

**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 Biology*

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 Agroforestry” / 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.

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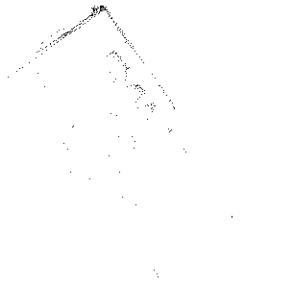
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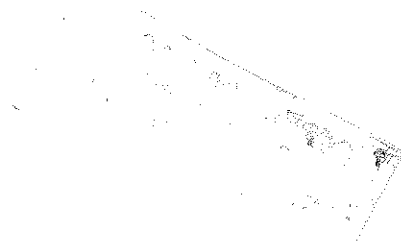
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LIST OF SYMBOLS AND ABBREVIATIONS

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	Adenosine triphosphate
GOGAT	Glutamine-2-oxoglutarate-amino-transferase
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	Polymrase chain reaction
REP	Repetitive extra genomic palindromic

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°C and 35°C and failed to grow at 40°C, 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

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 crops (Giller, 2001). They are categorized into 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 Viciae (*Lens*, *Pisum*, *Vicia faba*, *Lathyrus*), the recently separated tribe Ciceri (*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 within 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 *weng daga* or temperate zone between the

al., 1994). Moreover, it provides large cash for producers and foreign exchange for the country (Desta Beyene, 1988).

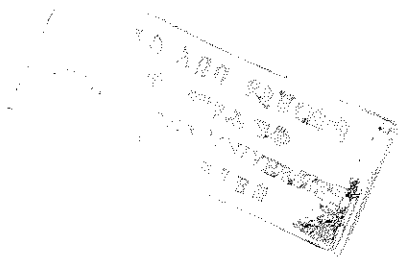
Ethiopia was a net exporter of faba bean. In the period of 1970-77, an average of 23000t were exported annually, to the Arabian Peninsula, Western Europe, and South-East Asia (Asfaw Telaye, 1979). Even though Ethiopia is the world's second largest producer of faba bean, next to the Peoples Republic of 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 N₂-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-input 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

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).



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 Mimosoideae (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 Viciae. 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,

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 3×10^9 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%.

Three major types of N₂ 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).

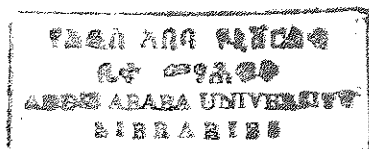
The predominant groups of N-fixers are Rhizobia (bacteria) and the others are cyanobacteria (blue green algae), which are found both as free-living species and in association with the variety of plants, most notably the aquatic fern *Azolla*. The third groups of important N₂ fixers are the actinomycetes *Frankia* species, which form symbiotic association with flowering plants from a number of different families. Almost all of their host plants are woody perennials. Fourth group of N₂-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 *Klebsiella* and *Azotobacter* live in the soil and fix N₂ where the forms N are unavailable.

3.3 Biochemistry of Nitrogen Fixation

In all N₂ fixing microorganisms the principle steps of this reaction are the same. The key enzyme complex, referred to as *Nitrogenase*, is unique to N₂-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

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 H₂-uptake (Hup) system synthesized by certain strains of rhizobia (Evans *et al.*, 1985). This H₂ 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 N₂-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-oxoglutarate-amino-transferase (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).

meliloti, *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 phylogenetic 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*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, *Allorhizobium*, and 28 species have been recognized (Zakhia and

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).

