ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY

Phenotypic characteristics and Symbiotic effectiveness of field pea (*Pisum Sativum* L.) *Rhizobia* from Some Central part of Ethiopia under greenhouse condition

By

Dinkinesh Miressa

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

Advisor

Dr. Fassil Assefa (PhD)

June, 2018

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Phenotypic characteristics and Symbiotic effectiveness of field pea (*Pisum Sativum* L.) Rhizobia from Some Central part of Ethiopia under greenhouse condition

By

Dinkinesh Miressa

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

Approved by examining Board:

Advisor	Signiture	Date
Chair Person	Signiture	Date
Internal Examinor	Signiture	Date
External Examinor	Signiture	Date

Declaration

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

Name: Dinkinesh Miressa

Signature__________________________

Date______________________________

Acknowledgement

I would like to express my heart-felt thanks and gratitude to my adviser Dr. Fassil Assefa, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, for his provision of essential references, valuable comments, constructive advice, patience, encouragement and inspiring guidance starting from the beginning of the work to its end. I would also thank him for his kindness, sociability, and friendly and flexible approach to all of his students.

I am thankful to Addis Ababa University, Department of Microbial, Cellular and Molecular Biology in accepting and educating me with provision of all necessary materials and facilities throughout the study.

I am grateful to National Soil Testing Center especially Ato Fikire Mekuria, for permitting me to use laboratory facilities, greenhouse and giving me all necessary supports during the study periods.

I am highly indebted to laboratory assistants and all workers of National Soil Testing Center in Microbiology Stream in assisting me during laboratory and Greenhouse work. I also thank Ehitemariam at NSTC who support me in mapping the study sites from the GPS reading.

I am also highly grateful to Holota Agricultural Research Center especially Dr. Gemechu Keneni for his cooperation for provision of seeds and farmers of Central Showa High Lands for their support and permission to collect nodule samples from their field pea seedlings.

Finally, my heartfelt thanks and love go to my family for their support, encouragement, patience and understanding during the period of this study. Without your prayers, love and support, this study would surely not have been possible.

Table of content

List of Tables page

List of Figures page

List of Annexes Page

List of Abbreviation

BCP	Bromo cresol purple
BNF	Biological Nitrogen Fixation
BTB	Bromothymole blue
CR	Congo red
IAR	Intrinsic antibiotic resistance
NSHFR	North Showa Fitche Route
NSHZDBR	North Showa Zone Debre Birhan Route
OD	Optical Density
OSZ	Oromiya Special Zone
PGA	Peptone-glucose agar
RCBD	Randomized Completed Block Design
SE	Symbiotic effectiveness
SNF	Symbiotic nitrogen fixation
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Nitrogen is one of the most abundant elements, and biological nitrogen fixation (BNF) is the main source of nitrogen for legume plants. Field pea is one of the most important legume plants which is grown in Central Showa High Lands. This study was initiated with the objectives of isolating and characterizing root nodule bacteria from three routes of Central Showa, and evaluating their symbiotic effectiveness on field pea under green house conditions in order to boost crop productivity. Root nodules were collected from young and healthy seedlings of *Pisum sativum* L. from farmer's fields at different locations (Debre birhan, Fitche and Ambo) of Central Showa. Twenty seven *Rhizobial* isolates were retrieved from the root nodules of *Pisum sativum* and characterized by standard biochemical tests. Average generation time was between 2.0 to 4hrs. All the isolates were gram-negative and did not absorb red color when cultured on Yeast Extract Manitol Agar containing Congo red. All the strains utilized glucose, D-mannitol, and sucrose. Only 17% *Rhizobium* isolates tolerated 6% NaCl. The isolates were tentatively identified as *Rhizobium leguminosarum*. All the isolates formed watery and mucoid colonies on YEMA medium, and failed to grow on peptone glucose agar medium and to solubilize inorganic phosphate. Almost all the isolates were tolerant to pH 5 to 9, salt concentration (1-2%), and temperature of 25^0C to 35^0C. The isolates were also tolerant to ampicillin, penicillin, erythromycin and chloramphenicol, but sensitive to gentamycin and tetracycline. The isolates were diverse in amino acid utilization in which about 22% of the isolates were utilized all the amino acids used and also they showed variation in heavy metal resistance in which most of the isolates resisted Al and Mn. About 34% of the isolates were highly effective and 48% effective whereas 7% were lowly effective and 11% ineffective. AAURFP35, AAURFP36, AAURFP37, AAURFP38 and AAURFP39 were the isolates that showed good physiological, eco-physiological and symbiotic characteristics and need further testing under different environmental conditions in the field to be used as effective bio inoculant in field pea cultivation.

Keywords: Field pea, Growth factors, Inoculant, Nitrogen fixation, Rhizobium

1. Introduction

Legumes are important crops in agricultural systems for they are important source of protein and improve soil fertility by fixing atmospheric nitrogen in a symbiotic association with root nodule bacteria known as rhizobia. Some of the important food and forage legumes are field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentils (*Lens culinaris*), chickpea (*Cicer arietinum),* grass pea (*Lathyrus sativum*), soybean (*Glycine max*) and common bean (*Phaseolus vulgaris),* etc. These crops are effective in biological nitrogen fixation with the capacity of converting nitrogen to the tune of 200-300 kg/ha/yr (Peoples, *et al*., 1995).

Pulse crops are grain legumes that are important sources of dietary protein for many people in developing countries. These legumes are cultivated in temperate, Mediterranean regions, and at high altitudes in sub-tropical and tropical countries (Aregu *et al*., 2012).

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

The nitrogen that the legume crop absorbs from the air is used for its own growth and is stored in the root nodules. When the crop is harvested the roots are left in the ground, where they decompose, releasing the nitrogen into the soil. This nitrogen can then be used by the next crop that is planted in the same field (Rienke and Joke, 2005).

Legumes take up nitrogen from the air and pass it into the soil, thereby improving soil fertility. The yields of crops grown on the same land after the legume crop will increase. In addition legumes are nutritious, and may provide income opportunities (Rienke and Joke, 2005).

Pea is a grain legume of world economic importance for feeds and human consumption. It relies on both soil mineral N absorption and symbiotic nitrogen fixation (SNF) (Gisele *et al*., 2007).

Pea nitrogen nutrition relies in part on mineral nitrogen absorption by roots and mostly on symbiotic fixation of atmospheric nitrogen occurring in nodules. Nitrogen-fixing activity varies during the pea growth cycle, increasing during the vegetative phase up to flowering (Viginie *et al*., 2007).

Field pea (*Pisum sativum*) is the second most important pulse crop in Ethiopia after faba bean in terms of both area coverage and production. It is widely cultivated in the different regions of the country at altitudes between 1,800 and 3,000 meter above sea level with annual average rainfall of 700-900 mm (Fano, 2010). It is a cash crop and a good source of protein and energy in the diet of the majority of the population (Aregu *et al*., 2012).

Field pea production in Ethiopia is generally low and mainly produced for subsistence due to low soil fertility and inefficient traditional agronomic practices. For many years, many researchers have undertaken nation-wide studies to improve field pea cultivars (Mussa, *et al*., 2006), and insect pest management (Kemal, 2006). However, many of the studies were not adequate with quantification of biological nitrogen fixation, and limited to soil plant interaction and fertilizer trials of these legumes in different agricultural research institutes (Aregu *et al*., 2012).

The present study, therefore, was designed with the objective of isolating and characterizing root nodule bacteria from several Woredas of Central Showa, 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:

The general objective of this study was to evaluate symbiotic effectiveness and phenotypic characterization of *rhizobia* nodulating field pea from Central Showa, Ethiopia.

The specific objectives of the present study were to:

- To isolate and characterize *Rhizobia* nodulating field pea *(Pisum sativum)* based on different phenotypic characters and biochemical tests
- To screen out efficient isolates under greenhouse conditions based on their symbiotic effectiveness

3. Literature review

3.1. The Legumes

Legumes are members of the bean family, Fabaceae, which includes all types of beans and peas as well as soybeans, peanuts, alfalfa, and clover. This large, widely distributed family also includes various trees and ornamentals such as black locust, wisteria, lupine, and the Texas bluebonnet. Most members of this dicot family share a very similar flower and fruit structure (Levetin, 2008).

Legumes are very important both ecologically and agriculturally because they are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms such as ammonia, nitrate, and organic nitrogen (Zahran, 1999).

The seeds of many legumes are an important food staple worldwide because they are rich in both oil and protein. They are higher in protein than any other food plant and are close to animal meat in quality. In fact, they are often called “poor man’s meat” because they are an inexpensive source of high-quality protein. The high protein content of legumes is correlated with the presence of root nodules, which contain nitrogen-fixing bacteria**.** These bacteria, which are species of the genus *Rhizobium*, are able to convert free atmospheric nitrogen into a form that can be used by plants in the making of protein and other nitrogen-containing compounds (Levetin, 2008).

Plants that belong to the legume family (*Leguminosae*) have pods in which beans grow. Legumes possess an important characteristic, which is their ability to absorb nitrogen from the air. Many crops that are unable to do this are dependent on the nitrogen that is present in the soil. Most soils in tropical areas do not contain sufficient nitrogen, an important nutrient. For this reason growing legumes (in addition to other staple crops such as potatoes, maize and rice) is a good way for farmers in tropical areas to enrich the soil. The legume crops also provide extra food for the daily diet of both humans and animals (Rienke and Joke, 2005).

Because of the presence of nitrogen-fixing bacteria, the cultivation of legumes enriches the soil. For this reason farmers often rotate legumes with crops that deplete soil nitrogen (Levetin, 2008). Grain legumes are a cost-effective option for improving the diets of low-income consumers who cannot easily afford meat, dairy products and fish. Grain legumes also generate substantial benefits to the well-being of smallholder farm families. Often a crop cultivated by women, harvests are consumed at home and sold to generate family income (ICRISAT *et al*., 2012).

Legumes generally do not require N fertilizer because of their symbiotic relationship with *Rhizobium* bacteria. In this relationship, symbiotic N-fixing bacteria invade root hairs of host plants, where they multiply and stimulate formation of root nodules (enlargements of plant cells and bacteria in intimate association). Legumes and bacteria work together to extract atmospheric N (air is 78 percent $N_{2,}$ but unavailable to plants) and convert it to plant-available forms within legume roots. Bacteria inside nodules convert free N_2 to ammonia (NH_3), which the host plant utilizes to make amino acids and proteins (http://osufacts.okstate.edu).

Legume crops provide dried beans for human consumption and are grown all over the world. Some beans are a good source of oil (groundnuts and soya beans), others are good for cooking, either as whole beans or pulses or as split beans or peas. Some beans are ground into flour, which is used to prepare a number of foods. After the beans have been harvested the crop remains make a good source of animal feed. They can also be dug into the soil so that they improve the fertility of the soil. Some legume crops can be grown in combination with a grain crop, which helps to increase yields and soil fertility. Cowpeas are often grown together with millet or maize (Rienke and Joke, 2005).

In addition, grain legumes provide on-farm agronomic benefits. By complementing cereals, roots and tubers in farming systems of smallholder farmers, legumes can help intensify and diversify systems. Legumes intensify cropping systems by utilizing under-exploited production niches, serving as rotation-, double- and inter-crops. Increasing cropping intensity, the ability of legumes to fix nitrogen, and improve soil health can enhance overall farm productivity and smallholder incomes. Grain legumes also diversify farming systems, making them more nutrient-efficient, resilient and sustainable. Their fast growth not only improves soil-protective land cover, but also helps break pest, disease and weed cycles in cereal cropping systems. Furthermore, diversifying farm activities with legumes reduces risks of catastrophic farm-wide harvest losses, thereby increasing farm resilience to climate change. In summary, Grain Legumes focuses on the poorest sectors of society in order to generate a range of economic, social and environmental benefits (ICRISAT *et al*., 2012).

3.2. Field Pea (*Pisum sativum*)

The term pea, like bean, denotes dozens of different kinds of edible seeds that have been cultivated for millennia. The most familiar include green peas, split peas, black-eyed peas, lentils, chick-peas (garbanzos), and snow peas. Nutritionally, peas are also a good source of protein (21 %) are grown during the cooler seasons of the year in temperate zones. Biologically, the most famous pea is the garden pea (field pea) *Pisum sativum*, which Gregor Mendel used for his famous genetics experiments (Levetin, 2008).

Pea (*Pisum sativum L.*) of the family Papilionoideae is an important pulse legume and nutritive cool season vegetable crop. This crop is nodulated by *Rhizobium leguminosaurum* bv. *viciae* at the expense of C supplied by the host plant (Abdul kabirkhan, 2007).

3.3. The Rhizobia and their biology, physiology and taxonomy

Rhizobia or root nodule bacteria are medium-sized, rod-shaped cells, 0.5-0.9 µm in width and 1.2-3.0 µm in length. They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two to six peritrichous flagella. Rhizobia are predominantly aerobic chemo organotrophs and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been found to fix N in the free-living form except under special conditions (Somasegaran and Hoben, 1994).

Some strains of rhizobia require vitamins for growth. Optimal growth of most strains occurs at a temperature range of 25-30^0C and a pH of 6.0-7.0. Despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at O_2 tensions of less than 0.01 atm. Generally, most rhizobia Produce white colonies, only weakly absorb congo red (diphenyldiazo-bis-a-naphthylaminesulfonate) dye, which is included in culture media for isolating rhizobia. However, if the culture medium is not buffered, acid-producing rhizobia cause the dye to turn purple. Other interesting and useful characteristics of rhizobia are their growth reactions in the standard YM medium containing bromthymol blue as the pH indicator. Fast-growing rhizobia produce an acid reaction in the YM medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction (Somasegaran and Hoben, 1994).

Rhizobia are soil bacteria that fix nitrogen (diazotrophs) after becoming established inside root nodules of legumes (Fabaceae). In order to express genes for nitrogen fixation, rhizobia require a plant host; they cannot independently fix nitrogen. In general, they are Gram-negative, motile, non-sporulating rods (Oelke *et al*., 1991).

Rhizobium is a genus of soil bacteria whose members are best known for their ability to establish symbiotic relationships with legumes of agricultural and environmental importance, in a process of biological nitrogen fixation (BNF). The inoculation of cultivated leguminous plants with selected rhizobial strains is recommended in order to maximize the contribution of BNF to the nitrogen status of the host plant. In the legume production, the application of high quality rhizobial inoculants substantially contributes to the N cost efficiency of farming systems through inputs from biological N fixation. (Mihaela *et al*., 2007).

Rhizobia are bacteria that induce the root hairs of the plant to form nodules in which nitrogen is stored. Rhizobia are found in most soils, but they do not always form nodules. Sometimes there are not enough bacteria in the soil to form nodules, or they might not be the right type of rhizobium for the plants. Just as there are different sorts of legumes there are also different sorts of rhizobia. For nitrogen fixation to take place, the correct combination of rhizobium and legume is needed (Rienke and Joke, 2005).

Rhizobia are a paraphyletic group that fall into two classes of the proteobacteria—the alpha- and beta-proteobacteria. Most belong to the order Rhizobiales, but several rhizobia occur in distinct bacterial orders of the proteobacteria (Oelke *et al*., 1991).

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 α, β and gamma 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. huautl ense , 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 earth. However, it is one of the most limiting factors of growth and production of crops. Nitrogen can be utilized when it is reduced to ammonia by nitrogen fixation. It can be reduced by chemical fixation through industrial production and/or biological fixation involving microorganisms. Even in the presence of such process called biological nitrogen fixation, nitrogen is one of the usually deficient plant nutrients in soils. Despite its abundance in the atmosphere as a gas, it cannot be utilized directly by plants. Most plants utilize nitrogen in its ionic forms ammonium ($NH4^{+}$) and nitrate ($NO3^{-)}$ from soil. (Tamiru *et al*., 2012)

Legumes have a special characteristic, that they can absorb nitrogen from the air and use it for their own growth. They store the nitrogen in nodules on their roots, with the help of special bacteria (Rhizobia). As the root nodules grow they start to produce nitrogen. The root provides the rhizobium bacteria with food and shelter and in return the bacteria help the plant to store nitrogen (Rienke and Joke, 2005).

The increasing cost of fertilizers and their impact on the environment have forced people to look for other possible sources of plant nutrients. In this regard, nitrogen fixation which is a process by which elemental atmospheric nitrogen is changed to organic forms by biological nitrogen fixation both by symbiotic and asymbiotic microorganisms in soil has drawn much attention (Tamiru *et al*., 2012). The amount of nitrogen that a plant can fix depends on the variety, the productivity of the rhizobium bacteria, the soil and the climate (Rienke and Joke, 2005).

3.5. Biological nitrogen fixation (BNF)

Biological nitrogen fixation (BNF) is the process whereby atmospheric nitrogen (N=N) is reduced to ammonia in the presence of nitrogenase (Oelke *et al*., 1991). It is a natural process through which several species of bacteria convert atmospheric nitrogen into plant available nitrogen, usually ammonium (NH_4) (Nape Victoria Mothapo, 2011).

Nitrogen is an essential and often limiting plant nutrient in crop production. Nitrogen fixation resulting from mutual symbiosis of rhizobia and cultivated legume plants is therefore critical to food security as it directly affects agricultural production (Nape Victoria Mothapo, 2011). Biological nitrogen fixation is brought about both by free-living soil microorganisms and by symbiotic associations of microorganisms with higher plants (Oelke *et al*., 1991).

3.6. Symbiotic nitrogen fixation

Symbiotic nitrogen fixation is performed by rhizobia inside the nodules, which are the symbiotic organs formed on the roots of the host legume. The bacteria supply ammonium to the plant, while the sources of carbon and energy necessary for SNF are obtained from the plant photosynthates (Gisele *et al*., 2007).

The symbiotic nitrogen fixation is used to maximum advantage in case of leguminous crops. There is no doubt that specificity exists between rhizobial strain and the legume, and compatibility between the two is essential for successful nodulation. This necessitates using specific cultures for different legumes (Tamiru *et al*., 2012).

Leguminous plants fix atmospheric nitrogen by working symbiotically with special bacteria, rhizobia, which live in the root nodules. Rhizobia infect root hairs of the leguminous plants and produce the nodules. The nodules become the home for bacteria where they obtain energy from the host plant and take free nitrogen from the soil air and process it into combined nitrogen. In return, the plant receives the fixed N from nodules and produces food and forage protein (Oelke *et al*., 1991).

