Symbiotic and phenotypic characteristics of indigenous rhizobia nodulating faba bean *(Vicia faba* L.) growing in some parts of Wello, Northern Ethiopia

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Symbiotic and phenotypic characteristics of indigenous rhizobia nodulating faba bean *(Vicia faba* L.) growing in some parts of Wello, Northern Ethiopia

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DEDICATION

It is my great pleasure to dedicate this work to my cherished brother, Abebe Negash, for his invaluable helps, morally and financially during all my phase of study.

DECLARATION

I hereby declare that this MSc thesis is my original work and has not been presented for a degree in degree in any other university, and all sources of material used for this thesis have been duly acknowledged

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA Analysis Of Variance

BCP Bromocresol purple

BNF Biological Nitrogen Fixation

BTB Bromthymol blue

CR Congo red

FBW Faba bean Wello

Ha hectare

IAR Intrinsic antibiotic resistance

KNO_3Potassium nitrate (chemical fertilizer)

Mton metric tone

NADPH Nucotinamid diphosphate hydrogen

NH_3ammonia

NH_4^+ ammonium ion

PGA Peptone Glucose Agar (selective media)

Pi inorganic phosphate

SDW Shoot Dry Weight

SE Symbiotic Efficiency

YEMA Yeast manitol agar

YEMB Yeast manitol broth

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ABSTRACT

Nitrogen is one of the most abundant elements in the atmosphere, and BNF is the main source of N for Legumes plants. Faba bean is one of the most important Legumes plants in Ethiopia and widely grown in Amhara regional state.In view of the presence of heterogeneous agroecologies found in the country, more diversity and effective strains nodulating faba bean are expected. This study was initiated with the objectives of isolation, characterization, and selection of symbiotically efficient faba bean rhizobia from some part of Wello, Ethiopia. The result will contribute for utilizing BNF system of faba bean to increase productivity into low-input agriculture of the region and the country at large. A total of 132 endophytic bacteria found in the nodule of faba bean (V. faba L.) growing at different farmers' fields in Wello were isolated and characterized based on phenotypic features. Up on authentication, only 70 (53%) isolates were found to be rhizobia (the true symbiont) of faba bean, while the remaining 62 (47%) of the test isolates were found to be non-nodulating nodule endophytes of faba bean. The result of the study showed the presence of diversity in morphological, physiological and symbiotic properties among the tested isolates. Presumptive reactions of the result showed all isolates are fast growing rhizobia, Gram negative rods, except CR absorption by 8 isolates and moderate growth on PGA media by seven isolates. ANOVA of the result indicated that all of the parameters including; nodules number, root fresh and dry weight, shoot fresh and dry weight, symbiotic efficiency were significantly improved by inoculation ($p< 0.05$). Based on SE (expressed as the ratio of % of SDW of the plant inoculated with the test isolates to SDW of the plant that received KNO_3^- as nitrogen source), 12.9% of the isolates were found to be highly effective and increased the shoot dry weight of host plant by 80-117.6%. Two of the test isolates (FBW140 and 145) in the current study showed better performance in SE%, exceeding 100% over KNO_3 treated plants. Total nitrogen content of the faba bean plant, when inoculated with 21 selected test isolates, showed that four isolates (FBW107, 140, 144 and145) significantly exceeded that of the plant which received KNO_3 (positive control). The results also revealed that SDW showed significant positive correlation with Shoot and root fresh weight, Root dry weight and nodule number. The relative amount of nitrogen fixed by the majority of selected test isolates, when compared with the commercial inoculant strain (D/sina) was found to be higher as evidenced by accumulation of high shoot fresh and dry weight, root fresh and dry weight and nodule number. The isolates have exhibited diverse and interesting features such as ability to grow on wide range of carbon and nitrogen sources, tolerance to higher and lower temperature, high salt concentration, acidic and alkaline pH and almost all isolates were resistant to Erythromycin (5µg/ml) while sensitive to Kanamycin at (5 and 10µg/ml). The numerical analysis based on 55 phenotypic traits revealed the existence of diversity among the test isolates and categorized all isolates into 3 clusters when 63% similarity was taken as cut point. Generally, the present work while showing the physiological and symbiotic diversity of the test isolates in the study sites, field study aiming at screening of the best nitrogen fixing candidate would be essential before using them as inoculants in areas where the indigenous rhizobia fail to do so.

Key words: Biological nitrogen fixation, nodulation, faba bean, *R. leguminosarum,* Symbiotic effectiveness, Wello.

1. INTRODUCTION

Nitrogen (N) is a vitally important plant nutrient (Frank *et al*., 2003). Plants are predominantly made up of carbon, oxygen and hydrogen which are supplied by air and water. Beyond these three elements, nitrogen is required in the greatest quantity (Timothy, 1999). It is a major component of chlorophyll; the most important pigment needed for photosynthesis. Production of high-quality, protein-rich food is extremely dependent upon availability of sufficient N.It is also found in other important biological molecules, such as ATP and nucleic acids (DNA and RNA).

Despite the fact that, 78% of the Earth's atmosphere is covered by nitrogen, plant growth and their product frequently are limited in nitrogen (Frank *et al*., 2003). The paradox is due to N_2 is extremely unreactive (Frank *et al*., 2003). The nitrogen reserve of agricultural soils must be replenished periodically in order to maintain an adequate (non-growth limiting level for crop production). This replacement of soil nitrogen is generally accomplished by the addition of chemical fertilizers or by the activity of biological nitrogen fixation (BNF) systems (Vance, 2001).

The application of chemical fertilizers have played significant role to increase the productivity of soil. The advantages of using chemical fertilizers are nutrients are soluble and immediately available to the plants; therefore the effect is usually direct and fast. They are more competitive than organic fertilizer, which makes it more acceptable and often applied by users (Jen-Hshuan, 2006). However, the production of chemical fertilizer requires a great consumption of fossil fuels (1-2 % global fossil fuel) and is subjected to constant variations in prices (Vieira *et al*., 2010). They are less affordable and too expensive, thus most challenging for developing countries like Ethiopia. In addition to the ever increasing prices, chemical nitrogen fertilization is associated with environmental problems because watershed contamination by nitrogen leaching, volatilization and denitrification.

The challenge behind using chemical fertilizers orders the world to seek another approach that is economically less expensive and environmentally safe. These problems could be avoided

offering to farmers' low-cost biofertilizer technologies generally regarded as Biological Nitrogen Fixation (BNF) Technology. These are ecologically sound and their application could help to minimize the global warming as well as to reduce the fertilizer input in farming practices(Herridge *et al*., 2008a).The evaluation, in terms of economic and ecological costs, between chemical-fertilizers and biological-nitrogen fertilizers support that BNF represents an economic, sustainable and environmentally friendly resource to guarantee the nitrogen requirement of an agro-ecosystem (Maria*et al*., 2012).

Biological nitrogen fixation (BNF) is the biological process by which the atmospheric nitrogen (N_2) is reduced to usable forms such as (NH_4^+ or NO_3^-) by certain microorganisms that can secrete an enzyme called nitrogenase. These organisms utilize this enzyme to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants utilize NH_3 to convert certain precursor metabolites (e.g., alpha-ketoglutarate, phosphoenopyruvate) into amino acids, which, in turn, are synthesized into proteins. However, most living organisms cannot break the strong triple bond within N_2; since nitrogenase enzyme secretion is an exclusive property of certain prokaryotic organisms. The symbiotic interaction between legumes and nitrogen fixing rhizobia bacteria provides the majority of terrestrial biological nitrogen fixation (Sprent, 2001). The nitrogen fixation is achieved by bacteria inside the cells formed organs, the nodules, which usually develop on roots, and more occasionally on stems of legumes plants (Ragaa, 2013). This symbiotic relationship is beneficial for both partners, the plant supplying dicarboxylic acids as a carbon source to bacteria and receiving, in return, ammonium.

