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Phenotypic and Symbiotic characteristics of Rhizobia nodulating field Pea (*Pisum sativum L.*) in southern Tigray, Ethiopia.

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List of Symbols and Abbreviation

AAUFPR	Addis Ababa University field Pea rhizobia.
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCP	Bromocresol purple
BNF-	Biological nitrogen fixation
BTB	Bromo thymol blue
CR	Congo red
CSA	Central Statistical Agency
EEPA	Ethiopian Export Promotion Agency
GPA	Global Plan of Action
H/T/Haimanot	Hizba Tekle Haimanot
IAR	Intrinsic antibiotic resistant
M.a.s.l.	Meters above sea level 2
Mg	Magnesium
NADP	Nucotinamid diphosphate
NADPH	Nucotinamid diphosphate hydrogen
IBC	Institute of Biodiversity Conservation
MGT	Mean growth time
Pi	Inorganic phosphate
PGA	Peptone glucose agar
rRNA	Ribosomal RNA
rpm	Revolution per minute
SE	Symbiotic effectiveness
Tabia	Administrative region between Woreda and Kebele
TYEA	Tryptone yeast extract agar
v/v	volume per volume
W/V	weight per volume
YEMA	Yeast extract manitol agar
YEMB	Yeast extracts manitol broth

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“Through him all things were made, without him nothing was made that has been made”. He is good God who made things for good. Praise God!

Abstract

Nitrogen is one of the most abundant elements, and BNF is the main source of nitrogen for Legumes plants. Field Pea is one of the most important Legumes plants and widely grown in Tigray regional state. Although some studies have covered some areas of Tigray in relation to biological nitrogen fixation, there was no comprehensive study on the crop in the most important pulse growing regions of Southern Tigray. The present study, therefore, was designed with the objective of isolating and characterizing root nodule bacteria from three Woredas of southern Tigray, and evaluating their symbiotic effectiveness on field pea. The result will serve as base line data for future endeavor of utilizing biological nitrogen fixing system of field pea to increase productivity into low-input agriculture of the region and the country at large. Thirty three soil samples were collected from field pea (*Pisum sativum*) growing areas of Southern Tigray (Ofla, Endamokoni and North western parts of Alamata Woreda). The soil samples were brought to Applied Microbiology Laboratory for further processing. For induction process the soil samples mixed with the extensively washed and sterilized river sand and filled in to surface sterilized 3kg capacity plastic pots. Seed of the cultivar *Pisum sativum* Addi surface sterilized and sown into each pot and placed in the greenhouse condition. After 55 days plants were uprooted and collected nodules of all isolates were induced into the original host plants. All isolates characterized their morphological and physiological characteristics. All isolates formed watery and mucoid colonies on YEMA medium, their mean growth time mostly between 2 & 4 hours and failed to grow on peptone glucose agar medium and to solubilize inorganic phosphate. Almost all isolates were tolerate to pH 5 to 9, salt concentration, and at temperature of 15°C to 35°C. The isolates were also tolerant to ampicillin, penicillin, erythromycin and chloramphenicol, but sensitive to gentamycin and tetracycline. All isolates utilized to sucrose, glucose and D-manitol but few isolates utilized citrate, cellulose and starch, and the isolates utilized many amino acids as the source of nitrogen. The numerical analysis based on the 54 phenotypic characteristics of isolates was clustered into six groups. The mean nodule number, nodule dry weight and mean shoot dry weight of the host plants inoculated with different isolates showed variations. After in field experiments, the isolates (AAUFPR05, 06, 07, 11, 16, 18 and 30, 37, 39 and 43), and particularly AAUFPR 07 and 28 can be recommended as inoculants in the future. The above isolates showed good physiological, eco-physiological and symbiotic characteristics.

Key Words: Field pea, Tigray, phenotypic characters, symbiotic effectiveness, Rhizobium

1. Introduction

Nitrogen is one of the most abundant elements. Although 78% of the atmosphere planate Earth is nitrogen, it is one of the limiting elements to plant production. It can not directly be utilized until it is converted into an utilizable form such as NH_4 and NO_3 . Nitrogen can be fixed through different processes of which 90% is contributed by free living and symbiotic bacteria (Dixon, and Wheeler, 1986).

Biological nitrogen fixation (BNF) is an efficient source of nitrogen. It is estimated that 175 million tons of nitrogen yr^{-1} is fixed by symbiotic association of which more than 70% is contributed by the legume-*Rhizobium* association (Burns and Hardy, 1975). It represents the most important renewable resource of nitrogen in the terrestrial ecosystem.

Legumes are important crops in agricultural systems; some of the important legumes are field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentils (*Lens culinaris*), chickpea (*Cicer arietinum*), grass pea (*Lathyrus saivum*), soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*), etc. These legumes are effective in biological nitrogen fixation with the capacity of converting nitrogen to the tune of 200-300 kg/ha/yr (Peoples, 1995).

Field pea (*Pisum sativum*) is one of the best known highland crops in Ethiopia. It is widely cultivated at altitudes between 1800 and 3000 m.a.s.l. with annual average rainfall of 700-900 mm in the different regions of Oromia, Amara, Tigray and Southern Ethiopia (EEPA, 2004). It is the second most important pulse crops in the country after faba bean in terms of both area coverage and production. According to the Central statistics Agency (as cited by IBC, 2008), field pea covers over 254,000 hectares with total production of 230,000 tons that accounts to 17% of the total grain legume production.

It is one of the most important food and feed crops with high contents of protein and vitamins. Consequently, it is an inexpensive source of protein and cooked as sauces to supplement carbohydrate rich food for many people.

Field pea is integrated in different crop systems as a sole crop or an intercrop in crop rotation for its capability to fix nitrogen in endosymbiotic association with root nodule bacteria known as ;*Rhizobium leguminosarum biovar viciae*, the cross-inoculation bacteria that also inoculate faba bean, lentils, grass pea (Jordan,1984) . Several works have shown that field pea fulfills more than 80% of its nitrogen requirements that subsequently transfers nitrogen to non-fixing plants in the agricultural system (People, 1995) .

In Tigray regional state, field pea is widely grown as source of food, cash and a break crop in the cropping system. Field pea is grown in southern, eastern, and central highlands of Tigray. The most important areas are Ofla, Endamohoni, Alaje, Hintalo-wojerat, and North western parts of Alamata Woreda.

For many years now many researches have been undertaken nation-wide to improve field pea cultivars, (Amare Ghizaw and Adamu Molla, 1994).Many of the studies were limited to soil plant nutrition and fertilizer trials in different agricultural research institutes (Tekalign Mamo and Asgelil Dibabe, 1994).Recently; Aregu Amsalu (2007) collected root nodules from field pea with wide range of taxonomic and symbiotic diversity from different important pulse growing regions of the country.

Although some studies have covered some areas of Tigray in relation to biological nitrogen fixation (Aregu Amsalu,2007).They were also limited to studies on faba bean and few sampling areas in the region (Alemayehu Workalemahu,2009).There was no comprehensive study on the BNF of the crop in the most important pulse growing regions of Southern Tigray.

The present study, therefore, was designed with the objective of isolating and characterizing root nodule bacteria from several Woredas of southern Tigray, and evaluating their symbiotic effectiveness on field pea. The result will serve as base line data for future endeavor of utilizing biological nitrogen fixing system of field pea to increase productivity into low-input agriculture of the region and the country at large.

2. Objectives

2.1. General objective:-

To Evaluate symbiotic effectiveness and phenotypic characterization of *Rhizobium leguminosarum biovar viciae* Isolates and used to increase yield of field pea using BNFagent in southern Tigray.

2.2. Specific objective of this study are:-

- *To isolate *Rhizobium* strains nodulating *Pisum sativum*
- *To characterize the isolates based on different phenotypic characters.
- *To screen the most symbiotic efficient isolate under green house conditions

3. Literature Review

3. 1. The Legumes

The legumes are found in the third largest family of flowering plants the family *leguminosae*. They are found in various habits on herbs, shrubs and Trees and well grown through out temperate and Tropical regions. It is estimated that the family contains 19400 species in about 750 different genera (Steen, 1986), and sub-divided into three sub families; the *Papilionoideae*, the *Mimossoideae* and the *Caesalpinoideae* (Polhill and Raven 1981).

Legumes are multipurpose plants that provide food, Medicine, fire wood, timber and other products. It is estimated that 25% of the world's major crop production and more than one-third of human protein requirements are derived from legumes (Postgate, 1982).

3.2 Field Pea (*Pisum sativum*)

The Field Pea (*Pisum sativum L.*), which belongs to the family *Papilionaceae* (*Leguminosae*), has been traced to agriculture 7000-6000 B.C. and is now grown world-wide. However, pea is largely confined to temperate regions and the higher altitudes or cooler seasons of warmer regions (Steen, 1986). Historians believe the main centre of pea development was middle Asia, including northwest India and Afghanistan. A second area of development lies in the Middle East, and a third includes the plateau and mountains of Ethiopia (Kosher, 2004)

In Ethiopia Field Pea (*Pisum sativum*) is a highly consumed Pulse in the daily diet of the society in urban and rural areas. It is eaten whole, spilt or milled usually fresh, fried, boiled or mixed with other cereals to make various types of Stews and Soups (EEPA, 2004). It represents a useful complement to cereal-based diets as a relatively inexpensive source of high quality protein. It contains protein (21-25%), starch (33-50%), amino acids, but it is limiting in fat, Tryptophan and in the Sulfur-contain amino acids, Methionine and Cysteine (Lazanyi, 2002).

Field Pea like other legumes is capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium* bacteria at the root of the crop. *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and causes injury to soil properties (Lazanyi, 2002). This crop thus improves soil and economizes crop production not only for itself but also for the next cereals (non-legume crops) grown in the relation and there by reducing the requirement of added nitrogen fertilizers (Burns and Hardy, 1975).

Field Peas are used in crop rotation for improvement of soil fertility and yield of the succeeding crops. In crop rotation tests, spring wheat and durum wheat grown on pea stubble produced higher yields and a higher protein percentage compared to wheat grown on wheat stubble (Lazanyi, 2002).

3.3. The Rhizobia and their taxonomy

Rhizobia are gram negative, rod shaped soil bacteria that are capable to fixing atmospheric nitrogen in the root nodule of leguminous plants (Jordan, 1984). They can live either in the soil as free living or as symbionts within the root nodules of host legumes (Vincent, 1970). Free living bacteria can fix about 30% of biological nitrogen fixation (BNF) and they have different shape from the bacteria living in the root nodule (Willems, 2006).

The development of molecular techniques accelerated the systematic evolution and led to the identification of many new rhizobial genera. Based on the sequence of the 16S ribosomal RNA (rRNA) gene rhizobia could be grouped into the α , β and γ subdivision of the Proteobacteria (Young and Haukka, 1996) Recently rhizobial systematic consisting of 13 genera. 10-belonging to α - Proteobacteria: *Allorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ocrobacterium*, *Phyllobacterium*, *Rhizobium*, and *Ensifer* (Ochman *et al.*, 2005). *Burkholderia*, *Ralstonia*, and *Cupriavidus* in the β -Proteobacteria (Willems, 2006).

The genus *Rhizobium* comprises a group of fast growers which includes 17–species, *R. Cellulosilyticum*, *R. daejeonense*, *R. etli*, *R. galegae*, *R. gallicum*, *R. giardinii*, *R. hainanense*, *R. huautlense*, *R. indigoferae*, *R. leguminosarum*, *R. loessense*¹, *R. lusitanum*, *R. mongolense*, *R. sullae*, *R. tropici*, *R. undicola*, *R. yanglingense* (Yadav,2007). Each of these species is associated with a group of host plants and is distinguished from the other species mainly on the basis of DNA-relatedness values, 16S rRNA homology values, and some phenotypic characteristics cross-inoculation pattern (Chen *et al.*,1995).

3.4. Nitrogen fixation

Nitrogen is one of the most abundant elements on the earth atmosphere. Although 78% of the earth's atmosphere is nitrogen gas, it can not be utilized unless it is converted or fixed to utilizable forms such as NH_4^+ and NO_3^- . The latter are required by all living organisms for the synthesis of proteins, nucleic acids and other nitrogen-containing compounds. Nitrogen fixation takes place both through natural and artificial processes. It is estimated that 90% of it fixed by BNF (free living and symbiotic bacteria) (Dixon and Wheeler, 1986).

3.5. Biological nitrogen fixation (BNF)

Biological nitrogen fixation (BNF) is reduction of atmospheric nitrogen into ammonia, by nitrogenase enzyme, which is an exclusive property of prokaryotic organisms. It is categorized into free living and symbiotic nitrogen fixing systems (Marschner,1995).The relative contribution of symbiotic and free living microbes in their nitrogen fixation is 70% and 30% respectively (People and Crawswell,1992).

At present terrestrial symbiotic BNF is estimated at 175 million tones of N /year (Kellman, 2008). Of this, 48 million tones (29%) are fixed by agricultural field crops (Society for General Microbiology, 2002). Biological nitrogen fixation is the preferable source of nitrogen for future time in relation to environmental safety and saving energy with compared to industrial nitrogen fixation.

The energy that is required in biological nitrogen fixation is mostly derived from photosynthesis and therefore does not emit additional CO₂, in contrast to industrial nitrogen fixation. Biological fixed nitrogen is also directly and fully assimilated into biological material preventing the 50% loss of fixed nitrogen as in the case of cropping systems that are supplemented with industrial fixed nitrogen (Engelhard, 2004).

3.6. Symbiotic nitrogen fixation

Nitrogen in air exists as two nitrogen atoms that are triple-bonded together; the atoms are glued, stapled, and taped together, not to be undone without a great expenditure of energy. In symbiotic nitrogen fixation, Sugars or carbohydrates of a legume infected with Rhizobia are the energy and hydrogen source used by the bacteria to fix nitrogen at high energy costs to the plant. (ACMA, 2008).

Symbiotic Biological nitrogen fixation is considerable in environmental and agricultural importance, since they are responsible for estimated 175million tones of fixed nitrogen per year to global nitrogen economy (Kellman, 2008). This represents the highest amount of nitrogen fixed globally and it is 2 to 3 -folds greater than the input of nitrogen from nitrogen fertilizers, which is estimated at 65×10^6 tones per annum (Sahgal *et al* .2006).

3.7. Early Events of Nodulation

In nitrate-poor soil, symbiosis between rhizobia and leguminous plants leads to the development of nitrogen-fixing nodules, in which atmospheric nitrogen is fixed. Molecular signal transduction between rhizobia and their hosts triggers nodulation. The nodulation (nod) genes of rhizobia play important roles in the development of nodules (Relic *et al.*, 1994).

