR12, AUFR18, and AUFR28 tolerated NaCl concentration of 1% and 2%. The only isolate that resisted growing at 5% NaCl concentration was AUFR18 (Table 4).

5.2.3 pH tolerance

Differences in pH tolerance are shown in Table 4. All isolates except AURF13 and AUFR22 grew at pH 5.5. All were found to resist pH 8.0 and pH 9.0. None of the strains tested grew in medium of pH 4.0 and 4.5 except isolates AUFR12 and AUFR28, capable of surviving at pH5.0.

Table 4. Physiological characterization of the isolates (Temp. Salt and pH*)*

Character	AUFR 12	AUFR 13	AUFR 14	AUFR 15	AUFR 16	AUFR 17	AUFR 18	AUFR 19	AUFR 20	AUFR 21	AUFR 22	AUFR 23	AUFR 24	AUFR 25	AUFR 26	AUFR 27	AUFR 28	AUFR 29	AUFR 30	AUFR 32	AUFR 34	**No of (+) isolates**
Temperature																						
5^{o}C	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	2
10^{o}C	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	2
15^{o}C	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	20
35^{o}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21
40^{o}C	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	2
Salt tolerance																						
0.1%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21
0.3%	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	-	-	-	12
0.5%	+	+	-	-	-	-	+	-	-	-	+	-	-	-	+	-	+	+	-	-	-	7
0.8 %	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	4
1%	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	3
2%	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	3
3%	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
4%	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
5%	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
pH tolerance											-											
5.0	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	2
5.5	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	19
8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21
9.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21

(+) Growth (-) No growth

5.2.4 Utilization of Carbohydrates

Almost all of the faba bean rhizobia were able to catabolize a large variety of carbon substrates (Tables 5). All tested strains grew on D-xylose, Fructose, Adonitol, Cellobiose, Glycerol, Lactose, Sucrose, Sorbitol, Arabinose Rhaminose, Galactose, Mannose, Maltose, and Raffinose. None of them utilized Citrate. With regard to the remaining carbohydrates such as Gluconate and Tartarate, rhizobial isolates exhibited a large diversity. Only 29% and 33% of the isolates utilized Gluconate and Tartarate, respectively. Rhizobial isolates AUFR12, AUFR13, AUFR14, AUFR18, AUFR26 and AUFR28 utilized 96% of carbohydrates. Two isolates AUFR19 and AUFR22 utilized 91% and the rest of them catabolized only 86% of the tested carbohydrates (Appendix 1).

Table 5. Comparative analysis of *Rhizobium leguminosarum* isolates for utilization of different carbohydrates

Isolates (%) utilized carbohydrates			
Source	**1**	**2**	**3**
Xylose	100	100	90
Arabinose	100	100	80
Rhamnose	100	92	90
Glucose	ND	92	80
Fructose	100	100	90
Mannose	100	100	90
Galactose	96	80	90
Maltose	100	80	90
Sucrose	96	92	80
Lactose	96	92	90
Gluconate	29	ND	ND
Tartarate	33	ND	ND
Citrate	0	ND	20
Sorbitol	100	100	90
Raffinose	100	92	ND
Adonitol	100	ND	ND
Cellobiose	100	ND	ND
Glycerol	100	ND	ND

1= Current work (*Rhizobium leguminosarum* var *viceae*) (faba bean)
2=Graham and Parker (1964) (*Rhizobium leguminosarum* var *viceae*) (bv. *viceae*; field pea, faba bean, lentil, latyrus)
3= Lindstrom and Lehtomaki (1988) (*Rhizobium leguminosarum* (bv. *trifolli*, bv. *phaseoli*, bv. *viceae.*)

5.2.5 Intrinsic Antibiotic Resistance

95% of the tested isolates were resistance to Erythromycin and Chloramphenicol at all concentrations. Resistance to all concentrations of Ampcillin, Streptomycin, Rifampcin, Kanamycin, and Nalidixic acid was recorded for only 24 %, 14%, 38%, 38%, and 43% of the isolates, respectively. Higher percentages of isolates were able to tolerate lower concentrations of Ampcillin, Streptomycin, Rifampcin, Kanamycin, Erythromycin, Nalidixic acid, and Chloramphenicol. Isolate AUFR28 and AUFR26 were found to be tolerant to almost all antibiotics, whereas AUFR12 and AUFR32 were the most resistant strains on several antibiotics. Isolate AUFR21 was observed to be the most sensitive strain capable of growing on only Rifampcin, Erythromycin, and Chloramphenicol at all tested concentrations (Appendix 2). Streptomycin and Kanamycin were found to be the most potent antibiotics that allow the growth of a few isolates.