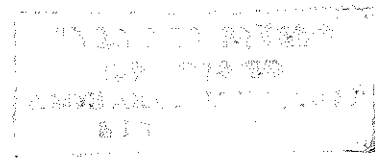
In response to nod factors, many of the developmental changes seen in the host plant early in the nodulation process including root hair deformation to establish a meristem and nodule primordium. Rhizobia continue to differentiate inside the nodule and synthesize proteins required for nitrogen fixation and for the maintenance of the mutualistic 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 N₂-fixation. According to Hungria and Vargas (2000), high temperature, drought and soil acidity constrain legume root-nodule formation in the tropics. Photosynthate deprivation, salinity, soil nitrate, heavy metals, and biocides are also typical environmental stresses faced by

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°C at 5 cm and 50°C 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

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 lipopolysaccharides composition, proton exclusion and extrusion accumulation of cellular polyamines, and synthesis of acid shock proteins (Zarhan, 1999), and composition and structure of outer membrane (Graham *et al.*, 1994), have been implicated with pH tolerance of endosymbionts.

Vlassak and Vanderleyden (1997) reported that, nodulation of legumes is reduced in 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

leguminous plants require a neutral or slightly acidic soil for growth, especially when they depended on symbiotic N₂ 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

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

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 inhabiting 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

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.leguminosarum* 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

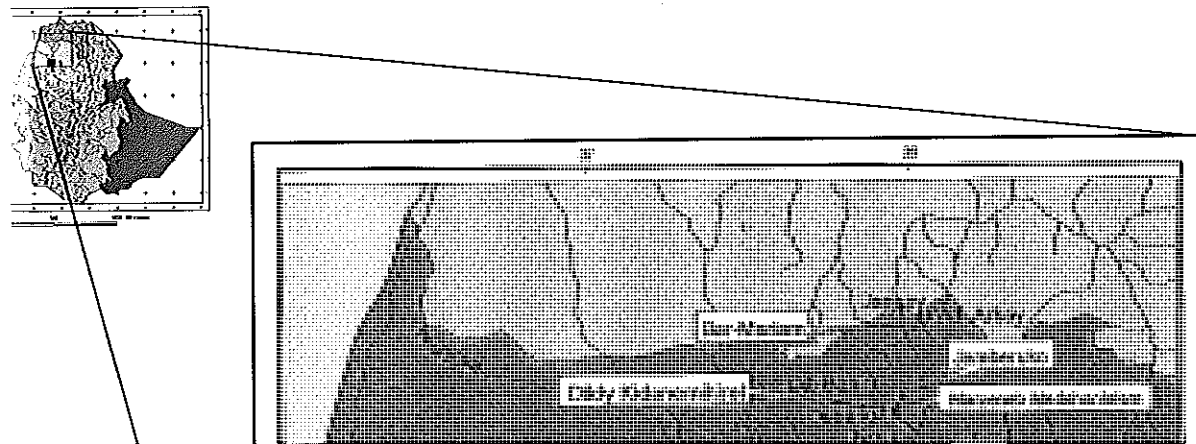
undertaken. Further more Ayneabeba Adamu *et al.* (2001), and Asefa Keneni (2002) studied the importance of Rhizobium inoculation and fertilizer treatment on growth and production of faba bean (*Vicia faba*) in some yield depleted and yield sustained regions of Semien Shewa.

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.

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 (*eriteb kola*) sub-moist and 3500m.a.s.l cold (*dega*) regions. The study sites are characterized by mean annual temperature of 10-20⁰C 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).



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 from 10-15 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