The *Rhizobium* signal molecules that play a key role in the induction of the initial stages of nodulation are lipochito-oligosaccharides known as Nod factors. The bacterial genes involved in Nod factor synthesis are the nod (nodulation) genes. These genes are not expressed in free-living bacteria, with the exception of nod-D, which is expressed constitutively. Nod- D has the ability to bind to specific flavonoids secreted by the roots of the host plant (Mylona *et al.*, 1995); upon flavonoid binding, it becomes a transcriptional activator of the other nod genes, which encode enzymes evolved in the synthesis of Nod factors.

In general, rhizobia have the ability to interact with only a limited number of host plants. However, some rhizobia, for example, *Rhizobium NGR234*, have a more promiscuous nature (Price *et al.*, 1992) . This *Rhizobium*, which can nodulate various tropical legumes, excretes 18 different Nod factors (Price *et al.*, 1992). The production of this variety of factors is thought to be the basis for its broad host range (Price *et al.*, 1992). In contrast, most rhizobia produce only a few different Nod factors (Mylona *et al.*, 1995).

3.8. Recognition and determinants of nodulation

Successful Symbiotic interaction requires compatibility at various stages starting from initial recognition, through successful differentiation to nitrogen fixation (Long and Ehrhardt,1989).The initial interaction between the host plant and free-living rhizobia is by the Plant roots secrete many different organic compounds into the soil, some of which allow microorganisms to grow in the rhizosphere and include carbohydrates, amino acids, organic acids, vitamins and phenolic derivatives (People and Crawswell,1992).

Reactions between certain compounds in the bacterial cell wall and the root surface are responsible for the rhizobia recognizing their correct host plant and attaching to the root hairs. Flavonoids secreted by the root cells activate the nod genes in the bacteria which then induce nodule formation because Nod genes direct the various stages of nodulation. The whole nodulation process is regulated by highly complex chemical

communications between the plant and the bacteria. (Society for General Microbiology, 2002)

Specificity genes determine which *Rhizobium* strain infects which legume. Even if a strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. Such rhizobia are termed ineffective. Effective strains induce nitrogen-fixing nodules. Effectiveness is governed by a different set of genes in the bacteria from the specificity genes (Ott , 2005).

3.9. Root infection and nodule formation

Root infection by rhizobia is a multistep process that is initiated by pre infection events in the rhizosphere. Compatible rhizobia are attracted by amino acids, dicarboxylic acids present in the exudates, and flavonoids (Kape *et al.*, 1991). Flavonoids secreted by the host plant into the rhizosphere function as inducers of the compatible rhizobial nod genes. Nod gene induction results in the secretion of lipo chitin oligosaccharides that are thought to bind to specific plant receptor kinases.

This initiates a complex signaling pathway involving calcium spiking in root hairs (Radutoiu, 2003) .The result is that root hairs curl and trap the rhizobia, which then enter the root hair through tubular structures known as infection threads that are formed by the plant. The infection threads then grow into the developing nodule tissue (Gage, 2004). Once a nodule is formed, the bacteria inside it change into a form known as bacteroids. The bacteroids produce the nitrogenase enzyme that converts nitrogen gas into ammonium that the plant can use. (Society for General Microbiology, 2002).

3.10. Biochemistry of nitrogen fixation

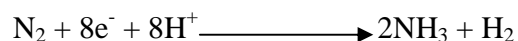
The process of changing atmospheric nitrogen into more reactive and useable form is done by special enzyme called Nitrogenase. Nitrogenase enzyme has two oxygen sensitive metallo-protein components (dinitrogenase reductase or component II) and Molybdenum-iron protein (dinitrogenase or Component-I) (Sprent and Raven, 1985). None of the components is active independently but they work synergistically (Benton *et al.*, 2002) .

The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe-protein complex is first reduced by electrons donated by ferredoxin. Then, the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2 .

The iron-protein also generates the reducing power (electron) for reducing nitrogen (Benton *et al.*, 2002).



The MoFe-protein uses this reducing power (electron) to actually reduce nitrogen (Benton *et al.*, 2002).



Net-reaction

$N_2 + 4\text{NADPH} + 4\text{H}^+ + 16\text{ATP} \longrightarrow 2\text{NH}_3 + \text{H}_2 + 4\text{NADP}^+ + 16\text{ADP} + 16\text{Pi}$
fixation consumes 8 ATP and 2 NADPH per single molecule of ammonium formed which is a high energy consuming process.

The reaction catalyzed by nitrogenase involves the Mg ATP-dependent reduction of nitrogen gas to yield two molecules of ammonia. The reduction of nitrogen to ammonia is a highly endergonic and energy consuming reaction. In the course of this reaction protons are also reduced. (Dixon and Wheeler, 1986)

The stoichiometry of the reaction showed below holds true in the laboratory conditions. In natural conditions up to 40 molecules of ATP can be hydrolyzed for the reduction of only one nitrogen molecule. (Hill,1992). Additionally for every reduced molecule of nitrogen the nitrogenase complex produces hydrogen molecule (H_2). The hydrogen production has been described as one of the major factors that affect the efficiency of symbiotic nitrogen fixation, however some rhizobia species have developed a system of hydrogenase that allows them to recycle the generated hydrogen (Baginsky *et al.*,2002), which oxidizes H_2 to H^+ and $2e^-$. This increasing the nitrogen fixation efficiency, as a result, symbiosis carried out by this system (Hup^+) is more efficient in nitrogen fixation. Actually, numerous reports have shown that legumes inoculated with Hup^+ strains have up to 30% plant dry matter increase as compared to non- Hup^+ strains (Evans *et al.*, 1985). Thus the equation for the whole reaction can be depicted as follows:

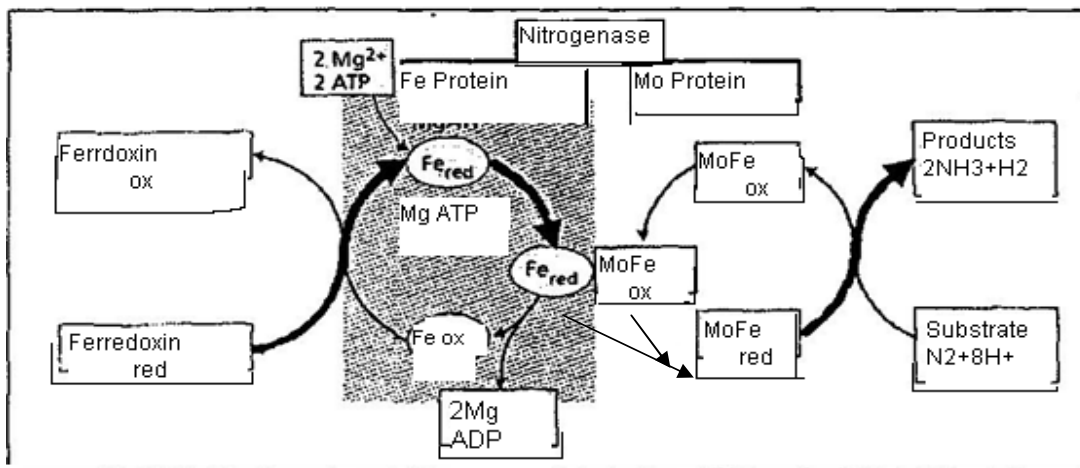


Fig-1 The reaction catalysed by nitrogenase. Ferredoxin reduces the Fe protein. Binding hydrolysis of ATP to the Fe is thought to cause a conformational change of the Fe protein, which facilitates the redox reaction. The Fe protein reduces the MoFe protein and the MoFe protein reduces the N_2 . It is possible for two Fe proteins to bind to the MoFe protein at one time, but only one is shown for clarity. The darker arrows trace the flow of electrons. Source: Marie, (2001).

The reaction sequence (Fig-1) starts by reduction of the Fe protein by the low-potential electron donor ferredoxin. Electrons are transferred, one at a time from the Fe protein to the MoFe protein in a process that involves Mg ATP hydrolysis. The cycle repeats until enough electrons have been provided for the complete reduction of the N_2 substrate (Marie, 2001).

3.11. Nitrogenase enzyme and its catalyzing activity

An enzyme called nitrogenase catalyses the conversion of nitrogen gas to ammonia in nitrogen-fixing organisms. In legumes it only occurs within the bacteroids. The reaction requires hydrogen as well as energy from ATP. The nitrogenase complex is sensitive to oxygen, because one of its components the MoFe co factor, is irreversibly denatured by oxygen with typical half decay time of 30 to 45 seconds (Dixon and Wheeler, 1986). On other hand the large amount of energy required for this reaction has to be generated by oxidative processes, there is high demand oxygen in nodules (Ott, 2005).

Rhizobium controls oxygen levels in the nodule with leghaemoglobin. Different strategies are used in different symbiotic interactions to cope with this paradox (Ott , 2005) .This red, iron-containing protein has a similar function to that of hemoglobin; binding to oxygen. This provides sufficient oxygen for the metabolic functions of the bacteroids but prevents the accumulation of free oxygen that would destroy the activity of nitrogenase (Society for General Microbiology, 2002) . A low oxygen tension in nodule is achieved by combination of a high metabolic activity of the microsymbionts and an oxygen diffusion barrier in the periphery of the nodules (Spink, 2000).

3.12. Ecological factors affecting Biological nitrogen fixation

Some of the ecological factors affecting biological nitrogen fixation are soil pH, soil moisture, salt stress, soil temperature and nutrient deficiencies (Zahran, 1999). All environmental limitations affect plant growth and nitrogen fixation in legumes (Giller, 2001). Nodulation is one of the most highly affected during the association. Therefore, selection and breeding of legume crops and micro-symbionts tolerant to environmental stresses is very important to improve yields of the affected areas (Giller, 2001).

3.12.1. Soil pH

Soil pH is one of the major constraints for agricultural production in many Legumes growing areas of the world. It reduces legumes productivity. Extreme low or high pH values affect the legume host survival and growth of the endosymbionts, and nodulation and nitrogen fixation (Evan *et al.*, 1980). 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 low pH than have slowly growing strains of *Bradyrhizobium* (Graham *et al.*, 1994).

Vassilava *et al.*, (1997) reported that, nodulation of legumes is reduced in acidic soil, mainly because of sensitivity of early nodulation events, such as attachment, root hair curling and initiation of infection thread formation. Evan *et al.*, (1980) found that nodulation of *Pisum sativum* was 10 times more susceptible to acidity than was either rhizobia multiplication or plant growth. There are two strategies that can solve the problems of soil acidity, (1) selecting acid tolerance plants and rhizobia, (2) limiting the acidic soils to ameliorate the effects of acidic conditions (Zahran,1999). The amelioration with lime increases the soil pH from 4.5 to 4.9 to decrease the concentration of extractable Al and Mn and improve growth and nitrogen fixation.

3.12.2. Aluminum and Manganese Toxicity

Acidic soil contains high concentration of H⁺, Al, Mn, or Fe that can be injurious to nodulation or growth of rhizobia; as well as deficiencies of phosphate, molybdenum and calcium. The solubility of Al⁺³ ions is very low in slightly acidic soil (pH >5.5) to neutral soil, but increases sometimes abruptly at pH value below 5.0. This condition inhibits calcium and phosphorous uptake (Taylor *et al.*, 1990).

Aluminum toxicity is a serious agricultural problem in acidic soils. Aluminum toxicity inhibits root growth and uptake of water and nutrients, results in decreased production (Kochian, 1995). Aluminum toxicity affects rhizobia by binding to DNA of both sensitive and tolerant strains (Jensen and Wood, 1990). The presence of aluminum in acidic soil is a limiting factor for development and functioning of *Rhizobium*-legume symbiosis. Manganese is another toxic element at low pH. Excess soil acidity allows manganese that is normally bound to soil particles to be released and taken up by the plant in very high concentrations, i.e., toxic levels (Alexander, 1985). Manganese toxicity mainly affects legume growth. Symptoms of manganese toxicity on plants are grouped into two. The most diagnostic feature is the darkening of leaf veins, usually on older foliage. A second less diagnostic symptom of manganese toxicity is interveinal chlorosis with leaf cupping or necrotic blotching of foliage (Jensen and Wood, 1990).

3.12.3. Soil temperature

Low and high temperature affects the symbiotic nitrogen fixation severely. Temperature regulates the metabolism of the bacteria, and the plant as well as bacteria-plant association. *Rhizobium* strains differ in their ability to grow, nodulate their host plants and expression of nitrogenase activity at extreme temperatures.

The low temperature limits for plants native to temperate zone is 2°C and for tropical species about 10°C (Jordan 1984). Sometimes the sensitivity of host towards low temperature affects the nitrogen fixation severely leading to abrupt off host plants at low temperatures where the bacterial cells can still grow and metabolize. Temperature affects N₂-fixation at any stage of nodulation from the attachment of bacteria to the host root hair up to the number of bacteroids within the host cell. Root hair infection is much more temperature sensitive than nodule development. Nodulation in *sativum* is affected to greater extent by low temperature. In extreme temperature in addition of nodulation and physiological disorders, rhizobial plasmid also loss and genomic rearrangements can be affected (Sobero-chavaz, *et al.*, 1986)

3.12.4. Soil moisture

In all living systems water determines the existence and productivity of organisms under severe environments. Low water activity impairs nodulation and affects survival of both partners. Even though water affects both partners. Different strains of rhizobia and different legume cultivars display differences in tolerance of low water activity (Bordeleau and Prevost, 1994, Zahran, 1999).

Water stress limits rhizobial survival, growth and population structure in the soil (Zahran, 1999). More over nodule formation, its longevity, leghemoglobin synthesis and nodule function is highly affected (Hungria and Vargas, 2000). Symbiotic N₂ 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 (Abdel wahab and Zahran, 1983).

The response of nodulation and nitrogen fixation to water stress depends on the growth stage of the plants. It was found that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation decreased about 26% than that imposed during the reproduction stage and there was little chance for recovery from water stress in the reproductive stage (Pena-cabriles and castellanoa, 1993).

Water logging (excess water) is an other factor that determines the function of symbiotic nitrogen fixation. When fields are water logged, the environments become anaerobic with high concentration of CO₂. This results respiration problem in rhizobia and nodules that result in reduced number of rhizobia and low nodulation (Eaglesham, 1984).

3.12.5. Salt stress

Salinity in the arid and semi-arid regions of the world as well as in irrigated lands is a serious threat to agriculture affecting plant growth and crop yields (Zahran, 1999). Current estimates that 10-35% of the world's agricultural land is affected with very significant areas becoming unusable each year. Increasing salt concentrations may have a 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 (Abdel-Wahab *et al.*, 1981). The legume-Rhizobium symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are rhizobia (Zahran, 1991).

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 *Ensifer meliloti* tolerate 200-300 mM NaCl, while nodulation and nitrogen fixation in their host can be inhibited at 50-100 mM. Strains of *R. leguminosarum biovar viciae* have been reported to be tolerant to NaCl concentrations up to 350 mM in growth cultures (Abdel-Wahab and Zahran, 1981). Rhizobium strains from *Vigna unguiculata* were tolerant to NaCl up to 5.5 (Abdel-Wahab *et al.*, 1981).