Table 6. Antibiotic resistance of isolates at different concentrations.

	% Resistance of isolates		
Antibiotics	2.5 µgml-1	5.0 µgml-1	10 µgml-1
Ampicillin	91	24	24
Chloramphenicol	95	95	95
Erythromycin	95	95	95
Kanamycin	52	38	38
Nalidixic acid	95	95	43
Rifampicin	67	67	38
Streptomycin	76	48	14

5.2.6 Numerical analysis

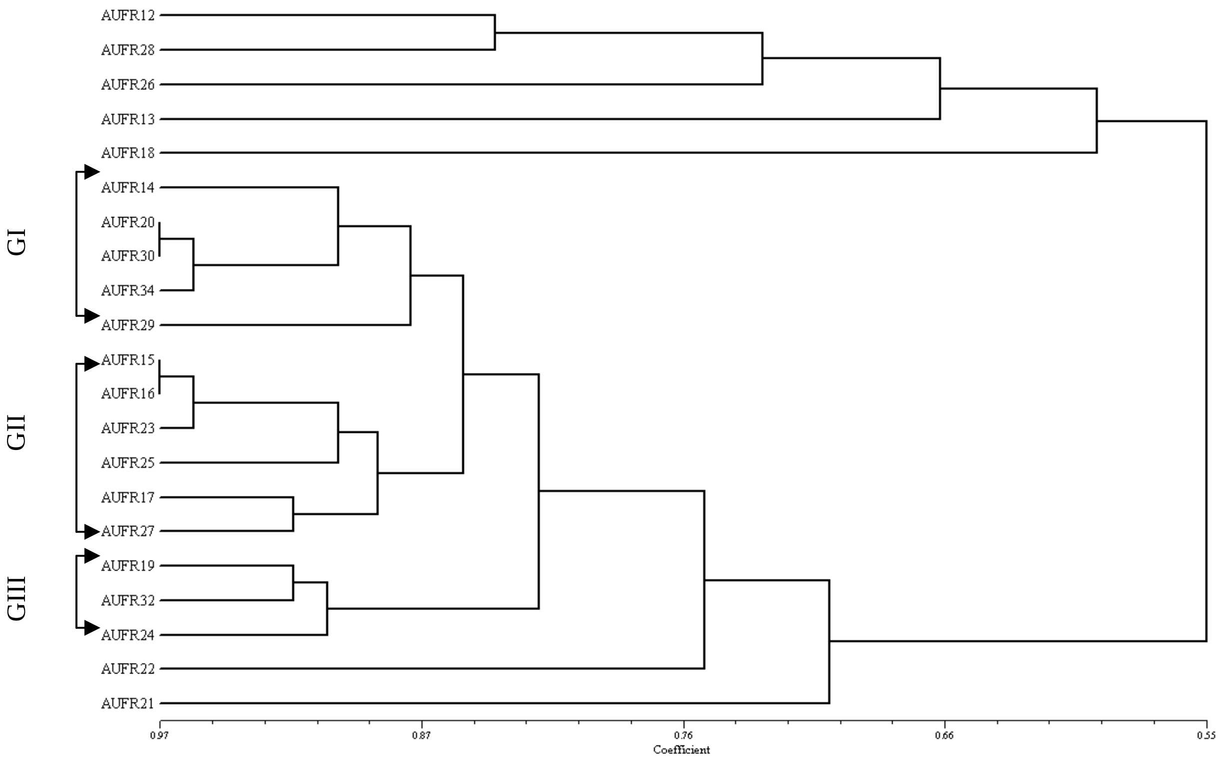

Figure 2. **Dendrogram highlighting the phenotypic similarities among faba bean rhizobia (*Rhizobium leguminosarum* bv. *viceae*) from different areas of Northern Gondar.**