Legumes are flowering plants that produce seedpods. They have colonized several ecosystems (from rain forests and arctic/alpine regions to deserts; Schrire *et al*., 2005), and have been found in most of the archaeological records of plants. They are very important crop both ecologically and agriculturally because they are responsible for substantial part of the global flux of nitrogen from N_2 to fixed form such as ammonia, nitrate and organic nitrogen. Atmospheric N_2 fixed symbiotically by association between Rhizobia and legumes represent renewable source of nitrogen for agriculture. Improving legume yield by inoculation with Rhizobia, increase soil organic matter, improve soil porosity and structure, recycle nutrients,

decrease soil pH, reduce soil compaction, diversify microorganisms and mitigate disease problems (USDA, 1998). In rotation with cereals, legumes provide a source of slow-release nitrogen that contributes to sustainable cropping systems. The improvement in the production of these crops will therefore contribute substantially to better human nutrition and soil health (Popelka *et al*., 2004).

Grain legumes also called pulses, which according to FAO (2011) are crops harvested exclusively for the dry seeds, play an important role in the nutrition of many people due to their high protein content in seeds. They represent a major source of protein in many developing countries, especially among the poorest population, and are rich in essential amino acids such as lysine, supplementing thus the nutritional value of cereal and tuber diets (Graham &Vance, 2000).The grain legumes such as soybeans, groundnuts, peas, faba beans, lentils, alfalfa and clover are a major source of protein for human and animal consumption. The percentage of nitrogen derived from the atmosphere estimated for various legume crops species are often impressive, commonly falling in the range of 200 to 300Kg of N/ha/year (Peoples *et al*., 1995).Among the grain legumes, faba bean is reported to derive the highest percentage of nitrogen from the atmosphere (McVicar*et al*., 2005). According to (Somasegaran and Hoben, 1994) the amount of nitrogen fixed by faba bean have been 240-325 kg/ha.

Faba bean (*Vicia faba* L.) is the most important cool season grain legume in Ethiopia in terms of hectarage, total production, foreign exchange earnings and soil amelioration (Amare Ghizaw, 1990). According to Somasegaran and Hoben (1994), it is the efficient N fixer (240-325 kg ha^{-1} yr^{-1}) when inoculated with *Rhizobium leguminosarum bv. Viciae.* Despite the importance of the crop in the traditional farming system in Ethiopia, the yield is generally low as compared to the temperate (Saxena *et al*., 1998). The average grain yields of faba bean in Ethiopia in 2008 and 2009 were 1.292 and 1.193 t ha^{-1}, respectively (CSA, 2010). On the other hand According to Central Statistics Agency of Ethiopia 2012/13, Faba bean takes over 30% (nearly half a million hectares) of cultivated land with an average national productivity of 1.5 tons ha^{-1}.

Rhizobia are a group of bacteria that have the capacity to form nodules on legume roots (and occasionally on stems) and can fix atmospheric nitrogen to partially or fully meet the nitrogen requirements of the plant. An effective symbiotic relationship between the bacteria and the plant hosts is crucial for the legume to achieve maximum growth efficiency. In the presence of available nitrogen, they can exist as free-living soil saprophytes. At a particular condition (in the absence of available nitrogen), these bacteria interact with the roots or stems of leguminous plants, inducing the formation of nodules in which the fixation of atmospheric nitrogen occurs. The interaction between host legume and rhizobia convert atmospheric nitrogen into ammonia providing the nitrogen requirements of both rhizobia and their host plants. In return, rhizobia receive a carbon source, typically dicarboxylates and other nutrients from the plants.

The interaction between the Rhizobia and host legume is so complex that a particular species of rhizobia only nodulate a selected number of plant genera. Some particular species of rhizobia may nodulate different genera of legumes. The host legumes that nodulated by common rhizobial species are called cross inoculation groups(Somasegaran and Hoben, 1994). For instance *Rhizobium leguminosarum biovar vicieae,* could nodulate pea(*Pisum spp.*), vetch(*Vicia spp.*), lentil(*Lens spp.*), *and* sweet pea(*Lathyrus spp.*)(Perret *et al.*, 2000).Specifity is determined by signal molecules which secreted from both host and bacteria (Gray *et al.*, 1996).Nevertheless, mere formation of nodules doesn't guarantee for effective symbiosis. Effective symbiosis can only be achieved when the nodules are formed between the right host and effective microsymbiont, accompanied with favorable environmental condition for both symbionts (Somasegaran and Hoben, 1994).

The tropics have the potential to be the most productive cropping environments in the world (Wani *et al.*, 1995). Despite of these natural advantages, yields in tropical cropping systems are often very small (Wani *et al.*, 1995). The unpredictability of the climate-in particular the timing of the rains-and the lack of nutrients for plant growth in many soils, combine to limit crop production in the tropics while we can do little to modify the climate we can use various approaches to solve the problems of soil fertility (Zahran, 1999). Ethiopia being part of tropics, most of the soils are N-deficient. The most obvious solution is to import nutrients in

the form of mineral fertilizers. However our farmers cannot afford the ever-increasing price of commercial fertilizer. The alternative is to increase the biological inputs of nutrients and it is here that biological fixation of atmospheric nitrogen (N_2) has a crucial role to play in increasing the sustainability of yields with minimal external inputs (Giller, 2001).

A number of research works made in recent years disclose that inoculation of faba bean with *R. leguminosarum spp* increase yield by 10-50% (Girmaye Kenasa *et al.,* 2014). Because of this potential benefits, screening of faba bean nodulating rhizobia were carried out during the past few years in the country (Ayneabeba Adamu *et al.*, 2001; Zerihun Belay, 2006; Getaneh Tesfaye, 2008; Abere Mnalku *et al*., 2009 and Assefa Keneni *et al*., 2010; Zerihun Belay and Fassil Assefa, 2011; Anteneh Argaw, 2012;Girmaye Kenasa*et al*., 2014). In view of the presence of heterogeneous agroecologies found in the country, more diversity and effective strains nodulating faba bean are expected. Therefore, this study was initiated with the objectives of isolation, characterization, and selection of symbiotically efficient faba bean nodulating rhizobia from some part of Wello, Ethiopia. The result will contribute for future endeavor of utilizing biological nitrogen fixing system of faba bean to increase productivity into low-input agriculture of the region and in chance the country at large.

1.1. Objectives

1.1.1. General objective;

To explore the diversity of rhizobia nodulating faba bean growing in Wollo, Ethiopia

1.1.2. Specific objectives;

- To isolate rhizobia from nodules of faba bean
- To characterize the isolates based on different phenotypic features
- To screen for the most symbiotically efficient isolate

2. LITERATURE REVIEW

2.1. Nitrogen

Most of the nitrogen on Earth is in the atmosphere. Approximately 78% of the molecules in Earth's atmosphere are made of two nitrogen atoms bonded together with triple bond, N≡N, (Bagali, 2012). All plants and animals need nitrogen to make amino acids, proteins and DNA, but the nitrogen in the atmosphere is not in a form that they can use. The molecules of nitrogen in the atmosphere can become usable for living things when they are broken apart during lightning strikes, volcanic activity, or fires, and by certain types of nitrogen-fixing bacteria (like those found in the root nodules of legumes). Other plants get the nitrogen they need from the soils or water in which they live mostly in the form of inorganic nitrate (NO_3^-).

Nitrogen is the most frequently found limiting to the growth of green plants (Graham and Vance, 2000). This is because either it is unreactive or subjected to continual loss through leaching, microbial denitrification and chemical volatilization processes. The nitrogen reserve of agricultural soils must therefore be replenished periodically in order to maintain an adequate (non-growth limiting) level for crop production (Socolow, 1999). This replacement of soil nitrogen is generally accomplished by the addition of chemically fixed nitrogen in the form of commercial inorganic fertilizers or by the activity of biological nitrogen fixation (BNF) systems (Socolow, 1999).