Many species of bacteria adapt to saline conditions by intracellular accumulation of low molecular weight organic solutes called osmolytes (Bordeleau and Prevost, 1994). 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 (Zahran *et al.*, 1997) examined that the rhizobial cells responded to high salt stress by changing their cellular morphology.

3.12.6. Soil nutrients

Nodulation and N₂-fixation by many legumes are limited by deficiencies in soil nutrients such as P, Mo, Ca, Fe and S, (Giller and Wilson, 1991). A group of these essential nutrients are required at specific stages in the development of legume symbiosis and the symbiotic N fixation to the extent that their deficiencies limit the productivity of host legumes in some agricultural systems (Aira, .2003).

Phosphorus is essential for both nodulation and N₂ fixation (Pereira and Bliss, 1989). Nodules are strong sinks for P and range in P content from 0.72 to 1.2% (Hart, 1989); consequently, plants engaged in symbiotic N₂ fixation generally have a higher requirement for P than those grown with N fertilization (Panda *et al.*, ,2002). P is required for signal transduction, membrane biosynthesis, nodule development and function (Grusak , 2000), and nitrogenase activity.

Molybdenum has a major role in symbiotic N fixation as a fundamental component for nitrogenase. Mo deficiency affects nodule development by reducing bacteroid multiplication and delaying or preventing the onset of nitrogenase activity (Voisin *et al.*, 2002) Calcium deficiency has specific effects on the legume symbiosis during the pre-infection and nodule development stages (O'Hara *et al.*, 1988). There appears to be a high Ca requirement for attachment of rhizobia to root hairs, (Grusak, 2000) for induction of nod genes in *R. leguminosarum* *bv. viciae* (Richardson *et al.*, 1988).

Iron is required for legume nodulation, possibly for the proliferation of the infecting rhizobia in the host root tissue (Panda *et al.*, 2002). Legumes are sensitive to Fe deficiency at an early stage of nodule initiation (O'Hara *et al.*, 1988) . The source of Fe from seed reserves or supplied from soil, during nodule development may be an important factor in the efficiency of transfer of Fe from host tissues to the infecting rhizobia (Aira .2003).

Sulfur deficiency in legume crops not only affects yield formation, but also the quality and the nutritional value of seeds, because methionine is usually the most limiting essential amino acid in legume seeds (Voisin *et al.*, 2002). With S deficiency amino acids and other N forms, accumulating due to a lack of sulphur being synthesized into proteins, may have a feed-back repression on nitrogen fixation. Furthermore S deficiency may affect N₂ fixation because of the relatively high S content of the nitrogenase enzyme (Panda *et al.*, 2002).

4. Materials and methods

4.1. Sampling site

The study Sites were important pulse growing areas of Ofla, Endamokhoni and North western parts of Alamata woreda in Southern Tigray

Administrative Woredas of Tigray Region, Ethiopia.

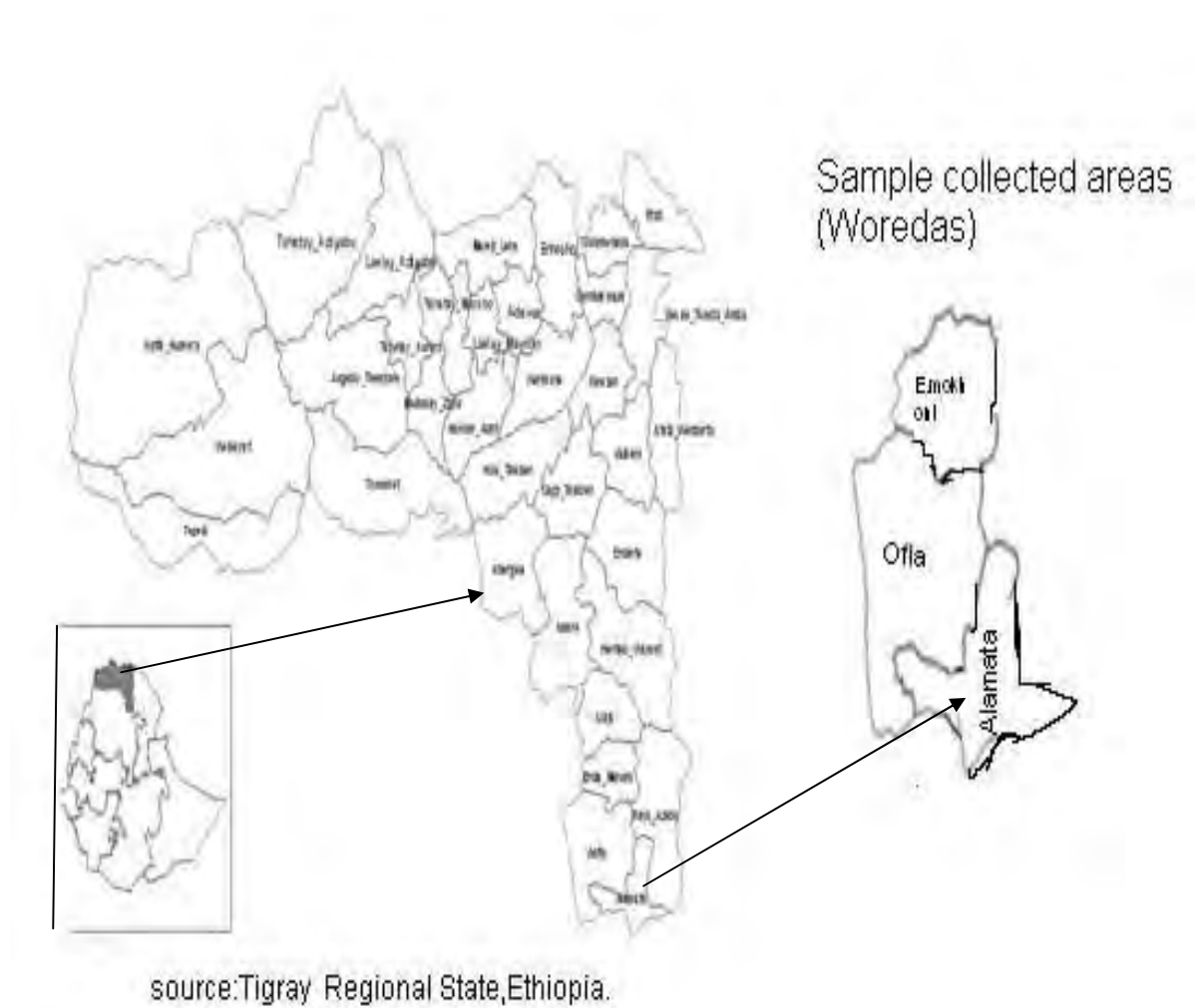


Fig.2.Location Map of Soil sample Sites

4.2. Soil sample Collection

Soil samples were collected from *Pisum sativum* growing field in October, 2009. The soil samples were randomly collected from Ofla, Endamohoni and North western part of Alamata Woreda and sub-sampled the soils in polyethylene plastic bags. The soil samples were brought to Applied Microbiology Laboratory of the Department of Biology, AAU for further processing. The specific sites and the soil pH indicated in Table-1 below.

4.3. Induction of Nodulation

The soil samples were mixed with the extensively washed and autoclave sterilized river sand and filled into surface sterilized 3Kg capacity plastic pots. Seeds of the cultivar '*Fabaceae Pisum sativum Addi*' provided by Holeta Agricultural Research Center was surface sterilized and 5 seeds were then sown in each pot and allowed to germinate. The pots were placed in green house and after germination seedlings thin down to three plants per pot. The plants were frequently (every two days) watered for 55 days. The plants were uprooted to collect healthy nodules.

4.4. Isolation of root nodule bacteria

The nodules (collected from induced host Pea) were surface sterilized with 95% ethanol for 10 seconds, and transferred to 3%(v/v) solution of sodium hypo chlorate for 3-minutes(Lupwayi and Haque,1994).The surface sterilized nodules were then rinsed with sterile distilled water six times to completely remove the sterilizing chemicals (Lupwayi and Haque ,1994). The nodules were crushed with sterile glass rods in 1 drop of sterilized 0.85% NaCl. The crushed nodules were transferred to YEM plate the contents of YEMA are shown below. They were then incubated at $28\pm 2^{\circ}\text{C}$ and periodically for colony formation.

YEMA Composition

chemicals	Amount (gm/l)
MgSO ₄ .7H ₂ O	0.2g
NaCl	0.2g
K ₂ HPO ₄	0.5
KH ₂ PO ₄	0.5g
Yeast extract	0.5g
D-manitol	10g
Agar	15g
Distil water	1000ml

Autoclaved at 121°C for 15 minutes, pH adjusted to 7, Taken from –Lupwayi and Haque. (1994)

4.5. Purification and preservation

Colonies of isolates picked with sterile inoculating loop and streaked on sterile YEMA plates and incubated at 28±2⁰C. The purify and uniformity of colony type carefully examined through repeated re-streaking and a single well isolated colony picked to YEMA slant containing 0.3% (W/V) CaCO₃ in a culture tube and incubated at 28±2⁰C . After sufficient growth the culture slant were preserved at 4⁰c (Somesagaran and Hoben, 1994).

4.6. Presumptive test

4.6.1. Gram staining test

All isolates were tested in gram stain for rapid means of identification of contaminants as indicated in Lupwayi and Haque, (1994).

4.6.2. Congo red absorption

To check the purity of the isolates tested on Congo -Red (CR-YEMA). Stock solution of Congo Red (CR) prepared by dissolving 0. 25g of CR in 100ml sterile distilled water. 10ml from this stock solution added to one liter of YEMA. The broth culture suspension inoculated into YEMA-CR medium, and the plates were wrapped with aluminum foil to provide a dark condition and incubated to at 28±2 C⁰ observe CR absorption or not (Somasegaran and Hoben, 1994).

4.6.3. Acid and alkaline production on BTB

The production of acid or alkaline determine by incorporating bromothymol blue (BTB) as reaction indicator on yeast extract manitol agar (YEMA)

YEMA-----1 liter

BTB-(0.5 % w/v in 95% ethanol) -----5ml

pH-----6.8

After 48 hours of growth, a loop full of *Rhizobium* culture (10^5 cells/ml) streaked on YEMA-BTB plate, fast growers changed the BTB in to yellow color, but slow growers changed into blue color, and results were recorded after 3-5 days of incubation (Somassegaran and Hoben, 1994).

4.6.4. Growth on Peptone-glucose agar (PGA) Medium

Isolates inoculated on PGA incorporated with bromocresol purple in order to check a change in pH of the medium associated with the presence of contaminants in the preserved culture further checked on PGA medium with 10 μ g/ml Bromocresol purple (BCP) dye.

Composition of, PGA medium

Glucose.....5g

Peptone.....10g

Agar.....15g

BCP stock solution.....-10ml

P^H.....6.7

Distilled H₂O.....1liter

Autoclaved at 1210C for 15 min. Source: Somassegaran and Hoben (1994)

The BCP (Bromo cresol purple) prepared as stock solution by dissolving 1g/100ml of ethanol (Somasegaran and Hoben, 1994). The pH adjusted at 6.7 by 1N NaOH and HCl (Lupwayi and Haque, 1994). The bacterial culture suspension was inoculated on the medium and incubated at 28 ± 2 °c; finally presence/absence of bacterial colonies was checked.

4.6.5. Authentication

River sand soaked in H₂SO₄ for 24 hours and extensively washed with tap water several times and sterilized in autoclave. Then the sterilized sand filled into surface sterilized 3kg capacity plastic pots. Healthy *Fabaceae Pisum sativum Addi* cultivar seeds of uniform size and color were surface sterilized with 95% ethanol for 10 seconds and then .02 % (w/v) Hgcl₂ for 4 minutes.

The seeds rinse six times in sterile water to remove any trace of sterilizing chemicals. The seeds were allowed to imbibe in water for 5 hours, and transfer into 0.75% water agar plates and incubates at 25c° (vincet,1970). After three days of germination, each seedlings inoculated with 1ml (10⁹cells/ml) broth suspension. Five seedlings transferred to each pot, later thinned down to three.

The experiment set up in randomized complete design with three blocks of replications. There were 33 treatments, three positive (with nitrogen but without inoculation) and three negative (neither fertilized nor inoculated) controls. At the beginning all plants given 100ml of 0.05% KNO₃ (W/V) once as starting nitrogen. The positive control were irrigated with 100ml of 0.05% KNO₃ (w/v) solution once a week for 4-weeks. The N-free nutrient solution gave to all plants 100ml/pot once a week as full strength for the four consecutive weeks and supply distilled water every 2 days (Somasegaran and Hoben, 1994).

Plants grown in green house with a12/12 light/ dark hrs cycle and an average25/18c° day/night temperature.55 days after planting, the whole plants uprooted and determined the number of nodules, nodule dry weight and shoot dry weight after drying at 70c° for 48hrs until constant weight.

N-free Nutrient Solution composition (Beattie *et al.*, 1989)(solution-1)

No	Chemicals	g/l
1	K ₂ HPO ₄	0.023
2	KH ₂ PO ₄	0.13
3	CaSO ₄ .2H ₂ O	3.425
4	MgSO ₄ .7H ₂ O	0.274
5	NaCl	0.04
6	FeCl ₃ .6H ₂ O	0.06

Add 1ml of solution-2/ liter of solution-1.

Solution-2

No	Chemicals	g/l
1	CU ₂ SO ₄ .5H ₂ O	0.15
2	ZnSO ₄ .7H ₂ O	0.44
3	MnSO ₄ .7H ₂ O	0.04
4	H ₃ BO ₃ (Boric acid)	1.43

Source: Beattie *et al.*, (1989) as cited in Mulisa Jida,(2005).

4.7. Growth, morphological and Cultural characteristics

4.7.1. Mean growth time

Each isolate inoculated into 10ml of YEMB test tube, and shaken on orbital shaker at 120 rpm (revolution per minute) for 48hrs at room temperature. One ml of each culture transferred into 250ml Erlenmeyer flasks containing 100ml of YEMB and placed on rotary shaker at 120 rpm (revolution per minute). After calibrating spectrophotometer (UV-7804C-Ultraviolet-Visible spectrophotometer) to zero with sterile uninoculated YEMB (3.5 ml blank), 3.5ml of the culture was transferred into cuvette to read optical density of rhizobium at 540 nm beginning from time of inoculation (0hr) and at every 6hrs interval for 72hours. Finally, the generation time (g) calculate from the logarithmic phase according White (1995),

The formula;
$$g = \frac{\log^2(t)}{\log^X - \log^{X_0}},$$

Where: g =generation time

t = time elapsed

X₀ =First OD,

X =second OD reading

OD=optical density

Generation time (g) =t/n

n=number of generations,

t= time elapsed

Viable cell count of isolates was also done by serial dilution (10⁻⁶) taking one ml of inoculums into 9ml of distilled water and 0.1ml of the samples were inoculated on sterile YEMA plates .The viable cell counts were estimated with the following formulae.