Numerical analysis based on different phenotypic characteristics puts the isolates in to different diversity groups (Fig.2). AUFR12, AUFR28, AUFR26, AUFR13, AUFR18, AUFR22 and AUFR21 were found to be separately clustered and the rest were clustered in to three main clusters at a boundary level of 0.85 average distances. Cluster I comprises AUFR14, AUFR20, AUFR30, AUFR34, and AUFR29, cluster II AUFR15, AUFR16, AUFR23, AUFR25, AUFR17, AUFR27, and cluster III AUFR19, AUFR32 and AUFR24.

5.3 Pot Experiment

5.3.1 Evaluation of Symbiotic Effectiveness on Sand Culture

The infectiveness and effectiveness of the twenty-one isolates were studied on sand pots under green house conditions Fig.3. All isolates, except AUFR13 were authenticated as root nodule bacteria by forming nodules on the test host plant Fig.4. Results of screening of the infective strains showed that they vary significantly in shoot dry mass productions ($P<0.05$) as shown in Table 7.

The nodule number record ranged from 67 for isolates AUFR27 to 168 for isolates AUFR24. Similarly nodules dry mass recorded between 37 mg/p and 111 mg/pl for isolates AUFR27 and AU FR18, respectively. Maximum mean shoot dry mass (2.4g/pl) was scored by positive nitrogen control and minimum (0.2g/pl) by the negative nitrogen control. The relative effectiveness expressed as percentage of shoot dry mass of inoculants over TN control, showed that 23.8%, 57%, 4.7% and 9.5% of the isolates were found to be highly effective (80-100%), effective (50-80%), lowly effective (35-50%) and ineffective (< 35%), respectively (Table 8). The highest scores of 88-100% effectiveness of symbiotic nitrogen fixation were displayed by AUFR14, AUFR15, AUFR24, AUFR28 and AUFR32 with shoot dry mass >2.0g/pl. Isolates AUFR12 was found to be lowly effective whereas AUFR27 and AUFR 29 obtained to be ineffective which were observed to be slightly higher than the ineffective equivalent of 0.2g/pl showed by uninoculated and non- N-fertilized negative control plant (Table 7).

Table 7. Nodulation and relative Effectveness of Nitrogen fixation of *Rhizobium leguminosarum* var *viceae* isolates of North Gondar tested on *Degaga* variety of faba bean on sand culture

Treatment	Nodule number/pl	Nodule dry mass (mg/pl)	Shoot dry mass (g/pl)	% Efficiency N fixation
AUFR12	133 ^{a}	108^{a}	1.0 ^{b}	42
AUFR13	-	-	-	-
AUFR14	148 ^{a}	91.1^{ab}	2.1 ^{a}	88
AUFR15	136 ^{a}	78.8^{a}	2.1^{a}	88
AUFR16	134 ^{a}	55.2^{ab}	1.7 ^{a}	71
AUFR17	151 ^{a}	88.4^{ab}	1.5 ^{a}	63
AUFR18	123^{a}	111.1 ^{a}	1.8 ^{a}	75
AUFR19	125 ^{a}	83 ^{a}	1.8 ^{a}	75
AUFR20	137^{a}	81.8 ^{a}	1.9 ^{a}	79
AUFR21	91^{ab}	54.5^{ab}	1.7^{ab}	71
AUFR22	154 ^{a}	55.2^{ab}	1.8 ^{a}	75
AUFR23	119 ^{a}	77.7^{ab}	1.6 ^{a}	67
AUFR24	168 ^{a}	78.8 ^{a}	2.3 ^{a}	96
AUFR25	128^{ab}	69.1^{ab}	1.4 ^{a}	58
AUFR26	156^{a}	79.2^{ab}	1.8 ^{a}	75
AUFR27	67^{b}	37.0^{b}	0.4^{b}	17
AUFR28	128 ^{a}	99.5^{ab}	2.1 ^{a}	88
AUFR29	78^{ab}	42.8^{b}	0.70^{b}	29
AUFR30	91^{ab}	53.7^{ab}	1.4 ^{b}	58
AUFR32	137 ^{a}	79.9^{ab}	2.2^{a}	92
AUFR34	88^{b}	74.9^{ab}	1.3^{ab}	54
N-	-	-	0.2 ^{b}	8
N+	-	-	2.4^{a}	100