The Nitrogen cycle is one of the biogeochemical cycles and is very important for ecosystems. The nitrogen cycle was schematically illustrated in Figure (1). Atoms of nitrogen don't just stay in one place. They move slowly between living things, dead things, the air, soil and water (Smil, 1999). These movements are called the nitrogen cycle. Understanding the N cycle help us make the best use of manure and fertilizers to meet crop needs while safeguarding the environment. In general, the N cycle processes of fixation, mineralization and nitrification increase plant available N. Denitrification, volatilization, immobilization, and leaching result in permanent or temporary N losses from the root zone (Paul, 1988). Fixation refers to the conversion of atmospheric N to a plant available form. This occurs either through an industrial

process, as in the production of commercial fertilizers, or a biological process, as with legumes such as alfalfa, faba bean and clover.

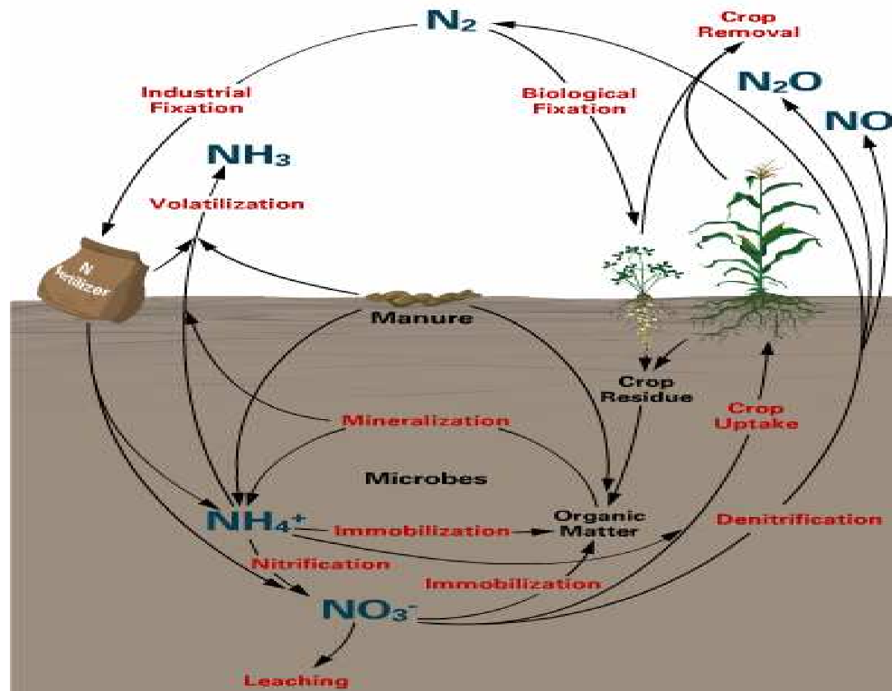


Figure 1: Schematic representation of N cycle

Some human actions are causing changes to the nitrogen cycle and the amount of nitrogen that is stored in reservoirs (Galloway *et al.*, 1995). The use of nitrogen-rich fertilizers can cause nutrient leaching in nearby waterways as nitrates from the fertilizer wash into streams and ponds. The increased nitrate levels cause plants to grow rapidly until they use up the nitrate supply and die (Zahran, 1999). The numbers of herbivores increase when the plant supply increases and then the herbivores are left without a food source when the plants die. In this way, changes in nutrient supply affect the entire food chain. Additionally, humans are altering the nitrogen cycle by burning fossil fuels and forests, which releases various solid forms of nitrogen. Farming also affects the nitrogen cycle. The waste associated with livestock farming releases a large amount of nitrogen into soil and water. In the same way, sewage waste adds nitrogen to soils and water (Smil, 1999).

2.1.1. Methods of determination and assessment of N-fixation

The measurement of atmospheric nitrogen (N_2) fixation in plant is an important step in effort to increase the contribution of atmospheric N_2 to plant nutrition and soil fertility. Although several methodologies have been developed for measuring atmospheric N fixation in plants, none of them measures N_2 fixed with absolute accuracy (Danso, 1985).

Various methods have been used to provide estimates of N fixed in forages, each of which has its own merits and disadvantages. Nodule number, weight and plant dry weight are among the earliest, most inexpensive and simplest methods (Heichel *et al*, 1984). Nodule parameters and plant yield, however, provide only indirect evidence of the extent of N_2 fixation. They do not give a measure of how much N_2 is fixed, neither do they always give an accurate indication of how much N_2 is fixed (Danso, 1985).

^{15}N isotope dilution technique

The ^{15}N methods are potentially accurate for measuring nitrogen fixation in plant. The only problem with those methods is, how to ensure $^{15}N/^{14}N$ ratio in the plant accurately reflects the integrated in $^{15}N/^{14}N$ ratio in soil which is variable in time and in soil depth, however the consequences of using inappropriate references plant varies with the level of nitrogen fixation. For instance, the error introduced in nitrogen fixation is higher at low levels of fixation and decrease with increase rates of fixation (Danso, 1985).

A great advantage with the use of ^{15}N isotope dilution technique to estimate N_2 fixed is its ability to give an integrated estimate of N fixation over a growing season or longer. It is the only method that can distinguish between soil, fertilizer and fixed N in field-grown crops. The technique has thus been extensively employed to quantify N-fixation in forage legumes, and there is an ever increasing interest in its use, as shown by recently published literature (Galal, 1997). Estimates made using the isotope dilution technique have established that most forage legumes derive a large proportion of their N from fixing atmospheric N_2 and, in general, this is in excess of 70 - 80 % of their total N requirements. The determination of the level of N-fixation by the ^{15}N isotope dilution technique requires an assessment of the $^{15}N/^{14}N$ ratio in

soil. This assessment is made by selecting an appropriate reference crop to assess the soil's $^{15}N/^{14}N$ ratio.

Principles of the ^{15}N-isotope-dilution method; as indicated by Danso, *et al.*(1985) stipulates that changes in ^{15}N enrichment result when two sources that differ in N isotopic composition are uniformly mixed. The extent of change that results will depend on the magnitude of the differences in the initial enrichments of the individual sources, as well as the relative amounts of each.

N-difference method

This method is quantification of %N in fixing plants and non-fixing reference crop plants. Although the need for Kjeldahl method N analysis (Giller *et al.*, 1986) of the samples makes this method more time consuming, it provide more information on the amount of nitrogen fixed. Recently infrared reflectance method calibrated against kjeldahl N have been used to determine plant N, resulting in considerable time saving and similar accuracy. The main disadvantage of this method is that legumes and reference crops must absorb similar amount of nitrogen from soil. When this is not the case erroneous estimate of N_2 fixation may result. Other Problems also occur when this method is employed under conditions thought to be free of mineral N. For example, vermiculite was used as ‘N-free’ growth medium for the study of associative N_2-fixation but later research showed that significant quantities of mineral N can be released from vermiculite when it is incubated under warm, moist conditions (Giller *et al.*, 1986).

N-difference method has the advantage of giving a measure of the total amount of N_2 fixed over the length of the experiment and is absolutely necessary for many laboratory-based studies. Screening of rhizobial strains has traditionally been conducted using this method in the ‘Leonard jar’ assay.

N-balance method

In N-balance experiments the amount of N in each of the various pools (i.e. the soil and in the plants) is measured both at the beginning and at the end of the experiment. Any gains unaccounted for are then attributed to N_2-fixation. Losses of N that are not measured (eg.due

to denitrification) will result in an underestimate of the amount of N_2-fixed. As the total of N in the soil is generally large compared with the amount of N_2-fixed, any error in the estimation of the amount of soil N will result in a large discrepancy in the estimate of N_2-fixation (Giller, 2001).

The ^{15}N natural abundance method.

Many soils are naturally enriched with ^{15}N compared with the atmosphere. Enrichment of soil N occurs due to isotopic discrimination during processes such as ammonia volatilization, denitrification and other transformations of N in soil. As the ^{15}N isotope is heavier than ^{14}N, compounds containing ^{15}N tend to react more slowly, particularly in reactions that lead to gaseous losses of N from the soil.

The principle is the same as that of the ^{15}N isotope dilution method except that ^{15}N-enriched fertilizers are not applied to the soil. Differences in enrichment of the N_2-fixing test and non-fixing reference plants reflect the dependence of the plant on atmospheric N_2, and are used to calculate N_2 fixation (Giller, 2001).