Number of cells=Number of colonies counted x dilution factor

1ml sample taken

4.7.2. Colony Morphology

The cultural characteristics of the isolates were performed according to (Lupwayi and Haque, 1994). Culture suspensions of each bacterial isolates were inoculated on the YEMA medium and incubated for 3-5 days. Single colonies of each isolate were characterized by colony appearance, diameter and extra cellular polysaccharide production. The record has taken as translucent, opeque, Large and Medium Mucoid and watery.

4.8. Physiological characteristics

4.8.1. Carbohydrate utilization.

The isolates streaked on different carbohydrates to evaluate their ability to use different carbon sources . A carbohydrates (both heat stable and labile) were prepared 10 % (w/v).Carbohydrate- free medium (basal medium) was prepared by omitting manitol and reducing the concentration of yeast extract from 0.5g/l to 0.05g/l, but incorporating all components of YEMA Some carbohydrates are heat stable (glucose, lactose,D- manitol, and sucrose), and sterilized with their basal medium.

The rest heat labile (galactose, maltose, Starch, Cellulose, sorbitol ,Tartarate, Glycerol, Arabinose and citrate,) were filter sterilized using 0.2µm Whatman filter paper and added to the autoclave sterilized medium. Isolates were grown in 10ml YEM broth for 48 hours at 28±2°c on 120 rpm (revolution per minute) shaker. 10⁹ cells/ml cultures inoculated in to media containing different carbon sources (Somasegran and Hoben, 1994).

Basal medium Composition

chemicals	Amount (gm/l)
MgSO ₄ .7H ₂ O	0.2g
NaCl	0.2g
K ₂ HPO ₄	0.5
KH ₂ PO ₄	0.5g
Yeast extract	0.05g
Agar	15g
Distil water	1000ml

Autoclaved at 121°C for 15 minutes, pH adjusted to 7, Taken from –Lupwayi and Haque .(1994)

4.8.2. Amino acid utilization

The growth of isolates was tested by different nitrogen sources: L-lysine, L-arginine, L-tyrosine, L-tryptophan, L-asparagines, Methionine and Glutamate. They were added at concentration of 0.5g /l to a basal medium (Amarger *et al.*, 1997).

Basal medium Composition

chemicals	Amount (gm/l)
MgSO ₄ .7H ₂ O	0.2g
NaCl	0.2g
K ₂ HPO ₄	0.5
KH ₂ PO ₄	0.5g
Yeast extract	0.05g
D-manitol	1g
Agar	15g
Distil water	1000ml

Autoclaved at 121°C for 15 minutes,pH adjusted to 7,Taken from –Lupwayi and Haque .(1994)

4.8.3. Phosphate solublizing ability

This was determined by inoculating the isolates on Pikovskaya agar medium (PA) containing: Glucose (10g/l),Tricalcium phosphate (5g/l),Ammonium phosphate (0.5g/l), Yeast extract (0.5g/l),Magnesium sulfate heptahydrate (0.1g/l),Sodium chloride (0.2g/l),Manganese sulfate (0.002g/l), Ferrous sulfate (0.002g/l) and Agar (15g/l). The pH of the medium adjusted to 7.00. This ability checked based on growth and the presence of clear zone around the colonies (Somasogaran and Hoben ,1994).Growth and clear zone qualitatively recorded as (+) for growth and clear zone ,(-) for no growth and clear zone

4.9 .Eco-physiological characteristics

4.9.1. Acid and alkaline (pH) tolerance

The pH tolerance test has been done on TYEA-medium according to Bernal and Grham,(2001).and inoculate using inoculation loop containing about 10^5 cells/ml and incubated at 28 ± 2 c^o.Growth determined qualitatively as (+) for growth and (-) for no growth. The pH range use between 4.0, 4.5, 5.0, 5.5, 8.5, 9.0, and 9.5.Tryptone yeast extract agar media is composed of:

Tryptone-----	5g/l
Yeast extract-----	3g/l
CaCl ₂ .2H ₂ O-----	0.89g/l
Agar-----	15g/l

Before sterilization pH adjusted to different pH using 0.1N NaoH and Hcl.

4.9.2. Salt tolerance

Tolerance to sodium chloride (NaCl) evaluated through determining growth on YEMA solid medium supplemented with 1, 2, 3, 4, 5, 6, and 7% (w/v), Nacl concentration. Growth was evaluated qualitatively as (+) for growth and (-) for no growth after 3-5 days (Lupiwayi and Haque,1994).

4.9.3. Temperature tolerance

The ability of bacterial strains to proliferate at varying temperatures was assessed on YEMA plates incubated at the temperatures of, 4, 15, 20, 25, 30, 35 and 40^oc (Lupiwayi and Haque,1994).Growth qualitatively recorded as (+) for growth and (-) for no growth .

4.9.4. Intrinsic antibiotic resistance (IAR)

The resistance of isolates to antibiotics was tested by streaking them on solid YEMA medium containing six different antibiotics. The stock of antibiotic solutions was first prepared as described in Lupiwayi and Haque,(1994). Each antibiotic sterilized by filtration using What man 0.2 micrometer pore size filter. The filter sterilized a liquor of each antibiotic (2.5, 5 and 10 µg/ml) was added aseptically in definite amount to sterile YEMA cooled to 50 °c and mixed thoroughly. The isolates tested in triplicate plates by streaking 1ml of the culture on each cooled plate. The antibiotics Ampicillin, Tetracyclin, Penicillin, Gentamycin, Chloramphenicol and Erythromycin (Lupiwayi and Haque, 1994).

4.10. Symbiotic effectiveness (SE)

Isolates effectiveness in accumulating plant shoot dry weight calculated according to the equation proposed by (Date et al., 1993 as cited in Purcino *et al.*, 2000)

$$SE = \frac{\text{Inoculated plant DM} \times 100\%}{\text{N-fertilized plant DM}}$$

DM=dry matter

SE=symbiotic effectiveness

Nitrogen fixing effectiveness classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

4.11. Numerical Analysis

Phenotypic similarities among *Pisum sativum Rhizobium (Rhizobium leguminosarum biovar viciae)* Numerically analyzed based on their 7 phenotypic characteristics, such as pH tolerance, temperature tolerance, Intrinsic antibiotic resistance, mean generation time, carbohydrate utilization, amino acid utilization and phosphate solubilizing abilities of isolates by using the PC-ORDv.5 program (Soft ware) (Lupiwayi and Haque, 1994).

4.12. Data Analysis Symbiotic effectiveness parameters such as nodule number, nodule dry weight, and shoot dry weight, data were analyzed by one-way ANOVA (Tukey's HSD test) SPSS. Version 13.0 (Somasogaran and Hoben ,1994).

5. Results

5.1. Growth, colony morphology and cultural characteristics

In this study, a total of 33 root nodule bacteria were induced and isolated from soils of some parts of Southern Tigray where field pea (*Pisum sativum*) is regularly growing (Table2). The soils were characterized by near neutral and slightly basic pH (pH 6.93-7.43). All isolates did not grow on Peptone –glucose agar medium and failed to absorb Congo-red on Yeast Extract Agar medium .

Almost all the isolates changed the green color BTB medium into yellow color. Almost half of the isolates (49%) displayed large mucoid (LM) translucent colonies; whereas (21%) showed large watery (LW), and 18% displayed medium mucoid, and 12% showed medium watery colonies with production of copious amount of exopolysaccharides on the medium. Many colonies (88%) were found to be fast growers with colony diameter of 2.0-4.0 mm after 3-5 days of growth. The largest colony diameter of 5.0 mm was recorded from the isolates AAUFPR06 and AAUFPR29 on YEMA medium (Table2).

The fastest growth rate was displayed by isolates AAUFPR40 AAUFPR38, AAUFPR37, AAUFPR28, AAUFPR22 with mean generation time of 2.2, 2.3, 2.4, 2.5 hours respectively. The relative slow growing ones were AAUFPR42, AAUFPR39, AAUFPR31, AAUFPR21 and AAUFPR16 with mean doubling times of 4 hours (Table2). There was no significant correlation between colony diameter and mean generation time of the isolates ($r=0.1$)(data/graph not shown).

Table-1- Growth, Colony morphology and Cultural characteristic

Isolates	Sample site	Colony characteristics	Colony size(mm)	YEM/ABTB	MGT in hours
AAUFPR01	Ofla	LW-transparent	4mm	Yellow green	2.6 hrs.
AAUFPR 03	Ofla	AAUFPR0LM-translucent	3.5mm	Yellow	2.6hrs.
AAUFPR05	Ofla	MW-transparent	3mm	Yellow	2.7
AAUFPR06	Ofla	LM-translucent	5mm	Yellow	3.4
AAUFPR07	Ofla	LM-translucent	4mm	Yellow	3.0
AAUFPR10	Ofla	MW-transparent	2mm	Yellow green	3.5
AAUFPR11	Ofla	MM-translucent	3mm	Yellow	3.2
AAUFPR13	Ofla	LM-translucent	3.5mm	Yellow	3.6
AAUFPR15	Ofla	LM-translucent	4.5mm	Yellow	3.7
AAUFPR16	Ofla	LM-translucent	4mm	Yellow	4.2
AAUFPR17	Ofla	MW-transparent	3mm	Yellow	3.0
AAUFPR18	Ofla	MM-translucent	3mm	Yellow	3.4
AAUFPR19	Ofla	LM-translucent	3.5mm	Yellow	3.3
AAUFPR21	Endamohoni	LW-transparent	4mm	Yellow	4.1
AAUFPR22	Endamohoni	LM-translucent	3.5mm	Yellow	2.5
AAUFPR23	Endamohoni	LM-translucent	4.0mm	Yellow	2.7
AAUFPR24	Endamohoni	LM-translucent	3.5mm	Yellow	3.8
AAUFPR25	Endamohoni	LM-translucent	4.0mm	Yellowgreen	2.8
AAUFPR26	Endamohoni	LM-translucent	4.0mm	Yellow	3.7
AAUFPR27	Endamohoni	LW-transparent	4.0mm	Yellow green	3.7
AAUFPR28	Endamohoni	L W-transparent	4.0mm	Yellow	2.5
AAUFPR29	Ofla	LM-translucent	5.0mm	Yellow	2.7
AAUFPR30	Ofla	MM-translucent	2.5mm	Yellow	3.0
AAUFPR31	Ofla	MM-translucent	2.0mm	Yellow	4.0
AAUFPR32	Ofla	LM-translucent	3.5mm	Yellow	3.0
AAUFPR36	Alamata	MW-transparent	3.0mm	Yellow	2.9
AAUFPR37	Alamata	L W-transparent	4.5mm	Yellow	2.4
AAUFPR38	Alamata	LW-transparent	4.0mm	Yellow	2.3
AAUFPR39	Alamata	LM-translucent	3.4mm	Yellow	4.0
AAUFPR40	Alamata	MM-translucent	3.0mm	Yellow	2.2
AAUFPR42	Ofla	LM-translucent	3.5mm	Yellow	4.1
AAUFPR43	Alamata	MM-translucent	3.0mm	Yellow	3.1
AAUFPR45	Alamata	L W-transparent	3.5mm	Yellow	3.5

Large mucoid (LM), large watery (LW), Medium watery (MW), Medium mucoid (MM), acid producer (yellow).

5.2. Physiological characteristics

5.2.1. Carbohydrate utilization

All isolates utilized sucrose, glucose and D-manitol as the sole source of carbon, and almost all isolates grew on YEMA medium containing maltose (96%), galactose (88%), lactose (81), and glycerol (81%) (fig.3). A few isolates utilized starch, cellulose and citrate. Isolate AAUFPR03 utilized a wide spectrum of carbohydrate, followed by isolates 24 and 43.

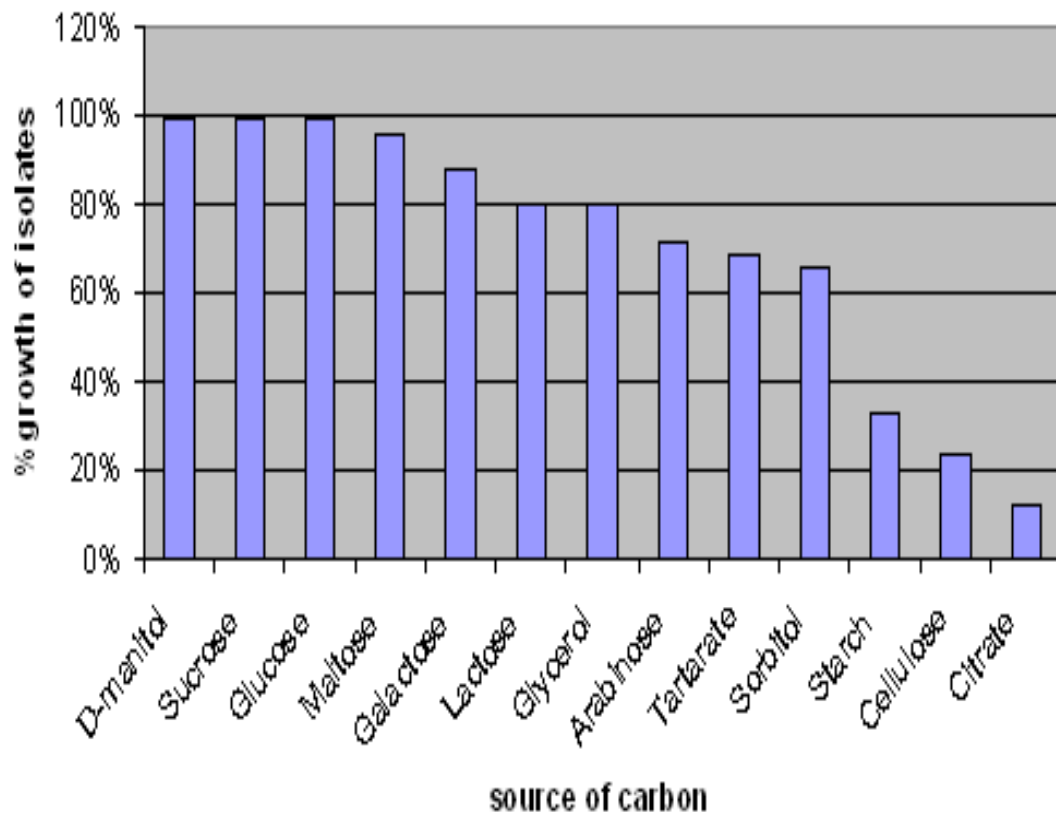


Figure-3- The pattern of carbohydrate utilization by isolates

5.2. 2.Amino acid utilization

Almost all of the isolates utilized the amino acid Tryptophan as a source of nitrogen followed by Glutamate, Lysine and Methionine (fig.4).Almost 40% of the isolates utilized all the amino acids. These isolates were AAUFPR 03, 06, 07, 13, 16, 21, 25,26,27,01,32,38 and 43.The most fastidious isolate was AAUFPR36 that metabolized only L-lysine.