3.7. Early Events of Nodulation

Molecular dialog or signal exchange between the legume and rhizobium is a complex process that involves both the legume symbiotic (*sym*) genes and the rhizobia nodulation (*nod*) genes. In the beginning of the signaling process, legumes exude flavonoid compounds into the rhizosphere, which then trigger soil dwelling rhizobia to release highly specific reverse signal molecules, *nod factors,* only comprehended by specific legume species to initiate nodule formation. Rhizobia strains have a defined group of legumes species, or host range, with which they can nodulate, and in parallel, legumes select for specific rhizobia partner species (Nape Victoria Mothapo, 2011).

It is possible to tell from the colour of the root nodules whether or not they are active and therefore fixing nitrogen. Active root nodules are pink inside. By cutting through a root nodule it is possible to see whether it is active or not. The best time to do this is when the plant is flowering (Rienke and Joke, 2005).

Root nodules that remain white or light green on the inside throughout the growth cycle of the plant are not active. Even if the soya receives nitrogen in the form of artificial fertilizer the root nodules remain small and white. Only once the nitrogen from the fertilizer has been used up do the root nodules become active and grow bigger. For this reason it is worthwhile giving soya extra nitrogen if it is grown on poor soil (Rienke and Joke, 2005).

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. 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

The legume-rhizobia symbiosis is highly specific and depends on complex signaling processes between the host plant and rhizobia partner. Symbiotic N fixation between legumes and rhizobia takes place in plant-derived root organ called nodules, and competent nodulation is critical for efficient BNF (Nape Victoria Mothapo, 2011).

The symbiotic relationship implies a signal exchange between both partners that leads to mutual recognition and development of symbiotic structures. Rhizobia live in the soil where they are able to sense flavonoids secreted by the roots of their host legume plant. Flavonoids trigger the secretion of nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses, such as ion fluxes. The best-known infection mechanism is called intracellular infection, in this case the rhizobia enter through a deformed root hair in a similar way to endocytosis, forming an intracellular tube called the infection thread. A second mechanism is called "crack entry"; in this case, no root hair deformation is observed and the bacteria penetrate between cells, through cracks produced by lateral root emergence. Later on, the bacteria become intracellular and an infection thread is formed like in intracellular infections (Oelke *et al*., 1991).

The infection triggers cell division in the cortex of the root where a new organ, the nodule, appears as a result of successive processes (Oelke *et al*., 1991).

Infection threads grow to the nodule, infect its central tissue and release the rhizobia in these cells, where they differentiate morphologically into bacteroids and fix nitrogen from the atmospheric, elemental N_2 into a plant-usable form, ammonium ($NH_3 + H^+ \rightarrow NH_4^+$), using the enzyme nitrogenase. The reaction for all nitrogen-fixing bacteria is:

$$N_2 + 8\,H^+ + 8\,e^- \rightarrow 2\,NH_3 + H_2$$

In return, the plant supplies the bacteria with carbohydrates, proteins, and sufficient oxygen so as not to interfere with the fixation process. Leghaemoglobins, plant proteins similar to human hemoglobins, help to provide oxygen for respiration while keeping the free oxygen concentration low enough so as not to inhibit nitrogenase activity (Oelke *et al*., 1991).

3.10. Biochemistry of nitrogen fixation

Biological nitrogen fixation (BNF) is the process of converting atmospheric di-nitrogen gas in to useable form which is made by nitrogenase enzymes. According to Sprent and Raven (1985), nitrogenase enzyme has two oxygen sensitive metallo-protien components Molybdenum-iron protein di nitrogenase (component-I) and dinitrogenase reductase (component II). Both of the components are not active independently but they work synergistically (Benton *et al*., 2002).

The reactions occur as N_2 is bound to the nitrogenise 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).

$$4\ NADPH + 16\ ATP \qquad\qquad 4NADP^{+} + 8e^{-} + 4\ H^{+} + 16\ ADP + 16\ Pi$$

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

$$N_2 + 8e^{-} + 8H^{+} \longrightarrow 2NH_3 + H_2$$

Net-reaction

$$N_2 + 4NADPH + 4H^{+} + 16ATP \longrightarrow 2NH_3 + H_2 + 4NADP^{+} + 16ADP + 16Pi$$

N_2 fixation consumes 8 ATP and 2 NADPH per single molecule of ammonium formed which is a high energy consuming process.

The reaction catalyzed by nitrogenise 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). In natural condition, up to 40 molecules of ATP can be hydrolyzed for the reduction of only one nitrogen molecule (Hill, 1992). Thus, the equation for the whole reaction can be depicted as follows:

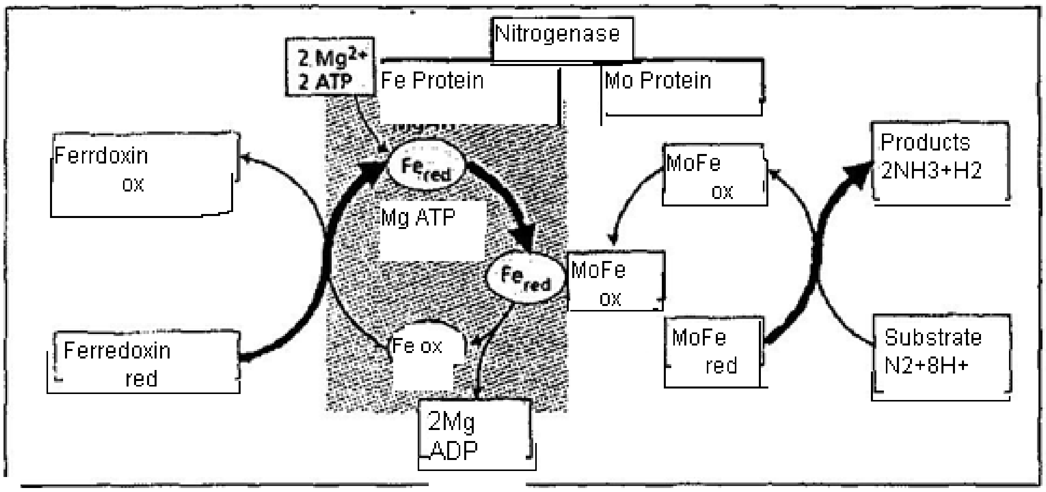
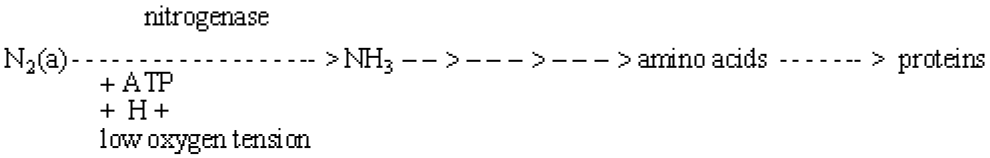


Figure 1. The reaction catalysed by nitrogenase, ferredoxin reduces the Fe protein. Binding hydrolysis of ATP to the Fe is thought to cause a conformation change of the Fe protein, which facilitates the redox reaction (Marie, 2001).

The reaction sequence starts by reduction of the Fe protein by the low-potential electron donor ferredoxin (Figure 1). 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).

The biochemical mechanism of N_2 fixation can be written in simplified form as follows:



$$\mathrm{N_2(a)} \xrightarrow[\substack{+\,\mathrm{ATP}\\ +\,\mathrm{H+}\\ \text{low oxygen tension}}]{\text{nitrogenase}} \mathrm{NH_3} \dashrightarrow \dashrightarrow \dashrightarrow \text{amino acids} \dashrightarrow \text{proteins}$$

The above mechanism indicates that N_2-fixing systems can thrive in soils poor in N. that they are a source of proteins, and that they provide N for soil fertility. Adenosine triphosphate (ATP) is the source of energy necessary for the cleavage and reduction of N_2 into ammonia. In *rhizobia*, for instance, ATP results from oxidative degradation of sugars and related molecules. These sugars are manufactured by the host-plant during photosynthesis and transferred to the nodules. In general, for each gram of N_2 fixed by *Rhizobium*, the plant fixes 1-20 grams carbon (C) through photosynthesis. This is an indication that symbiotic N_2 fixation requires additional energy which, in nitrate-fed plants, can be used to produce more photosynthates (products of photosynthesis). The extra energy cost of N_2 fixation can, however safely be carried by most field-grown legumes with little or no loss of production (Oelke *et al*., 1991).

3.11. Nitrogenase enzyme and its activity

Nitrogenase is a biological catalyst found naturally only in certain microorganisms such as the symbiotic *Rhizobium* and *Frankia*, or the free-living *Azospirillum* and *Azotobacter* (Oelke *et al*., 1991).

Nitrogenase is an oxygen sensitive enzyme. The low oxygen tension condition is realized through compartmentation in cyanobacteria (heterocysts in *Anabaena azollae*), active respiration (in *Azotobacter*), synthesis of leghemoglobin (in *Rhizobium* legume). Leghemoglobin is a macromolecule synthesized by both symbiotic partners, the rhizobia and the host plant. *Rhizobium* synthesizes the heme portion, and the plant the globine. Like human hemoglobin, leghemoglobin fixes O_2. It is responsible for the red or brown color of active (N_2-fixing) nodules. Non-N_2-fixing nodules have a white nodule content, or a green content when the globine has degenerated (Oelke *et al*., 1991)

Nitrogen fixation, which involves the chemical reduction of N_2 to NH_3 or NH_4, requires a source of electrons. Sources of electrons for the nitrogenase activity vary with the organism. They are all small proteins and highly reductive molecules such as flavodoxin, ferredoxin, nicotinamide, or adenine dinucleotide (phosphate) (Oelke *et al*., 1991).

All organisms which reduce dinitrogen to ammonia do so with the aid of an enzyme complex, nitrogenase. The nitrogenase enzymes are irreversibly inactivated by oxygen, and the process of nitrogen fixation uses a large amount of energy (Zahran, 1999).

3.12. Ecological factors affecting Biological nitrogen fixation

3.12.1. Soil pH

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N_2 fixation. (Zahran, 1999) reported that pasture and grain legumes acidify soil to a greater extent and that the legume species differ in their capacity to produce acids. Legumes and their rhizobia exhibit varied responses to acidity. Some species, like lucerne (*M. sativa*), are extremely sensitive to acidity, while others, such as *Lotus tenuis*, tolerate relatively low soil pH. Soil acidity constrains symbiotic N_2 fixation in both tropical and temperate soils, limiting *Rhizobium* survival and persistence in soils and reducing nodulation.

Soil acidity and related problems of Ca deficiency and aluminum and manganese toxicity adversely affect nodulation, N_2 fixation and plant growth . Research work on the identification of symbioses adapted to acid soil should focus on the host plant, because effective rhizobia adapted to- soil acidity can be found naturally and can be produced through genetic manipulations (Oelke *et al*., 1991).

3.12.2. Aluminum and manganese toxicity

Rhizobia showed varied responses to aluminum toxicity in acidic soils and cultures. Legume species vary markedly in their tolerance to Al^{3+} and Mn^{2+}, with some plants being significantly more strongly affected by these ions than are the rhizobia. Therefore, for acid soils with high Al content, improvement is achieved by manipulating the plant rather than the rhizobia (Zahran, 1999).

3.12.3. Soil temperature

Extreme temperatures affect N_2 fixation adversely. This is easy to understand because N_2 fixation is an enzymatic process. However, there are differences between symbiotic systems in their ability to tolerate high ($>35^0$C) and low ($<25^0$C) temperatures (Oelke *et al*., 1991).

High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops (Michiels et al., 1994). High root temperatures strongly affect bacterial infection and N_2 fixation in several legume species, including soybean, guar, peanut, cowpea, and beans (Hungria and Franco, 1993; Piha and Munnus, 1987). Critical temperatures for N_2 fixation are 30^0C for clover and pea and range between 35 and 40^0C for soybean, guar, peanut, and cowpea (Michiels *et al.*, 1994). Nodule functioning in common beans (*Phaseolus* spp.) is optimal between 25 and 30^0C and is hampered by root temperatures between 30 and 33^0C (Piha and Munnus, 1987). Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to the plant cultivar (Arayankoon *et al.*, 1990; Munevar and Wollum, 1982). Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (Roughley, 1970).

3.12.4. Soil moisture

Excessive moisture and waterlogging prevent the development of root hair and sites of nodulation, and interfere with a normal diffusion of O_2 in the root system of plants. *Sesbania rostrata* and *Aeschynomene* sp. can actively fix N_2 under these conditions because they are located on the plant stems, rather than on the roots (Oelke *et al.*, 1991).

The occurrence of rhizobial populations in desert soils and the effective nodulation of legumes growing therein emphasize the fact that rhizobia can exist in soils with limiting moisture levels; however, population densities tend to be lowest under the most desiccated conditions and to increase as the moisture stress is relieved (Zahran, 1999).

Drought reduces the number of rhizobia in soils, and inhibits nodulation and N_2 fixation. Prolonged drought will promote nodule decay. Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing N_2 when the soil is drying (Oelke *et al.*, 1991).

3.12.5.Salt stress

Salinity is a serious threat to agriculture in arid and semiarid regions. Nearly 40% of the world's land surface can be categorized as having potential salinity problems (Cordovilla *et al.*, 1994); most of these areas are confined to the tropics and Mediterranean regions. Increases in the salinity of soils or water supplies used for irrigation result in decreased productivity of most crop plants and lead to marked changes in the growth pattern of plants (Cordovilla *et al.*, 1994). Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate, 1995).

Soil salinity and acidity are usually accompanied by mineral toxicity (specific ion toxicity), nutrient deficiency, and nutrient disorder. Salt damage to non halophytic plants grown in nutrient solution is often due to the effect of ion imbalance (disorder) rather than the osmotic potential. This disorder might occur by specific toxicity of ions such as Na^+ and Cl^- and might be balanced by increasing the concentration of counter ions, like K^+ and Ca^{2+}, against Cl^- (Zahran, 1999).

The salinity response of legumes varies greatly and depends on factors; climatic conditions, soil properties, and the stage of growth. The legume-Rhizobium symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Rao *et al*., 2002). Salt stress inhibits the initial steps of Rhizobium-legume symbioses. The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leg hemoglobin, by nodules. The depressive effect of salt stress on N_2 fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Rao *et al.*, 2002). The salt-induced distortions in nodule structure could also be reasons for the decline in the N_2 fixation rate by legumes subject to salt stress. Although the root nodule-colonizing bacteria of the genera Rhizobium and Bradyrhizobium are more salt tolerant than their legume hosts, they show variation in salt tolerance.

3.12.6. Soil nutrients

Nodulation and N_2-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 deficiency is commonplace in tropical Africa and reduces nodulation, N_2 fixation and plant growth. Identification of plant species adapted to low-P soils is a good strategy to overcome this soil constraint (Oelke *et al*., 1991). Phosphorus is one of several elements which affects N_2 fixation, and, along with N, it is a principal yield-limiting nutrient in many regions of the world (Zahran, 1999). Plants engaged in symbiotic N_2 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.

The addition of P-solubilizing microorganisms, particularly of the general *Psemdamaias, Bacillus, Penicillium,* and *Aspergillus* can solubilize rock phosphate and organically bound soil P (which constitutes 95 - 99% of the total phosphate in soils). However, the use of these microorganisms is not widespread (Oelke *et al*., 1991).

Mineral N inhibits the *Rhizobium* infection process and also inhibits N_2 fixation. The former problem probably results from impairment of the recognition mechanisms by nitrates, while the latter is probably due to diversion of photosynthates toward assimilation of nitrates. Application of large quantities of fertilizer N inhibits N_2 fixation, but low doses (<30 kg N ha^{-1}) of fertilizer N can stimulate early growth of legumes and increase their overall N_2 fixation. The amount of this starter N must be defined in relation to available soil N (Oelke *et al*., 1991).

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).

Various microelements (Cu, Mo, Co, B) are necessary for N_2 fixation. Some of these are components of nitrogenase for example Mo (Oelke *et al*., 1991).

3.13, Formulation methods and inoculants development

The agricultural benefits are achievable from the use of selected, efficient N_2 fixing strains of rhizoid. This can be realized only when farmers get and suitably use high-quality inoculants on their legume seeds or soil before planting. Technology on growing rhizoid, preparing inoculants with suitable carrier materials, and distributing viable inoculants to farmers is essential (Somasegaran and Hoben, 1994).

3.13.1, Culturing rhizobia/ Rhizobial Inoculant Production

It is easy to grow rhizobia in the laboratory. These bacteria are aerobic and also mi-croaerophilic. They need aeration, which may be provided by using a mechanical shaker or by bubbling sterile air through the medium. Rhizobia grow best at 25-30^{0}C. The medium must provide energy, nitrogen source, certain mineral salts, and growth factors. Most commonly used is a yeast extract mannitol mineral salts medium, but if cost or ease of use is a concern, sucrose or glycerol may be substituted (Somasegaran and Hoben, 1994).

Inoculant production starts with a pure slant culture. This culture is used to inoculate yeast-mannitol broth (YMB) in a small flask. The resulting broth culture will serve as inoculum (starter culture) for a greater volume of broth or medium contained in a large flask or a 2-4 liter glass fermentor. The volume of a starter culture should be a minimum of 1% of the broth volume in the fermentor. Thus, a l liter starter culture would be required to inoculate 100 liters of medium in a steel fermentor. Commonly, a larger inoculum is used to reduce the incubation time needed to obtain 1 X 10^9 rhizobial cells ml^{-1}. This population level is considered necessary, particularly when using non sterile carrier material. Aseptic conditions are maintained throughout the production period. The broth culture is checked regularly for purity (Somasegaran and Hoben, 1994).