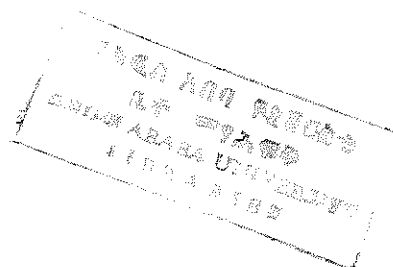
Sample number	Name of the sites	Altitude (m.a.s.l)	Location		Type of legume on the soil field	Soil pH H ₂ O (1:2.5)
			Latitude (N)	Longitude (E)		
12	Infranz	ND	12°15'	037°37.529'	Faba bean	6.7
13	Maksegnit	1929	12°39.856'	037°33.122'	Faba bean	7.2
14	Aba Matabo	2264	13°43.151'	037°28.355'	Faba bean	5.8
15	Sabian	2419	12°45.233'	037°30.083'	Faba bean	6.7
16	Janhoy	2693	12°47.433'	037°29.511'	Faba bean	6.6
17	Kosaye	2898	12°51.82'	037°32.41'	Faba bean	6.3
18	Ambasege	2694	12°56.831'	037°41.104'	Faba bean	6.8
19	Godabage	2767	13°01.257'	037°43.988'	Faba bean	7.4
20	Dabat	2634	13°04.463'	037°44.839'	Faba bean	6.7
21	Dala	2672	13°01.257'	037°47.351'	Faba bean	6.7
22	Wokin	2758	13°04.463'	037°49.790'	Faba bean	6.5
23	Debark	2795	13°07.912'	037°53.484'	Faba bean	6.3
24	Jemberko	3196	13°11.704'	037°58.440'	Faba bean	6.9

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 28⁰C for 3-5 days.

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

Mannitol	10 g/l
K ₂ HP0 ₄	0.5 g/l
MgSO ₄ .7H ₂ O	0.2 g/l
NaCl	0.1 g/l
Yeast Extract	0.5 g/l
Agar	15 g/l



4.4 Characterization of the Isolates

All colonies were checked on YEMA containing $25 \mu\text{gml}^{-1}$ Congo red to evaluate their ability to absorb the dye. Isolates were also inoculated on YMA containing $25 \mu\text{g ml}^{-1}$ 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°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 Gyrotory shaker at $100 \text{ rev. min}^{-1}$, 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 $3.3\mu\text{l}$ calibrated inoculation loop containing ($10^5 \text{ cells ml}^{-1}$), against control plates and incubated at 28°C (Lupwayi and Haque, 1994). Growth was determined qualitatively and recorded as (+) for growth or (-) for no growth.

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 ₂ O	0.504g/l
ZnSO ₄ .7H ₂ O	0.277g/l
CuCl ₂ .2H ₂ O	0.034g/l
NaMO ₂ .2H ₂ O	0.008g/l

B. Phosphate stock solute 1.0ml

KH ₂ PO ₄	1.36g/l
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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 SO ₄	0.131g

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 Ampicillin, Streptomycin, Rifampicin, 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

as described before (Vincent, 1970) and transferred to 0.75% (w/v) water agar plates for germination and incubated at 25°C 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	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	294.1
P	KH_2PO_4	136.1
Fe	Fe-Citrate	6.7
Mg	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	123.3
K	K_2SO_4	87.0
Mn	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0338
B	H_3BO_3	0.247
Zn	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.288
Cu	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.100

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) [$100 \times \text{inoculated plant DM} / \text{N-fertilized plant DM}$] 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 Ambagiorgis (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 ₂ O 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

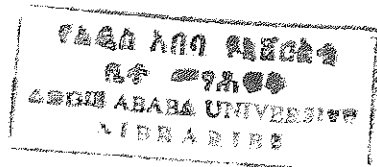
bean were surface sterilized as described before (Vincent, 1970) and allowed to germinate on a water agar for three days at 25°C. Based on their symbiotic efficiency selected rhizobial isolates were prepared by growing in YEM nutrients broth media and adjusted their number approximately to be 10^9 cells 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 sulphuric 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



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 1mm in diameter after 3-5 days growth. All isolates showed no color absorption on congo red except AUFR12 that completely absorbed congo red 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

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

5.2 Physiological Characterization

5.2.1 Temperature tolerance

As indicated in Table 4, all rhizobial isolates grew at 15⁰C and 35⁰C except isolate AUFR13 that showed no growth at temperature of 15⁰C. Isolates AUFR22 and AUFR28 were found to grow at a temperature of 40⁰C whereas two isolates AUFR18 and AUFR26 showed growth at temperatures of 5⁰C and 10⁰C.

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 AUFR13 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 pH 5.0.

Symbiotic and Phenotypic Diversity of Rhizobium leguminosarum var viceae spp...