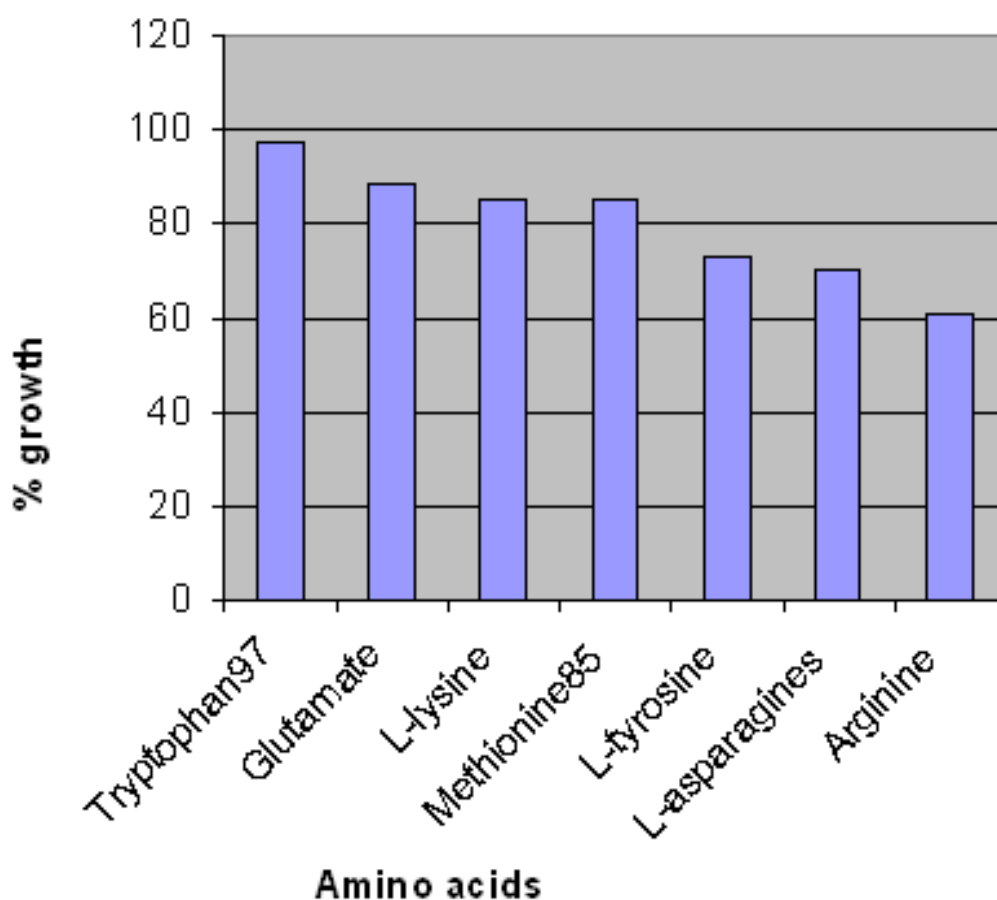


Figure-4- Amino acid utilization test of isolates

5.3. Eco-physiological characteristics

5.3. 1. Acid and alkaline (pH) tolerance

Almost all of the isolates were tolerant to pH 5-9.0. Although 39% of the isolates grew at pH 9.5, only isolate AAUFPR13 grew at pH 4.5. Isolates AAUFPR01, 03, 05, 13, 10, 18, 19, 21, 23, 25, 26, 27, 32 and 39 showed a wide range of pH tolerance.

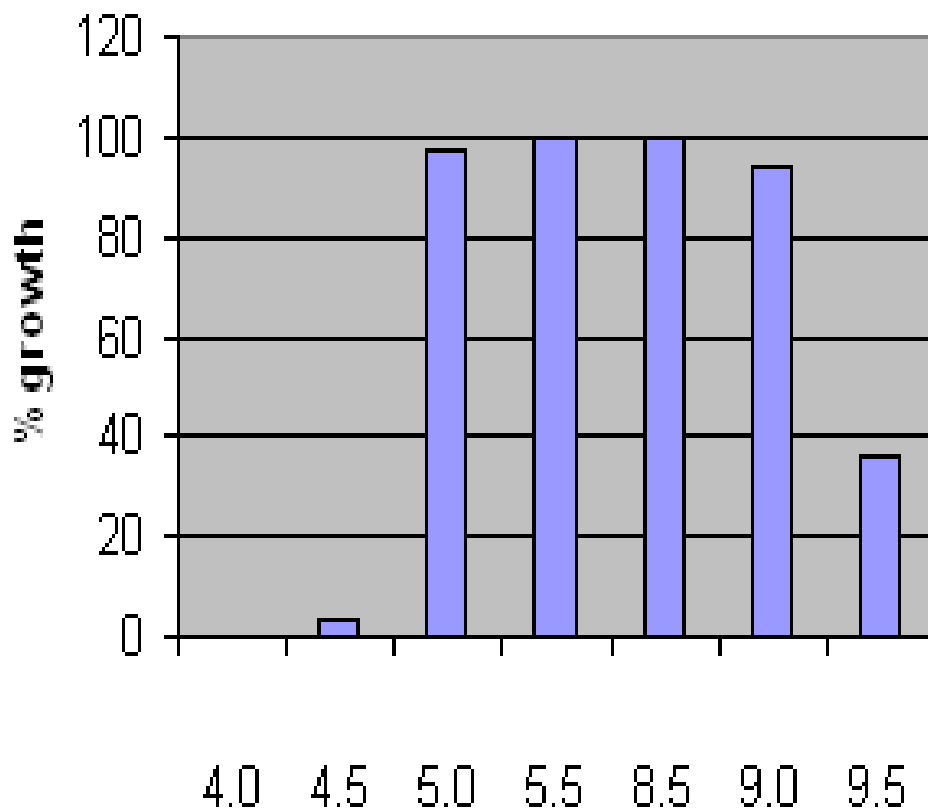


Figure-5- pH tolerance of isolates

5.3.2. Salt tolerance

As indicated in figure-6, isolates showed variations in tolerance to different concentrations of salt. 91% of the isolates did grow at medium salt concentration of 1 % (figure-6). The number of isolates growing decreased as the concentration of salt increased. There was a drastic reduction in salt tolerance by the isolates, when the salt was increased to 2%. Isolates AAUFPR07, AAUFPR16, AAUFPR27, AAUFPR28 and AAUFPR31 were found to tolerate 7% salt and isolates AAUFPR01, AAUFPR22 and AAUFPR32 did not grow at any salt concentrations (1-7%).

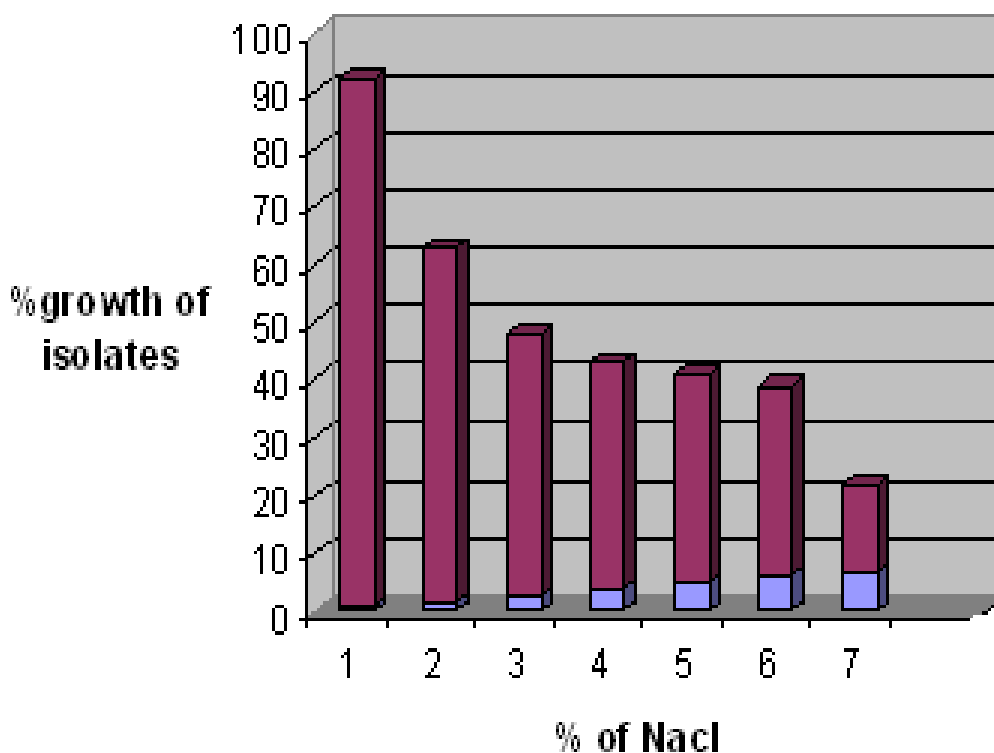


Figure-6- Salt Tolerance test of the isolates

5.3. 3. Temperature tolerance

All isolates were found to grow at incubation temperature of 25-35°C and none of them grew at 40°C. Isolates AAUFPR10, AAUFPR13 and AAUFPR21 were found to be tolerant to temperature of 4°C and these isolates were characterized by a wide range of temperature tolerance (4°C-35°C).

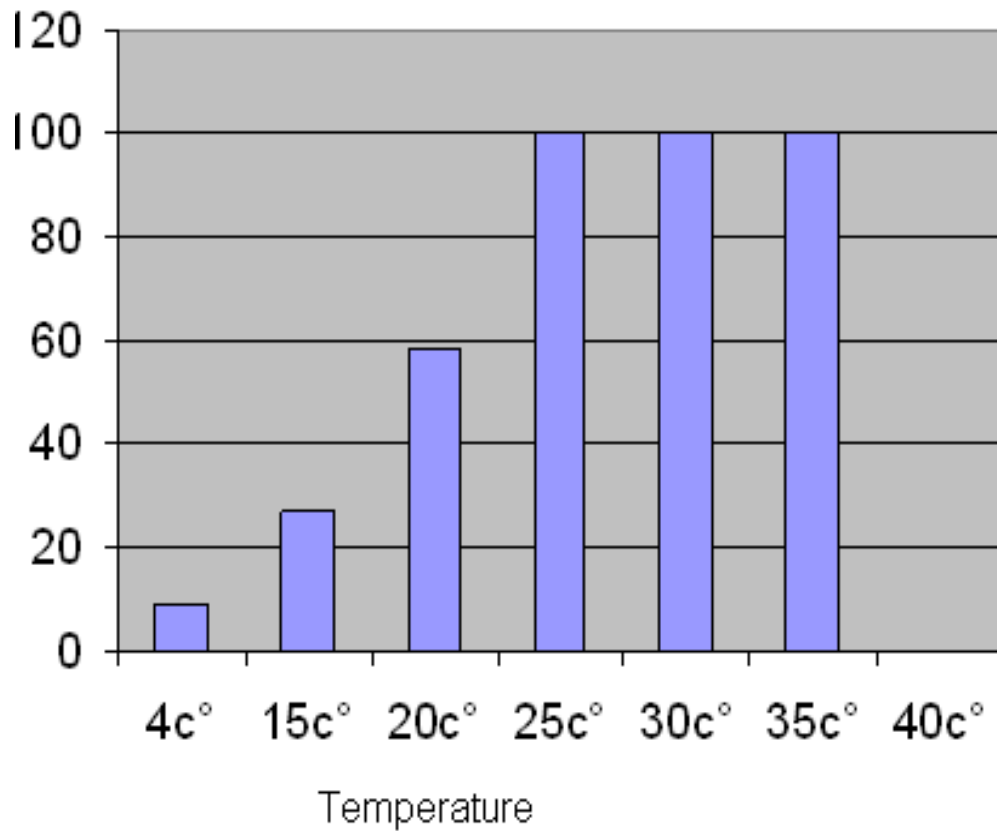


Figure-7- Temperature Tolerance test of the isolates

5.3.4. Intrinsic antibiotic resistance (IAR)

Isolates were found to be diverse in tolerance to different types and concentrations of antibiotics. Generally most isolates were tolerant to erythromycin, penicillin, chloramphenicol and ampicillin, but sensitive to gentamycin, tetracycline, and high concentration of chloramphenicol. The growth pattern showed that isolates grew well on erythromycin (75%), penicillin (71%), chloramphenicol (59%), but low growth on gentamycin and tetracycline and as concentration increased (2.5 to 10 µg/ml) growth decreased (Table-2) . Isolates 05,07,26,28,31,37,40 and 43 were the most resistant to different antibiotics, where as isolates 01, 19, 25, 42 and 45 were the most antibiotic sensitive strains (Table-3).

Table-2-Antibiotics and total growth of isolates

Antibiotics	% of resistant isolates to antibiotics			
	2.5 µg/ml	5 µg/ml	10 µg/ml	Average %
Ampicillin	76%	64%	21%	54%
Tetracyclin	9%	6%	0%	5%
Penicillin	97%	85%	30%	71%
Gentamycin	9%	6%	0%	5%
Chloramphenicol	91%	81%	6%	59%
Erythromycin	97%	91%	36%	75%
Total %	63	56	16	45

Table-3-Summary of antibiotic sensitivity test by isolates

Isolates	Total number of tests	Positive growth	Growth %
AAUFPR01	18	3	17%
AAUFPR 05	18	10	56%
AAUFPR 07	18	11	61%
AAUFPR 19	18	4	22%
AAUFPR 25	18	3	17%
AAUFPR 26	18	10	56%
AAUFPR 28	18	11	61%
AAUFPR 31	18	10	56%
AAUFPR 37	18	9	50%
AAUFPR 40	18	10	56%
AAUFPR 42	18	5	28%
AAUFPR 43	18	9	50%
AAUFPR 45	18	4	22%

5.4. Numerical Analysis

Numerical analysis based on 54 different phenotypic characteristics such as mean growth time, cultural characteristics, acid-alkaline tolerance, salt tolerance, temperature tolerance, IAR, carbohydrate and amino acid utilization clustered the isolates into different diversity groups (fig.8).

The 33 isolates were grouped into six clusters, except isolates 15 and 23 that did not show any affinity to other groups. The cluster contained different number of isolates. Groups 5 and 1 included the largest number of 9 (3,7,11,13,16,21,27,31 and 40) and 7 (1,10,22,25,29,39 and 45) isolates respectively. The other groups showed the same pattern groups 2,3 and 4 having 4 isolates in each cluster. Group-2 contained isolates 18, 32, 38 and 43. Group-3 included isolates, 5,6,26 and 37. Group 4 included isolates 17, 19, 36, and 42, Likewise, Group 6 included isolates 24, 28 and 30.