Somewhat it is easy to grow rhizobia in liquid medium. Because rhizobia are not competitive with other microorganisms, it is very necessary to sterilize the whole of growth vessel and medium as well as ensuring inoculation of the fermenter with rhizobial starter culture under sterile environment. The purpose of the production is to have high density of rhizobia in the broth culture. This can be influenced by culture medium, rhizobial strain, temperature and aeration. Rhizobia are aerobic bacteria and want oxygen for growth. Long experience in rhizobial inoculant production has shown that rhizobia need aeration of 5-10 litre of air for 1 litre medium in 1 hour. Optimum temperature for rhizobial growth is 28-30^{0}C. The medium supplies energy, nitrogen, certain mineral salts and growth factor. General Yeast Manitol (YM) medium is used in rhizobial broth culture (FNCA Biofertilizer Project Group, 2006).

Production of liquid inoculant (For scale 1,500 ml) includes the following steps: Step 1. Inoculate a loop full of rhizobia into 500 ml Erlenmeyer flask containing sterilized 150 ml of YM broth. Step 2. Culture on rotary shaker at 28^{0}C, 200 rpm until late log phase (cell concentration about 10^8 cells/ml). This culture will be used as starter culture for liquid inoculant production. Step 3. Inoculate 150 ml of starter culture into 2,000 ml Erlenmeyer flask as simple fermenter containing sterilized 1,500 ml of a modified YEM media, which is used as basal media for liquid inoculant formulated with selected appropriate additive for each genus of rhizobia. Step 4. Culture at 28^{0}C by using air continuously pump through a 0.45 µm filter into the medium until reach maximum cell concentrations of 10^9 cells/ml. Step 5. Inoculate 20 ml aliquot of cell culture into sterile polypropylene bag and heat sealing. Liquid inoculant should be stored at appropriate temperature before used (4^{0}C for long-term storage). Step 6. Use 20 ml of liquid inoculant for inoculation 1 kg of seed (for medium seed size, such as soybean), without using sticker, before sowing (FNCA Biofertilizer Project Group, 2006).

3.13.2, Inoculant carriers

Most inoculants are a mixture of the broth culture and a finely milled, neutralized carrier material. The properties of a good carrier material are: (1) nontoxic to rhizobia, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8) good pH buffering capacity.

The best researched and most frequently used carrier material for inoculant production is peat. A large number of studies have shown that peat provides better protection for the rhizobia in the package and on inoculated seed than other carriers (Somasegaran and Hoben, 1994).

Peat is not available in some countries. A wide range of substitutes, e.g., coal, charcoal, bagasse, filter mud, vermiculite, polyacrylamide, mineral soils, vegetable oils, and ground plant residues have been tested as alternative carriers. in spite of of which type of carrier is chosen, a rhizobia-carrier interaction cannot be avoided. A particular rhizobial strain may survive well in one carrier but not another. Therefore, it is important that the producer knows the strain-carrier interactions for all of his strains of rhizobia (Somasegaran and Hoben, 1994).

Carrier processing, e.g., mining, drying, and milling, are the most capital-intensive aspects of inoculant production. Generally, the wet peat is mined, drained, and screened to remove stones and roots, then shredded and dried. The peat is then ground in a high- speed hammer mill and passed through a sifting machine. Material with a particle size of 10-40 µm (0.001-0.004 mm) is collected for seed coating. Peat with a particle size of 500-1500 µm (0.5-1.5 mm) is used for the production of soil implant (granular) inoculant. The carriers are neutralized with precipitated calcium carbonate (pH 6.5-7.0). Both sterilized and non sterilized peat are used in commercial production systems (Somasegaran and Hoben, 1994).

Peat carrier selected for sterilization is prepackaged in thin-walled polyethylene bags. The sealed bags are then gamma-irradiated at 5.0 Mrads. Alternatively, the carrier may be autoclaved in partially opened, thin-walled, polypropylene bags for 60 min at 15 Ib/in^2 pressure and 121^{0}C. However, heat sterilization of some peats has been found to produce undesirable changes and to release toxins. Gamma-irradiation sterilization is preferred (Somasegaran and Hoben, 1994).

3.13.3, Adding broth culture in to carriers

In the United States, under commercial conditions, quality-tested broth cultures are added into peat at the rate of 1 liter per kilogram of peat. After a curing period, the mixture is packaged in thin-gauge (0.05 mm) polyethylene bags. Bags of this specification allow gas exchange even as minimizing moisture loss from the inoculant. The expiration date for inoculants based on non sterile carriers is usually 6 months (Somasegaran and Hoben, 1994).

Sterilization of carrier material is crucial to maintain high number of inoculant bacteria on carrier for long storage period. Gamma-irradiation is the most proper way of carrier sterilization, because the sterilization process makes almost no change in physical and chemical properties of the material. In short, carrier material is packed in thin-walled polyethylene bag, and then gamma-irradiated at 50 kGy (5 Mrads). Another way of carrier sterilization is using autoclave. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121^0C. It should be noted that during autoclaving, some materials changes their properties and produce toxic substance to some bacterial strains (FNCA Biofertilizer Project Group, 2006).

In some countries, such as South Africa, Australia, and New Zealand, inoculant producers produce inoculants with sterilized carriers. In this case, the carrier is first packaged and then sterilized by gamma-irradiation or autoclaving. Thin-gauge (0.05 mm) polyethylene bags are used for carriers to be gamma-irradiated. Carriers to be autoclaved are packaged in polypropylene bags of equal gauge. The rhizobial broth culture is aseptically injected into the packaged carrier with a manually operated motorized syringe (Somasegaran and Hoben, 1994).

Inoculants based on sterile carriers are typically of higher quality than the non sterile carrier type. The number of viable rhizobia per gram can be between 10^9-10^{10} cells in inoculants produced with sterilized carriers. In non sterile carriers, like in non sterile peat, the initial number of viable rhizobia tends to be lower by at least one log after curing. The number of rhizobia added to most sterile carriers remains high during shelf life or storage because there are no other microorganisms in the carrier competing with the rhizobia. The quality of such inoculants may still be acceptable after 6-12 months, depending on the temperature during storage (Somasegaran and Hoben, 1994).

Inoculants are cured for about 2 weeks at 25-30^0C to gain maximum numbers in excess of 10^8 and 10^9 cells g^{-1} for non sterile and sterile carrier-based inoculants, respectively. After that, inoculants are stored in a refrigerated or air-conditioned environment, protected from direct light (Somasegaran and Hoben, 1994).

3.13.4, Production of sterile carrier-based inoculants

The production requires a completely sterile carrier in sterile package. The simplest way is to mix the sterile carrier with bacterial liquid culture. Each pre-sterilized carrier bag is injected aseptically with culture by means of a syringe fitted with a sterile needle. For mass production, an automatic dispensing machine (auto syringe) may be used. The area of puncture must be disinfected with ethanol. Quantity of broth should be adequate to wet 40% of the carrier by carrier weight. If the carrier material contains nutrient available for the incorporating bacteria to grow (e.g. mineral soil), injection into the carrier package of starter culture of the bacterial cells together with sterile water for moisture adjustment will be sufficient. The puncture hole is then immediately sealed with preprinted self-sticking label. The bags are then kneaded by hand or by shaker until the liquid inoculum has been uniformly absorbed in to the carrier. The final moisture of inoculants should be 45-50%. After the injection, the carrier package should be placed in temperature-controlled area for appropriate period to let the bacterial cells grow up to maximum population. The inoculant is ready for use after 2 weeks (FNCA Biofertilizer Project Group, 2006)

4. Materials and Methods

4.1. Description of the sampling sites

Sampling Sites were some important Field pea growing areas of Central Showa, Ethiopia (Figure 2).

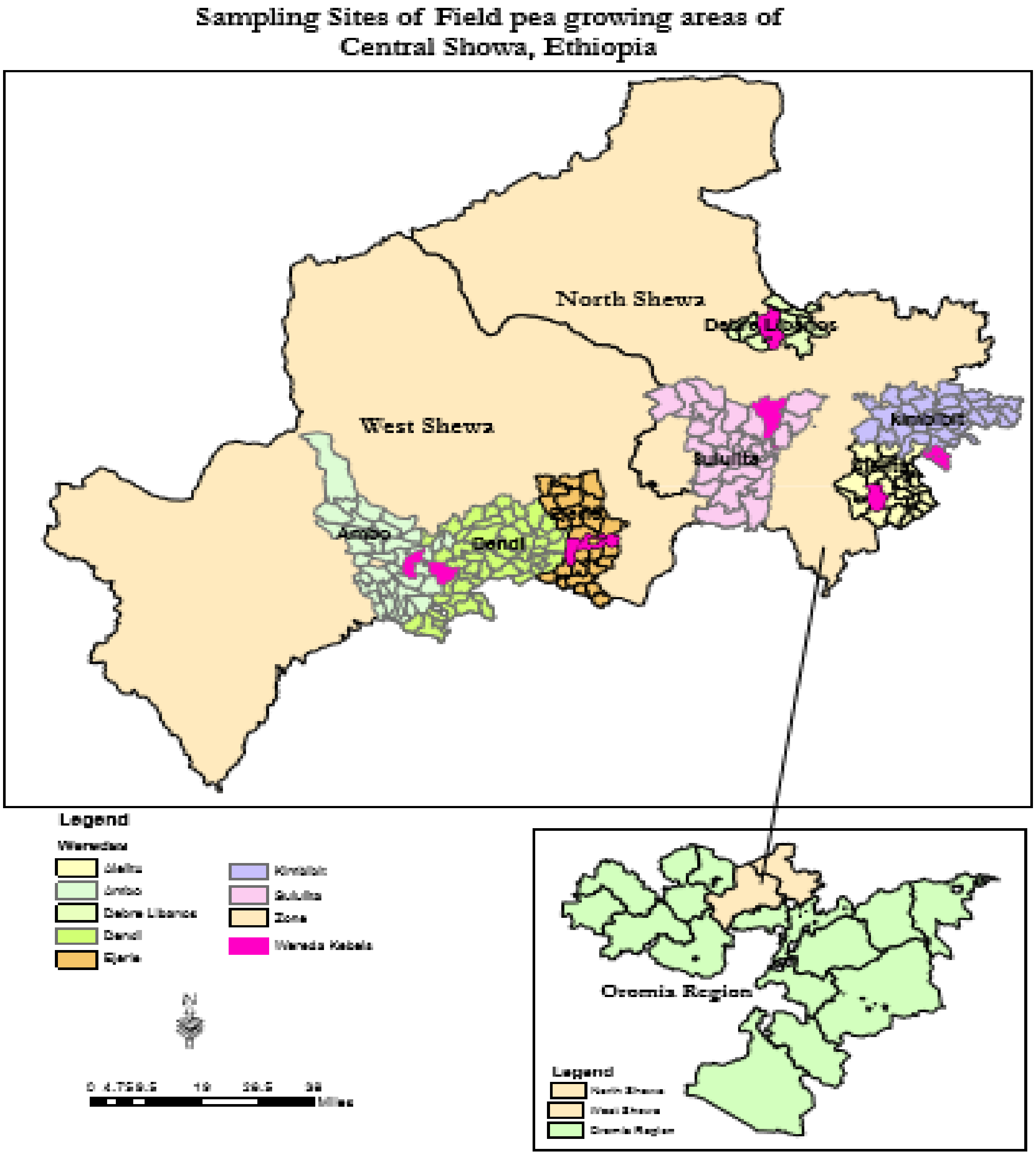


Figure 2. Map of samples collection Sites

4.2. Sample Collection

A total of 44 nodule samples were randomly collected among which a total of 17 samples from North Showa of Debre Birhan route (3 from Laga Bolo Woreda, 4 from Alaltu Woreda, and the rest 10 from Sheno/Kimbibit Woreda). Other 16 nodule samples were from North Showa of Fitche (12 samples from Sululta woreda, 2 from Wuchale and 2 from Debre Libanos Woredas). The rest 11 samples were from West Showa zone of which; 5 from Ejere Woreda, 3 from Dandi and 3 from Ambo Woredas. These all nodule samples were randomly collected from farmers' field and immediately kept in sealed vials containing a desiccant (Silica gel) covered with 1cm of cotton wool for isolation of rhizobia (Somasegaran and Hoben, 1994) in September 2015/ 2016 and taken to National Soil Testing Center for further processing.

4.3. Isolation of root nodulating bacteria

Dehydrated or desiccated root nodules were immersed in sterile distilled water overnight in labeled sets of flasks to imbibe water. The imbibed nodules were surface-sterilized with 70% ethanol for 10 seconds and then transferred to 3% (v/v) solution of sodium hypochlorite ($NaHClO_3$) for 3 minutes according to Somasegaran and Hoben (1994). The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse off and remove the sterilizing chemicals. The nodules were crushed with sterile glass rods in1 ml drop of normal saline solution (0.85% Nacl). The crushed nodules were streaked on to yeast extract mannitol agar plates with congo red. The plates were then incubated at 28 ± 2^0C and periodically observed for colony formation. *Rhizobium* colonies were remained white, translucent, elevated and mucilaginous, after 24-72 hrs, whereas contaminants turned red. The colony were picked up and re-transferred to YEMA plates for further purification. This re-inoculation on YEMA took place many times and purified colonies were preserved on YEMA slant at 4^0C (Vishal and Abhishek, 2014).

Yeast extract manitol agar medium (g/l) was prepared by mixing: $MgSO_4.7H_2O$ (0.2), NaCl (0.2), K_2HPO_4 (0.5), KH_2PO_4 (0.5), Yeast extract (0.5), D-mannitol (10), Agar (15), Distilled water (1000 ml) and Congo red (10 ml/l). This mixture with pH adjusted to 7 was autoclaved at 121^0C for 15 minutes (Lupwayi and Haque, 1994).

4.4. Purification and preservation

Different colonies were picked with sterile inoculating loop and streaked on sterile YEMA (Yeast Extract Mannitol Agar) plates and incubated at 28±2^{0}C for 3-5 days. The purity and uniformity of colony type was carefully examined through repeated re-streaking and a single well isolated colony was picked and transferred to YEMA slant containing 0.3% (W/V) $CaCO_3$ (used to prevent the dryness of slant medium) in a culture tube and incubated at 28±2^{0}C. After sufficient growth the culture slant was preserved at 4^{0}C (Somasegaran and Hoben, 1994).

4.5. Presumptive test

All the isolates were examined for presumptive purity using gram staining, Peptone Glucose Test (PGT), and growth response to Yeast Extract Manitol Agar with Congo Red (YEMA-CR) medium (Somasegaran and Hoben, 1994).

4.5.1. Gram staining test

All the isolates were gram stained for rapid means of identification of contaminants as indicated in Lupwayi and Haque, (1994). The procedure was as follows:

Using a sterile loop, a loopful of the samples was transferred on two different slides. The specimens were allowed to dry on the slide at room temperature. After the specimen was dried, it was heat-fixed. The Stain bacterial smears were stained by Gram's Method as follows:

Crystal Violet (1 minute) - washed gently in tap water for 2-3 seconds. Gram's Iodine (I_2-KI) (1 minute) - washed gently in tap water, shake off excess water. Alcohol-acetone 95% (10 seconds) - washed gently in tap water, shake off excess water. Safranin (counter stain) (20 seconds) - washed in tap water and blot dried.

Then the gram stained smear was examined with oil immersion objective using compound light microscope. Gram-negative organisms observed as pink to red whereas gram positive bacteria showed purple color /retain the primary dye.

4.5.2. Congo red absorption

To check purity, the isolates were tested on Congo -Red (YEMA-CR). Stock solution of Congo Red (CR) was prepared by dissolving 0.25 g of CR in 100 ml sterile distilled water. A 10 ml from this stock solution was added into one liter of YEMA. The broth culture suspension was inoculated into YEMA-CR medium, and incubated at 28 ± 2^0C for 3-5 days.

4.5.3. Acid and alkaline production on Bromothymol blue (BTB)

The ability of isolates to produce acid or alkali was determined on YEMA-BTB (YEMA1 liter; BTB-(0.5 % w/v in 95% ethanol), 5 ml; pH 6.8). After 48 hours of growth, a loopful of *Rhizobium* culture was streaked on YEMA-BTB and incubated at 28^0C for 3-5 days (Somasegaran and Hoben, 1994). The isolates were considered as fast growers when the color changed to yellow and slow growers when the color was changed into blue.

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

Isolates were inoculated to PGA amended with bromocresol purple in order to check a change in pH of the medium associated with the presence of contaminants in the preserved culture which further checked on PGA medium with 10 μg/ml Bromocresol purple (BCP) dye with composition (g/l): Glucose 5, Peptone 10, Agar 15, BCP stock solution 10 ml, pH 6.7, distilled H_2O 1 liter. The medium was autoclaved at 121^0C for 15 min (Somasegaran and Hoben, 1994). The BCP (Bromo cresol purple) was prepared as stock solution by dissolving 1 g in 100 ml of ethanol (Somasegaran and Hoben, 1994). The pH was adjusted at 6.8 by 1N NaOH and HCl (Lupwayi and Haque, 1994). The bacterial culture suspension was inoculated on the medium and incubated at $28\underline{+}2^0$C for 3-5 days. The isolates were designated as AAURFP (Addis Ababa University Rhizobia of Field pea).

4.5.5. Authentication

The isolates were tested for their symbiotic effectiveness under greenhouse conditions using sterile sand culture with *Pisum sativum*, variety *Burkitu*. River sand was soaked in 1N H_2SO_4 for 24 hours and extensively washed with tap water several times, and filled into alcohol sterilized (70%) 3 kg capacity plastic pots.

Field pea seeds were treated with 95% ethyl alcohol for 10 seconds and left for the complete evaporation of alcohol and then transferred into sodium hypochlorite solution of 3% for 5 minutes and rinsed six times with distilled water. Five sterilized seeds were sown into each pot and when germinated, they were thinned to three seedlings per pot after a week of planting.

One milliliter of liquid inoculum (10^9 rhizobia cells/ml) of collected isolates was used separately to inoculate each seedling. The pots were arranged in randomized completed block design (RCBD) with three replicates, along with un inoculated (negative control) and N-fertilized (positive control) pots. At the beginning, all the plants were given 100 ml of 0.05% KNO_3 (W/V) once as starting nitrogen. The positive control plants were fertilized with 100 ml of 0.05% KNO_3 once every week. The N-free nutrient solution (Jensen's nitrogen-free nutrient) was given to all plants 100 ml/pot once a week as full strength for the four consecutive weeks and supply tap water every 2 days (Somasegaran and Hoben, 1994).