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
Temperature													
5°C	-	-	-	-	-	-	+	-	-	-	-	-	-
10°C	-	-	-	-	-	-	+	-	-	-	-	-	-
15°C	+	-	+	+	+	+	+	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+	+	+	+	+	+	+
40°C	-	-	-	-	-	-	-	-	-	-	+	-	-
Salt tolerance													
0.1%	+	+	+	+	+	+	+	+	+	+	+	+	+
0.3%	+	+	+	-	-	+	+	-	-	+	+	-	-
0.5%	+	+	-	-	-	-	+	-	-	-	+	-	-
0.8 %	+	-	-	-	-	-	+	-	-	-	-	-	-
1%	+	-	-	-	-	-	+	-	-	-	-	-	-
2%	+	-	-	-	-	-	+	-	-	-	-	-	-
3%	-	-	-	-	-	-	+	-	-	-	-	-	-
4%	-	-	-	-	-	-	+	-	-	-	-	-	-
5%	-	-	-	-	-	-	+	-	-	-	-	-	-
pH tolerance													
5.0	+	-	-	-	-	-	-	-	-	-	-	-	-
5.5	+	-	+	+	+	+	+	+	+	+	-	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+	+
9.0	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) 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

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 Ampicillin, 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 Ampicillin, 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

5.2.6 Numerical analysis

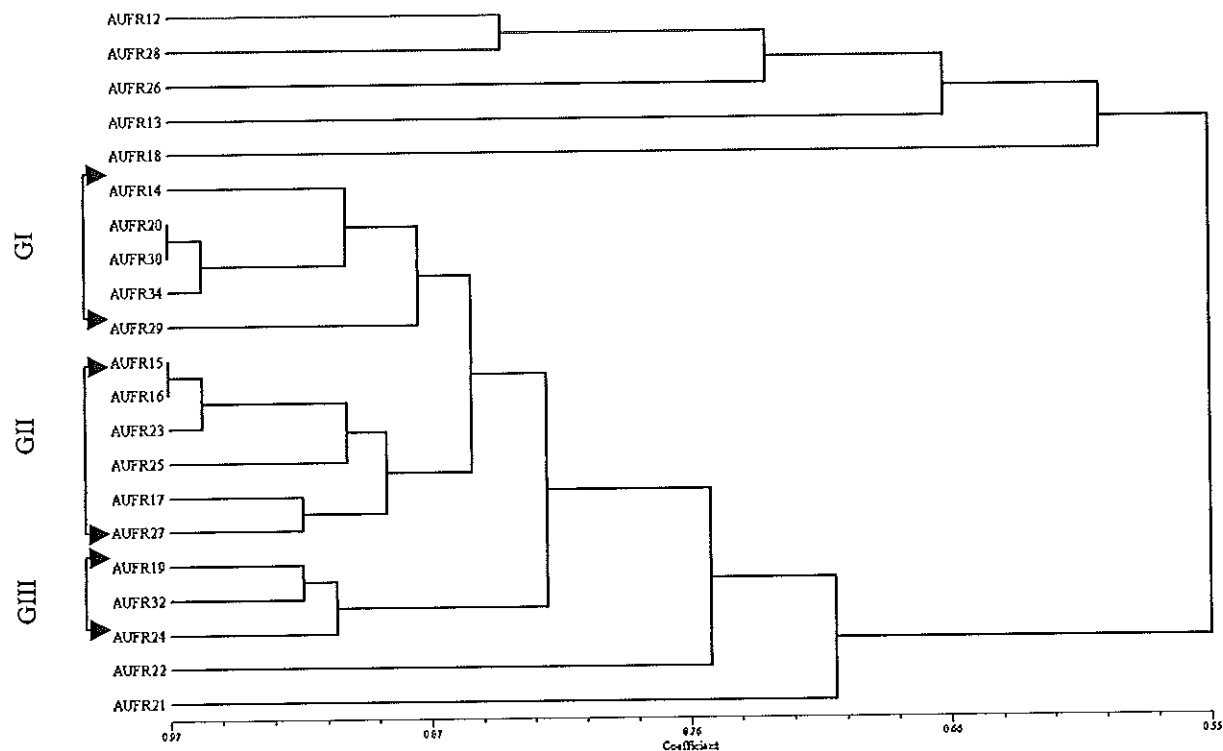


Figure 2. Dendrogram highlighting the phenotypic similarities among faba bean rhizobia (*Rhizobium leguminosarum* bv. *viceae*) from different areas of Northern Gondar.

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

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 > 2.0 g/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 Effectiveness 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



Fig.3 Showing the shoot height of the different treatment 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% 9.52	4.76%	57% 57.14%	23.8% 23.81

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

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.

loleta					Ambagiorgis			
reatment	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 (%)
UFR32	73 ^a	45.3 ^a	2.2 ^a	1.9 ^a	99 ^a	43.6 ^a	2.7 ^a	2.6 ^a
UFR28	86 ^a	46 ^a	2.3 ^a	2.9 ^a	104 ^a	34 ^a	3± ^a	3.3 ^a
UFR24	78 ^a	49.3 ^a	2.5 ^a	2.7 ^a	84 ^a	31.6 ^a	2.6 ^a	3.9 ^a
UFR15	41 ^{ab}	28.3 ^{ab}	2.3 ^a	2.8 ^a	67 ^a	27 ^a	2.6 ^a	3.5 ^a
UFR14	51 ^{ab}	54.0 ^a	2.4 ^a	2.3 ^a	122 ^a	26 ^a	2.8 ^a	3.9 ^a