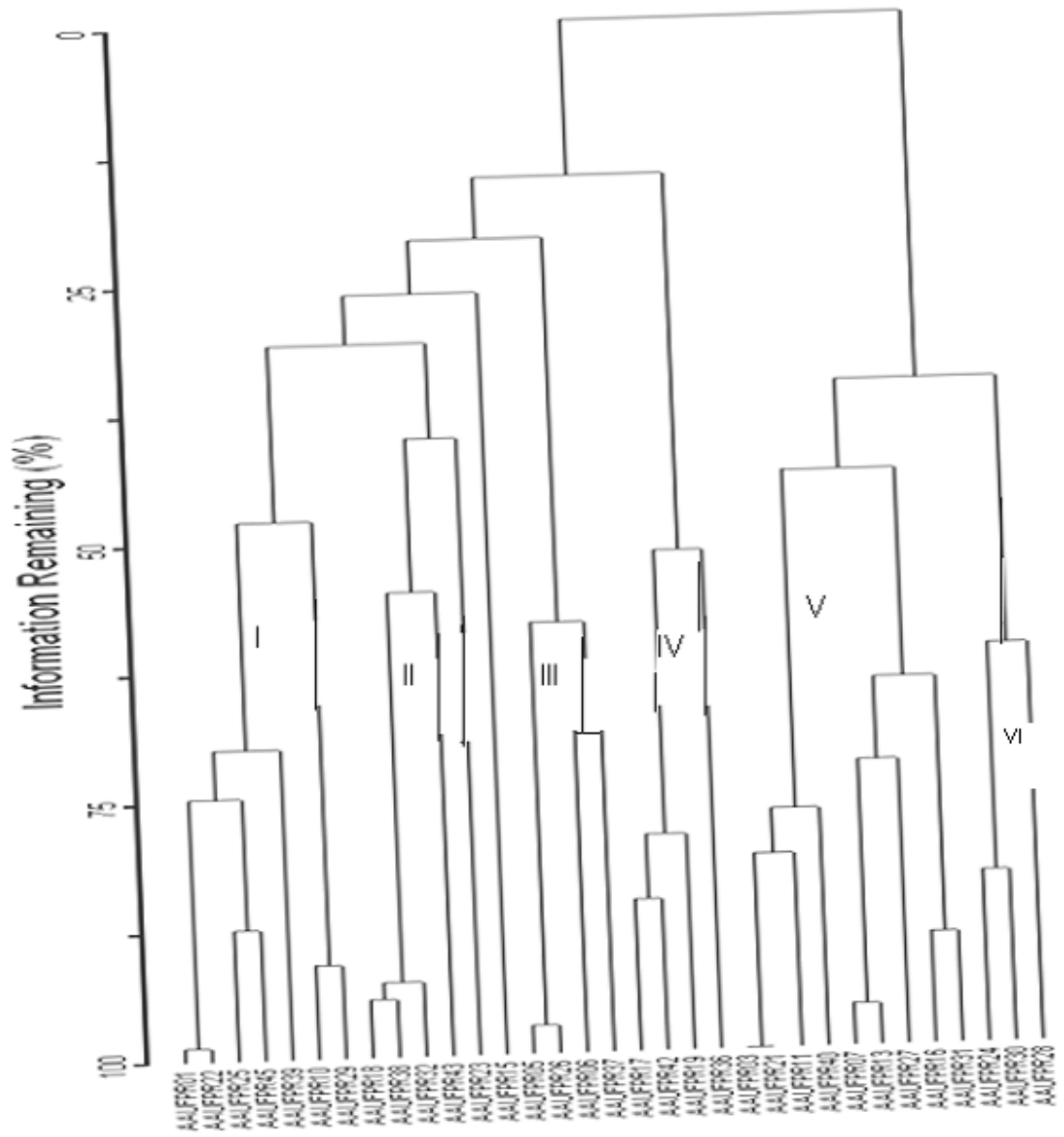


Figure-8-Dendrogram highlighting the phenotypic similarities among *pisum sativum* rhizobium (*Rhizobium leguminosarum biovar viciae*) from different areas of **southern** Tigray high lands (Ofla, Endamokoni and Alamata woreda)

Numerical analysis showing that the soil of the sampling sites harbor diverse field pea rhizobia. Group I had almost common physiological characteristics, these isolates had, highly pH-tolerant, moderate temperature tolerant, antibiotic sensitive, good carbohydrate utilization and moderate to utilize nitrogen sources. Isolates clustered under group II also had common physiological characteristics such as sensitive to high pH, salt concentration and temperature, but highly efficient in utilization of many carbohydrates and nitrogen sources, and moderately tolerant to antibiotics. Isolates clustered under group III had unique physiological characters such as moderately tolerant to antibiotics and pH, well utilizes of carbohydrates and amino acids, but highly sensitive to salt.

Isolates clustered under group IV showed similar phenotypic characteristics these characteristics were highly sensitive to alkaline, salt, and antibiotic and weakly assimilate to source of nitrogen and carbohydrates, but groups V and VI had almost similar characteristics and these characteristics were to opposite of group IV characteristics of group V and VI were highly utilized amino acids and carbohydrates, Highly tolerant to low pH, salt, temperature and antibiotics

The different clusters were also related whether there was any affinity with soil pH. Isolates clustered in group I have similar soil pH, isolates AAUFPR01 and 27 have 7.20 and 7.21 soil pH (0.1 difference) respectively. And isolates 25, and 45 have 7.35 and 7.36 soil pH (0.1 difference).

Isolates clustered under group II their site collection was very apart away from each other, and they did not show similarity in soil pH and site location, but isolates from group III, AAUFPR05 and 06 have almost similar soil pH (7.09 and 7.07). Isolates under group IV did not show in soil pH and site location, but isolates 13, 27 and 40 from group V showed similarity in soil pH. Isolates that were clustered under groups VI and the ungrouped ones did not show any relation with site collection and soil pH.

Table-4-clustered analysis of isolates based on geographic locations

Cluster	Isolates	Sample site		Original soil pH	Degree of co-relation
		Woreda	Kebele/Field		
I	AAUFPR01	Ofla	Tinbuyo	7.20	Isolate 01 and 22,25 &45Showed similar soil pH
	AAUFPR 10	Ofla	Maedokorem	7.43	
	AAUFPR22	Endamohoni	Hadish Addi	7.21	
	AAUFPR 25	Endamohoni	Libalib	7.35	
	AAUFPR 29	Ofla	Tinbuyo	7.31	
	AAUFPR 39	Alamata	Metakshim	7.16	
	AAUFPR 45	Alamata	Metakshim	7.36	
II	AAUFPR 18	Ofla	Hawla	7.29	No similarity in their soil pH.
	AAUFPR 32	Ofla	Tinbuyo	7.00	
	AAUFPR 38	Alamata	Garda	7.31	
	AAUFPR 43	Alamata	Metakshim	7.13	
III	AAUFPR 05	Ofla	Tsigea	7.09	Isolates05&06 almost Collected from similar soil pH
	AAUFPR 06	Ofla	Sibhale	7.07	
	AAUFPR 26	Endamohoni	Tsehafti	7.16	
	AAUFPR 37	Alamata	Garda	7.01	
IV	AAUFPR 17	Ofla	Hawla	7.27	Didn't show any relation in soil pH
	AAUFPR 19	Ofla	Hawla	7.14	
	AAUFPR 36	Alamata	Garda	6.86	
	AAUFPR 42	Ofla	Kidana	7.17	
V	AAUFPR 03	Ofla	Tinbuyo	6.93	Isolates 13,27&40 showed similar soil pH .
	AAUFPR 07	Ofla	Maedokorem	7.38	
	AAUFPR 11	Ofla	Maedokorem	7.16	
	AAUFPR 13	Ofla	Tsilia	7.20	
	AAUFPR 16	Ofla	Hawla	7.26	
	AAUFPR 21	Endamohoni	Hadish Addi	7.16	
	AAUFPR 27	Endamohoni	Tsehafti	7.21	
	AAUFPR31	Ofla	Tinbuyo	7.32	
AAUFPR 40	Alamata	Metakshim	7.20		
VI	AAUFPR 24	Endamohoni	Libalib	7.38	No relation in soil pH and site location
	AAUFPR 28	Endamohoni	Tsehafti	7.21	
	AAUFPR 30	Ofla	Tinbuyo	7.36	
Unclust ered	AAUFPR 15	Ofla	Tsilia	7.02	No site location and soil pH relation
	AAUFPR 23	Endamohoni	Hadish Addi	7.40	

5.5. Authentication and Symbiotic effectiveness (SE)

All isolates were authenticated as root nodule bacteria by forming nodules on the original field pea host plant (*Pisum sativum L.*) on sand culture under greenhouse conditions. The physical appearance of inoculated plants showed clear differences between the highly efficient and inefficient isolates). Most of the inoculated plants showed deeper green leaves, long and branched stems and pink nodules.

The inoculated plants also showed differences in nodule number; nodule dry weight and shoot dry weight (Table 5) The mean nodule number ranges from 25 ± 1 to 93 ± 6 . The highest nodule number of 93 was recorded from the isolate 29, followed by isolates 43,39,37 with nodule number of 88, 86 and 82 nodules/plant, respectively. The mean nodule dry weight of inoculated plants was 30-90mg/plant.

The mean shoot dry weight of the inoculated plants showed variations among the treatments. The highest shoot dry weight of 1.76 gm/plant was recorded from the plant inoculated with isolate 29 followed by plants treated with isolates 37, 01, 06 with shoot dry matter accumulation of 1.63gm/plant, 1.61gm/plant and 1.55gm/plant, respectively.

Most of the isolates were found to accumulate slightly high shoot dry matter. Based on the shoot dry matter values of inoculated plants in relation to the nitrogen fertilized control plants, the symbiotic effectiveness of each isolate was determined (Table 5). Accordingly, 36% of the isolates were found to be highly effective with shoot dry mass accumulation of 80-100% of the nitrogen fertilized control plant. These isolates were 1,5,7,11,17,18,25,26,30,38,42, and 43. Likewise, 30% of the isolates were effective with shoot dry matter accumulation of 50-80% of the nitrogen fertilized control plants. In general 67% of the isolates were effective and highly effective; where as 33% of them were lowly effective and ineffective nitrogen fixers with shoot dry matter accumulation less than 50%.

Table- 5-Symbiotic effectiveness of isolates (SE) collected from southern Tigray.

ISOLATES	Nodule number Mean ±S.E	Nodule dry wt. Mean mg/plant ±S.E	Shoot dry weight Mean gm/plant ±S.E	Symbiotic effectiveness %	Rate
AAUFPR01	35±4 ^{k-o}	30±0.6 ^{d-i}	1.61±0.9 ^{abc}	102	HE
AAUFPR03	28±2 ^{no}	30±0.7 ^{d-i}	0.52±0.17 ^{f-h}	33	I
AAUFPR05	61±4 ^{efg}	60±0.2 ^{a c-g}	1.07±0.11 ^{a-h}	68	E
AAUFPR06	68±0.3 ^{def}	20±0.3 ^{bhi}	1.55±0.16 ^{a-d}	98	HE
AAUFPR07	50±2 ^{g-j}	70±0.1 ^{a-d}	1.48±0.6 ^{b-e}	94	HE
AAUFPR10	38±1 ^{i-o}	20±0.6 ^{e-i}	0.59±0.36 ^{e-h}	16	I
AAUFPR11	49±0.3 ^{g-k}	30±0.5 ^{d-i}	1.38±0.9 ^{a-f}	88	HE
AAUFPR13	36±3 ^{j-o}	30±0.1 ^{d,f,i}	0.75±0.10 ^{bh}	47	LE
AAUFPR15	35±2 ^{k-o}	10±0.28 ^a	0.77±0.17 ^{b-h}	49	LE
AAUFPR16	52±2 ^{ghi}	90±0.1 ^{abc}	1.35±0.24 ^{a-g}	86	HE
AAUFPR17	44±2 ^{h-m}	50±0.4 ^{b-j}	1.32±0.28 ^{a-g}	84	HE
AAUFPR18	48±3 ^{g-l}	50±0.9 ^{b-i}	1.22±0.25 ^{a-g}	77	E
AAUFPR19	48±1 ^{g-l}	50±0.4 ^{b-i}	1.05±0.4 ^{a-h}	66	E
AAUFPR21	32±4 ^{mno}	30±0.4 ^{d-h}	0.5±0.5 ^{fgh}	31	I
AAUFPR22	37±2 ^{j-o}	24±0.24 ^{e-i}	0.78±0.3 ^{b-h}	50	LE
AAUFPR23	41±2 ⁱ⁻ⁿ	30±0.9 ^{d-i}	0.73±0.3 ^{c-h}	46	LE
AAUFPR24	61±1 ^{efg}	60±0.4 ^{a-e}	1.39±0.8 ^{a-f}	88	HE
AAUFPR25	46±0.3 ^{h-m}	30±0.4 ^{d-i}	1.32±0.7 ^{a-g}	83	HE
AAUFPR26	37±3 ^{j-o}	20±0.7 ^{f-i}	0.66±0.3 ^{d-h}	42	LE
AAUFPR27	37±2 ^{j-o}	30±0.5 ^{e-i}	0.96±0.12 ^{a-h}	60	E
AAUFPR28	34±1 ^{i-o}	32±0.5 ^{d-i}	1.03±0.7 ^{a-h}	65	E
AAUFPR29	93±6 ^a	60±0.1 ^{a-f}	1.76±0.19 ^a	112	HE
AAUFPR30	56±3 ^{fgh}	65±0.3 ⁱ	1.11±0.27 ^{a-h}	70	E
AAUFPR31	27±2 ^o	30±0.3 ^{d-i}	0.49±0.5 ^{a-h}	30	I
AAUFPR32	26±1 ^o	26±0.3 ^{d-i}	0.78±0.4 ^{b-h}	49	I
AAUFPR36	25±1 ^o	20±0.1 ^{g-i}	0.24±0.7 ^h	15	I
AAUFPR37	82±6 ^{abc}	90±0.2 ^{ab}	1.63±0.6 ^{ab}	103	HE
AAUFPR38	45±3 ^{h-m}	40±0.1 ^{c-i}	0.75±0.17 ^{b-h}	47	LE
AAUFPR39	86±2 ^{abc}	80±0.4 ^{abc}	1.15±0.4 ^{a-g}	73	E
AAUFPR40	88±2 ^{ab}	60±0.6 ^{b-h}	0.94±0.9 ^{a-h}	59	E
AAUFPR42	73±4 ^{cde}	80±0.7 ^{abc}	1.51±0.7 ^{a-d}	96	HE
AAUFPR43	77±1 ^{bcd}	50±0.3 ^{b-i}	1.5±0.6 ^{a-d}	95	HE
AAUFPR45	39±2 ^{i-o}	30±0.8 ^{d-i}	1.07±0.17 ^{a-h}	68	E
+Ve-control	-----	-----	1.57±0.33 ^{a-h}	-----	HE

HE – Highly effective, E-Effective, LE-Lowly effective, I-Ineffective, S.E-Standard error

*Means in columns followed by the same letters are not significantly different at p<0.05

(Tukey's HSD test) -SPSS 13.0 Version.