Plants of all pots were harvested at 55 days after sowing and nodulation status, nodule number, shoot and root length, nodule dry weight, and shoot dry weight were recorded after drying at 70^0C for for 48 hrs until constant weight.

N-free Nutrient Solution-1 composition (g/l): K_2HPO_4-0.023, KH_2PO_4-0.13, $CaSO_4.2H_2O$-3.425, $MgSO_4.7H_2$-0.274, NaCl-0.04, $FeCl_3.6H_2O$-0.06. N-free Nutrient Solution-2 composition (g/l): $CUSO_4.5H_2O$-0.15, $ZnSO_4.7H_2O$-0.44, $MnSO_4.7H_2O$-0.04, H_3BO_3 (Boric acid) - 1.43. N-free nutrient solution was prepared by adding 1 ml of solution-2 per a liter of solution-1 (Beattie *et al.*, 1989).

4.6. Growth, morphological and Cultural characteristics

4.6.1. Generation time

Generation time was determined by inoculating rhizobial isolates into 50 ml sterilized YEM broth in 150 ml conical flask and was incubated at $28\underline{+}2^0$C on a shaker at (150 rpm) for 24 hrs. Growth was measured by taking optical density (OD) at 610 nm of the culture at every 4 hrs interval. The generation time was calculated using the following formula:

$$\text{Generation time} = \frac{(T_2\text{-}T_1)}{3.3(\log_{10}OD_2-\log_{10}OD_1)}$$

Where T_2-T_1 **is** the difference of two time intervals at any two points in log phase in growth curve; ($\log_{10}OD_2$-$\log_{10}OD_1$) is the difference between the $\log_{10}$ value of OD_2 at time T_2 and OD_1 at time T_1 **(**Vishal and Abhishek, 2014). Other way of evaluating generation time was by using colony forming unit (CFU).

4.6.2. Colony Morphology

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

4.7. Physiological characteristics

4.7.1. Carbohydrate utilization

The isolates tested on different carbohydrates to evaluate their ability of using different carbon sources. The two types of carbohydrates (heat stable and labile) were prepared. Basal medium (carbohydrate- free medium) was prepared by reducing the concentration of yeast extract from 0.5 g/l to 0.05 g/l and all components of YEMA were added except mannitol. Some carbohydrates are heat stable (glucose, lactose, D-mannitol, and sucrose), and sterilized with the basal medium. The heat labile carbohydrates (galactose, maltose, starch, cellulose, sorbitol, tartarate, glycerol, arabinose and citrate) were filter sterilized using 0.2 µm filter paper and added to the sterilized medium. Isolates were grown in 10 ml YEM broth for 72 hours at 28 ± 2^0C on 120 rpm (revolution per minute) rotary shaker. The broth cultures inoculated into medium (plate) prepared containing different carbon sources and observed whether the growth took place or not (Somasegaran and Hoben, 1994). The basal medium consisted of (g/l): 0.2 $MgSO_4.7HO2$, 0.2 NaCl, 0.5 K_2HPO_4, 0.5 KH_2PO_4, 0.05 Yeast extract, 15 Agar and 1000 ml distilled water.

The medium was autoclaved at 121^0C for 15 minutes. The pH of the medium was adjusted to 7 (Lupwayi and Haque, 1994).

4.7.2. Amino acid utilization

Different types of amino acids including L-arginine, L-glutamate, L-leucine, L-phenylalanine, L-tryptophane, urea and L-tyrosine were used in this experiment in order to determine the ability of the isolates to utilize the amino acids as sole nitrogen source. These amino acids were added at a concentration of 0.5 g/l to a basal medium (g/l: 0.2 $MgSO_4$.7H2O, 0.2 NaCl, 0.5 K_2HPO_4, 0.5 KH_2PO_4, 0.05 Yeast extract 1 D-manitol, 15 Agar and 1000 ml distilled water) source that lack ammonium sulfate and supplemented with 1g/l of mannitol. The membrane filter sterilized amino acids were added to the autoclaved and cooled (approximately 55^0C) basal medium as indicated in Amargar *et al*., (1997). The medium was autoclaved at 121^0C for 15 minutes by adjusting the pH to 7 (Lupwayi and Haque, 1994). The prepared medium was poured in to plates asepticaly in laminar air flow hood and solidified. Finally, 72 hrs old rhizobial suspensions were inoculated into these basal media and incubated at $28±2^0$C for 3-5 days. Then growth of the colonies on plates were observed and taken as (+) if growth was there and (-) if colonies did not grew.

4.7.3. Phosphate solubilization test

The ability of isolates to solubilize phosphate was tested by inoculating them on Pikovskaya agar medium (PA) containing: Glucose (10 g/l), Tricalcium phosphate (5 g/l), $NH_4(SO_4)_2$-(0.5 g/l), Yeast extract (0.5 g/l), Magnesium sulfate heptahydrate (0.1 g/l), Sodium chloride (0.2 g/l), Manganese sulfate (0.002 g/l), Ferrous sulfate (0.002 g/l) and Agar (15 g/l). The pH of the medium was adjusted to 7.00. Phosphate solubilizing abilities of the isolates was checked based on growth and the presence of clear zone around the colonies (Somasogaran and Hoben, 1994).

4.8. Eco-physiological characteristics

4.8.1. Acid and alkaline (pH) tolerance

The capacity of each rhizobial isolates to grow on acidic and alkaline medium was determined by inoculating each isolate on YEMA adjusted at a pH of 4.0, 4.5, 5.0, 8.0, 8.5, 9.0, 9.5 and 10.0, using 1N NaOH and HCl as indicated by Bernal and Graham (2001).

4.8.2. Salt tolerance

The ability of the isolates to grow at different level of salt concentrations was determined by inoculating each isolate on YEMA medium containing 1%, 4%, 5%, 6%, 7%, 8%, 9% and 10% of NaCl as indicated in Lupwayi and Haque (1994).

4.8.3. Temperature tolerance

The growth of each isolate at different incubation temperatures was evaluated by inoculating each isolate on YEMA plates. The inoculated plates were incubated at a temperature of 4^0C, 10^0C, 15^0C, 20^0C, 25^0C, 30^0C, 35^0C, 40^0C, 45^0C and 48^0C as described in Lupwayi and Haque (1994). Then the growth was observed if the isolates formed colonies on the plates or not.

4.8.4. Intrinsic antibiotic resistance (IAR)

The resistance of isolates to different antibiotics at different concentration was evaluated by streaking each isolate on YEMA containing freshly prepared filter sterilized antibiotics using 0.2 micrometer pore size filter. The antibiotics were Tetracycline, Erythromycin, Ampicillin, Chloroamphinicol, and Penicillin. Each antibiotic was tested at the following concentrations in µg/ml (2.5, 5 and 10). Erythromycin was dissolved in ethanol, whereas others were dissolved in sterilized distilled water. The stock solution of each antibiotic was prepared by dissolving 2 g of each antibiotic in 100 ml of water according to Lupwayi and Haque (1994). The required concentration was aseptically added to the medium using a different pipette for each antibiotic. The sterilized solution of each antibiotic was aseptically added to autoclaved YEMA (kept at 50^0C approximately) and shaken well. Then isolates were streaked on the plates prepared with antibiotics and incubated at 28 + 2^0C for 3-5 days and observed whether growth was there or not.

4.8.5. Heavy metal resistance of the isolates

All the isolates were also tested on solid YEMA medium containing the following filter (0.2 µm pore size membrane) sterilized heavy metals (at the concentrations in µg ml^{-1} in the parenthesis): AlK_2SO_4 (250), $CoCl_2$ (20), $CuCl_2$ (50), $HgCl_2$ (10), $MnSO_4$ (500), and $ZnCl_2$ (50) at pH 6.8 (Maatallah *et al*., 2002). Then the rhizobial culture suspensions of 72 hrs old were streaked on the media (plates) and the growth of colonies were observed and recorded as (+) for growth and (-) for no growth.

4.9. Symbiotic effectiveness (SE)

Twenty nine isolates of *rhizobia* tested for this experiment. The experiment was statistically laid out in a randomized complete block design with a total of 29 treatments with three replications. Two uninoculated treatments, one with nitrogen fertilizer (positive control, 0.05%) and other without nitrogen (negative control) were included (Somasegaran and Hoben, 1994). The isolates were tested for their infectivity (nodule formation) and effectivity in nitrogen fixation ability using sterile sand culture with *Pisum sativum*, variety *Burkitu*. River sand was soaked in $1NH_2SO_4$ for 24 hours and extensively washed with tap water several times, and filled into alcohol sterilized (70%) 3 kg capacity plastic pots. The seeds were surface sterilized with 95% ethanol for 3 minutes followed by sodium hypochlorite solution of 3% and subsequently washed with 5 changes of sterile distilled water, and the pots were planted with five seeds, two uprooted after germination and the left three seedlings were inoculated with 1 ml (10^9 cells ml-1) of the test *rhizobia.*

After fifty five days of planting upon re-inoculation, the plants were uprooted to measure nodule number, nodule dry weight and shoot dry weight. The effectiveness of isolates in accumulating plant shoot dry matter was calculated as described in Date *et al.* (1993) cited in Purcino *et al.* (2000) as follows:

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

Where, D.M. = dry matter, S.E. = symbiotic effectiveness

The rate of nitrogen fixing effectiveness is evaluated as: Highly effective > 80%, Effective 50-80%, Low effective 35-50% and Ineffective <35% (Date *et al.,* 1993).

4.10. Numerical Analysis

Phenotypic similarities among *Pisum sativum Rhizobium* (*Rhizobium leguminosarum biovar viciae)* numerically analyzed based on their phenotypic characteristics, such as pH tolerance, temperature tolerance, salt tolerance, intrinsic antibiotic resistance, mean generation time, carbohydrate utilization, amino acid utilization and intrinsic heavy metal resistance abilities of isolates by using the Un weighed Pair Group Methods with Average.

4.11. Data Analysis

Analysis for comparisons between the treatments with shoot dry weight, nodule number and nodule dry weight per plant was done by Duncan multiple range tests at alpha /0.05/ using the statistical program SAS version 9.3. Means separation was calculated using the LSD test value when the F-test was significant at p =0.05%.

5. Results and Discussion

5.1. Growth, colony morphology and cultural characteristics

Among 44 rhizobial isolates, 27 of the isolates (root nodulating Rhizobia) 14 (from North Showa D/Birhan route), 3 (from North Showa Fitche route) and 10 (from West Showa) were authenticated as root nodule bacteria and fully characterized (Table 1). The rest 15 of them (3 from Sheno/Kimbibit, 8 from Sululta, 2 from Wuchale, 1 from D/Libanos and 1 from Ejere) were rejected during presumptive test and 2 (from Sululta) during authentication test under greenhouse conditions (Table 1).

The root nodulating isolates were gram negative rods, did not grow on Peptone –glucose agar medium with Bromocresol Purple (PGA-BCP) and failed to absorb Congo-red on Yeast Extract Agar medium (Table 1). Failure of these isolates to absorb Congo red from YEMA-CR medium and to grow on peptone glucose agar medium presumptively characteristics of root nodule bacteria (Vincent, 1970). All the isolates changed the green color BTB medium into yellow color indicating that they were acid producing bacteria (Jordan, 1984). There were reports from previous studies (Aregu *et al*., 2012; Fanno, 2010; Kassa *et al*., 2015) that showed similar pattern of growth characteristics for rhizobia isolated from field pea.

About 67% of the isolates displayed large mucoid (LM) translucent colonies, 33% showed large watery (LW) with production of high amount of exopolysaccharides on the medium (Table 1). Similarly Fano (2010) reported that 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.

All the isolates showed colony diameter within the range of 2 mm to 4.5 mm (Table 1). This implies that all of the isolates were fast growers based on their generation time (Table 1). The fastest growth rate was displayed by isolates: AAURFP20 (Sululta), AAURFP29 (Sululta), AAURFP38 (Ejere), AAURFP1 (Laga Bolo) and AAURFP2 ((Laga Bolo) with mean generation time of 2.4 and 2.5 hours, respectively (Table 1). About 85% of the isolates were found to have colony diameter of 2.0-4.0 mm after 3-5 days of growth, whereas 15% showed colony diameter of 4.5 (AAURFP37, AAURFP38, AAURFP41, AAUFP42). There were reports which have similarities with the present study on field pea nodulating rhizobia colony characteristics and generation time (Aregu *et al*., 2012; Kassa *et al*., 2015; Fanno, 2010; Vishal and Abhishek, 2014).

Table 1. Sample site, Growth, colony morphology and cultural characteristics of authenticated rhizobia isolates from different sampling sites in central parts of Ethiopia after 3-5 days of growth on YEMA medium.

Isolates	**Sample Sites(Woredas)**	**Kebele**	**Zone**	**Colony Characteristics**	**Colony Size(mm)**	**YEMABTB**	**MGT in Hrs**
AAURFP1	Laga Bolo(OSZ)	Kijari	Oromiya Special Zone	LM- translucent	3.5	Yellow	2.5
AAURFP2	Laga Bolo	Kijari	Oromiya Special Zone	LM-translucent	3.0	Yellow	2.5
AAURFP3	Laga Bolo	Kijari	Oromiya Special Zone	LM-translucent	2.5	Yellow	2.9
AAURFP4	Alaltu	CholleSonkolle	NSHZDBR	LM-translucent	2	Yellow	3.2
AAURFP5	Alaltu	CholleSonkolle	NSHZDBR	LM-translucent	2.5	Yellow	3.0
AAURFP6	Alaltu	CholleSonkolle	NSHZDBR	LM- translucent	4	Yellow	3.3
AAURFP7	Alaltu	Mikiwa	NSHZDBR	LM-translucent	2.5	Yellow	3
AAURFP8	Sheno/Kimbibit	Zengo	NSHZDBR	LW-transparent	2	Yellow	3.4
AAURFP9	Sheno/Kimbibit	Zengo	NSHZDBR	LW-transparent	2	Yellow	3.5
AAURFP10	Sheno/Kimbibit	Zengo	NSHZDBR	L W-transparent	3	Yellow	4
AAURFP11	Sheno/Kimbibit	GolbafKafelo	NSHZDBR	LM-translucent	3.5	Yellow	2.8
AAURFP12	Sheno/Kimbibit	MogorofGaraDaga	NSHZDBR	LM-translucent	2.5	Yellow	3.2
AAURFP13	Sheno/Kimbibit	Ontu	NSHZDBR	LM-translucent	4	Yellow	3.1
AAURFP15	Sheno/Kimbibit	Aragesa	NSHZDBR	LW-transparent	2	Yellow	4
AAURFP20	Sululta	MoyeGajo	Oromiya Special Zone	L W-transparent	3	Yellow	2.4
AAURFP29	Sululta	Gorfo	Oromiya Special	L W-transparent	3	Yellow	2.4

Isolates	**Sample Sites(Woredas)**	**Kebele**	**Zone**	**Colony Characteristics**	**Colony Size(mm)**	**YEMABTB**	**MGT in Hrs**
			Zone				
AAURFP32	D/Libanos	Wakene	NSHFR	L W-transparent	3.5	Yellow	3.4
AAURFP35	Ejere(AR)	Chirri	West Showa	LM-translucent	4	Yellow	2.6
AAURFP36	Ejere	Chirri	West Showa	LM-translucent	4	Yellow	3.2
AAURFP37	Ejere	Kimoye	West Showa	LM-translucent	4.5	Yellow	3.2
AAURFP38	Ejere	Kimoye	West Showa	L W-transparent	4.5	Yellow	2.4
AAURFP39	Dandi	Golole Bolo	West Showa	LM-translucent	4	Yellow	2.6
AAURFP40	Dandi	Golole Bolo	West Showa	LW-transparent	2.5	Yellow	3
AAURFP41	Dandi	Golole Bolo	West Showa	LM-translucent	4.5	Yellow	3.8
AAURFP42	Ambo	Amaro	West Showa	LM-translucent	4.5	Yellow	3.4
AAURFP43	Ambo	Amaro	West Showa	LM- translucent	4	Yellow	2.8
AAURFP44	Ambo	Amaro	West Showa	LM-translucent	2	Yellow	3.6

Key: OSZ=Oromiya Special Zone, NSHZDBR=North Showa Zone Debre Birhan Route, NSHFR= North Showa Fitche Route

5.2. Physiological characteristics

5.2.1. Carbohydrate utilization

All isolates utilized glucose, sucrose and D-manitol and maltose as the sole source of carbon, and many isolates grew on YEMA medium containing galactose and glycerol (85%), arabinose (78%), lactose and sorbitol (74%), tartarate (70%) (data not shown). They were limited to grow on starch (33%), citrate (22%) and cellulose (11%) (Figure 3). AAURFP39 utilized all the carbohydrates used followed by AAURFP37 and AAURFP9, respectively (data not shown).

The general pattern of carbon utilization showed that the isolates were capable of utilizing the majority of the carbon sources indicating that the isolates were adaptable in assimilating different carbon sources and heterotrophically competent that ensured their capability to survive in the soil. These results were similar with the previous works that rhizobial isolates from Shewa, Gojam, Gondar, Wollo and Tigray (Aregu *et al*., 2012) and southern Tigray (Fano, 2010) able to utilize diverse carbohydrate sources.

Those isolates which utilized starch, citrate and cellulose may produced their own enzymes which helped them to catabolize these carbon sources. These isolates will be capable of competing with other enzyme producing organisms in the soil if inoculated to field pea on field trials.