+	-	-	2.7 ^a	3.2 ^a	31 ^b	14.0 ^a	2.9 ^a	3.5 ^a
-	-	-	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 congo red, 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

In the present study it is observed that maximum growth temperatures of the isolates were obtained at 15°C and 35°C and only isolates AUFR22 and AUFR28 survived at temperature of 40°C. 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°C 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 pH 4.5 and pH 5, except isolates AUFR12 and AUFR28 that could survive at pH 5. 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

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 rhizobium 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). However, it must be experimentally supported before this isolate is identified as contaminant or genetically defective rhizobia.

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 Ambaghiorgis 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

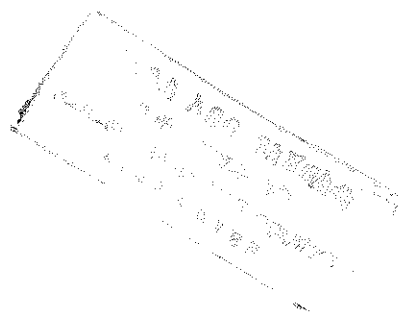
pronounced (47-67%) than Ambagiorgis soil (8-25%) compared to the negative (N-) control. The result also showed that N+ and N- treatments of the Holetta soil failed to produce nodules whereas sufficient on N- and reduced number of nodules on N+ were produced in the treatments of Ambagiorgis soil. Soil analysis showed that, Holetta 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

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°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.

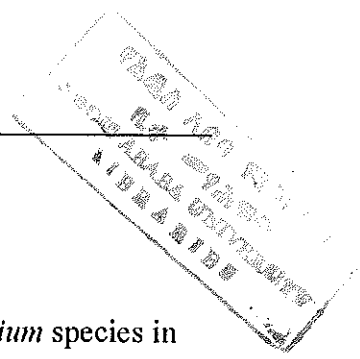


7. CONCLUSIONS AND RECOMMENDATION

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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e growth of rhizobial isolates

in		Refampcin (µg/ml)			Kanamycin (µg/ml)			Erythromycin (µg/ml)			Nalidixic acid (µg/ml)			Chloramphenicol (µg/ml)		
	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+
	+	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+
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	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+
	16	8	14	14	8	8	11	20	20	20	9	20	20	20	20	20

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