The symbiotic efficiencies of isolates were also compared among the sampling sites (Woredas) (Table-6). The data showed that most of the highly effective isolates (44%) were collected from Ofla woreda; whereas 29% and 25% of the highly effective isolates were from Alamata and Endamohoni Woredas, respectively. In general, Alamata, Ofla and Endamohoni Woreda soils harboured 72%, 66% and 63% of the effective and highly effective rhizobial isolates inoculating field pea.

Table-6- Symbiotic efficiency of isolate among the sampling Woredas.

Woreda	Ineffective	Lowly effective	Total	Effective	Highly effective	Total
Alamata	14%	14%	28%	43%	29%	72%
Ofla	17%	17%	34%	22%	44%	66%
Endamohoni	12%	25%	37%	38%	25%	63%
Total	14 %	19 %	33 %	34 %	33 %	67 %

Isolates which displayed good performance (Highly effective and effective) in symbiotic relation showed diverse phenotypic characters. According to their phenotypic characteristics 29% (AAUFPR07, 11, 16, 24, 28 and 40) of the isolates were tolerant to many eco-physiological factors. The remaining isolates (71%) did not show persistent pattern even though they showed good tolerance ability to some tests (Table-7).

Table-7-Selected isolate in relation to symbiotic effectiveness and Eco-physiological tests

Isolate	Site (Woreda)	Symbiotic efficiency (%)	pH tolerance	Salt tolerance at (%)	Temperature Tolerance	IAR (%)
AAUFPR 01	Ofla	102	5-9.5	No growth	25-35	17%
AAUFPR 05	Ofla	68	5-9.5	1	25-35	56%
AAUFPR06	Ofla	98	5-9	1	20-35	44%
AAUFPR07	Ofla	94	5-9	1-7	15-35	61%
AAUFPR 11	Ofla	88	5-9	1-6	20-35	44%
AAUFPR 16	Ofla	86	5-9	1-7	20-35	44%
AAUFPR17	Ofla	84	5-9	1	20-35	39%
AAUFPR18	Ofla	77	5-9.5	1	25-35	44%
AAUFPR 19	Ofla	66	5-9.5	1	15-35	22%
AAUFPR 24	Endamohoni	88	5-9	1-6	15-35	44%
AAUFPR 25	Endamohoni	83	5-9.5	1-3	15-35	17%
AAUFPR 27	Endamohoni	60	5-9,5	1-7	15-35	33%
AAUFPR28	Endamohoni	65	5-9	1-7	15-35	61%
AAUFPR29	Ofla	112	5-9	1	20-35	31%
AAUFPR30	Ofla	70	5-9	1-3	25-35	44%
AAUFPR37	Alamata	103	5-9	1	25-35	50%
AAUFPR 39	Alamata	73	5-9	1	25-35	39%
AAUFPR 40	Alamata	59	5-9	1-5	15-35	56%
AAUFPR42	Ofla	96	5-9	1-2	25-35	28%
AAUFPR 43	Alamata	95	5.5-8.5	1-2	25-35	50%
AAUFPR45	Alamata	68	5-9	1	20-35	22%

6. Discussion

In this study, a total of 33 root nodule bacteria were collected and characterized using different methods. All isolates did not absorb Congo-red from CRYEMA medium and failed to grow on peptone-glucose agar medium. All of the isolates were authenticated as root nodule on sand culture on the host plant (*Pisum sativum L.*).

Almost all of the isolates changed the BTB medium into yellow, indicating that they are acid producing bacteria (Jordan, 1984), and showed generation times of 2-4 hours with large colony diameter of 2-5 mm with production of exopolysaccharides characteristics of fast growing rhizobia. Other researchers from Ethiopia (Aregu Amsalu, 2007), and Bangladesh (Talukder *et al.*, 2008) showed the same pattern of colony and growth features from rhizobia isolated from field pea. However, some isolates (17%) from Ethiopia were found to be slow growers with generation times of 4-12 hours (Aregu Amsalu, 2007). These characteristics are common for root nodule bacteria that are categorized within cross-inoculation group known as *Rhizobium leguminosarum biovar viciae*.

Many isolates utilized many monosaccharides and disaccharides as sole source of carbon. Unlike the previous report of Aregu Amsalu (2007) on field pea rhizobia, and Assefa Keneni *et al.*, (2010) on the same inoculation group, faba bean, a few isolates in this study utilized starch and citrate (fig 3).

Almost 40% of the isolates utilized all the amino acids and many could metabolize Tryptophan, Glutamate, Lysine and Methionine as source of nitrogen. Shewakena Beyene (2009) reported similar results on *Rhizobium leguminosarum biovar viciae* of *Lens culinaris*. According to his report isolates utilized well to amino acids of methionine, tryptophan, glutamate and asparagines. No isolate was able to solubilize inorganic phosphate in this study unlike the work of Alikahani *et al.*, (2006) that reported *Rhizobium leguminosarum biovar viciae* from *Pisum sativum* produced large halo (clear) zone characteristics of phosphate solubilization.

All isolates were tolerant to pH 5.5 to 8.5, and several isolates were able to grow at pH 4.5 and 9.5, showing similar pattern of pH tolerance by field pea rhizobia isolated from Ethiopia (Aregu Amsalu, 2007). Furthermore, Negash Demissie, (2006) reported that the *Rhizobium leguminosarum biovar viciae* of *Pisum sativum* and *Vicia faba* from North western Ethiopia, grew at pH more than 4.75 and Assefa Keneni *et al.*, (2010) showed that faba bean rhizobia from Wollo, Northern Ethiopia, could not tolerate pH 4.

Isolates were also diverse in the tolerance of different salt concentrations. Twelve percent of the isolates (07, 16, 27 and 28) were found to grow on YEMA medium containing 7% NaCl. Others also were tolerant to higher concentration more than 1%. Different reports showed variations in tolerance to salt concentration of 0.5 to 7%. Aregu Amsalu, (2007) reported similar results on *Pisum sativum* according to his report isolates tolerate to 0.5-6% of NaCl, the isolates tolerate to 6% were 34%, out of this 6% isolates were from Tigtay. Furthermore, Alemayehu Workalemahu, (2009) reported that faba bean rhizobia were assessed on salt concentrations 0.5, 1, 1.5 and 2% and the isolates grew on these concentrations. On the contrary, Assefa Keneni *et al.*, (2010) reported much lower tolerance (0.5-2%) by isolated from Wollo.

All isolates were growing 25 to 35°C but none of the isolates grew at 40°C. This is similar to the work of Naeem *et al.*, (2008) who reported that *Pisum sativum* rhizobia from Pakistan grew between temperatures of 20 and 35°C. On the contrary, Zerihun Belay, (2006) reported that a few rhizobia from faba bean in Northern Gonder were able to grow at the temperature of 40°C.

The data on inherent antibiotic resistance of isolates showed that they were tolerant to penicillin, erythromycin and ampicillin. This pattern of resistance is similar to the report of Turco and Berdcek, (1987) which showed pea rhizobia from soils of eastern Washington grew well on the same type and concentration. Assefa Keneni *et al.*, (2010) also reported on penicillin resistant faba bean rhizobia, from Wollo, Northern Ethiopia. The antibiotic resistance pattern also showed that the isolates were sensitive to gentamycin and tetracycline. A report on intrinsic antibiotic resistance test pattern on faba bean rhizobia from Egypt soils also showed that gentamycin has the most

suppressive effect on *Rhizobium leguminosarum biovarviciae* (Hosney *et al.*, 2005). Aregu Amsalu, (2007) on field pea *Rhizobium leguminosarum biovarviciae* from different parts of Ethiopia also showed that the isolates tolerate to chloramphenicol and grew 90% and 88% at concentration of 5 and 10µg/ml respectively. Especially isolates collected from Tigray out of the seven isolates only five tolerate to 5 and 10 µg/ml of chloramphenicol.

The inoculated plants showed diversity in their physical appearances, nodule number, nodule dry weight, and shoot dry weight. Ali *et al.*,(2008) from Bangladesh reported that all varieties of field pea receiving *Rhizobium* inoculums produced higher nodule number/plant, nodule dry weight/plant and shoot dry weight /plant over un inoculated plants. In this study, there was strong coefficient correlation ($r=0.6$) amongst nodule number, nodule dry weight and shoot dry weight. Different reports showed that shoot dry weight and nodule dry weight are usually highly correlated, thus shoot dry weight is used routinely as an indicator of relative effectiveness (Somasagaran and Hoben, 1985; Solaiman and Rabbiani ,2004).

Generally 33% of the isolates were highly effective, 34% were effective, indicating that 67% of the isolates have the potential to be good candidates for future screening of inoculants. These effective inoculants were distributed in different Woredas; where 72%, 66%, and 63% of them were collected from Alamata, Ofla, and Endamohoni, respectively.

Isolate AAUFPR07 displayed unique characteristics in physiological tests from the above isolates .It was highly effective (94%), tolerant to Gentamycin antibiotic and generally highly antibiotic resistant (61%), highly salt stress resistant (7%), moderately pH tolerance (5-9), tolerate to moderate temperature (15-35^oc) and efficiently utilize many carbon and nitrogen sources. This isolate can be recommended for further investigation .

7. Conclusion and Recommendations

The present study shows the physiological and symbiotic diversity of *Rhizobium leguminosarum biovar Viciae* population on Field pea from Southern Tigray. Some of the isolates showed remarkable physiological characteristics such as resistance to antibiotics, high salt tolerance, high carbohydrate assimilation, optimum pH tolerance, and tolerate to optimum temperature. Most isolates were also effective in nitrogen fixation (67%)

After in field experiments, the isolates (AAUFPR05, 06, 07,11,16,18 and 30, 37, 39 and 43), and particularly AAUFPR 07 can be recommended as inoculants in the future. The above isolates showed good physiological, eco-physiological and symbiotic characteristics.

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9. Annexes

Annex-1 Soil sample site and Soil pH.

Soil sample	Woreda	Tabia	Kebele	Soil PH
01	Ofla	Wonberet	Tinbuyo	7.20
03	Ofla	Wonberet	Tinbuyo	6.93
05	Ofla	Hayalo	Tsigea	7.09
06	Ofla	Adigolo	Sibhale	7.02
07	Ofla	Adigolo	Maedo-Korem	7.38
10	Ofla	Adigolo	Maedo-Korem	7.43
11	Ofla	Adigolo	Maedo-Korem	7.16
13	Ofla	Wonberet	Tsilia	7.20
15	Ofla	Wonberet	Tsilia	7.02
16	Ofla	Wonberet	Hawla	7.26
17	Ofla	Wonberet	Hawla	7.27
18	Ofla	Wonberet	Hawla	7.29
19	Ofla	Wonberet	Hawla	7.14
21	Endamokhoni	Mekan	Hadish-Adi	7.16
22	Endamokhoni	Mekan	Hadish-Adi	7.21
23	Endamokhoni	Mekan	Hadish-Adi	7.40
24	Endamokhoni	H/T/Haimanot	Libalib	7.38
25	Endamokhoni	H/T/Haimanot	Libalib	7.35
26	Endamokhooni	Shimta	Tsehafti	7.16
27	Endamokhoni	Shimta	Tsehafti	7.21
28	Endamokhoni	Shimta	Tsehafti	7.21
29	Ofla	Wonberet	Tinbuyo	7.31
30	Ofla	Wonberet	Tinbuyo	7.36
31	Ofla	Wonberet	Tinbuyo	7.32
32	Ofla	Wonberet	Tinbuyo	7.00
36	Alamata	Awdikulu	Garda	6.86
37	Alamata	Awdikulu	Garda	7.01
38	Alamata	Awdikulu	Garda	7.31
39	Alamata	Awdikulu	Metakshim	7.16
40	Alamata	Awdikulu	Metakshim	7.20
42	Ofla	Kidana	Adi-Hasti	7.17
43	Alamata	Awdikulu	Metakshim	7.13
45	Alamata	Awdikulu	Metakshim	7.36

Annex -2-C carbohydrate utilization test

Isolates	D- mannitol	sucrose	galactose	mallose	lactose	glucose	sorbitol	starch	citrate	cellulose	Tartaric acid	Glycerol	Arabinose
AAUFPR01	+	+	+	+	+	+	+	-	-	-	+	+	-
AAUFPR03	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUFPR05	+	+	+	+	+	+	+	+	-	-	-	+	+
AAUFPR06	+	+	+	+	+	+	+	-	-	-	+	-	+
AAUFPR07	+	+	+	+	+	+	+	-	-	-	-	+	-
AAUFPR10	+	+	+	+	+	+	+	-	-	-	+	+	+
AAUFPR11	+	+	+	+	+	+	-	-	-	+	+	+	+
AAUFPR13	+	+	+	+	+	+	+	-	-	+	+	-	-
AAUFPR15	+	+	-	+	-	+	-	+	-	-	-	+	+
AAUFPR16	+	+	+	-	+	+	-	-	-	-	-	+	+
AAUFPR17	+	+	+	+	-	+	+	+	-	-	+	+	+
AAUFPR18	+	+	+	+	-	+	+	-	-	-	-	+	+
AAUFPR19	+	+	+	+	-	+	+	-	-	-	+	+	+
AAUFPR21	+	+	+	+	+	+	+	-	-	+	+	+	-
AAUFPR22	+	+	+	+	+	+	+	+	-	-	-	+	+
AAUFPR23	+	+	+	+	+	+	+	+	-	-	+	+	+
AAUFPR24	+	+	+	+	+	+	-	+	+	+	+	+	-
AAUFPR25	+	+	+	+	+	+	+	-	-	-	+	+	+
AAUFPR26	+	+	+	+	+	+	+	+	-	-	-	-	+
AAUFPR27	+	+	+	+	+	+	+	-	-	+	+	+	-
AAUFPR28	+	+	+	+	+	+	-	-	+	+	+	-	+
AAUFPR29	+	+	-	+	-	+	-	-	-	-	+	+	+
AAUFPR30	+	+	+	+	+	+	-	+	+	-	-	+	-
AAUFPR31	+	+	+	+	+	+	-	-	-	-	-	+	+
AAUFPR32	+	+	+	+	+	+	+	-	-	-	+	+	+
AAUFPR36	+	+	-	+	-	+	+	-	-	-	+	+	+
AAUFPR37	+	+	-	+	-	+	+	+	-	-	+	+	+
AAUFPR38	+	+	+	+	+	+	-	-	-	-	+	+	+
AAUFPR39	+	+	+	+	+	+	-	-	-	-	-	+	-
AAUFPR40	+	+	+	+	+	+	+	-	-	+	+	-	+
AAUFPR42	+	+	+	+	-	+	+	-	-	-	+	+	+
AAUFPR43	+	+	+	+	+	+	+	+	-	-	+	+	+
AAUFPR45	+	+	+	+	-	+	-	-	-	-	+	+	-
Total	33	33	29	32	27	33	22	11	4	8	23	27	24
%	100%	100%	88%	96%	81%	100%	66%	33%	12%	24%	69%	81%	72%