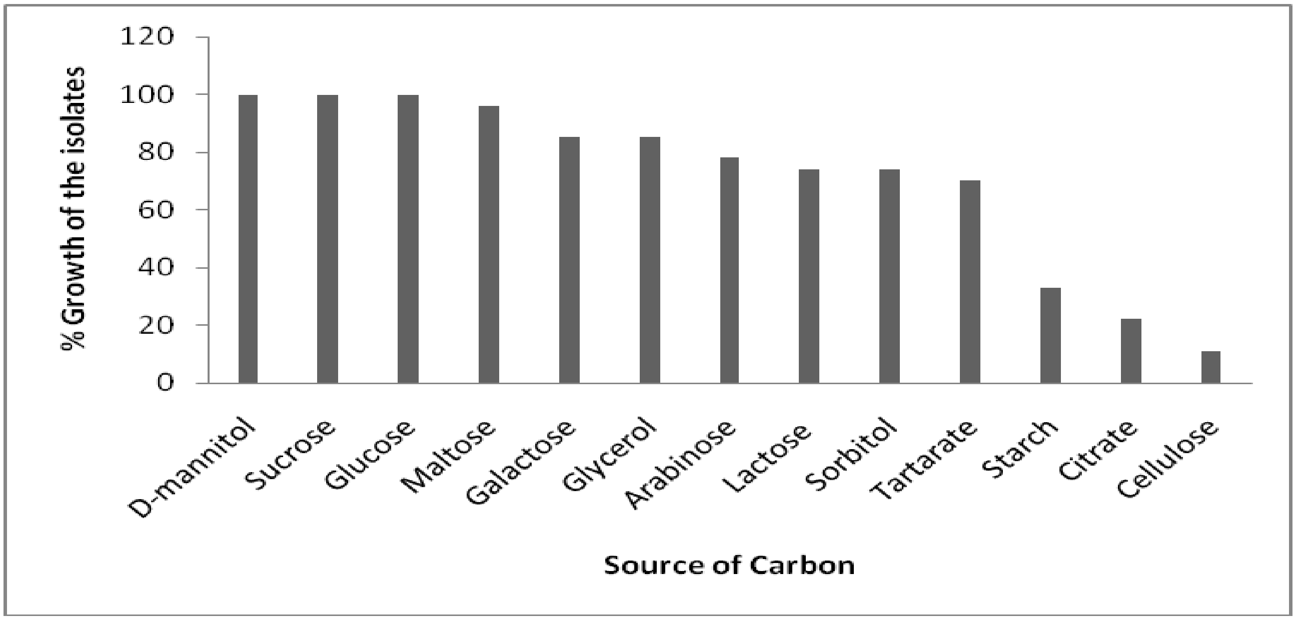


Figure 3. The pattern of carbohydrate utilization by isolates

5.2.2. Amino acid utilization

Almost all of the isolates utilized the amino acid tryptophan as a source of nitrogen (96%) followed by lysine (93%), glutamate (85%), methionine (81%), l-asparagines and tyrosine (70%) and few isolates utilized l-arginine (Figure 4). Almost, 22% of the isolates (AAURFP1, AAURFP13, AAURFP15, AAURFP32, AAURFP36 and AAURFP39) utilized all the amino acids, whereas AAURFP37 utilized only L-lysine (data not shown).

Similarly, Fanno (2010) has reported that 40% of the *rhizobial* isolates utilized all the amino acids and many did metabolize tryptophan, glutamate, lysine and methionine as source of nitrogen.

None of the isolates utilized inorganic phosphate in this study and the same result was indicated by Fano (2010), whereas Alikahani *et al*., (2006) reported that *Rhizobium leguminosarum biovar Viciae* from *Pisum sativum* produced large halo (clear) zone characteristics of phosphate solubilization.

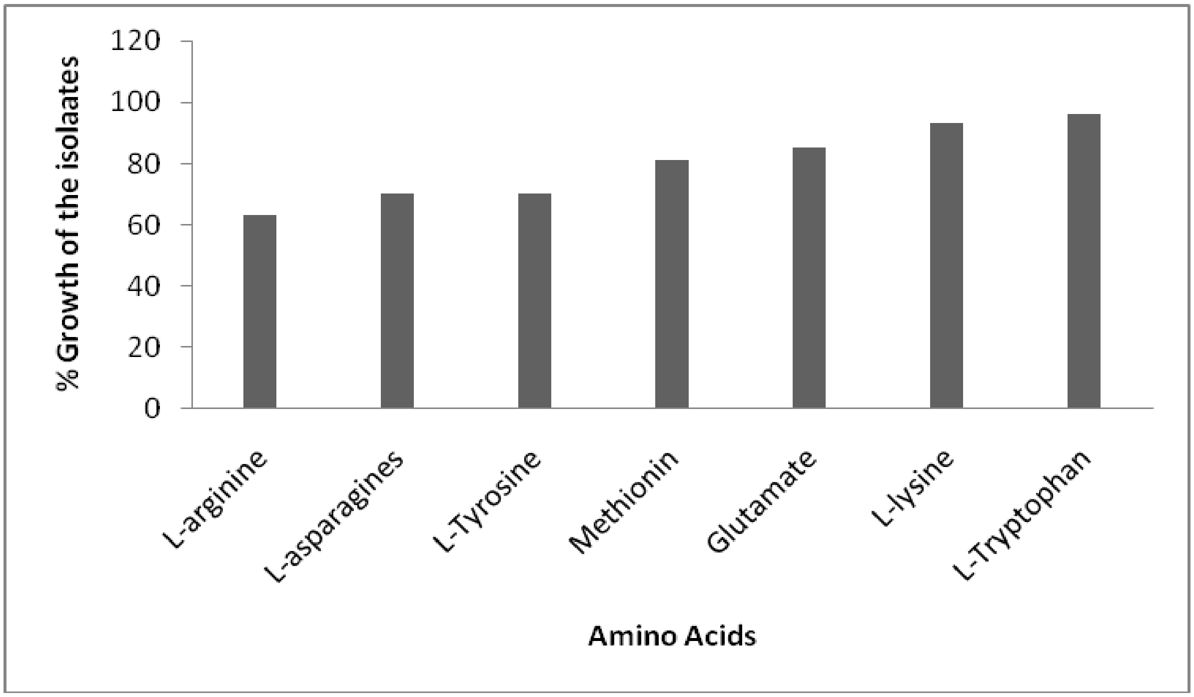


Figure 4. Amino acid utilization ability of the isolates

Eco-physiological characteristics

5.2.3. Acid and alkaline (pH) tolerance

Almost all of the isolates were tolerant to pH 5-9.0 (Figure 5). About 59% of the isolates grew at pH 4.5, whereas 41% of the isolates grew at pH 9.5 but no growth was observed at pH 4.0 and 10.0. Isolates AAURFP2, AAURFP3, AAURFP6, AAURFP8, AAURFP29, AAURFP35, AAURFP36, AAURFP37, AAURFP38 and AAURFP39 showed a wide range of pH tolerance (data not shown).

This result indicated that almost more than half of the isolates (59%) were able to survive in acidic media and about 41% of the isolates were with the capacity to resist basic media whereas about 37% of the isolates were able to adapt in acidic basic and neutral media (Figure 5).

Similar reports indicated by (Kassa *et al*., 2015) 4.5 to 9.0 pH ranges; (Aregu *et al*., 2012) 5 to 9.5 pH range (Fanno, 2010) demonstrated that 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.

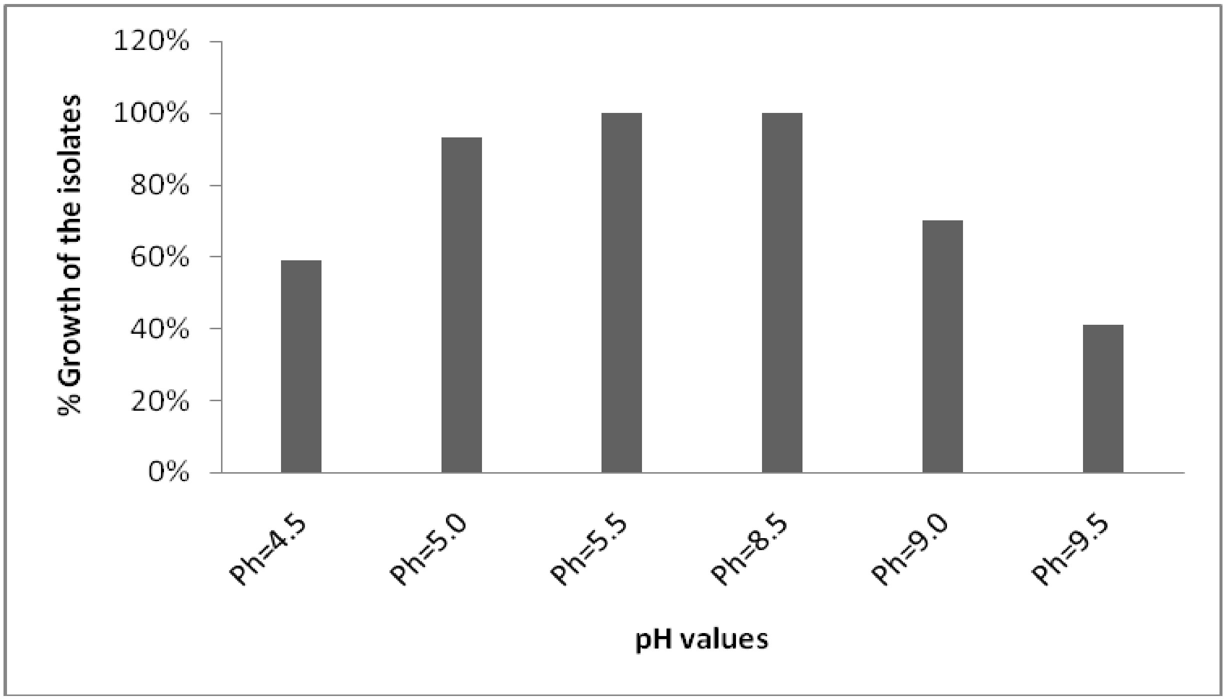


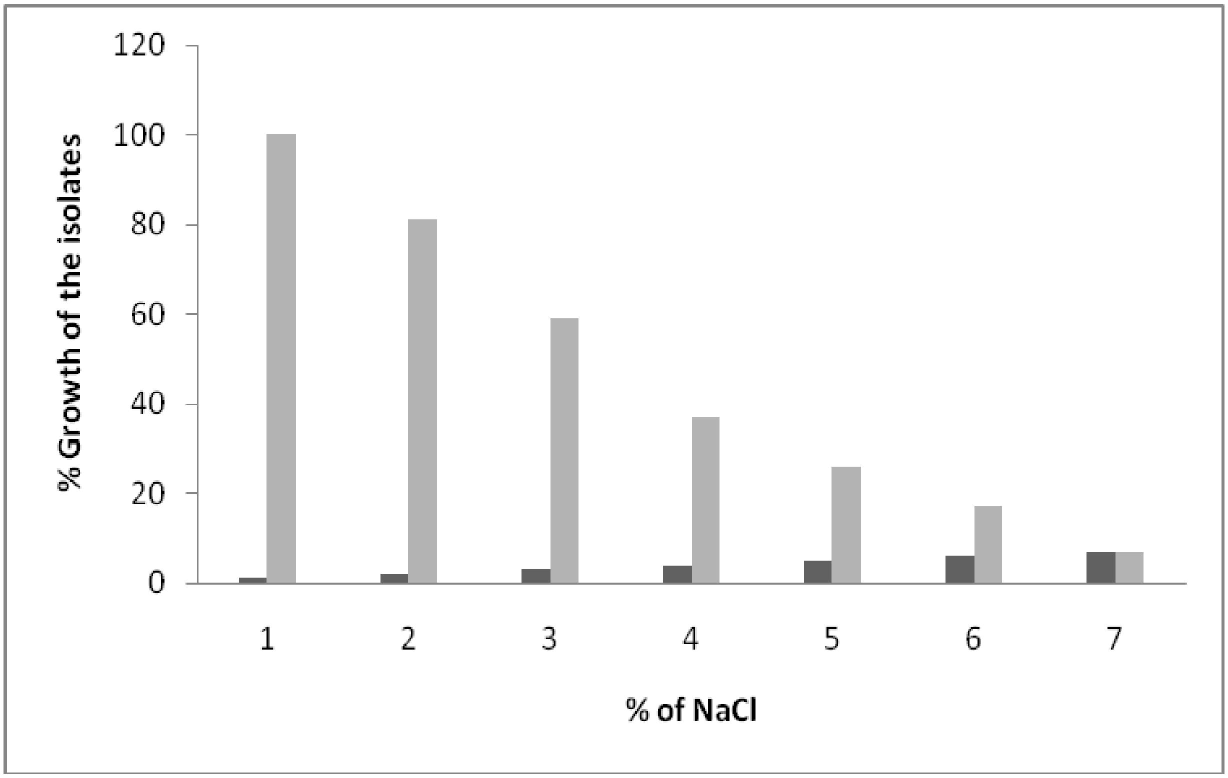
Figure 5. pH tolerance of the isolates

5.2.4. Salt tolerance

The isolates showed variations in tolerance to different concentrations of salt (Figure 6). All of the isolates (100%) were grown at salt concentration of 1%, whereas 81% grew at salt concentration of 2% and 59% of the isolates grew at 3% salt concentration. Only 17% and 7% of the isolate were grown at 6% and 7% salt concentration, respectively which showed that as salt concentration increased tolerance of the isolates decreased.

This result indicated that all the isolates were tolerant to 1% NaCl, and a few isolates were very tolerant up to 7% salt concentration (Figure 6) which implied that the isolates varied in salt tolerance. Those isolates grew at salt concentration of 3%, 4%, 5%, 6% and 7% were able to compete in salt stressed soils. Similar report was given from earlier work on the isolates of field pea by Fanno, (2010) who has stated that 12% of the isolates were found to grow on YEMA medium containing (7%) NaCl and all the isolates grew at salt concentration of 1%.

However, Aregu *et al*. (2012) have reported that most of the isolates (80%) were sensitive to salt as they were limited to grow at 0.1% NaCl, whereas only 20% of the isolates were resistant to 0.52% salt concentration. In addition, Kassa *et al*. (2015) have stated that the salt tolerance of the test isolates showed that all the isolates displayed growth only at 0.1% NaCl concentration of the growth medium. However, all the isolates were sensitive to 0.5, 1 and 1.5% NaCl concentrations.



.

Figure 6. Salt tolerance test of the isolates grown on YEMA media and incubated at 28 ^{0}C

5.2.5. Temperature tolerance

All the isolates were found to grow at incubation temperature of 25^{0}C -35^{0}C, and a number of them (44%) also grew at 20^{0}C and 26% grew at 15^{0}C, but only 15% of the isolates were tolerant to 40^{0}C (Figure 7). This result indicated that even though all of the isolates were grown at optimum temperature (25^0C-35^0C), there were few isolates which resist temperature stressed environments (those which grown at (15^0C, 20^0C and 40 ^{0}C; Figure 7).

Similarly, Fanno, (2010) has reported that all the isolates grew at 25 ^{0}C to 35^0C but none of the isolates grew at 40^0C. This is similar to the work of Naeem and Yusuhaeef (2008) who have reported that *Pisum sativum* rhizobia from Pakstan grew between temperatures of 20 and 35^0C. Furthermore Kassa *et al*. (2015) have showed that all of the tested isolates grew well within 20-40^0C. However, all of the isolates (100%) were not tolerant to low temperatures (4^0C and 10^0C) likewise Aregu *et al*. (2012) have demonstrated that all the isolates were found to grow within 20-35^0C, which is the optimum growth temperature for most strains of *rhizobia* and 20% of the isolates grew at 5^0C - 38^0C.

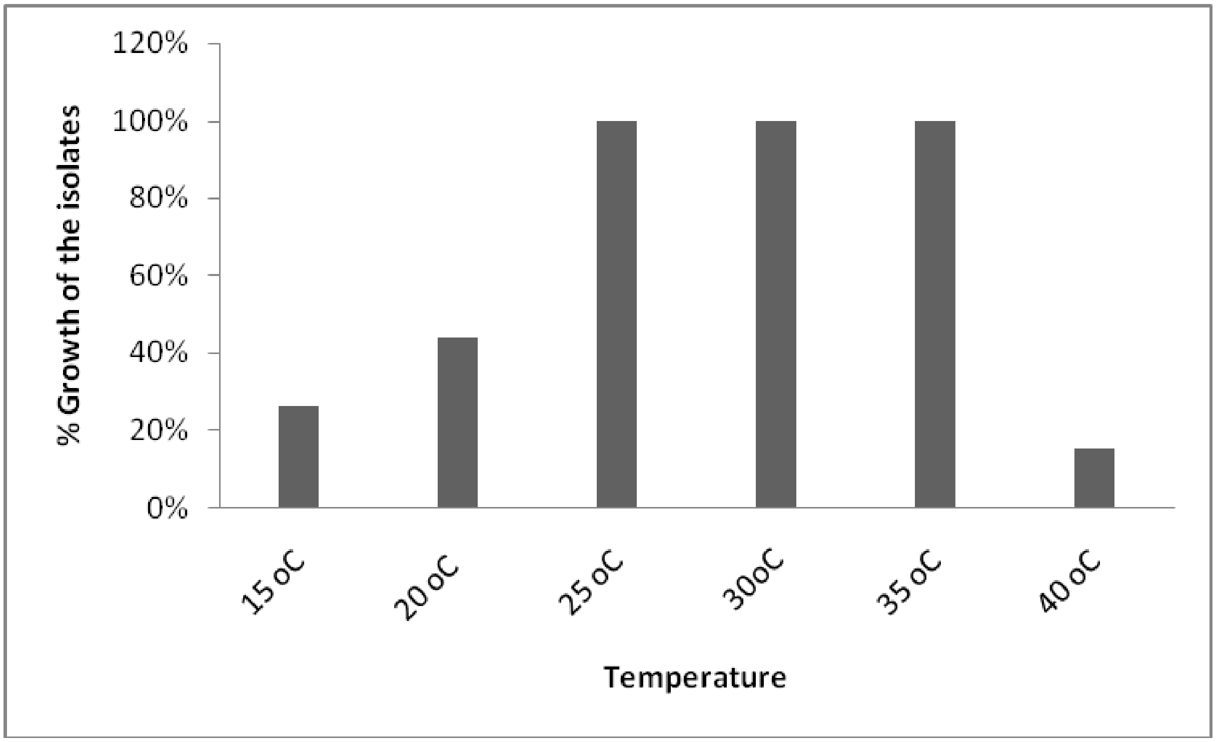


Figure 7. Temperature tolerance test of the isolates

5.2.6. Intrinsic antibiotic resistance (IAR)

The isolates were diverse in tolerance to different types and concentrations of antibiotics (Table 2). Generally, most of the isolates were tolerant to erythromycin (75%), penicillin (71%), ampicillin (58%) and chloramphenicol (42%), but sensitive to gentamycin, tetracycline, and high concentration of chloramphenicol (Table 2). Isolates AAURFP3, AAURFP6, AAURFP35, AAURFP36, AAURFP37, AAURFP38, AAURFP39 and AAURFP43 were the most resistant of all the isolates to different antibiotics, whereas isolates AAURFP2, AAURFP10, AAURFP11, AAURFP12, AAURFP13, AAURFP20, AAURFP29, AAURFP32, AAURFP42 and AAURFP44 were the most antibiotic sensitive strains (data not shown).