Ureide technique

In recent years, ureide technique has been developed for measuring N_2 fixation. Ureides are a group of nitrogenous compounds including allantoin and allantoic. The allantoin and allantoic acid compounds are the products of N_2 fixation from their nodules to the shoots of legumes. In these legumes, the ratio of ureide N to total N in xylem sap or stem segments is highly correlated with %Ndfa. Some legumes produce large quantities of ureides when N is fixed symbiotically, but not when assimilated from soil mineral sources (Somasegaran and Hoben, 1994). Although not applicable to all legumes, and to no other N_2-fixing associations, the technique has been widely used with both experimental and non-experimental (farmer) crops. The analytical procedures are simple with minimal requirements for sophisticated or expensive equipment.

2.2. Nitrogenfixing microorganisms

Organisms that can fix nitrogen, i.e., convert the stable nitrogen gas in the atmosphere into a biologically useful form; all belong to a biological group known as prokaryotes. 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 (Dixon and Wheeler, 1986). The ability to reduce and siphon out such appreciable amounts of nitrogen from the atmospheric reservoir and enrich the soil is confined to bacteria and Archaea (Young, 1992). These include, symbiotic nitrogen fixing (N_2-fixing) forms rhizobia with, the obligate symbionts in leguminous plants and Frankia in non-leguminous trees, and Non-symbiotic (free-living and associative) N_2-fixing forms such as *cyanobacteria, Azospirillum, Azotobacter, Acetobacter diazotrophicus, Azoarcus*, etc.

2.2.1. Free-living micro-organisms

Bacteria living in the soil are called free-living as they do not depend on root exudates for their survival. Free living microorganisms (non-symbiotic nitrogen fixers) are known to be of great agronomic significance. Free living bacteria can fix about 30% of biological nitrogen fixation (BNF) and they have different shape from the bacteria living in the root nodule (Willems, 2006). The main limitation to non-symbiotic nitrogen fixation is the availability of carbon and energy source for the energy intensive nitrogen fixation process. This limitation can be compensated by moving closer to or inside the plants, within diazotrophs present in rhizosphere, rhizoplane or those growing endophytically.

Some important nonsymbiotic nitrogen-fixing bacteria include, *Achromobacter, Acetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Azomonas, Bacillus, Beijerinckia, Clostridium, Corynebacterium, Derxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas, Rhodospirillum, Rhodopseudomonas and Xanthobacter* (Saxena, 1998). Despite the fact that free living microorganisms are out in number but *Azotobacter, Beijerinckia* and *clostridium* species, pseudomonas and bacillus are the best known from free living nitrogen fixing microorganisms (Saxena, 1998).The nitrogen fixing activity of free-living, non-photosynthetic, aerobic bacteria is strongly dependent on favorable moisture conditions, oxygen, and an organic food source. As opposed to this anaerobic representatives (*Clostridium*) predominate in grassland and waterlogged soils and soil aggregates where

moisture conditions and organic substrates are available but oxygen supply to the micro-environment of the bacteria is severely restricted.

The amounts of nitrogen fixed by free-living non-photosynthetic bacteria in the soil may achieve an approximate maximum of 15 kilograms per hectare per year (Tilak *et al.*, 2005). This relatively low estimated contribution is the result of limited availability of suitable organic substrates (energy sources) and low bacterial populations in the soil environment.Nitrogen fixation is characteristically higher in environments such as tropical soils, where such factors as substrate availability, temperature and moisture are more favorable to the maintenance and activity of a high bacterial population. A minor improvement of soil with a readily used organic substrate generally results in some increase in nitrogen fixation. The increased population of N-fixing bacteria resulting from inoculation is temporary and will rapidly die back to the original number found in an unamended soil, where no provision has been made to create environmental changes which will favor a higher microbial population. The increase number of rhizobia in the rhizosphere is in response to the excretion of nutrient by plant roots, especially in host legume.

Living plant roots release a wide variety of simple organic compounds, which may be used as food by free-living soil bacteria. This continuous supply of food supports a higher microbial population in the soil immediately surrounding the plant root (rhizosphere). Evidence indicates that native nitrogen-fixing bacteria are common in the rhizosphere of certain plants and that they may fix significant amounts of nitrogen in some cases. This effect may be related to the closeness of the root-microorganism association. Food material released from the roots would be available in greater concentration to those microorganisms more closely associated with the root surface (*Azospirillum*). A striking example is seen in certain combinations of bacteria with some tropical grasses, which have a high photosynthetic efficiency and grow under environmental conditions favoring high photosynthetic activity. The roots of such plants may supply the nitrogen-fixing microorganisms with a relatively high and sustained supply of food (photosynthetic) which is available in limited supply in the rhizosphere of most plants (Perret *et al.*, 2000).

2.2.2. Symbiotic N-fixing micro-organisms

The most common feature of rhizobia is symbiotic living with legume plants. rhizobia are rod shaped Gram-negative, nitrogen-fixing bacteria that form nodules on host plants, Which are mobile by single polar flagellum or two to six pertrichious flagella. They don't form endospore and are predominantly aerobic chemoorganism and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrate and amino compounds (Somasegaran and Hoben, 1994).

Rhizobia have symbiotic relationships with legume plants, which play major role for essential nitrogen-fixing processes together with hosts. In nodules, the rhizobia bacteroids use carbon and energy from the plant in the form of dicarboxylic acids. Recent studies have suggested that the bacteroids do more than just provide the plant with ammonium (through nitrogen fixation). It was shown that a more complex amino-acid cycle is needed for rhizobia to fix nitrogen successfully in nodules. Rhizobia can use the amino acids from the plant to shut down their ammonium assimilation; however, the bacteria must provide the plant with ammonium in order to obtain the amino acids. This alone would mean that the plant could regulate the amount of decarboxylase that the bacteroids use by amino acid supply and dominate the relationship. This is not the case, however, because the bacteroids "act like plant organelles to cycle amino acids back to the plant for asparagine synthesis," making the plant dependent on them.

2.3. Biological nitrogen fixation

Biological N_2 fixation represents the major source of N input in agricultural soils. The major N_2-fixing systems are the symbiotic systems, which can play a significant role in improving the fertility and productivity of low-N soils. The rhizobium-legume symbioses have received most attention and have been examined extensively. Approximately, half of the 23 million metric tons of nitrogen consumed as human food sources (grains and livestock) comes from biological nitrogen fixation by prokaryotes (Socolow, 1999). Out of this, rhizobia in root nodules are estimated to take away between 50-70% of the world's biological nitrogen fixation (Burris and Roberts, 1993).

The annual input of fixed nitrogen was calculated to be 2.95 Mton for the pulses and 18.5 Mton for the oilseed legumes, being the soybean the dominant crop legume (50% global crop legume area and 68% global production). In addition to the annual legume nitrogen fixation inputs of 12-15 Mton (pasture and fodder legumes), there is an input by nitrogen fixation in rice (5 Mton), sugar cane (0.5 Mton), non-legume crop lands (<4 Mton) and extensive savannas (<14 Mton). Thus, the total overall estimated in agricultural systems is of 50–70 Mton biologically fixed nitrogen (Herridge *et al*., 2008a). These numbers show that the process of BNF is an economically attractive and eco-friendly alternative to reduce the external nitrogen (chemical fertilizers) input, which improves the quality and quantity of crop resources. Having this characteristics, they are particular important in countries where the cost of nitrogen fertilizer is high and/ or availability is limited.

The effect of BNF also vary depending on survival of the rhizobial strains and legumes under different soil conditions like salinity, drought, acidity, soil temperature (Zaharan, 1999). O'Hara *et al*., (2002), reported that the abundance of diversity in the soil populations of rhizobia provides a large resource of natural germplasm to screen for desired characteristics present in the natural pool. This requires rigorous screening for efficient rhizobial strains with adaptation to different soil conditions (Zaharan, 1999). To achieve this, indigenous rhizobial strains can be characterized under different conditions in the laboratory and tested in the field for their effectiveness.BNF commonly categorized into free living and symbiotic nitrogen fixing systems (Druille *et al*., 2012).