Annex-3-Aminoacid utilization test

Isolates	L-lysine	L-asparagines	L-arginine	L-Tyrosine	Glutamate	L-Tryptophan	Methionin
AAUFPR01	+	+	-	+	+	+	+
AAUFPR03	+	+	+	+	+	+	+
AAUFPR05	+	-	-	+	-	+	+
AAUFPR06	-	+	-	+	+	+	+
AAUFPR07	+	+	+	+	+	+	-
AAUFPR10	+	-	+	+	+	+	+
AAUFPR11	+	+	+	-	+	+	+
AAUFPR13	+	+	+	+	+	+	+
AAUFPR15	+	-	+	+	+	+	+
AAUFPR16	+	+	+	+	+	+	-
AAUFPR17	+	-	+	-	+	+	+
AAUFPR18	+	-	-	+	+	+	+
AAUFPR19	+	-	-	-	+	+	+
AAUFPR21	+	+	+	+	+	+	-
AAUFPR22	+	+	-	-	+	+	+
AAUFPR23	-	+	+	-	+	+	+
AAUFPR24	+	+	-	+	+	+	+
AAUFPR25	+	+	+	+	-	+	+
AAUFPR26	+	+	+	+	+	+	+
AAUFPR27	+	+	+	+	+	+	+
AAUFPR28	+	+	+	-	+	+	+
AAUFPR29	+	-	-	-	+	+	+
AAUFPR30	+	+	+	+	+	+	+
AAUFPR31	+	+	+	+	-	+	-
AAUFPR32	+	+	+	+	+	+	+
AAUFPR36	+	-	-	-	-	-	-
AAUFPR37	-	-	-	+	+	+	+
AAUFPR38	+	+	+	+	+	+	-
AAUFPR39	-	-	+	+	-	+	+
AAUFPR40	+	+	-	+	+	+	+
AAUFPR42	+	+	-	-	+	+	+
AAUFPR43	+	+	+	+	+	+	+
AAUFPR45	+	+	-	+	+	+	+
Total	28	23	20	24	29	32	28
%	85%	70%	61%	73%	88%	97%	85%

Annex-4- pH-Tolerance test

Isolates	Ph=4	Ph=4.5	Ph=5.0	Ph=5.5	Ph=8.5	Ph=9.0	Ph=9.5
AAUFPR01	-	-	+	+	+	+	+
AAUFPR03	-	-	+	+	+	+	+
AAUFPR05	-	-	+	+	+	+	+
AAUFPR06	-	-	+	+	+	+	-
AAUFPR07	-	-	+	+	+	+	-
AAUFPR10	-	-	+	+	+	+	+
AAUFPR11	-	-	+	+	+	+	-
AAUFPR13	-	+	+	+	+	+	-
AAUFPR15	-	-	+	+	+	+	-
AAUFPR16	-	-	+	+	+	+	-
AAUFPR17	-	-	+	+	+	+	-
AAUFPR18	-	-	+	+	+	+	+
AAUFPR19	-	-	+	+	+	+	+
AAUFPR21	-	-	+	+	+	+	+
AAUFPR22	-	-	+	+	+	+	-
AAUFPR23	-	-	+	+	+	+	+
AAUFPR24	-	-	+	+	+	+	-
AAUFPR25	-	-	+	+	+	+	+
AAUFPR26	-	-	+	+	+	+	+
AAUFPR27	-	-	+	+	+	+	+
AAUFPR28	-	-	+	+	+	+	-
AAUFPR29	-	-	+	+	+	+	-
AAUFPR30	-	-	+	+	+	+	-
AAUFPR31	-	-	+	+	+	+	-
AAUFPR32	-	-	+	+	+	+	+
AAUFPR36	-	-	+	+	+	-	-
AAUFPR37	-	-	+	+	+	+	-
AAUFPR38	-	-	+	+	+	+	-
AAUFPR39	-	-	+	+	+	+	+
AAUFPR40	-	-	+	+	+	+	-
AAUFPR42	-	-	+	+	+	+	-
AAUFPR43	-	-	-	+	+	-	-
AAUFPR45	-	-	+	+	+	+	-
Total growth	0	1	32	33	33	31	12
% growth	0%	3%	97%	100%	100%	94%	36%

For growth(+),for no growth(-)

Annex-5- Salt Tolerance test

isolates	Concentration of salt (NaCl)							Total
	1%	2%	3%	4%	5%	6%	7%	
AAUFPR01	-	-	-	-	-	-	-	0
AAUFPR03	+	+	+	+	+	-	-	5
AAUFPR05	+	-	-	-	-	-	-	1
AAUFPR06	+	-	-	-	-	-	-	1
AAUFPR07	+	+	+	+	+	+	+	7
AAUFPR10	+	+	-	-	-	-	-	2
AAUFPR11	+	+	+	+	+	+	-	6
AAUFPR13	+	+	+	+	+	+	-	6
AAUFPR15	+	+	+	+	+	+	-	6
AAUFPR16	+	+	+	+	+	+	+	7
AAUFPR17	+	-	-	-	-	-	-	1
AAUFPR18	+	-	-	-	-	-	-	2
AAUFPR19	+	-	-	-	-	-	-	1
AAUFPR21	+	+	+	+	+	-	-	5
AAUFPR22	-	-	-	-	-	-	-	0
AAUFPR23	+	+	-	-	-	-	-	2
AAUFPR24	+	+	+	+	+	+	-	6
AAUFPR25	+	+	+	-	-	-	-	3
AAUFPR26	+	-	-	-	-	-	-	1
AAUFPR27	+	+	+	+	+	+	+	7
AAUFPR28	+	+	+	+	+	+	+	7
AAUFPR29	+	-	-	-	-	-	-	1
AAUFPR30	+	+	+	-	-	-	-	3
AAUFPR31	+	+	+	+	+	+	+	7
AAUFPR32	-	-	-	-	-	-	-	0
AAUFPR36	+	+	-	-	-	-	-	2
AAUFPR37	+	-	-	-	-	-	-	1
AAUFPR38	+	+	+	+	+	+	-	6
AAUFPR39	+	-	-	-	-	-	-	1
AAUFPR40	+	+	+	+	+	+	-	6
AAUFPR42	+	+	-	-	-	-	-	2
AAUFPR43	+	+	-	-	-	-	-	2
AAUFPR45	+	-	-	-	-	-	-	1
Total growth	30	20	15	13	12	11	5	109
%growth	91%	61%	45%	39%	36%	33%	15%	

+ For growth, -for no growth

Annex-6- Temperature tolerance test

Isolates	4c ⁰	15c ⁰	20c ⁰	25c ⁰	30c ⁰	35c ⁰	40c ⁰
AAUFPR01	-	-	-	+	+	+	-
AAUFPR03	-	-	-	+	+	+	-
AAUFPR05	-	-	-	+	+	+	-
AAUFPR06	-	-	+	+	+	+	-
AAUFPR07	-	+	+	+	+	+	-
AAUFPR10	+	+	+	+	+	+	-
AAUFPR11	-	-	+	+	+	+	-
AAUFPR13	+	+	+	+	+	+	-
AAUFPR15	-	-	-	+	+	+	-
AAUFPR16	-	-	+	+	+	+	-
AAUFPR17	-	-	+	+	+	+	-
AAUFPR18	-	-	-	+	+	+	-
AAUFPR19	-	-	+	+	+	+	-
AAUFPR21	+	+	+	+	+	+	-
AAUFPR22	-	-	-	+	+	+	-
AAUFPR23	-	+	+	+	+	+	-
AAUFPR24	-	+	+	+	+	+	-
AAUFPR25	-	-	+	+	+	+	-
AAUFPR26	-	+	+	+	+	+	-
AAUFPR27	-	+	+	+	+	+	-
AAUFPR28	-	+	+	+	+	+	-
AAUFPR29	-	-	+	+	+	+	-
STPSR30	-	-	-	+	+	+	-
AAUFPR31	-	-	+	+	+	+	-
AAUFPR32	-	-	-	+	+	+	-
AAUFPR36	-	-	-	+	+	+	-
AAUFPR37	-	-	-	+	+	+	-
AAUFPR38	-	-	-	+	+	+	-
AAUFPR39	-	-	-	+	+	+	-
AAUFPR40	-	-	+	+	+	+	-
AAUFPR42	-	-	-	+	+	+	-
AAUFPR43	-	-	-	+	+	+	-
AAUFPR45	-	-	+	+	+	+	-
Totalgrowth	3	9	19	33	33	33	0
%growth	9 %	27 %	58 %	100 %	100 %	100 %	%

Annex-7-Antibiotic Tolerance test

isolates	Antibiotic concentration in µg/ml															Total	% of growth	
	Ampicillin			Tetracycline		Penicillin			Gentamicin		Chloramphenicol			Erythromycin				
	2.5µg/ml	5 µg/ml	10 µg/ml	2.5 µg/ml	5 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	2.5 µg/ml	5 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	2.5 µg/ml	5 µg/ml			10 µg/ml
AAUFPR01	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	3	17
AAUFPR3	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	7	39
AAUFPR05	+	+	+	-	-	+	+	+	-	-	+	-	-	+	+	+	10	56
AAUFPR06	+	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	8	44
AAUFPR07	+	+	+	-	-	+	+	+	+	-	+	-	-	+	+	+	11	61
AAUFPR10	+	+	-	-	-	+	+	-	-	-	+	+	-	+	+	-	8	44
AAUFPR11	+	-	-	-	-	+	+	-	-	-	+	+	-	+	+	+	8	44
AAUFPR13	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	+	8	44
AAUFPR15	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	7	39
AAUFPR16	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	+	8	44
AAUFPR17	+	-	-	+	-	+	-	-	-	-	+	-	-	+	+	-	7	39
AAUFPR18	+	+	-	-	-	+	+	+	-	-	+	-	-	+	+	-	8	44
AAUFPR19	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	4	22
AAUFPR21	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	+	8	44
AAUFPR22	+	+	-	-	-	+	+	+	-	-	+	-	-	+	+	-	8	44
AAUFPR23	-	-	-	-	-	+	+	+	-	-	+	-	-	+	-	-	5	28
AAUFPR24	+	+	-	-	-	+	-	-	-	-	+	+	+	+	+	-	8	44
AAUFPR25	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	3	17
AAUFPR26	+	+	-	-	-	+	+	+	-	-	+	+	-	+	+	+	10	56
AAUFPR27	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	+	6	33
AAUFPR28	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	-	11	61
AAUFPR29	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	7	39
AAUFPR30	+	+	+	-	-	+	+	-	-	-	+	-	-	+	+	-	8	44
AAUFPR31	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	10	56
AAUFPR32	+	+	+	-	-	+	+	-	-	-	+	-	-	+	+	-	8	44
AAUFPR36	+	-	-	-	-	+	+	+	-	-	+	-	-	+	+	-	7	39
AAUFPR37	+	+	+	-	-	+	+	-	-	-	+	-	-	+	+	+	9	50
AAUFPR38	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	7	39
AAUFPR39	+	-	-	-	-	+	+	+	-	-	+	-	-	+	+	-	7	39
AAUFPR40	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	10	61
AAUFPR42	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	5	28
AAUFPR43	+	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	9	50
AAUFPR45	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	4	22
Total	25	21	7	3	2	32	28	10	3	2	30	6	2	32	30	12	246	
%	76	64	21	9	6	97	85	30	9	6	91	81	6	97	91	36	91	

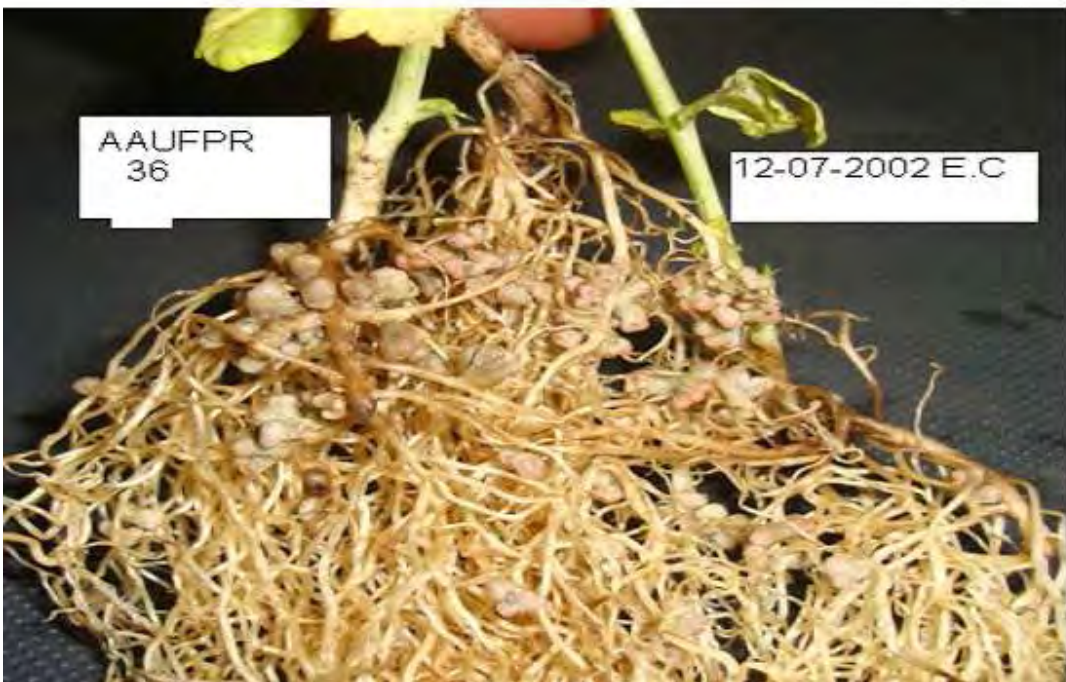
Annex-8-Symbiotic effectiveness of Field pea plants in green house





Annex-9- Nodules from Field pea plants





Declaration

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

Name: Fano Berhe

Place: Addis Ababa University

Signature-----

Date-----

This Thesis has been submitted for examination with our approval as

Advisor: Fassil Assefa (Ph.D)

Signature-----

Date-----