Similar report from Fanno (2010) has indicated that 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) who have showed that pea rhizobia from soils of eastern Washington grew well on the same type and concentration.

A report on intrinsic antibiotic resistance test pattern on faba bean rhizobia by Assefa *et al*., (2010) also reported on penicillin resistant faba bean rhizobia, from Wollo, Northern Ethiopia showed that the isolates were sensitive to gentamycin and tetracycline. Report from Egypt also showed that gentamycin has the most suppressive effect on *Rhizobium leguminosarum biovar viciae* (Husney *et al*., 2005). There was also report on field pea Rhizobium leguminosarum *biovar viciae* from different parts of Ethiopia which also showed that the isolates tolerate chloramphenicol (Aregu *et al*., 2012).

Table 2. Intrinsic antibiotic resistance of the isolates

	% of resistant isolates to antibiotics		
Antibiotics	2.5 *µg/ml*	5 *µg/ml*	10 *µg/ml*
Ampicillin	89%	67%	19%
Tetracycline	15%	11%	0%
Penicillin	96%	81%	37%
Gentamycin	15%	7%	0%
Chloramphenicol	89%	26%	11%
Erythromycin	93%	85%	48%
Total average %	66	46	19

5.3.5. Heavy metal resistance

Almost all the isolates grew on YEMA medium containing Al (89%) and most of the isolates were resistant to Mn (81%) whereas only about half of the isolates were resistant to Zn (56%) and few of them were resistant to Co (37%), Cu (26%) but almost all the isolates were sensitive to Hg except AAURFP35, AAURFP36 and AAURFP39 (11% resistant) (Table 3).

AAURFP2 and AAURFP12 resisted all the tested heavy metals except Zn and Hg. AAURFP3 and AAURFP4 resisted all the tested heavy metals except Hg and Cu. AAURFP7 and AAURFP38 resisted all but Hg and Co. AAURFP35, AAURFP36 and AAURFP39 resisted all but Co and Cu (Table 3).

Similarly Aregu *et al*., (2012) has reported that the isolates were resistant to manganese, and sensitive to copper chloride, zinc chloride and mercuric chloride. But Kassa *et al*., (2015) has reported in another way that evaluation of the intrinsic resistance to heavy metals showed that all the tested isolates exhibited high resistance to ZnCl and MnCl.

The effectiveness of isolates under controlled environment may not necessarily be correlated to their performance in the field because of the environmental factors that manage the process of nitrogen fixation (Sanginga *et al*., 1995). Therefore, it is essential to assess the effectiveness of the different isolates in relation to their performance in the field and tolerance to different eco-physiological factors, to use them as microbial inoculants. Although most of the *rhizobial* isolates from the different sampling sites were effective and very effective in nitrogen fixation of field pea, all of them may not be competitive if they are inoculated in the soil under different environmental stresses (Aregu *et al*., 2012).

Table-3- Heavy metal resistance of field pea rhizobial isolates obtained from different parts of Central Showa, Ethiopia

Heavy metals	Concentration (μgml^{-1})	Resistant isolates (%)	Resistant isolates
AlK(SO4)3.12H2O	250	89	All but AAURFP11, AAURFP20 and AAURFP29
$ZnCl_2$	100	56	AAURFP3, AAURFP4, AAURFP5, AAURFP6, AAURFP7, AAURFP8, AAURFP9, AAURFP10, AAURFP35, AAURFP36, AAURFP37, AAURFP38, AAURFP39, AAURFP41 and AAURFP43
$HgCl_2$	10	11	AAURFP35, AAURFP36 and AAURFP39
$MnCl_2$	500	81	All but AAURFP1, AAURFP20, AAURFP29, AAURFP32 and AAURFP42
$CoCl_2$	20	37	AAURFP1, AAURFP2, AAURFP3, AAURFP4, AAURFP11, AAURFP12, AAURFP29, AAURFP40, AAURFP42 and AAURFP44
$CuCl_2$	100	26	AAURFP2, AAURFP7, AAURFP12, AAURFP20, AAURFP32, AAURFP38, AAURFP42

5.4. Numerical Analysis

The physiological traits studied indicated wide diversity among these *rhizobia* and the dendogram obtained from the numerical analysis of (27 isolates) on the basis of phenotypic traits categorized into four distinctive clusters (Figure 8).

Similarly, Ahmed Seid (2010) on chickpea (*Cicer arietinum*) has reported that the twenty seven isolates were clustered in to 4 groups based on the phenotypic features used in physiological test. In the same way, Musa Adal (2009) on grass pea (*Lathyrus sativus*) has reported that the numerical analysis based on phenotypic features allowed the grouping of all isolates in to four similarity groups plus one ungrouped isolate whereas Wubayehu Gebremedhin (2016) on chickpea has indicated that the dendogram obtained from the numerical analysis of phenotypic traits placed the strains in five distinctive clusters.

Cluster I was the largest with 15 (AAURFP1, AAURFP2, AAURFP3, AAURFP4, AAURFP5, AAURFP8, AAURFP9, AAURFP10, AAURFP11, AAURFP12, AAURFP13, AAURFP15, AAURFP40, AAURFP42 and AAURFP44) isolates originating from various locations. The isolates exhibited all levels of effectiveness even though the highest effective isolates were dominantly included in this cluster. All the isolates in Cluster I commonly shared characteristics such as their potential to resist ampicilline, penicilline, erythromycin, Al and Mn in their growth media. They also had ability to catabolize D-mannitol, sucrose, glucose and maltose as well as L-tryptophan as their carbohydrate and amino acid sources, respectively. All these isolates were tolerant to salt concentration of 1% and unable to grow at (5-7) % salt. On the other hand, all of them were not able to grow at high temperatures (40^0C and 45^0C).

Strains belonging to cluster II (AAURFP6, AAURFP7, AAURFP35, AAURFP36, AAURFP37, AAURFP38, AAURFP39, AAURFP41 and AAURFP43) had an average and high effectiveness except AAURFP7 which had low effectiveness. This cluster commonly shared catabolization of almost half of the tested carbohydrates, had Mn resistance from heavy metals and most of them resisted ampicilin, penicillin and erythromycin from antibiotics. They showed better growth at 15^0C (78% grown) and all of them grew at a temperature of (20^0C-35^0C). This cluster was sensitive to pH4 and pH10, 7% salt concentration (except AAURFP37 and AAURFP39) and high temperatures 40^0C (15%) and 45^0C.

Cluster III contained two (AAURFP20 and AAURFP32) fast-growing isolates originating from different locations. They displayed good symbiotic efficiency, catabolized almost all the amino acids used and utilized almost 50% of the carbohydrates used. This cluster was sensitive to most of the antibiotics and heavy metals used.

Cluster IV (AAURFP29) utilized more than half of the tested carbohydrates and N sources. Sensitive to pH 4 and 10, and also susceptible to most of the antibiotics and heavy metals tested, whereas grew at a temperature of 25-35^0C and resisted salt concentration of only 1 and 2%.

This numeric analysis showed the clustering of isolates did not correlate with their geographical origin because the isolates from same origin were found in different clusters and isolates from different areas were included in the same group (Figure 8).

Similarly Aklil Gebremedhin (2016) has showed that numerical analysis of field pea isolates based on different phenotypic characteristics puts the isolates into diverse groups. Additionally, Wubayehu Gebremedhin (2016) has indicated that the numeric analysis of chickpea *rhizobial* strains showed that the clustering of isolates did not correlate with their geographical origin since isolates from same origin were included in different clusters and vice versa.

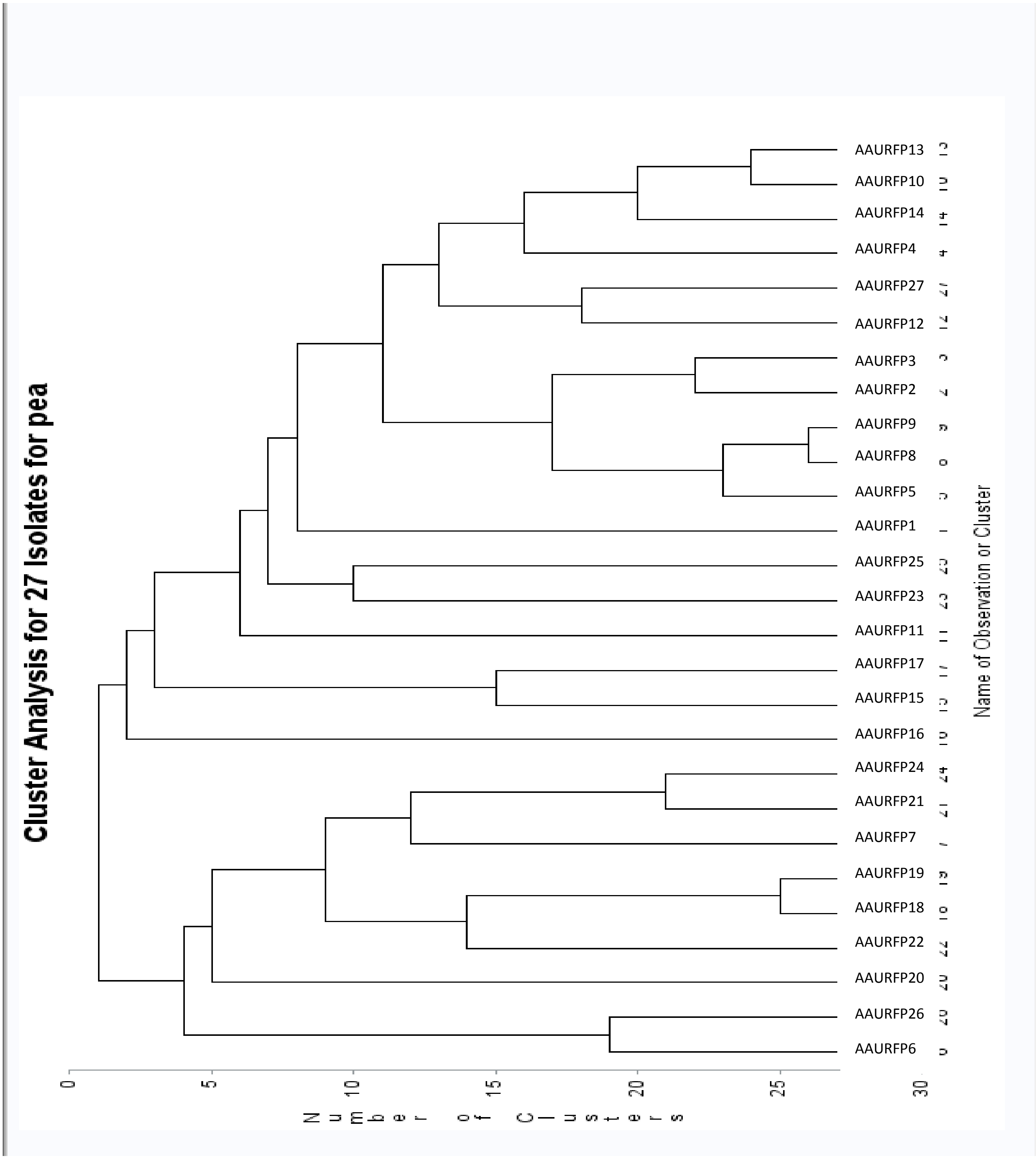


Figure 8. Dendrogram highlighting the phenotypic similarity among the test isolates

5.5. Symbiotic effectiveness (SE) test on sand culture

All the 27 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 compared to negative control (Figure 9).

Negative control at harvest date

positive control at harvest date

Inoculated plants at harvest date

Inoculated plants at harvest date

Inoculated plant at harvest date

Inoculated plant at harvest date

Inoculated plants and their nodules

Inoculated plants and their nodules

Figure 9. Comparison of negative control, positive control and some inoculated plants

The inoculated plants also showed differences in nodule number; nodule dry weight and shoot dry weight (Table 5). The isolates induced nodulation with nodule numbers ranging from 41 to 150 nodules per plant (Figure 10).

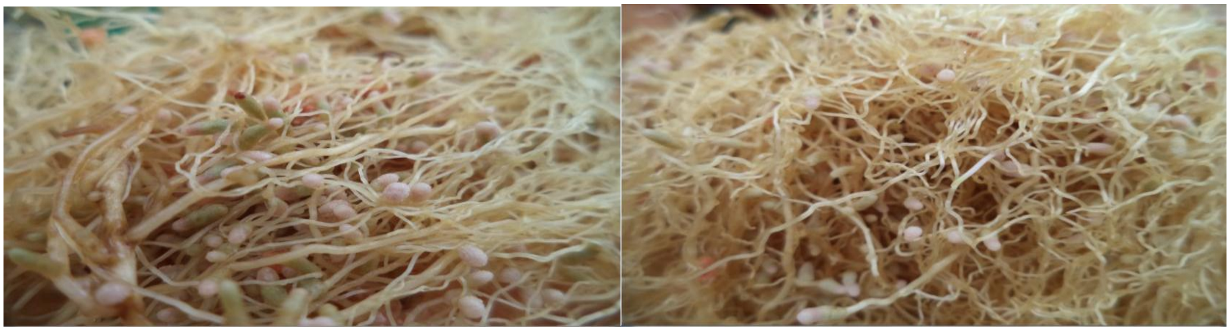


Figure 10. Nodule numbers per plant ranging from 41 (AAURFP9) to 150 (AAURFP39)

The isolates also showed variation in nodule dry weight between that ranged from 0.02(AAURFP9 and AAURFP15)-0.13(AAURFP39) g/plant (Table-4). The highest shoot dry weight (1.7 g/plant) was recorded from the plant inoculated with AAURFP39, whereas the least shoot dry weight of (0.33 g)/plant was obtained from plants nodulated by isolates: AAURFP8, AAURFP 9, AAURFP 11, AAURFP 20 and AAURFP 32 (Table 4).

Table-4-Symbiotic performance of the isolates

No	Isolates	NN/plant	NDW (gm/plant)	SDW (gm/plant)	SE (%)	Rate
1	AAURFP1	77.667d	0.05667edc	0.70000ihg	68.9	E
2	AAURFP2	75.667ed	0.05667edc	0.62000ih	62.2	E
3	AAURFP3	66.333egdf	0.04333ed	0.77667efg	75.6	E
4	AAURFP4	69.333edf	0.04333ed	0.87667ed	86.7	HE
5	AAURFP5	66.000egdf	0.05667edc	0.60000ih	57.8	E
6	AAURFP6	65.667egdf	0.04333ed	0.57667i	56.7	E
7	AAURFP7	66.667egdf	0.04333ed	0.67667ihg	65.6	E
8	AAURFP8	55.667eghf	0.04333ed	0.35333j	35.6	LE
9	AAURFP9	44.333h	0.02333ef	0.34333j	34.4	IE

10	AAURFP10	68.667edf	0.05667edc	1.01000d	101.1	HE
11	AAURFP11	79.000d	0.07000bdc	0.35333j	35.6	LE
12	AAURFP12	69.333edf	0.04333ed	0.87667ed	86.7	HE
13	AAURFP13	97.667c	0.08000bc	1.20000cb	117.8	HE
14	AAURFP15	44.667h	0.02333ef	0.85333ef	33.33	IE
15	AAURFP20	53.000ghf	0.03000ef	0.33000j	66.7	E
16	AAURFP29	65.667egdf	0.04333ed	0.58667i	58.9	E
17	AAURFP32	47.000gh	0.03000ef	0.33000j	33.3	IE
18	AAURFP35	69.333edf	0.04333ed	0.60000ih	58.9	E
19	AAURFP36	71.667edf	0.04333ed	0.60000ih	58.9	E
20	AAURFP37	68.667edf	0.05667edc	0.72000ihfg	71.1	E
21	AAURFP38	119.000ba	0.10000ba	1.01000d	101.1	HE
22	AAURFP39	133.333a	0.12000a	1.64333a	162.2	HE
23	AAURFP40	79.000d	0.07000bdc	0.75333ehfg	75.6	E
24	AAURFP41	111.333bc	0.09000bac	1.32000b	131.1	HE
25	AAURFP42	99.667c	0.09000bac	1.22000cb	122.2	HE
26	AAURFP43	110.000bc	0.09000bac	1.16667c	113.3	HE
27	AAURFP44	75.667ed	0.08000bc	0.57667i	56.7	E
28	+ve control	0.000i	0.00000f	1.00000d	100	HE
29	-ve control	0.000i	0.00000f	0.57667i	0	IE

Legend - NN/plant= Nodule Number per plant NDW(g/plant)= Nodule Dry Weight in gram per plant SDW(g/plant)= Shoot Dry Weight in gram per plant SE=Symbiotic Effectiveness HE= Highly Effective E= Effective LE= Less Effective IE= Ineffective.

Means with the same letter in the same column are not significantly different.

A moderate correlation was observed between nodule number and nodule dry weights with correlation coefficient (r^2= 0.4096) value of (r= 0.64) and low correlation (r^2=0.2704) with value of (r=0.52) between nodule number and shoot dry weight. Whereas a strong correlation with correlation coefficient (r^2= 0.5776) value of (r= 0.76) was obtained between nodule dry weights and shoot dry weight (Table 5).

Table-5- Correlating table of nodule number, nodule dry weight and shoot dry weight

Variables	Nodule No	Nodule dry Wt	Shoot dry Wt
Nodule number	1	0.64	0.52
Nodule dry weight		1	0.76
Shoot dry weight			1

The symbiotic effectiveness (SE) of inoculated plants in relation to the positive and negative control plants showed difference (p=0.05) from the lowest effective isolates (AAURFP15) and (AAURFP32) having SE of about 33% to that of the most effective isolate AAURFP39 with symbiotic effectiveness rate of above 100% (Table 4) under greenhouse conditions.