2.3.Symbiotic Nitrogen fixation

Many microorganisms fix nitrogen symbiotically by partnering with a host plant. The plant provides sugars from photosynthesis that are utilized by the nitrogen-fixing microorganism for the energy it needs for nitrogen fixation. In exchange for these carbon sources, the microbe provides fixed nitrogen to the host plant for its growth (Wagner, 2012).

Even though the symbiotic partners many microbes play an important role in the worldwide ecology of nitrogen fixation, by far the most important nitrogen-fixing symbiotic associations are the relationships between legumes and *Rhizobium* and *Bradyrhizobium* bacteria. These

microbes mostly associated with legumes such as; alfalfa, beans, clover, cowpeas, lupines, peanut, soybean, and faba bean (Vance, 2001).

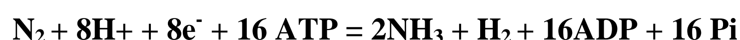
2.4. Mechanism of symbiotic nitrogen fixation

Symbiosis begins with the exchange of chemical signals between the plant and the bacteria. The exchange of diffusible signaling molecules between rhizobia and host plant takes place prior to physical contact. The chemical mediators involved in the molecular dialogue include flavonoids, Nod factors, surface polysaccharides, and extra cellular proteins (Perret*et al*., 2000). Legumes excrete a number of secondary metabolites released into their rhizosphere. Flavonoids and/or bioflavonoids are two such metabolite released from the root of the legume host that induce transcription of nodulation genes on compatible rhizobia, leading to the formation of lipochitooligosaccharide molecules that, in turn, signal thehost plant to begin nodule formation (Long, 1996). Flavonoids concentrations in the rhizosphere increase in response to compatible rhizobia (Recourt *et al.,* 1991; Schmidt*et al.,* 1994; Zuanazzi*et al.,* 1998).

The initial recognition between compatible partners is crucial for the successful development of a symbiotic nodule and it seems logical that surface interactions between the two partners may be involved in this complicated recognition process (Long, 1996). That is through specific binding of particular polysaccharide structures present on the bacterial cell surface to host plant lectins (Bohlool and Schmidt, 1974).Several steps need to be successfully completed before effective symbiotic biological nitrogen fixation can occur. Nodulation is a complex process orchestrated by a multitude of bacteria and plant signals (Ferguson *et al*., 2010). The process is initiated by plant roots secreting flavonoid molecules into the soil. This attracts compatible rhizobia and concomitantly stimulates them to synthesize a highly specific signal molecule called Nod factor. The nodulation (nod) genes of rhizobia play important roles in the development of nodules. The plant perceives Nod factor via LysM receptors on the root (Indrasumunar *et al*., 2011). Nod factor perception triggers a subsequent signaling cascade that is required for proper nodule establishment (Ferguson *et al*., 2010).

The presence of the rhizobia together with their Nod factor signal molecule initiates the nodulation infection process. Root hair penetration is the most common form of rhizobia invasion. The bacteria attach to emerging root hairs, which begin to deform and eventually encapsulate some of the bacteria, which are continuously dividing. This process happens in as little as 6 to 8 hrs post-inoculation (Turgeon and Bauer, 1985). Specialized structures, called infection threads, begin to form and provide a passage way for the bacteria to enter the root reviewed by Gage (2004). These infection threads are predominately comprised of plant cell wall components and they permit the bacteria to continue proliferating within the host plant. As the process of rhizobia infection occurs, cortical cells in the root begin to divide and eventually give rise to the nodule primordium (Mathews *et al.*, 1989). Infection threads initiating in the root hair eventually grow and extend towards the dividing nodule primordium located in the root cortex. Once there, rhizobia located at the tip of the infection threads are released into an infection droplet that separates and is released into the Cytoplasm of the host cell. Within the cytoplasm, the rhizobia are encapsulated by a specialized plant derived membrane, known as the peribacteroid membrane, making what is commonly referred to as the symbiosome (Udvardi and Day, 1997).Ultimately, the dividing bacteria differentiate into what are known as bacteroids, which are highly specialized and whose main purpose is to fix atmospheric di-nitrogen gas. Inside the mature nodule, the bacteroids use a nitrogenase enzyme complex to fix the di-nitrogen into forms of nitrogen that the plant can use, such as ammonia. The ammonia, which is toxic to the plant, is then quickly converted into compounds such as glutamate or ureides that are non-toxic and are safely transported throughout the plant. Legume nodules provide the ideal setting for this process as they establish a peripheral oxygen barrier, via physical and metabolic barriers, to create a low-oxygen environment that is essential for nitrogenase activity to occur.

Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of 8 electrons and 8 protons (Giller, 2001).



$$\mathbf{N_2 + 8H+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

This reaction is performed exclusively by some prokaryotes (the bacteria and related organisms), using an enzyme complex termed **nitrogenase** involves the Magnesium ATP-dependent reduction of nitrogen gas to yield two molecules of ammonia.This enzyme consists of two proteins - an iron-sulfur protein and a molybdenum-iron-sulfur protein. The reduction of nitrogen to ammonia is a highly endergonic and energy consuming reaction. In the course of this reaction protons are also reduced (Dixon and Wheeler, 1986).

The enzyme nitrogenase is a complex of two enzymes, Fe-containing protein and Fe-Mo protein. It is responsible for the conversion (reduction) of atmospheric N intoNH_4^+, and is synthesized in the cytosol of the bacteroids; bacteria inside the nodule are called bacteroids. The legume utilizes NH_4^+ toconvert certain precursor metabolites (e.g., a-ketoglutarate, phosphoenopyruvate) intoamino acids, which, in turn, are synthesized into proteins. The complex biochemical reaction whereby the inert atmospheric nitrogen is enzymatically reduced into an utilizable form for the plant by the nitrogenase enzyme complex of the bacteroids is called biological nitrogen fixation (BNF).

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

The reaction starts by reduction of the Fe protein by the low-potential electron donor ferredoxin. Electrons are transferred, one at a time from the Fe protein to the MoFe protein in a process that involves Mg ATP hydrolysis. The cycle repeats until enough electrons have

been provided for the complete reduction of the N_2 substrate (Marie, 2001). The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein 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, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to H_2N-NH_2, and this in turn is reduced to 2moles NH_3. Depending on the type of microorganism, the reduced ferredoxin, which supplies electrons for this process, is generated by photosynthesis, respiration or fermentation.

2.5.Significance of biological nitrogen fixation to soil fertility

Deficiency in mineral nitrogen often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Freiberg *et al.*, 1997). For optimum plant growth, nutrients must be available in sufficient and balanced quantities (Chen, 2006). Soil infertility is the most important constraint limiting crop yield in developing nations worldwide, and especially among resource-poor farmers. Unless the fertility is restored in these areas, farmers will gain little benefit from the use of improved varieties and more productive cultural practices. Soil fertility can be restored effectively through adopting the concept of integrated soil fertility management (ISFM) encompassing a strategy for nutrient management-based on natural resource conservation, biological nitrogen fixation (BNF) and increased efficiency of the inputs (Vlek and Vielhauer , 1994).

Microorganisms that carryout biological nitrogen fixation have great importance for soil fertility. The process of BNF performed by symbiotic fixing bacteria with legume species, commonly known as alpha and beta rhizobia, this provides high sustainability for ecosystem. These microorganisms can help promote plant growth not only by supplying nitrogen but also by other mechanisms, such as production of siderophores, exopolysacharide and phytohormones; phosphate solibilizatin; protection against phytopathogenic fungus (Serraj and Adu-Gyamfi, 2004).

An examination of the history of BNF shows that interest generally has focused on the symbiotic system of leguminous plants and rhizobia, because these associations have the

greatest quantitative impact on the nitrogen cycle.A tremendous potential for contribution of fixed nitrogen to soil ecosystems exists among the legumes (Brockwell *et al*., 1995; Peoples *et al*., 1995a; Tate, 1995). There are approximately 750 genera and about 19,000 species of legumes, only a portion of which (about 20%) have been examined for nodulation and shown to have the ability to fix N_2(Sprent, 2001). Atmospheric N_2 fixed symbiotically by the association between rhizobia and legumes represents a renewable source of N for agriculture (Peoples *et al*., 1995b). Estimates are that the rhizobial symbioses with the somewhat greater than 100 agriculturally important legumes contribute nearly half the annual quantity of BNF entering soil ecosystems (Tate, 1995). Whatever the true figure, legume symbioses contribute at least 70 million tonnes of N per year, approximately half deriving from the cool and warm temperature zones and the remainder deriving from the tropics (Brockwell *et al*., 1995). Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N_2 fixation.