-: Not found

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

Fig.3 Showing the shoot height of the different treatment on sand culture

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Figure 4. Showing proliferation nodulation occurring for isolate AUFR24 on sand culture

Table 8. Comparative analysis of relative symbiotic effectiveness of the isolates

Absence of nodulation **(GI)**	Ineffective **(GII)**	Mildly effective **(GIII)**	Effective **(GIV)**	Highly effective **(GV)**
AFUR13	AFUR27 AFUR29	AFUR 12	AFUR17, AFUR16, AFUR18, AFUR19, AFUR20, AFUR21, AFUR22 AUR23, AFUR25, AFUR26, AFUR30, AFUR34	AFUR14, AFUR15, AFUR24, AFUR28, AFUR32
4.76%	9.5%	4.76%	57%	23.8%

5.3.2 Evaluation of Symbiotic Effectiveness of the Strains on Different Soils

Based on the total shoot dry mass production, five top strains were selected as inoculants for faba bean grown on the acidic Holeta soil and the slightly neutral Ambagiorgis soil. The symbiotic effectiveness of the strains was evaluated based on shoot dry mass, nodule number and dry mass, and total nitrogen of the plant. Although, all strains accumulated 2.2-2.5 g/pl dry matter on Holeta soil, and 2.6-3.0 g/pl on Ambagiorgis soil, there was no significant inter-strain difference in the shoot dry mass, nodule dry mass and nitrogen content of all treatments. Strains generally increased shoot dry mass accumulation by 0-14% for Holeta and 14-43% for Ambagiorgis compared to the shoot dry weight obtained from their respective sand cultures. Compared to their negative control they were found to increase shoot dry matter by 47-67% on Holeta soil and only 8-25% on Ambagiorgis on their respective soil type. The result showed that N+ and N- treatment of the Holeta soil failed to produce nodules whereas sufficient amount of nodules were produced in both treatments of Ambagiorgis soil. Nitrogen treated control faba bean of Ambagiorgis soil produced significantly less number of nodules compared to all other treatments.

Table 9. Comparative effectiveness of selected strains of *Rhizobium leguminosarum* var *viceae* from North Gondar on the growth and nitrogen fixation of faba bean on soil culture.

Holeta					Ambagiorgis			
Treatment	Nodule number/pl	Nodule dry mass (mg/pl)	Shoot dry mass (g/pl)	Total nitrogen (%)	Nodule number (no, /plant)	Nodule dry mass (mg/pl)	Shoot dry mass (g/pl)	Total nitrogen (%)
AUFR32	73^{a}	45.3^{a}	2.2^{a}	1.9^{a}	99^{a}	43.6^{a}	2.7^{a}	2.6^{a}
AUFR28	86^{a}	46^{a}	2.3^{a}	2.9^{a}	104^{a}	34^{a}	3±^{a}	3.3^{a}
AUFR24	78^{a}	49.3^{a}	2.5^{a}	2.7^{a}	84^{a}	31.6^{a}	2.6^{a}	3.9^{a}
AUFR15	41^{ab}	28.3^{ab}	2.3^{a}	2.8^{a}	67^{a}	27^{a}	2.6^{a}	3.5^{a}
AUFR14	51^{ab}	54.0^{a}	2.4^{a}	2.3^{a}	122^{a}	26^{a}	2.8^{a}	3.9^{a}
N +	-	-	2.7^{a}	3.2^{a}	31^{b}	14.0^{a}	2.9^{a}	3.5^{a}
N -	-	-	1.5^{b}	1.9^{a}	79^{a}	19.33^{a}	2.4^{a}	3.3^{a}

-: Not found

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

6. DISCUSSION

Twenty-one isolates of root nodule bacteria from North Gondar showed diversity in phenotypic and symbiotic characteristics. As shown on Table 3, all isolates were found to be similar, in colony size, except AUFR13. The isolates showed fast growth on attaining 2-4 mm in diameter with in 3-5 days incubation and growth is usually accompanied by copious exopolysaccharides. Isolate AUFR13 was characterized by a colony size of 1mm with the production of less gum.