Generally about 34% of the isolates were highly effective, 48% effective, 7% low effective and 11% ineffective based on their Symbiotic effectiveness. Thus, 82% of the isolates displayed symbiotic efficiency of very effective and effective level that were able to accumulate 50-100% of shoot dry weight compared to the nitrogen-fertilized control.

Previously there were some studies on nodulation and symbiotic effectiveness of field pea nodulating *Rhizobia* for different parts of Ethiopia. These reports showed that there were clear differences in nodule number, nodule dry weight and shoot dry weight (Table 6).

Table 6. Symbiotic effectiveness comparison among the present and previous studies

Crop	Variety	NNR/pl	NDWR(g/pl)	ShDWR	SER(%)	SE (%)	Reference
Field Pea	Burkitu	41-150	0.02-0.13	0.33-1.7	33-162	81	Present study
Field Pea	Markose	29-108		0.68-3.35	27-133	76	Aregu *et al*., 2012
Field Pea	Addi	25-93	0.03-0.09	0.24-1.76	15-112	67	Fanno, 2010

Where: NNR/pl= Nodule number range per plant, NDWR(g/pl)= Nodule dry weight range in gram per plant, ShDWR= Shoot dry weight range, SER(%)= Symbiotic effectiveness range in percent, SE (%)= Symbiotic effectiveness in percent

The data showed that AAURFP10, AAURFP13, AAURFP38, AAURFP39, AAURFP41, AAURFP42, AAURFP43 were the most effective isolates based on their symbiotic effectiveness (greater than 100%) under greenhouse conditions (Table 4). But based on their eco-physiological properties: AAURFP6, AAURFP35, AAURFP36, AAURFP37, AAURFP38, AAURFP39, AAURFP41 and AAURFP43 were the best tolerant isolates (Table 7).

Table 7. Summary of Eco-physiological and symbiotic properties of the isolates

Isolate	Tolerated pH levels	Tolerated Temp. (^{0}C)	Tolerated salt conc. (%)	Utilized N source (%)	Utilized C source (%)	Tolerated IAR (%)	Tolerated IHMR (%)
AAURFP1	5-9.5	20-35	43	100	62	50	33
AAURFP2	4.5-9.5	25-35	43	86	77	44	67
AAURFP3	4.5-9.5	25-35	29	86	62	63	67
AAURFP4	5-9	25-35	14	71	69	50	67
AAURFP5	5-9	25-35	57	71	69	56	50
AAURFP6	4.5-9.5	15-35	29	57	54	63	50
AAURFP7	5-9	20-35	71	86	77	50	67
AAURFP8	4.5-9	25-35	57	71	69	56	50
AAURFP9	4.5-9	25-35	57	71	85	50	50
AAURFP10	5.5-9	25-35	29	86	54	38	50
AAURFP11	5.5-9	25-35	14	86	77	38	33
AAURFP12	5-9.5	25-35	14	86	85	31	67
AAURFP13	5-8.5	25-35	29	100	77	38	33
AAURFP15	5-9.5	20-35	43	100	77	56	33
AAURFP20	4.5-9	25-35	14	86	62	24	17
AAURFP29	4.5-9.5	25-35	29	57	54	38	17
AAURFP32	5-9	25-35	43	100	54	25	33

AAURFP35	4.5-9.5	15-40	86	71	69	75	67
AAURFP36	4.5-9.5	15-40	86	100	77	70	67
AAURFP37	4.5-9.5	15-35	100	14	92	63	50
AAURFP38	4.5-9.5	20-40	86	86	62	63	67
AAURFP39	4.5-9	15-40	100	100	100	70	67
AAURFP40	5-8.5	25-35	43	57	69	44	50
AAURFP41	4.5-9	15-35	71	71	69	50	50
AAURFP42	4.5-8.5	20-35	14	86	69	38	50
AAURFP43	4.5-9	15-35	43	86	85	63	50
AAURFP44	4.5-9	25-35	29	86	69	38	50

Legend: IAR= Intrinsic Antibiotic Resistance, IHMR= Intrinsic Heavy Metal Resistance, Temp. (^{0}C) = Temperature in Degree Centigrade

Although the study was not exhaustive and limited to a number of isolates, dependent on one host variety (*Burkitu*), and to a few sampling sites of the central parts of the country, it gave an evidence that most of the pulse growing areas of the country harbored symbiotically effective rhizobia that nodulate field pea. The best performing isolates can then be recommended to enhance biological nitrogen fixation and field pea production. In general, if the selection of rhizobial isolates from pea were properly followed with the appropriate genetic, competitiveness, and environmental studies, the isolates can partly contribute to integrated soil fertility management in the low-input agriculture in the country.

Conclusion and Recommendations

This study shows the physiological and symbiotic diversity of *Rhizobium leguminosarum biovar Viciae* population on Field pea from Central Showa of Ethiopia under greenhouse condition. Some of the isolates (AAURFP35, AAURFP36, AAURFP37, AAURFP38 and AAURFP39, AAURFP41 and AAURFP43) showed important physiological characteristics such as resistance to antibiotics, high salt tolerance, high carbohydrate assimilation, optimum pH tolerance, and tolerate optimum temperature.

After field experiments under different environmental conditions, the isolates (AAURFP35, AAURFP36, AAURFP37, AAURFP38 and AAURFP39, AAURFP41 and AAURFP43) can be recommended as inoculants in the future. These listed isolates showed good physiological, eco-physiological and symbiotic characteristics under greenhouse and in laboratory conditions.

Since using bio inoculants reduces the adverse effects of chemical fertilizers, further researches and findings will be recommended in different parts of the country to get the novel rhizobial inoculants that nodulate field pea and effectively maximize the productivity of field pea.

6. References

Abdul Kabirkhan Achakzai (2007). Effect of various levels of nitrogen fertilizer on nodulation of pea cultivars. *Pak. J. Bot.*, **39**(**5**): 1673-1680.

Ahmed Seid (2010). Symbiotic Effectiveness and phenotypic Characterization of Rhizobia Nodulating Chickpea (*Cicer arietinum*) from Some growing areas of South and North Wollo zones. MSc Thesis. Addis Ababa University, Ethiopia.

Aira Päivöke (2003). Responses of *Pisum sativum* to soil Arsenate, Lead and Zinc greenhouse study of Mineral elements, Phytase activity, ATP and chlorophylls. Academic dissertation, University of Helsinki. Pp. 34-47.

Aklil Gebremedhin Meressa (2016). Characterizing of Symbiotic Effectiveness and Phosphate Solubilising Abilities of *Rhizobium* Nodulating Field pea (*Pisum Sativum var. Abyssinicum*) Isolated from Southern Tigray, Ethiopia. MSc Thesis. HaramayaUniversity, Ethiopia.

Alikhani, S., Rastin N. and Antoun H. (2006). Phosphate solublization activity, of Rhizobia Native to Iranian Soils .*J. Soil Microbiol* .**287**: 35-41.

Amargar, N., Macheret, V. and Aguerre, G. (1997). *Rhizobum gallicum sp. Nov*, and *Rhizobum giardinii sp. Nov*. from *Phaseolus vulgaris* nodules. *Int. J. syst. Bacteriol*. **47**: 996-1006.

Arayankoon T., Schomberg H. H. and Weaver R. W. (1990). Nodulation and N_2 fixation of guar at high root temperature. *Plant Soil*. 126: 209–213.

Aregu A., Fassil A. and Asfaw H. (2012). Symbiotic and phenotypic characterization of *Rhizobium* isolates of field pea (pisum Sativum L.) Fabaceae from central and southern Ethiopia. *Ethiop. J. Biol. Sci*. **11**(2): 163-179.

Assefa Keneni, Fassil Assefa and Prabu P.C. (2010). Characterization of acid and salt tolerant Rhizobial strains Isolated from Faba bean Fields of Wollo, Northern Ethiopia. *J.Agr.Sci.Tech*. **12**: 365-376.

Beattie, G. A; Clayton, M. K., and Handelsman,J. (1989).A rapid method for the isolation and identification of Rhizobium from root nodules. *Microbiol Methods* **9**: 29-33.

Benton, P. M. C; Sen., S. Peters, J. W. (2002). Nitrogenase structure. **In**: Nitrogen fixation at the millennium, pp. 35-68, (Leigh, G.J., ed.). Elsevier, Amsterdam.

Bernal, G. and Graham, P. H. (2001). Diversity in the Rhizobia associated with *Phaseolus vulgaris L*. in Ecuadore, and comparisons with Mexican bean *Rhizobia*. *Can. J. Microbiol*. **47**: 526-534.

Burns RC, Hardy RWF. (1975). Nitrogen fixation in bacteria and higher plants. Springer-Verlag, Berlin.

Chen, W., Wang E.,S.G; Li,Y; Chen,X.and Li.Y. (1995). Characterization of *Rhizobium tianshanense sp.nov*., a moderately and slowly growing root nodule bacterium isolated from an arid saline Enviroment in Xinjiang, People's Republic of China *J.App.Microbiol*. **45**: 153-159.

Cordovilla M. P., Ligero F. and Lluch C. (1994). The effect of salinity on N fixation and assimilation in *Vicia faba*. *J Exp Bot.* 45: 1483–1488.

Dixon, R. O. D., Wheeler, C. T. (1986). *Nitrogen fixation in plants*. Department of Soil and Crop Sciences, Texas A&M University, UK, pp. 234-278.

Fano Berhe (2010). Phenotypic and Symbiotic characteristics of Rhizobia nodulating field Pea (*Pisum sativum* L.) in southern Tigray, Ethiopia. MSc Thesis, School of Graduate Studies. Addis Ababa University.

FNCA Biofertilizer Project Group, (2006). Biofertilizer Manual. Forum for nuclear cooperation in Asia (FNCA). Japan Atomic Industrial Forum (JAIF).

Giller, K. E. and Wilson, K. J. (1991). *Nitrogen fixation tropical cropping systems*. C. A. B. international, Wallingford, USA. PP. 423.

Gisele, L., Geraldine, D., Virgine, B. and Gerard, D. (2007). *Rhizobium leguminosarum bv. viciae* genotypes interact with pea plants in developmental responses of nodules, roots and shoots. *New phytologist* **176**: 680–690.

Grusak M. A. (2000). Strategies for improving the iron nutritional quality of seed crops: Lessons learned from the study of unique iron-hyper accumulating Pea mutants. *Pisum genet* .**32**: 1-6.

Hill S. (1992). Physiology of nitrogen fixation in free living hetro-trophs. **In**: *Biological nitrogen fixation*. Stacey G.,Burris R. H.,Evans H. J.(eds).New York, Chaman and Hall: pp. 87-134.

Hungria, M. and Franco A. A. (1993). Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. *Plant Soil*. 149: 95–102.

Husney, M.A.K.H.and Fatma H.A., (2005). Ecological studies on soil indigenous rhizobia in Egypt. *J.Biol.Sci*. **6**: 67-76.

ICRISAT, CIAT, ICARDA, and IITA, (2012). Leveraging legumes to combat poverty, hunger, malnutrition and environmental degradation. CGIAR Research program on Grain Legumes.

Jordan, D. C., (1984). Family III. Rhizobiaceae. **In:** *Bergey's Manual of Systematic Bacteriology*, pp.234-254, (krieg, N. R. and Holt, J. G. eds). The Williams and Wilkins, Baltimore.

Kassa B., Ameha K. and Fassil A., (2015). Isolation and Phenotypic Characterization of Field Pea nodulating Rhizobia from Eastern Ethiopia Soils. *World Appl. Sci. J.,* **33** (**12**): 1815-1821.

Kemal Ali (2006). Insect pest management research of faba bean and field pea in Ethiopia. **In**: Food and Forage Legumes of Ethiopia: Progress and Prospects, pp. 247-259 (Kemal Ali, Seid Ahmed, Beniwal, S., Gemechu Keneni, Malthora, R.S., Makkouk, K. and Haila, M.H., eds.). ICARDA, Addis Ababa.

Levetin-McMahon (2008). Plants and Society, Fifth Edition. Legumes. The McGraw-Hill Companies.

Lupwayi, N. and Haque, I. (1994**).** *Legume-Rhizobium Technology Manual*. Environmental Sciences Division International Livestock Center for Africa. Addis Ababa, Ethiopia.pp. 1-93.

Maatallah, J., E.B. Berrah, J. Sanjuan and C. Lunch. C. (2002). Phenotypic characterization of *Rhizobia* isolated from chickpea (*Cicer arietinum*) growing in Moroccan soils. *Agron.* **22**: 321-329.

Marie A. S. (2001). Exploring nitrogen fixation and assimilation in symbiotic Field pea root nodules, by in vivo ^{15}N NMR Spectrophotometery. Ph.D.Thesis, Roskiilde University, Roskilde, Denmark.Pp. 30-43.

Michiels J, Verreth C, Vanderleyden J. (1994). Effects of temperature stress on bean nodulating *Rhizobium* strains. *Appl Environ Microbiol.* 60:1206–1212.

Mihaela B., Sanja S., Darko U., Dubravko M., Sulejman R. (2007). Genotypic Characterisation of Indigenous *Rhizobium leguminosarum bv. viciae* Field Population in Croatia. *Agric. conspec. Sci.* **72**(2): 153-158.

Munevar F., Wollum A. G. (1982). Response of soybean plants to high root temperature as affected by plant cultivar and *Rhizobium* strain. *Agron J.* 74: 138–142.

Musa Adal (2009). Phenotypic and Symbiotic characterization of grass pea (*Lathyrus sativus*) rhizobial isolates from some major growing areas of South Wollo and West Shoa, Ethiopia. MSc Thesis. Addis Ababa University, Ethiopia.

Mussa Jarso, Tezera Welabu and Gemechu Keneni (2006). Review of field pea (*Pisum sativum*) genetics and breeding research in Ethiopia. **In**: Food and Forage Legumes of Ethiopia: Progress and Prospects, pp. 247-259 (Kemal Ali, Seid Ahmed, Beniwal, S.,Gemechu Keneni, Malthora, R.S., Makkouk, K. and Haila, M.H., eds.). ICARDA, Addis Ababa.

Naeem F.A and Yusuhaeef .F. (2008). *Pisum sativum-rhizobium* interactions under different Environmental stress. *Pak. J. Bot*., **6:** 260-261.

Nape Victoria Mothapo (2011). Nodulation and *Rhizobia* Diversity Associated with Distinct Hairy Vetch Genotypes. MSc thesis, North Carolina State University. Raleigh, North Carolina.

Ochman, H., Lerat, E. and Daubin,V. (2005).*Proc.Natu.Acad Sci*. **102**: 6595– 6599.

Oelke A. E., Oplinger S. E., Hanson V. C., Davis W. D., Putnam H. D., Fuller I. E., and Rosen J. C. (1991). Field Pea, University of Minnesota, St. Paul.

Ott, T. (2005). Functional Genomics of nodulins in the model Legume *lotus japonicas*. PhD. Dissertation, Postdam University ,Germany. Pp. 45-60.

Panda B., and Panda K. (2002) .Genotoxicity and mutagenicity of metals in plants **In**: Prasad MNV, Strzalka K (eds) Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants, pp. 395-414. Kluwer Academic Publishers, Dordrecht.

Peoples, M. B and Craswell, E. T. (1992). Biological nitrogen fixation: Investments, expectations and actual contributions to agriculture. J. *Plant Sci*.. **141**: 13-39.

Peoples, M. D. Heridge and J.Ladha **(**1995**).** BNF: An efficient source of nitrogen for Sustainable Agricultural production. *J. Soil Sci.* **174**: 3-8.

Piha M. I., Munnus D. N. (1987). Sensitivity of the common bean (*Phaseolus vulgaris* L.) symbiosis to high soil temperature. *Plant Soil*. 98: 183–194.

Purcino, H. M. A., Festin, P. M. and Elkan, G. H. (2000). Identification of effective strains of *Brady rhizobium* for *Archis Pintoi*. *Trop. Agric*. 77: 226-231.

Rao, D. C. N. and Sharama, P. C. (1995). Effectiveness of rhizobial strains for chickpea under salinity stress and recovery of nodulation on desalinization. *Int. J. Exp. Boil*. **33**: 500-504.

Rao, D., K. Giller, A. Yeo, and T. Flowers. (2002). The effects of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum*). *Annals of botany.* **89**: 563-570.

Rienke N. and Joke N. (2005). Cultivation of soya and other legumes. Agrodok 10, Wageningen, The Netherlands.

Roughley R. J. (1970). The influence of root temperature, *Rhizobium* strain and host selection on the structure and nitrogen-fixing efficiency of the root nodules of *Trifolium subterraneum*. *Ann Bot*. 34: 631–646.

Sanginga, N., Vanlaue, B. and Danso, S.K.A. (1995). Management of biological nitrogen fixation in alley cropping system: Estimation and contribution to N-balance. *Plant Soil* **174**: 119-141.

Society for General Microbiology (2002). *Rhizobium*, Root Nodules and nitrogen Fixation. *J. Microbiol*. **5**: 11-25.

Somasegaran, P. and Hoben, H. J. (1994**).** Handbook for Rhizobia. Methods in Legume-Rhizobium Technology. Springer-Verlag, New York, pp. 1-441.

Sprent J.I. and Raven J.A. (1985). Evolution of nitrogen fixing symbiosis. *Proceedings of Rural Sociol. Edin*. **85**:215-235.

Tamiru S., Lalit M., P. and Tsige A. (2012). Effects of Inoculation by *Bradyrhizobium japonicum* Strains on Nodulation, Nitrogen Fixation, and Yield of Soybean (*Glycine max L. Merill*) Varieties on Nitisols of Bako, Western Ethiopia. *ISRN Agronomy*, 2012 :2012.

Tate R. L. (1995). *Soil microbiology (symbiotic nitrogen fixation*) New York, N.Y: John Wiley & Sons, Inc.; pp. 307–333.

Turco,R., and Benzadicek, D. F. (1987). Diversity within two serogroups of *Rhizobium leguminosarum biovar viciae* native to soils in the Palouse of Eastern Washington, *Annu .Appl.Biol*. **20**: 29-33.