Most of the attention is directed toward N_2 fixation inputs by legumes because of their proven ability to fix N_2 and their contribution to integral agricultural production systems in both tropical and temperate climates. Successful *Rhizobium*-legume symbioses will definitely increase the incorporation of biologically fixed nitrogen into soil ecosystems. *Rhizobium*-legume symbioses are the primary source of fixed nitrogen in land-based systems and can provide well over half of the biological source of fixed nitrogen (Tate, 1995).

Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range of 200 to 300 kg of N ha^{-1} $year^{-1}$ (Peoples *et al*., 1995a). Yield increases of crops planted after harvesting of legumes are often equivalent to those expected from application of 30 to 80 kg of fertilizer-N ha^{-1}. Inputs of fixed N for alfalfa, red clover, pea, soybean, cowpea, faba bean and vetch were estimated to be about 23 to 335 kg of N ha^{-1} $year^{-1}$ (Tate, 1995). However, the measured amounts of N fixed by symbiotic systems may differ according to the method used to study N_2fixation (Brockwell *et al*., 1995). Inputs into terrestrial ecosystems of BNF from the symbiotic relationship between legumes and their rhizobia amount to at least 70 million tons of N per year (Brockwell *et al*., 1995); this enormous quantity will have to be augmented as the world's population increases and as the natural resources that supply fertilizer-N diminish. This objective will be achieved through the

development of superior legume varieties, improvements in agronomic practice, and increased efficiency of the nitrogen-fixing process itself by better management of the symbiotic relationship between plants and bacteria.

The symbioses between rhizobia and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crop and pasture production than the application of fertilizer-N. The introduction of legumes into pastures is seen as the best strategy to improve nitrogen nutrition of the grasses. Large contributions (between 75 and 97 kg of N ha^{-1} in 97 days of growth) by *Stylosanthes guianensis* were found (Viera-Vargas *et al.*, 1995). ^{15}N data suggested that over 30% of the N accumulated by the grass in mixed swards could be derived from nitrogen fixed by the associated legume (Viera-Vargas *et al.*, 1995). Mandimba (1995) revealed that the nitrogen contribution of Arachis hypogaea to the growth of Zea mays in intercropping systems is equivalent to the application of 96 kg of fertilizer-N ha^{-1} at a ratio of plant population densities of one maize plant to four groundnut plants. This indicates the significance of rhizobium-legume symbioses as a major contributor to natural or biological N_2 fixation.

2.6. Rhizobia and their current taxonomy

Rhizobia are soil bacteria which are capable of forming nitrogen-fixing symbiosis with different leguminous plants and have a significant role in nutrient cycling due to biological nitrogen fixation, and enhancing crop productivity (Stocker *et al.*, 2008). They are a genetically diverse and physiologically heterogeneous group of bacteria despite their single grouping by virtue of their ability to nodulate members of the *Leguminosae* (Somasegaran and Hoben, 1994). So, many nodule-forming bacteria are of significant agricultural and ecological importance. The symbiotic relationships between rhizobia and leguminous plants provide rich soil for legumes cultivation. In the presence of available nitrogen, they can exist as free-living soil saprophytes. At a particular condition (in the absence of available nitrogen), these bacteria interact with the roots or stems of leguminous plants, inducing the formation of nodules in which the fixation of atmospheric nitrogen occurs(Pongsilp, 2012).

It is important to understand the level of genetic diversity of *Rhizobium* species and how this affect soil fertility and crop productivity in response to agricultural practices and climate (Smalla *et al*., 2007).For many years, the characterization of rhizobia species was based on specific ability of the bacteria to nodulate a host plant, recognizing the selective interaction between the symbionts (Pongsilp, 2012). The taxonomy based on the cross inoculation concept defined plant species based on their shared symbionts. However, this idea has been challenged because many overlapping host range have been noticed and discordant plant-bacteria reactions cast doubt on its validity. Due to the short coming of this method of classification, there was a need to look for another means of classification which as a result, gave rise to the numeric classification (taxonometrics).

It is now agreed that bacterial classification must reflect the phylogenetic relationship between them, mainly witnesses being the sequences of ribosomal RNA (Fredric and philippe, 2001).The genetic relationships among rhizobial populations are of interest because they can provide the information on the gene transfer and the adaptation of bacteria to environments. Despite increasing studies on rhizobial diversity and their importance in sustainable agriculture world-wide, the gathering information is quite rare (Pongsilp, 2012). The current validly published names for rhizobia based on phylogenetic studies comprised of 92 species found in 12 genera (Weir, 2011). However, recent research suggests that there are many other rhizobial species; these new species arose through lateral gene transfer of symbiotic genes (Weir, 2011).

The diversity of rhizobia provides valuable bio-resource for the searches of bacterial isolates in attempt to find isolates that maximize legume crop productivity (Binde *et al*., 2009). Many techniques were developed and widely used to detect polymorphism including Restriction fragment length polymorphism (RFLP), and sequencing of 16S rRNA gene (Lafay and Burdon 2001; Romdhane *et al.,* 2006; Neto *et al*., 2010). One of the most advantages of 16S rRNA region is that, it is highly conservative hence, supports the well-established subdivision of rhizobia into species and genera (Sun *et al*., 2001). The sequencing of 16S rRNA gene has been previously used to detect novel taxa and new isolates (Heyndrickx *et al*., 1996). So, it is anticipated to infer the phylogenetic association among rhizobium isolates (Bala *et al*., 2003).

Additionally, the DNA sequence analysis of 16S rRNA gene is a powerful tool in discriminating among strains and also known to exhibit a great deal of sequence and length variation (Mutch and Young 2004; Neto *et al*., 2010). It is worth mentioning that although microsatellites genetic markers (SSR) are highly variable; yet it is less common in the prokaryotes genomes (Ellegren, 2004).

Many studies have addressed the diversity level of *V. faba* rhizobia; mainly focusing on rhizobial populations from the same location (Laguerre *et al*., 1994), or for comparison with R. leguminosarum isolates from other legume species(Moreira *et al*., 1987).In Ethiopia, attempts have been made to conduct research on rhizobiology of cool season legumes such as faba bean and field bean for the last two decades (Anteneh Argaw, 2012). However, there is still a scarcity of information about the taxonomic and symbiotic diversity of rhizobia nodulating faba bean from different agro ecological zones of the country.

2.6. Faba bean production and N-fixation in faba bean

Faba bean (*Vicia faba* L.) is believed to be originated in the Near East quickly spread to Europe, North Africa, along the Nile to Ethiopia (Asfaw Tilaye *et al*., 1994). China has been the main producing country, followed by Ethiopia, Egypt, Italy and Morocco (Hawitin and Hebblewaite, 1993). It is the first among pulse crops cultivated in Ethiopia and leading protein source for the rural people and used to make various traditional dishes. According to Central Statistics Agency of Ethiopia 2013, Faba bean takes over 30% (nearly half a million hectares) of cultivated land with an average national productivity of 1.5 tons per ha.