All isolates, except AUFR12 did not absorb congored, and all changed the BTB-YEMA medium to yellow color indicating the production of acid. The mean doubling time of most isolates was found to be 2-4 hrs with the exception of AUFR20 that displayed shortest generations time of 1.9 hrs compared to others. It was also observed that four strains AUFR16, AUFR17, AUFR22 and AUFR32 scored generation times more than 4h, which were slower in their growth rate than the other isolates.

The fact that almost all isolates displayed large colonies, doubling time with in the range of 2-4hrs and acid production on BTB-YEMA medium corroborates their characteristics with fast growing rhizobia (Jordan, 1984;Somasagaran and Hoben, 1994). Since they were isolated from faba bean, they are characterized in to a cross-nodulation group of ***Rhizobium leguminosarum*** var. ***viceae*** (Jordan, 1984).

With regard to salt tolerance the isolates showed that 57%, 33% and 19% of them found to be resistance to 0.3%, 0.5% and 0.8% NaCl, respectively and only isolates AUFR12, AUFR18, and AUFR28 could resist NaCl of 2%. Although, reports of previous workers (Abdul-wahab and Zahran, 1979; Zerhari ***et al.,*** 2000), showed that fast growing ***Rhizobium,*** in general, grew well at NaCl concentration between 3-5%, none of the isolates, except AUFR18 could tolerate more than 2% NaCl. This suggests that, most of the isolates were observed to be sensitive to salt concentrations greater than 0.3%. Similar to this, the report of Graham and Parker (1964) indicated that out of ***Rhizobium leguminosarum*** strains tested, none could tolerate NaCl concentrations of 2-3%. The only isolate that continued to grow in 5% NaCl was AUFR18 and can be considered as osmotolerant strain (Bouhmouch ***et al***., 2001).

In the present study it is observed that maximum growth temperatures of the isolates were obtained at 15^0Cand 35°C and only isolates AUFR22 and AUFR28 survived at temperature of 40^0C. The result is within the range of T max; 32.5-34.5°C reported for *Rhizobium leguminosarum* var *viceae* HAMBI499, HAMBI 1125, and MPI 6001 isolated from faba bean and field pea from USA, UK, and the Netherlands (Lindstrom and Lehtomaki, 1988). Although isolate AUFR26 that tolerated 5^0C was isolated from relatively highest altitude, no such correlation between climatic region and low or high temperature was observed in the other isolates(r:0.34).

All tested isolates were tolerant to alkaline medium of pH 8 and pH 9, which is similar to the report of Graham and parker (1964) and Jordan (1984). All isolates were sensitive to pH4.5 and pH5, except isolates AUFR12 and AUFR28 that could survive at pH5. Several findings showed that strains of *Rhizobium leguminosarum* are generally sensitive to low pH and grow on near neutral and basic pH (Graham and Parker, 1964; Jordan, 1984). This tendency might be related to neutral pH that characterized most of the soils of the tested isolates (Table 1).

Almost all of the rhizobial isolate showed 100% growth on the tested carbohydrates except gluconate and tartarate on which the isolates showed large diversity. None of the tested isolates grew on citrate. As reported by Jordan (1984) and Stowers (1985), all fast growing rhizobia including *Rhizobium leguminosarum* var *viceae* utilize a wide range of carbohydrate as a carbon source with out gas formation. Lindstrom and Lehtomaki (1988) reported that 20% of their isolates from *Rhizobium leguminosarum* were capable of growing on citrate. This may indicate that failure of utilizing citrate may be restricted to the *Rhizobium* subgroup *viceae* but not on subgroups *R. leguminosarum* var *phaseoli* and *trifolli*. The fact that the variation in carbohydrate utilization was very limited indicated that catabolism of monosaccharide and disaccharides are widespread in fast growing rhizobia including *Rhizobium leguminosarum* var *viceae* (Jordan, 1984).

