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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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â€œThrough him all things were made, without him nothing was made that has been

madeâ€. He is good God who made things for good. Praise God!

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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 5to 9, salt concentration, and at temperature of 15oc to 35oc. 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 07and 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

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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 NH4 and NO3. 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:-
T o Evaluate symbiotic effectiveness and phenotypic characterization of Rhizobium leguniosarum 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 worldâ€™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 16Sribosomal RNA(rRNA) gene rhizobia could be grouped into the Î±,Î² and Î³ subdivision of the Proteobacteria (Young and Haukka, 1996) Recently rhizobial systematic consisting of 13 genera.10-belonging to Î±- Proteobacteria: ,Allorhizobium, Bradyrhizobium, Azorhizobium, Devosia, Mesorhizobium, Methylobacterium, Ocrobacterium, Phyllobacterium, Rhizobium ,and Ensifer (Ochman et al., 2005). Burkholderia, Ralstonia, and Cupriavidus in the Î²-Proteobacteria (Willems, 2006).

The genus Rhizobium comprises a group of fast growers which includes 17â€“species , R. Cellulosilyticum, R. daejeonense , R. etli , R. galegae, R. gallicum , R. giardinii , R. hainanense, R. huautl ense , R. indigoferae, R. leguminosarum, R. loessense1 , 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 earthâ€™s atmosphere is nitrogen gas, it can not be utilized unless it is converted or fixed to utilizable forms such as NH4+ 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 175milliom 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 65x106, 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
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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).

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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-protien 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 N2 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 N2.

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

4 NADPH + 16 ATP 4 NADP+ +8e- + 4 H+ + 16 ADP + 16 Pi

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

N2 + 8e- + 8H+ 2NH3 + H2

Net-reaction

N2 + 4NADPH + 4H+ +16ATP 2NH3 + H2 + 4NADP+ + 16ADP + 16PiN2 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)
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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 (H2). 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 H2 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:
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 N2 substrate (Marie, 2001).
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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 breading of legume crops and micro-symbionts tolerant to environmental stresses is very important to improve yields of the affected areas (Giller, 2001).

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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 to4.9 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 series agricultural problem in acidic soils. Aluminum toxicity inhibits root growth and uptake of water and nutrients, results in decrease production (Kochian, 1995). Aluminum toxicity affects rhizobia by binding to DNA of both sensitive and tolerant strains (Jonsen 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 (Jonsen 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 2oC and for tropical species about 10oC (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 sever 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, leghmoglobin synthesis and nodule function is highly affected (Hungria and Vergas, 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 serious threat to agriculture affecting plant growth and crop yields (Zahran ,1999).Current estimate that 10-35% of worldâ€™s agricultural land is affected with very significant areas becoming unusable each year. 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 (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- 300mM NaCl, while nodulation and nitrogen fixation in their host can be inhibited at 50-100mM. Strains of R.leguminosarum biovar viciae have been reported to be tolerant to Nacl concentrations up to 350mM in growth cultures (Abdel wahab and Zahran1981).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 nitrongenase activity (Voisin et al., 2002) Calcium deficiency has specific effects on the legume symbiosis during the pre-infection and nodule development stages (Oâ€™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 (Oâ€™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).

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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
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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 â€˜â€™Fabaceae Pisum sativum Addiâ€™ 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 in1 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 28Â±2oc and periodically for colony formation.
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YEMA Composition
chemicals Amount (gm/l)
MgSO4.7H2O 0.2g
NaCl 0.2g

K2HPO4 0.5
KH2PO4 0.5g
Yeast extract 0.5g
D-manitol 10g
Agar 15g
Distil water 1000ml
Autoclaved at 121oC for 15 minutes, pH adjusted to 7, Taken from â€“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 28Â±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 at28Â±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 28Â±2 C0 observe CR absorption or not (Somasegaran and Hoben, 1994).

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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 (105cells/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 Âµ g/ml Bromocresol purple (BCP) dye.

Composition of, PGA medium
Glucoseâ€¦.5g
Peptoneâ€¦10g
Agarâ€¦.15g
BCP stock solution.....-10ml
PHâ€¦6.7
Distilled H2Oâ€¦..1liter
Autoclaved at 1210C for 15 min. Source: Somasegaran and Hoben (1994)

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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 28Â±2 oc; 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 25co (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 average25/18co 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 70co for 48hrs until constant weight.

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

No	Chemicals	g/l
1	K2HPO4	0.023
2	KH2PO4	0.13
3	CaSO4.2H2O	3.425
4	MgSO4.7H2O	0.274
5	NaCl	0.04
6	FeCl3.6H2O	0.06

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

Solution-2

No	Chemicals	g/l
1	CUSO4.5H2O	0.15
2	ZnSO4.7H2O	0.44
3	MnSO4.7H2O	0.04
4	H3BO3 (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-Ultraviolat-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),

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The formula; $g = \log 2 (t) / \log X - \log Xo$,

Where: g =generation time
t = time elapsed
XO =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.