Faba bean is the most important legume crops worldwide because N fixing ability, offering high quality protein, capable of returning atmospheric nitrogen to the soil. Its seed not only provide a cheap source of protein but also a food of high calorific and nutritive value especially in the diet of low income people (Senayit Yethebarek and Asrat Wondimu, 1994). Its acreage has declined from 4.8 million ha in1961 to 2.4 in 2008 with the reduction in production from 4.8 tons per hectares to 4.4 tons per hectares. However the productivity is increased from 0.8 tons per hectares to1.7 tons per hectares globally (FAOSTAT, 2008). Many justifications have been given for the decline in the productivity of faba bean, such as

its susceptibility to biotic (Sillero *et al*., 2010) and abiotic stresses (Link *et al*., 2010). Broomrape, one of the serious constraints of faba bean in North Africa and Nile Valley and sub-Saharan Africa countries where more than 30% of faba bean is produced (Makkouk *et al*., 1994). Chocolate spot (Botrytis fabae) and faba bean rust (Uromyces viciae-fabae) became the important diseases worldwide. Viruses were also one of the major enemies of this crop mainly Faba bean leaf roll virus (FBLRV) and Faba bean necrotic yellow virus (FBNYV) (Makkouk *et al*., 1994).

Faba bean grown as field crop throughout the highlands and is most common in Wayena Dega between the altitudes 1800m a.s.l and 2400m a.s.l in Ethiopia (Asfaw Telaye, 1985).Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean(Asfaw Telaye *et al*., 1994).As the faba bean is familiar in Ethiopian feeding culture, the majority of the seed produced would be consumed domestically and only a smaller percentage of the crop is delivered to the export market. However, still this small portion of export volume put Ethiopia among the top broad bean exporting countries of the world (Biruk Bereda, 2009). Amhara and Oromia regions' are the major faba bean producing regions in Ethiopia. Within the regions some zones such as West Shoa, North Shoa, South Wello and East Gojjam are identified as major production areas of faba bean (Biruk Bereda, 2009). The growing importance of faba bean as an export crop in Ethiopia has led to a renewed interest by farmers to increase the area under production (Samuel *et al*., 2008).Despite the importance of the crop in the traditional farming system in Ethiopia, the yield is generally low as compared to the temperate (Saxena *et al*., 1991). Such failures were attributed to poor nodulation (Desta Beyene, 1988) and ineffectiveness of the indigenous rhizobia (Alemayehu Workalemahu, 2009)

Faba bean grows during cool weather when other vetches and clovers are relatively dormant, but does not tolerate heat well.In contrast to chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* L.) which have considerable drought tolerance (Morgan *et al*., 1991), faba bean has a shallow root system, little osmoregulation, and is very sensitive to high temperatures and water stress, particularly during flowering period and pod filling (Bond *et al*., 1994). Drought and heat waves are having significant effects on the productivity of faba bean in rained areas

with a Mediterranean type of climate (Maalouf *et al*., 2010). Faba bean can grow on a wide range of soils, from loams to clays, and under a variety of drainage conditions. However, it does not tolerate extended periods of saturated soils; and drought, especially at flowering, reduces seed production drastically. Fava bean tolerates a wide range of pH (4.5 to 8.3), although low pH may delay the development of root nodules, thus preventing the plant from converting atmospheric nitrogen to plant-available forms (Sattell, 1998).

Faba bean used as a winter or spring cover crop, green manure; silage, forage, hay, and vegetable. It is capable of producing large amounts of dry matter and accumulating large quantities of nitrogen (N), part of which is available to subsequent crops. Dry matter and N accumulation in fava bean depends on the variety and can be highly variable (Hauggaard-Nielsen *et al*., 2011).There is a distinct difference between faba bean and other typical grain legume species in their proportion of shoot N derived from fixation (Ndfa) in the order faba bean >lentil = soybean > pea > chickpea> common bean(Hauggaard-Nielsen *et al*., 2011). From a quantitative point of view faba bean and soybean fixes around the same amount of N (120 kg N ha^{-1}) followed by pea and lentil (85 kg N ha^{-1}) and chickpea and common bean (50 kg N ha^{-1}) (Hauggaard-Nielsen *et al*., 2011). High levels of soil nitrate, induced by such factors as excessive tillage, long periods of bare fallow and applications of fertilizer N are known to delay the formation of nodules and the onset of N_2 fixation reducing %Ndfa (Peoples *et al*., 2001). Strategies that reduce soil mineral N availability to faba bean e.g. reduces soil tillage (Peoples *et al*., 2001) and increased competition for soil mineral N such as intercropping legumes with cereals (Hauggaard-Nielsen *et al*., 2011), generally increase %Ndfa. However, several studies haves shown that faba bean can maintain a higher dependence on N_2 fixation for growth and fix more N than species like chickpea under the same soil N supply (Jensen *et al*., 2010).The amount of N_2-fixed by faba bean is estimated to be between 240 and 325 kg ha^{-1} (Somasegaran and Hoben, 1994; Maidl *et al*., 1996).

Faba bean roots need to be colonized by an appropriate strain of rhizobia bacteria to be able to convert atmospheric nitrogen into plant-available forms. Inoculating seed with the proper rhizobia bacteria ensures that the bacteria will be present when the seed germinates. It is a legume capable of fixing nitrogen in an endosymbiotic association with *Rhizobium*

leguminosarum biovar viciae thus improves soil fertility (Solomon and Fassil, 2014). It is the most efficient nitrogen fixer of all cool season pulse crops (McVicar *et al.,* 2005).

The ability of faba bean to fix the desired amount of nitrogen depends on many factors, such as the effectiveness of the rhizobium strain, the genetic variation of the host plant and other environmental and agronomic factors (Nutman, 1976). In order to obtain a very effective faba bean *R. leguminosarum biovar viciae* system, it is necessary to search for effective indigenous rhizobia with the host under laboratory and greenhouse conditions.As with most legumes, there is substantial variation in ability to form nodules and ineffectiveness of N_2-fixation with rhizobial strains, both between species and among genotypes of the same plant species (Somasegaran and Martin, 1986). Inoculation of faba bean with local rhizobial isolates at planting is generally recommended to maximize the potential for nodulation and N fixation and hence yield of the crop (Vessey, 2004).

2.7.Factors affecting symbiotic nitrogen fixation

Environmental stress affecting Symbiotic Nitrogen Fixation (SNF) may be either abiotic factors such as drought, salinity, waterlogging, temperature, soil acidity and inadequate mineral nutrients or biotic factors; insects, pests and diseases(Zahran, 1999). Most stress factors influence all physiological process in plants as stress develops. They influence all aspects of nodulation and symbiotic N_2-fixation; in some case reduce rhizobial survival and diversity in the soil. It is often difficult to isolate the effect of the stress factors in success of inoculation from their effect symbiosis functioning and N_2-fixation (Zahran, 1999).

In the Rhizobium-legume symbiosis, which is a N_2-fixing system, the process of N_2 fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigor of the host legume (Zahran, 1999) quoting (Brockwell *et al.*, 1995; Peoples *et al.*, 1995).

A critical question with regard to N_2-fixation is whether the stress first affect other physiological process, which then influence N_2-fixation, or whether stress initially and directly affects N_2-fixation mechanism(Sinclair *et al.*, 1987). Physiological understanding of most stress sensitive steps also important for establishing strategies for crop improvement and adequate management practice to optimize legume N_2-fixation and increase in its cropping system. For instance, N_2-fixation has been found to be more sensitive to soil dehydration than leaf gas exchange and nitrate assimilation (Purcell and King, 1996) and dry matter accumulation (Sinclair *et al.*, 1987; Wery *et al.*, 1994).

2.7.1. Salinity of soils

Salinity is a serious threat to agriculture in arid and semiarid regions (Rao and Sharma, 1995). Salinization is known to limit nodulation and nitrogen fixation. Response of legumes to salinity varies greatly; some legumes, e.g. *Vicia faba, Phaseolus vulgaris* and *Glycine max* are more salt tolerant than others such as, e.g. *Pisum sativum*. Other legumes like *Prosopis, Acacia and Medicago sativa* are salt tolerant, but their rhizobia are more salt tolerant than the host plants (Zahran, 1999). Significant variations are also observed among salt tolerance of different species of rhizobia. Strains of *Bradyrhizobium japonicum* is inhibited at less than 100 mM NaCl, while various strains of *Sinorhizobium meliloti* and *R. leguminosarum* grow at more than 300 mM NaCl. On the other hand rhizobia isolated from woody legumes like *Hedysarum, Acacia, Prosopis* and *Leucaena* can tolerate up to 500 to 800 mM of NaCl. Many species of rhizobia adapt to salinity stress by intracellular accumulation of compatible solutes (Serraj and Drevon, 1998).