In the present study of intrinsic antibiotic resistance (IAR) all isolates exhibited random susceptibility to different concentrations and types of antibiotics particularly Ampicillin Streptomycin, Rifampcin, Kanamycin and Nalidxic acid. Since the isolates were found to be similar in most of the physiological responses to pH, NaCl, temperature, carbohydrate utilization, IAR was found to be the best of all to delineate the rhizobial isolates into specific diversity groups of *Rhizobium leguminosarum* var *viceae* from North Gondar (Table 6). The pattern of antibiotic resistance is generally similar to the findings of the same antibiotics on isolates of *Rhizobium leguminosarum* var *viceae* ranging from Streptomycin (14%) to that of Erythromycin 80% (Josey *et al*, 1979). Some of these antibiotics (Kanamycin and Streptomycin) were also used to define the different clusters of faba bean rhizobiam isolated from the USA (Brockman and Bezdicek, 1989). IAR was also reported to be useful in the preliminary screening of taxonomic groups of other root nodule bacteria (Shishido and Pepper, 1990; Chen *et al*, 2002). Exceptionally, the resistance of isolates AUFR28 to all and AUFR26 to all but two of the tested antibiotics at concentration of 10µg/ml showed that, they were well adapted to antibiotic producing microorganisms in the natural habitat.

Two features, infectivity (the ability to form nodules) and symbiotic effectiveness (capacity to fix nitrogen), are commonly used to assess the ecological and evolutionary relationship between rhizobia and their host (Brockwell, 1998). Thus, all of the rhizobial isolates except AUFR13 assessed in this study showed a capacity to induce nodule formation on the host plant. Although AUFR13 showed different colony morphology (Table 3) and failed to nodulate the parent host (Table 7), most of its physiological characteristics were found to be similar to the other isolates. Therefore it is possible to conclude that the isolate may be either rhizobia that loss its nitrogen fixing capacity due to the loss of its plasmids (Segovia *et al.,* 1991) or some other rhizobia that penetrate the nodules (Johnston and Beringer, 1976). How ever, it must be experimentally supported before this isolate is identified as contaminant or genetically defective rhizobia.

The infective strains of the faba bean rhizobia showed significant difference in shoot dry weight. Several reports showed that shoot dry mass is a good indicator of relative strain effectiveness, and there is a good correlation between shoot dry matter production and nitrogen fixation capacity of legumes (Somasegaran and Hoben 1994; Sorwil and Mytton 1986; Peoples *et al.,* 2002).

Based on the percentage differences of shoot dry weight of inoculated and nitrogen-fertilized plants as a measure of effectiveness (Date *et al.,* 1993 cited in Purcino *et al.,* 2000; Lupwayi and Haque, 1994), 80% of the isolates were found to be effective nitrogen fixers and almost a quarter of them performed as good as the positive control on the sand culture. The highest scores of 88-96% effectiveness of symbiotic nitrogen fixation were displayed by AURF14, AURF15, AUFR24, AURF28 and AUFR32 that were isolated from Aba Matabo, Sabian, Jemberko, Dib-bahir, and Amba-giorgis sites, respectively. Previously, Desta Beyene and Angaw Tsigie (1987) only managed to isolate 23 symbiotically effective strains (11%) from 108 isolates from Central Shewa, Ethiopia. Such variability in symbiotic effectiveness of faba bean rhizobium was found to be widespread in Ethiopia (Van Berkum *et al.,* 1995). Ayneabeba Adamu *et al.* (2001) also reported 66-87% effectiveness in nitrogen fixation on sand culture of ***Rhizobium leguminosarum*** var *viceae* isolates from Ankober, Molale, Keyt, and Mehalmeda sites from Northern Shewa, Ethiopia. Generally the result of this study suggests that, the highly performed strains are worthy of further glasshouse and field-testing in order to select effective and competent strains.