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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 0.2Âµm Whatman filter paper and added to the autoclave sterilized medium. Isolates were grown in 10ml YEM broth for 48 hours at 28Â±2oc 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)
MgSO4.7H2O	0.2g

NaCl 0.2g
K2HPO4 0.5
KH2PO4 0.5g
Yeast extract 0.05g
Agar 15g
Distil water 1000ml
Autoclaved at 121oC for 15 minutes, pH adjusted to 7,Taken from â€“Lupwayi and Haque .(1994)

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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)
MgSO4.7H2O 0.2g
NaCl 0.2g
K2HPO4 0.5
KH2PO4 0.5g
Yeast extract 0.05g
D-manitol 1g
Agar 15g
Distil water 1000ml
Autoclaved at 121oC for 15 minutes,pH adjusted to 7,Taken from â€“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

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4.9 .Eco-physiological characteristics
4.9.1. Acid and alkaline (pH) tolerance
The pH tolerance test has be done on TYEA-medium according to Bernal and Grham,(2001).and inoculate using inoculation loop containing about 105cells/ml and incubated at28Â±2 c0.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
Yeastextract----------3g/l
CaCl2.2H2o----------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 40oc (Lupiwayi and Haque,1994).Growth qualitatively recorded as (+) for growth and (-) for no growth .

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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 Âµg/ml) was added aseptically in definite amount to sterile YEMA cooled to 50 0c 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)

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

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) .

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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 (Tukeyâ€™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 â€“glucose agar medium and failed to absorb Congo-red on Yeast Extract Agar medium .

Almoost 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).

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Table-1- Growth, Colony morphology and Cultural characteristic

Isolates	Sample site	Colony characteristics	Colony size(mm)	YEMA BTB 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).
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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

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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

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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

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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, AAUFPR22and AAUFPR32 did not grew at any salt concentrations (1-7%).

Figure-6- Salt Tolerance test of the isolates

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5.3. 3.Temperature tolerance

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

Figure-7- Temperature Tolerance test of the isolates

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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, chlor amphenicol 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 Âµg/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
% of resistant isolates to antibiotics

Antibiotics	2.5 Âµg/ml	5 Âµg/ml	10 Âµg/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

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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 solates into different diversity groups (fig.8).

The 33 isolates were grouped in to 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 (3,7,11,13,16,21,27,31and40) 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 and43.Group-3-included isolates, 5,6,26 and37. Group 4 included isolates 17, 19, 36, and 42, Likewise, Group 6 included isolates 24, 28 and 30.

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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)

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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 (0.1difference).

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, AAUFPR05and 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 isolates13,27 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.

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

		Sample site			
Cluster	Isolates	Woreda	Kebele/Field	Original soil pH	Degree of co-relation
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 Didnâ€™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

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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 25Â±1 to 93Â±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 82nodules/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 Â±S.E	Nodule dry wt. Mean mg/plant Â±S.E	Shoot dry weight Mean gm/plant Â±S.E	Symbiotic effectiveness %	Rate
AAUFPR01	35Â±4 k-0	30Â± 0.6d-i	1.61Â± 0.9abc	102	HE
AAUFPR03	28Â±2no	30Â±0.7d-i	0.52Â±0.17 f-h	33	I
AAUFPR05	61Â±4efg	60 Â±0.2a c-g	1.07Â± 0.11a-h	68	E
AAUFPR06	68Â±0.3def	20 Â±0.3bhi	1.55Â±0.16 a-d	98	HE
AAUFPR07	50Â±2 g-j	70Â±0 .1 a-d	1.48 Â±0.6 b-e	94	HE
AAUFPR10	38Â± 1 i-o	20Â±0.6e-i	0.59 Â±0.36 e-h	16	I
AAUFPR11	49Â± 0.3 g-k	30Â±0.6d-i	1.38 Â±0.9 a-f	88	HE
AAUFPR13	36.Â± 3 j-o	30Â±0.1d,f-i	0.75Â±0.10bh	47	LE
AAUFPR15	35Â± 2 k-o	10Â±0.28 a	0.77 Â±0.17 b-h	49	LE
AAUFPR16	52Â± 2ghi	90 Â±0.1abc	1.35Â± 0.24 a-g	86	HE
AAUFPR17	44 Â±2 h-m	50 Â±0.4 b-j	1.32Â±0.28 a-g	84	HE
AAUFPR18	48 Â±3 g-l	50Â±0.9b-i	1.22Â± 0.25 a-g	77	E
AAUFPR19	48Â± 1g-l	50Â± 0.4b-i	1.05Â±0.4 a-h	66	E
AAUFPR21	32 Â±4mno	30Â±0.4 d-h	0.5 Â±0.5fgh	31	I
AAUFPR22	37Â±2 j-o	24Â± 0.24e-i	0.78Â±0.3 b-h	50	LE
AAUFPR23	41Â± 2 i-n	30Â± 0.9d-i	0.73 Â±0.3c-h	46	LE
AAUFPR24	61Â±1efg	60Â±0.4 a-e	1.39Â±0.8 a-f	88	HE
AAUFPR25	46 Â±0.3 h-m	30Â± 0.4d-i	1.32Â±0.7 a-g	83	HE
AAUFPR26	37 Â±3 j-o	20 Â±0.7f-i	0.66Â±0.3 d-h	42	LE
AAUFPR27	37 Â±2 j-o	30Â±0.5e-i	0.96Â± 0.12 a-h	60	E