Viginie, B., Gisele, L., Geraldine, D., Anne-Sophie, V.,Christophe, S. and Gerard, D. (2007). Genetic Variability in Nodulation and Root Growth Affects Nitrogen Fixation and Accumulation in Pea. *Annals of Botany,* **100**: 589–598.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root-Nodule Bacteria.* IBP Handbook 15. Blackwell Scientific Publications, Oxford. pp. 164-190.

Vishal K. and Abhishek Ch. **(**2014). Isolation and Characterization of *Rhizobium leguminosarum* from Root nodule of *Pisum sativum* L. *J Academia and Industrial Research (JAIR)* **2**(8): 2278-5213.

Voisin, A., Salon C., Munier-Jolain, N. and Ney, B. (2002) .Effect of mineral nitrogen on nitrogen nutrition and biomass partitioning between the shoot and roots of pea (*Pisum sativum L*.). *J. Plant and Soil Sci*. **242**: 251-262.

Willems, A. (2006).The taxonomy of rhizobia : An overview. *Plant and Soil,* **287**: 3-14.

Wubayehu Gebremedhin (2016). Phenotypic and Symbiotic effectiveness of Chickpea (*Cicer arietinum* L.) Root nodulating Rhizobia from Some Selected parts of Southeastern Ethiopia. MSc Thesis. Addis Ababa University, Ethiopia.

Yadav, K. A. (2007). The Changing Face of Rhizobial Taxonomy .*Biofertiliser Newsletter*. **15**: 1-30.

Young, J., P. W., and Haukka, K .E. (1996). Diversity and physiology of rhizobia. *New Phytol.* **133**: 87-94.

Zahran H. H. (1999). *Rhizobium*-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. *Microbiol Mol Biol Rev*. **63**(4): 968–989.

7. Annexes

Annex -1 Carbohydrate utilization test

Isolates	**D-mannitol**	**Sucrose**	**Galactose**	**Maltose**	**Lactose**	**Glucose**	**Sorbitol**	**Starch**	**Citrate**	**Cellulose**	**Tartarate**	**Glycerol**	**Arabinose**
AAUR FP1	+	+	+	+	+	+	_	_	_	_	_	+	+
AAUR FP2	+	+	+	+	+	+	+	_	_	_	+	+	+
AAUR FP3	+	+	_	+	_	+	+	_	_	_	+	+	+
AAUR FP4	+	+	_	+	_	+	+	+	_	_	+	+	+
AAUR FP5	+	+	+	+	+	+	_	_	_	_	+	+	+
AAUR FP6	+	+	+	+	+	+	_	_	_	_	_	+	_
AAUR FP7	+	+	+	+	+	+	+	_	+	_	+	_	+
AAUR FP8	+	+	+	+	_	+	+	_	_	_	+	+	+
AAUR FP9	+	+	+	+	+	+	+	+	_	_	+	+	+
AAUR FP10	+	+	+	+	_	+	_	_	_	_	+	+	_
AAUR FP11	+	+	+	+	+	+	+	+	_	_	_	+	+
AAUR FP12	+	+	+	+	+	+	+	+	_	_	+	+	+
AAUR FP13	+	+	+	+	_	+	+	+	_	_	+	+	+
AAUR FP15	+	+	+	+	_	+	+	+	_	_	+	+	+
AAUR FP20	+	+	_	+	_	+	+	_	_	_	+	+	+
AAUR	+	+	+	_	+	+	_	_	_	_	_	+	+

Isolates	**D-mannitol**	**Sucrose**	**Galactose**	**Maltose**	**Lactose**	**Glucose**	**Sorbitol**	**Starch**	**Citrate**	**Cellulose**	**Tartarate**	**Glycerol**	**Arabinose**
FP29													
AAUR FP32	+	+	_	+	_	+	_	+	_	_	_	+	+
AAUR FP35	+	+	+	+	+	+	+	_	+	_	+	_	_
AAUR FP36	+	+	+	+	+	+	_	_	+	_	+	+	+
AAUR FP37	+	+	+	+	+	+	+	_	+	+	+	+	+
AAUR FP38	+	+	+	+	+	+	+	_	_	_	_	+	_
AAUR FP39	+	+	+	+	+	+	+	+	+	+	+	+	+
AAUR FP40	+	+	+	+	+	+	+	_	_	_	+	_	+
AAUR FP41	+	+	+	+	+	+	+	_	+	_	_	+	_
AAUR FP42	+	+	+	+	+	+	+	_	_	_	+	_	+
AAUR FP43	+	+	+	+	+	+	+	+	_	+	_	+	+
AAUR FP44	+	+	+	+	+	+	+	_	_	_	+	+	_
Total	27	27	23	26	20	27	20	9	6	3	19	23	21
%	100	100	85	96	74	100	74	33	22	11	70	85	78

Annex-2 Amino acid utilization test

Isolates	**L-lysine**	**L-asparagines**	**L-arginine**	**L-Tyrosine**	**Glutamate**	**L-Tryptophan**	**Methionin**
AAURFP1	+	+	+	+	+	+	+
AAURFP2	+	_	+	+	+	+	+

Isolates	**L-lysine**	**L-asparagines**	**L-arginine**	**L-Tyrosine**	**Glutamate**	**L-Tryptophan**	**Methionin**
AAURFP3	+	+	+	+	+	+	–
AAURFP4	+	–	+	-	+	+	+
AAURFP5	+	–	–	+	+	+	+
AAURFP6	+	–	–	-	+	+	+
AAURFP7	+	+	+	+	+	+	–
AAURFP8	+	+	–	–	+	+	+
AAURFP9	–	+	+	–	+	+	+
AAURFP10	+	+	–	+	+	+	+
AAURFP11	+	+	–	+	+	+	+
AAURFP12	+	+	+	+	–	+	+
AAURFP13	+	+	+	+	+	+	+
AAURFP15	+	+	+	+	+	+	+
AAURFP20	+	+	+	–	+	+	+
AAURFP29	+	–	–	–	+	+	+
AAURFP32	+	+	+	+	+	+	+
AAURFP35	+	+	+	+	–	+	–
AAURFP36	+	+	+	+	+	+	+
AAURFP37	+	–	–	–	–	–	–
AAURFP38	+	+	–	+	+	+	+
AAURFP39	+	+	+	+	+	+	+
AAURFP40	+	–	–	+	–	+	+
AAURFP41	–	+	–	+	+	+	+
AAURFP42	+	+	+	+	+	+	–
AAURFP43	+	–	+	+	+	+	+
AAURFP44	+	+	+	–	+	+	+

Isolates	**L-lysine**	**L-asparagines**	**L-arginine**	**L-Tyrosine**	**Glutamate**	**L-Tryptophan**	**Methionin**
Total	**25**	**19**	**17**	**19**	**23**	**26**	**22**
%	**93**	**70**	**63**	**70**	**85**	**96**	**81**

Annex- 3- pH-Tolerance test

Isolates	**Ph=4**	**Ph=4.5**	**Ph=5.0**	**Ph=5.5**	**Ph=8.5**	**Ph=9.0**	**Ph=9.5**	**Ph=10**
AAURFP1	-	–	+	+	+	+	+	-
AAURFP2	-	+	+	+	+	+	+	-
AAURFP3	-	+	+	+	+	+	+	-
AAURFP4	-	–	+	+	+	+	–	-
AAURFP5	-	–	+	+	+	+	–	-
AAURFP6	-	+	+	+	+	+	+	-
AAURFP7	-	–	+	+	+	+	–	-
AAURFP8	-	+	+	+	+	+	–	-
AAURFP9	-	+	+	+	+	+	–	-
AAURFP10	-	-	–	+	+	+	–	-
AAURFP11	-	-	–	+	+	+	–	-
AAURFP12	-	–	+	+	+	+	+	-

Isolates	**Ph=4**	**Ph=4.5**	**Ph=5.0**	**Ph=5.5**	**Ph=8.5**	**Ph=9.0**	**Ph=9.5**	**Ph=10**
AAURFP13	-	–	+	+	+	–	–	-
AAURFP15	-	–	+	+	+	+	+	-
AAURFP20	-	+	+	+	+	+	–	-
AAURFP29	-	+	+	+	+	+	+	-
AAURFP32	-	–	+	+	+	+	–	-
AAURFP35	-	+	+	+	+	+	+	-
AAURFP36	-	+	+	+	+	+	+	-
AAURFP37	-	+	+	+	+	+	+	-
AAURFP38	-	+	+	+	+	+	+	-
AAURFP39	-	+	+	+	+	+	–	-
AAURFP40	-	–	+	+	+	–	–	-
AAURFP41	-	+	+	+	+	+	–	-
AAURFP42	-	+	+	+	+	–	–	-
AAURFP43	-	+	+	+	+	+	–	-
AAURFP44	-	+	+	+	+	+	-	-
Total	0	16	25	27	27	19	11	0

Isolates	**Ph=4**	**Ph=4.5**	**Ph=5.0**	**Ph=5.5**	**Ph=8.5**	**Ph=9.0**	**Ph=9.5**	**Ph=10**
%	0	59	93	100	100	70	41	0

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

Annex- 4 Salt Tolerance Test

	Salt (NaCl) Concentration (%)							
Isolates	1	2	3	4	5	6	7	Total
AAURFP1	+	+	+	–	–	–	–	3
AAURFP2	+	+	+	–	–	–	–	3
AAURFP3	+	+	–	–	–	–	–	2
AAURFP4	+	–	–	–	–	–	–	1
AAURFP5	+	+	+	+	–	–	–	4
AAURFP6	+	+	–	–	–	–	–	2
AAURFP7	+	+	+	+	+	–	–	5
AAURFP8	+	+	+	+	–	–	–	4
AAURFP9	+	+	+	+	–	–	–	4
AAURFP10	+	+	–	–	–	–	–	2
AAURFP11	+	–	–	–	–	–	–	1
AAURFP12	+	–	–	–	–	–	–	1
AAURFP13	+	+	–	–	–	–	–	2
AAURFP15	+	+	+	–	–	–	–	3
AAURFP20	+	–	–	–	–	–	–	1
AAURFP29	+	+	–	–	–	–	–	2
AAURFP32	+	+	+	–	–	–	–	3
AAURFP35	+	+	+	+	+	+	–	6

	Salt (NaCl) Concentration (%)							
AAURFP36	+	+	+	+	+	+	–	6
AAURFP37	+	+	+	+	+	+	+	7
AAURFP38	+	+	+	+	+	+	–	6
AAURFP39	+	+	+	+	+	+	+	7
AAURFP40	+	+	+	–	–	–	–	3
AAURFP41	+	+	+	+	+	–	–	5
AAURFP42	+	–	–	–	–	–	–	1
AAURFP43	+	+	+	–	–	–	–	3
AAURFP44	+	+	–	–	–	–	–	2
Total growth	27	22	16	10	7	5	2	
% Growth	100	81	59	37	26	17	7	

Annex – 5 Temperature Tolerance Test

Isolates	**4c^0**	**15 c^0**	**20 c^0**	**25 c^0**	**30 c^0**	**35 c^0**	**40 c^0**	**45 c^0**
AAURFP1	–	–	+	+	+	+	–	–
AAURFP2	–	–	–	+	+	+	–	–
AAURFP3	–	–	–	+	+	+	–	–
AAURFP4	–	–	–	+	+	+	–	–
AAURFP5	–	–	–	+	+	+	–	–
AAURFP6	–	+	+	+	+	+	–	–
AAURFP7	–	–	+	+	+	+	–	–
AAURFP8	–	–	–	+	+	+	–	–
AAURFP9	–	–	–	+	+	+	–	–
AAURFP10	–	–	–	+	+	+	–	–

Isolates	**4c^{0}**	**15 c^{0}**	**20 c^{0}**	**25 c^{0}**	**30 c^{0}**	**35 c^{0}**	**40 c^{0}**	**45 c^{0}**
AAURFP11	–	–	–	+	+	+	–	–
AAURFP12	–	–	–	+	+	+	–	–
AAURFP13	–	–	–	+	+	+	–	–
AAURFP15	–	–	+	+	+	+	–	–
AAURFP20	–	–	–	+	+	+	–	–
AAURFP29	–	–	–	+	+	+	–	–
AAURFP32	–	–	–	+	+	+	–	–
AAURFP35	–	+	+	+	+	+	+	–
AAURFP36	–	+	+	+	+	+	+	–
AAURFP37	–	+	+	+	+	+	–	–
AAURFP38	–	–	+	+	+	+	+	–
AAURFP39	–	+	+	+	+	+	+	–
AAURFP40	–	–	–	+	+	+	–	–
AAURFP41	–	+	+	+	+	+	–	–
AAURFP42	–	-	+	+	+	+	–	–
AAURFP43	–	+	+	+	+	+	–	–
AAURFP44	–	–	–	+	+	+	–	–
Total growth	0	7	12	27	27	27	4	0
Growth (%)	0	26	44	100	100	100	15	0

Annex- 6 Antibiotic tolerance test

	Antibiotic concentration in µg/ml																	
	Ampicilline			**Tetracycline**		**Penicilline**			**Gentamicine**		**Chloramphenicol**			**Erythromycin**			**Total**	**% of growth**
Isolates	25	5	10	25	5	25	5	10	25	5	25	5	10	25	5	10		
AAURFP1	+	+	–	–	–	+	+	+	–	–	–	+	–	+	–	+	8	50
AAURFP2	+	+	–	–	–	+	+	+	–	–	+	–	–	+	–	–	7	44
AAURFP3	+	+	+	–	–	+	+	+	–	–	+	–	–	+	+	+	10	63
AAURFP4	+	–	–	–	–	+	+	–	–	–	+	+	–	+	+	+	8	50
AAURFP5	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	+	9	56
AAURFP6	+	+	+	+	–	+	+	–	–	–	+	–	–	+	+	+	10	63
AAURFP7	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	+	8	50
AAURFP8	+	+	–	–	–	+	+	+	–	–	+	+	–	+	+	–	9	56
AAURFP9	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	–	8	50
AAURFP10	+	–	–	–	–	+	+	–	–	–	+	–	–	+	+	–	6	38
AAURFP11	+	+	–	–	–	–	+	–	+	–	–	–	–	+	+	–	6	38
AAURFP12	–	–	–	–	–	+	–	+	–	–	+	–	–	+	+	–	5	31
AAURFP13	+	–	–	–	–	+	+	–	–	–	+	–	–	+	+	–	6	38
AAURFP15	+	+	+	–	–	+	+	–	–	–	+	+	–	+	+	–	9	56
AAURFP20	–	–	–	–	–	+	–	–	–	–	+	–	–	–	+	–	3	24
AAURFP29	+	–	–	+	–	+	–	+	–	–	+	–	–	–	+	–	6	38
AAURFP32	–	–	–	–	–	+	–	–	+	–	–	–	–	+	+	–	4	25
AAURFP35	+	+	–	+	–	+	+	–	+	+	+	+	–	+	+	+	12	75
AAURFP36	+	+	–	+	–	+	+	–	–	+	+	+	–	+	+	+	11	70
AAURFP37	+	+	+	–	–	+	+	–	–	–	+	+	–	+	+	+	10	63

	Antibiotic concentration in μg/ml																	
	Ampicilline			**Tetracycline**		**Penicilline**			**Gentamicine**		**Chloramphenicol**			**Erythromycin**			**Total**	**% of growth**
AAURFP38	+	+	–	–	–	+	+	+	–	–	+	–	+	+	+	+	10	63
AAURFP39	+	+	–	+	+	+	+	–	–	–	+	–	+	+	+	+	11	70
AAURFP40	+	+	+	–	–	+	+	–	–	–	+	–	–	+	–	–	7	44
AAURFP41	+	–	–	–	–	+	+	+	–	–	+	–	–	+	+	+	8	50
AAURFP42	+	–	–	–	–	+	+	–	+	-	+	–	–	+	-	-	6	38
AAURFP43	+	+	–	+	–	+	+	–	–	–	+	–	+	+	+	+	10	63
AAURFP44	+	+	–	–	–	+	–	–	–	–	+	–	–	+	+	–	6	38
Total	24	18	5	6	1	26	22	10	4	2	24	7	3	25	23	13		
%	89	67	19	15	11	96	81	37	15	7	89	26	11	93	85	48		

Annex- 7 Heavy metal resistance of the isolates

	Heavy metals in μg/ml						
Isolates	Al	Zn	Hg	Mn	Co	Cu	Resistance (%)
AAURFP1	+	_	_	_	+	_	33
AAURFP2	+	_	_	+	+	+	67
AAURFP3	+	+	_	+	+	_	67
AAURFP4	+	+	_	+	+	_	67
AAURFP5	+	+	_	+	_	_	50
AAURFP6	+	+	_	+	_	_	50
AAURFP7	+	+	_	+	_	+	67
AAURFP8	+	+	_	+	_	_	50
AAURFP9	+	+	_	+	_	_	50

	Heavy metals in μg/ml						
AAURFP10	+	+	_	+	_	_	50
AAURFP11	_	_	_	+	+	_	33
AAURFP12	+	_	_	+	+	+	67
AAURFP13	+	_	_	+	_	_	33
AAURFP15	+	_	_	+	_	_	33
AAURFP20	_	_	_	_	_	+	17
AAURFP29	_	_	_	_	+	_	17
AAURFP32	+	_	_	_	_	+	33
AAURFP35	+	+	+	+	_	_	67
AAURFP36	+	+	+	+	_	_	67
AAURFP37	+	+	_	+	_	_	50
AAURFP38	+	+	_	+	_	+	67
AAURFP39	+	+	+	+	_	_	67
AAURFP40	+	_	_	+	+	_	50
AAURFP41	+	+	_	+	_	_	50
AAURFP42	+	_	_	_	+	+	50
AAURFP43	+	+	_	+	_	_	50
AAURFP44	+	_	_	+	+	_	50
Total	24	15	3	22	10	7	
(%)	89	56	11	81	37	26	

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

AAURFP43

Collection of pots from Green house

Collection of pots from Green house

AAURFP41, AAURFP42, AAURFP43

Observing shoot biomass

Observing shoot length

Pot collection from greenhouse

AAURFP37

AAURFP36

AAURFP35

AAURFP38

AAURFP39

Nodule and shoot collection

Nodule collection