All strains were found to accumulate 82-92% on Holeta soil and 90-103% on Ambaghiorgis soil, of their shoot dry weight compared to their respective N-fertilized controls. However, compared to their negative controls, they were found to increase shoot dry matter by 47-67% on Holeta soil and only 8-25% on Ambagiorgis soil. This shows that there were no significant inter-strain differences in nodule number, nodule dry mass, shoot dry weight, and nitrogen contents on the tested soils and cultivar.

All isolates were also found to increase shoot dry weight by 0-43% higher than their respective sand cultures. This value is lower than the 45-50% increase in shoot dry weight between sand culture and soil culture experiments on faba bean production of Molale, Mehalmeda, Keyt, and Ankober soils of Northern Shewa (Ayeneabeba Adamu *et al*., 2001), and 45% of Mehalmeda isolate evaluated for its effectiveness on soil culture together with phosphate solubilizing bacteria (Assefa Keneni, 2002). The increase in shoot dry weight in soil culture compared to a sand culture may be attributed to the high nitrogen content of the soil, additional nodulation by the background indigenous strains of the tested soils, and other rhizosphere effects on plant growth (Kang and Mills, 2004).

Although both soils were once treated with fertilizers as recommended by Somasegaran and Hoben (1994), response of the Holetta soil to inoculation by selected effective strains was much pronounced (47-67%) than Ambaghiorgis soil (8-25%) compared to the negative (N-) control. The result also showed that N+ and N- treatments of the Holetta soil failed to produce nodules whereas sufficient on N- and reduced number of nodules on N+ were produced in the treatments of Ambagiorgis soil. Soil analysis showed that, Holeta is characterized by high nitrogen and low in phosphorous contents where as Ambagiorgis contained very high nitrogen and high phosphorous in the soil according to characterization of Desta Beyene and Angaw Tsige (1989); Negeborg (1986). Both nutrients observed to be the main limiting growth factors in field crops of Ethiopia (Desta Beyene and Angaw Tsige, 1989). This indicates that the low pH, low nitrogen and phosphorus status of the Holetta soil severely affected the survival of the indigenous rhizobia and their nodulation and nitrogen fixation.

It has been reported that there is a strong correlation between soil pH and survival and persistence of rhizobia and nodule activity (Coventry and Evans, 1989; Brockwell *et al.,* 1991; Lapinskas *et al.,* 2005), and failure of nodulation of faba bean at lower pH <5.5 was reported on French soil (Evans *et al.,* 1980), and reduced growth at <pH 5.4 in Germany (Schubert *et al.*, 1990), and <pH 6 in Australia (Tang and Thomson, 1996). Desta Beyene and Angaw Tsigie (1988) also reported limited nodulation on faba bean at Bekoji, Southeastern Ethiopia with a pH of 5.1.

The fact that the selected strains on Holetta soil managed to increase shoot dry matter and accumulated as much as 2/3 of the nitrogen content of plants grown on Ambagiorgis soil, partly reflects that the selected strains could yet manage to fix nitrogen on acidic soil despite the fact that the strains were originally isolated from a mildly acidic or near neutral pH. This shows that it is possible to reverse failed nodulation of indigenous rhizobia and extend the growth of legumes on acidic soils by primarily selecting acid and soil acid tolerant rhizobia.

Selection and application of acid tolerant strains of faba bean was used to effectively nodulate faba bean (Carter *et al.,* 1994), and medics (Howieson *et al.*, 1988; Dilworth *et al.*, 2001) and enhance their establishment in marginalized acidic soils of Western Australia. A work on Bekoji (pH 5.1) in the central part of Ethiopia showed an increase of 24% of shoot dry matter of the control plot with inoculation of effective rhizobia and fertilizer treatment (Amanuel Gorfu *et al.*, 2000).