AAUFPR28 34Â± 1 l-o 32Â±0.5d-i 1.03Â±0.7 a-h 65 E
AAUFPR29 93Â±6 a 60Â± 0.1a-f 1.76Â± 0.19 a 112 HE
AAUFPR30 56 Â±3fgh 65Â±0.3i 1.11Â±0.27 a-h 70 E
AAUFPR31 27Â± 2 o 30Â± 0.3d-i 0.49 Â±0.5 a-h 30 I
AAUFPR32 26 Â±1o 26Â±0.3d-i 0.78Â±0.4 b-h 49 I
AAUFPR36 25 Â±1 o 20 Â±0.1g-i 0.24 Â±0.7 h 15 I
AAUFPR37 82Â±6abc 90Â±0.2ab 1.63 Â±0.6ab 103 HE
AAUFPR38 45Â±3 h-m 40Â± 0.1c-i 0.75Â± 0.17 b-h 47 LE
AAUFPR39 86Â± 2abc 80Â±0.4abc 1.15Â±0.4 a-g 73 E
AAUFPR40 88Â± 2ab 60 Â±0.6 b-h 0.94Â±0.9 a-h 59 E
AAUFPR42 73 Â±4cde 80Â±0.7abc 1.51Â±0.7 a-d 96 HE
AAUFPR43 77 Â±1bcd 50 Â±0.3b-i 1.5 Â±0.6 a-d 95 HE
AAUFPR45 39Â±2 i-o 30Â±0.8d-i 1.07Â±0.17 a-h 68 E
+Ve-control---------- ---------- 1.57 Â±0.33a-h --------- HE
HE â€“ 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 $p<0.05$ (Tukeyâ€™s HSD test) -SPSS 13.0 Version.
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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, 11,1 6, 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).

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

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 where 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 2-4hours with large colony diameter of 2-5mm 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 4-12hours (Aregu Amsalu, 2007).These characteristics are common for root nodule bacteria that are categorized within cross-inoculation group known as Rhizobium leguminosarum biovarviciae

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 leguminosaarum biovarviciae of Lens culinaris . According to his report isolates utilized well to aminoacids of methionine,tryptophan ,glutamate and asparagines. No isolate was able to solubilze 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 Vcia 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 and28) 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 fromTigtay. 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 35oc but none of the isolates grew at 40oc. This is similar to the work of Naeem et al., (2008) who reported that Pisum sativum rhizobia from Pakstan grew between temperatures of 20 and 35oc. 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 40oc.

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 Kenenie 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 10Âµg/ml respectively. Especially isolates collected from Tigray out of the seven isolates only five tolerate to 5 and 10 Âµg/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 (15-35oc) 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

71

Annex -2-C carbohydrate utilization test
Isolates D-ma
nit
ol
suc
ros
e
ga
lac
tos
e ma
lto
se
lac
tos
e
glu
co
es
sor
bit
ol
sta
rch

cit
rat
e
cel
lul
os
e Ta
rta
rat
e Gl
yc
ero
l Ar
ab
ino
se

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
%
10
0%

88
%
96
%
81
%
10
0%

66
%
33
%
12
%

24
%
69
%
81
%
72
%

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%

73

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(-)

74

Annex-5- Salt Tolerance test

	Concentration of salt (NaCl)						
isolates	1%	2%	3%	4%	5%	6%	7% Total

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

75

Annex-6- Temperature tolerance test
Isolates 4c0 15c0 20c0 25c0 30c0 35c0 40c0
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 % %

76

Annex-7-AntibioticTolerance test

Antibiotic concentration in Âµg/ml
Ampicillin Tetracycli
ne
Penicillin Gentamicin Chloramphenicol Erythromycin
isolates
2.5
Âµg
/m
l

5 Âµ
g/m
l

10
Âµg
/m
l

2.5
Âµg
/m
l
5 Âµ
g/m
l

2.5
 Âµg
/m
l

5 Âµ
g/m
l

10
 Âµg
/m
l

2.5
 Âµg
/m
l
5 Âµ
g/m
l

2.5
 Âµg
/m
l
5 Âµ
g/m
l

10
 Âµg
/m
l
2.5
 Âµg
/m
l

5 Âµ
g/m
l

10
 Âµg
/m
l

To
tal

%
 of

gr
ow
th

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

77

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

78

79

Annex-9- Nodules from Field pea plants

80

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Declaration

I the under signed declare that this Thesis is my original work. It has never been submitted in any institution and 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----------