The nitrogen treated control faba beans of Ambagiorgis soil produced significantly less number of mean nodule number compared to all the other treatments indicating, that high concentration of nitrogen may have inhibited nodulation. Although high concentrations of nitrogen negatively affect nodulation and nitrogen fixation of faba bean (Nelson, 1987; Mukhtar, 1989) and common bean (Leidi and Rodriguez- Navarro, 2000), high availability of both nitrogen and phosphorus together with relatively higher pH in Ambagiorgis (7.3) may have induced more favorable environment than Holetta soil (4.8) for effective legume-*Rhizobium* symbiosis.

Although correlation of tolerance to different environmental factors and symbiotic effectiveness was not clearly indicated in the present work, the ability of AUFR28 to grow at 40^{o}C, pH5, NaCl 2%, resistance to all antibiotics and displaying one of the highest shoot dry mass (96%) is a remarkable feature for future selection. Similarly the ability of other strains such as AUFR12 and AUFR18 to resist low pH, high salt concentration and most antibiotics, is very important in the screening of elite strains as inoculants for pulse production under extreme environmental conditions provided that their performance corroborate under different stressed soil environments in field trials.

7. CONCLUSIONS AND RECOMMENDATION

The present study shows physiological and symbiotic diversity of *Rhizobium leguminosarum* var *viceae* population on faba bean from Northern Gondar. Some of the strains showed remarkable physiological characteristics such as resistance to antibiotics, high salt tolerance and survival at low pH. In addition to these some of these isolates scored highest percentage in effectiveness of symbiotic nitrogen fixation, which may qualify them as respective candidates for inoculants under adverse environmental conditions. The result also demonstrate that selection of adapted strains under stress conditions in agar culture is possible and pure culture evaluation may be a useful tool in the search for rhizobial strains better suited for soil environments where high NaCl and pH constitute a limitation for symbiotic nitrogen fixation.

Given that North Gondar is one of the several important regions of faba bean production, it may well be that more effective strains of *Rhizobium leguminosarum* var *viceae* can be isolated from/for other common cross-inoculation group of cool season legumes of the tribe viceae that nodulate field pea, lentil and latyrus from other parts of Ethiopia.

Considering these observations, it remains to be a follow-up research to screening using molecular techniques such as REP/PCR or RFLP/PCR, sequencing of 16S rDNA genes and DNA/DNA hybridization to study the competitiveness of the strains in terms of nodule occupancy, and further investigation to evaluate their effectiveness under different environmental conditions both in the glass house and field trials.

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Appendix 1. Utilization of carbohydrates by rhizbial isolates

Isolates	D-xylose	gluconate	Fructose	Citrate	Lactose	Sucrose	Sorbitol	Tartarate	Arabinose	Rhaminose	Glactose	Mannose	Maltose	Adontoil	Cellobiose	Raffinose	Glycerol
AUFR12	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR13	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+
AUFR14	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR15	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR16	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR17	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR18	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR19	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR20	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR21	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR22	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR23	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+
AUFR24	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR25	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR26	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR27	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR28	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR29	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR30	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR32	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
AUFR34	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+

(+) Growth
(-) No growth

Appendix 2. Effect of different antibiotics on the growth of rhizobial isolates

Isolates	Ampcillin (μg/ml)			Streptomycin (μg/ml)			Refampcin (μg/ml)			Kanamycin (μg/ml)			Erythromycin (μg/ml)			Nalidixic acid (μg/ml)			Chloramphencol (μg/ml)		
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
AUFR12	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR13	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
AUFR14	-	-	+	-	-	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+
AUFR15	-	-	+	-	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+
AUFR16	-	-	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	+
AUFR17	-	-	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
AUFR18	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+
AUFR19	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR20	-	-	+	-	-	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+
AUFR21	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
AUFR22	-	-	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+
AUFR23	-	-	+	-	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+
AUFR24	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
AUFR25	-	-	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+
AUFR26	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
AUFR27	-	-	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
AUFR28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AUFR29	-	-	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
AUFR30	-	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+
AUFR32	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
AUFR34	-	-	+	-	+	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+
Total (+) isolates	5	5	19	3	10	16	8	14	14	8	8	11	20	20	20	9	20	20	20	20	20

(+) Growth (-) No growth