The legume-Rhizobium symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Zahran, 1999).Salt stress inhibits the initial steps of Rhizobium-legume symbioses (Zahran, 1999). Bacterial colonization and root hair curling of *V. faba* were reduced in the presence of 50 to 100 mM NaCl; the proportion of root hairs containing infection threads was reduced by 30 and 52% in the presence of NaCl and polyethylene glycol, (Zahran, 1986).

Singleton *et al*., (1982) reported that rhizobia can generally tolerate a high level of salinity than the host legume. Fast growing rhizobia strains are more salt tolerant than slow growing one. Subbarao *et al*., (1990) observed marked differences among pigeon pea (*Cajanas Cajan)* rhizobial strains in their ability to nodulate and fix nitrogen with pigeonpean genotype under saline condition, and further observed that nodule initiation was salt-susceptible aspect of pigeonpean. The mechanism that pigeonpean tolerate salt stress by excretion of sodium and chloride ion and inversely by maintenance of potassium level.

There are also significant positive correlation between salt tolerance and adaptation of rhizobial strains in alkaline condition. *Rhizobium* nodulating *Phaseolus vulgaris* isolated from Morocco were able to resist a sodium chloride concentration up to 4% NaCl (680 mM NaCl) in liquid culture (Berrada*et al.,* 2012). There are some evidence that rhizobia strains isolated from alkaline soils are rather tolerant to high temperature, pH, and salt stress (Johnson and Wood, 1990).

2.7.2. Soil acidity

Acid soil poses major challenge to sustainable agriculture and especially to the establishment of N_2-fixating symbioses. Symbiotic nitrogen fixation can be seriously reduced in such soils, due to its effect of high Hydrogen ion, toxic level of Aluminum and Manganese and induces deficiency of Calcium, Phosphorous and Molybdenum (Mo). Mo is an essential micronutrient to all plants and is required for the formation and function of the nitrogenase enzyme complex. Soils deficient in Mo produce poor and ineffectively nodulated legumes. Soil acidity limits rhizobial growth and survival in the soil, as well as root nodule development. Acidity also affects several steps of in the development of symbiosis, including the exchange molecular signal between legume and microsymbiont (Hungria and Vargas, 2000).

Mechanism of governing competition between rhizobial strains for nodule formation under acidic condition poorly understood and the genetic basis of acid tolerance in rhizobia has yet to be elucidated. Large vibration in tolerance of acidity factors are found both within and between rhizobium species. Fast growing Rhizobia are generally considered as more acid sensitive than *Bradyrhizobium,* but low pH tolerant strains exist in many species (Glenn *et al*.,

1998).Liming is effective in overcoming soil acidity and Aluminum toxicity (Johnson and Wood, 1990).

2.7.3. Soil temperature

Nodulation and nitrogen fixation are observed under wide range of temperature with the optimum between 25˚c -30˚c. Elevated temperature delay nodule initiation and development interfere with nodule structure and function in temperate legume, whereas tropical legumes nitrogen fixation efficiency is mainly affected. Low temperature affects nodule formation and nitrogen fixation in temperate legume; however, in extreme environment of high arctic, native legumes can nodulate and fix nitrogen at rate comparable those observed with legume temperate climates, indicating that both plants and rhizobia have successfully adapted to arctic climatic condition (Wani *et al*., 1995).

Root temperature has a marked influence on the development and function of the legume-rhizobium symbiosis. The infection of the root hairs, the rate of nodule appearance, and the number of nodules formed, the amount and rate of nitrogen fixation, and the distribution of fixed nitrogen from the nodules are affected by root temperature (Gibson, 1971). Root temperature effects on legume nodulation and N_2 fixation are subject matter under consideration to choice of time of sowing of winter-grown legumes. Optimum temperatures for nodulation are often higher than for N_2 fixation (Gibson, 1971). Legumes grown at low temperature shows delay in nodule formation and the onset of N_2 fixation even in the presence of adequate populations of rhizobia.

2.7.4. Soil nutrients

Soil nutrient status has an impressive influence on the symbiosis, as well as on the independent growth and survival of both partners. It should be noted, however, that in some cases, nutrient stresses are indirectly caused by changes in soil matric potential or acidity, which limit nutrient bioavailability, rather than to the lack of the presence of nutrients. When considering nutrient limitations to symbiotic nitrogen fixation, one must clearly separate factors affecting growth of the host from those influencing the microbe or the symbiotic interaction. For example, acid and water stress causes alterations in root growth, which can

indirectly affect both nodulation and nitrogen fixation (Zhang *et al*., 2001).The legume-rhizobia symbiosis imposes additional nutritional requirements apart from minerals need for plant growth, as whole. Nutrient that affect symbiotic nitrogen fixation include; NO_3^- N, P, B, Zn, S, Molybdenum (Mo) and cobalt (Serraj and Adu-Gyamfi, 2004).

P is second only to N as the most limiting element for plant growth (Vance *et al*., 2000). The amount of P in plants ranges from 0.05% to 0.30% of total dry weight (Vance, 2001). Crop yield on 40% of the world's arable land is limited by P availability. P is unavailable because it rapidly forms insoluble complexes with cations and is incorporated into organic matter by microbes (Vance, 2001). Phosphorous supply and availability remains a severe limitation to nitrogen fixation and symbiotic interactions.Nodulation and N_2 fixation are strongly influenced by P availability. Nodule establishment and function are important sinks for P and nodules usually have highest P content in plant (Sinclair and Valdez, 2002). Nitrogen fixing plants have an increased requirement for P over that receiving direct nitrogen fertilization, owing perhaps to nodule development and signal transduction (Graham and Vance, 2000). About 33% of the arable land in the world is limited by P availability (Graham and Vance, 2000). This situation is especially true in soils impacted by low pH.

It has long been known that P plays a major part in the build-up and maintenance of soil fertility through its effect on legume growth (Donald and Williams, 1954). More recently, there has been an accumulation of evidence of a specific effect of P on the growth and survival of rhizobia and their capacity for nodulation and N_2 fixation (expression of differential symbiotic effectiveness (Serraj and Adu-Gymafi, 2004). Therefore, p deficiency condition result in reduced SNF potential and P fertilization will usually result in enhanced nodule number and mass, as well as greater N_2-fixation activity per plant.

Stress conditions apparently increase requirements for Ca^{2+}. Ca^{2+} might, in some instances, offset the deleterious influence of low pH on root growth and ion uptake (Torimitsu *et al*., 1985) and increase nod-gene induction and expression (Richardson and Simpson,1989). Calcium deficiency, with or without the confounding influence of low pH, also affects attachment of rhizobia to root hairs (Smit *et al*., 1992), and nodulation and nodule

development (Alva *et al*., 1990). To sum up, a calcium-spiking phenomenon is initiated in root-hair cells of legumes by nodulation factors and rhizobia (Wais *et al*., 2002), suggesting that Ca2+ plays a pivotal role in symbiotic interactions at the molecular level.

Molybdenum has a major role in symbiotic N fixation as a fundamental component for nitrogenase. Mo deficiency affects nodule development by reducing bacteroids multiplication and delaying or preventing the onset of nitrogenase activity (Voisin *et al*., 2002). Iron is required for legume nodulation, possibly for the proliferation of the infecting rhizobia in the host root tissue. Legumes are sensitive to Fe deficiency at an early stage of nodule initiation (Panda and Panda, 2002).

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

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

2.7.5. Drought stress

Legume productivity in semi-arid tropics (SAT) is largely limited by low moisture availability in addition to nutrient deficiencies. The relative high nitrogen and biomass accumulation to soil dehydration was demonstrated for soybean (*Glycine* max) grown on a soil with virtually no mineral N reserve (Sinclair *et al*., 1987), with essentially an N uptake resulting from N_2 -fixation, a comparison of biomass accumulation and N accumulation rates offered an index of the relative sensitivity to water deficit conditions under which the plants were grown. Sinclair *et al*., (1987) conclude from their study on soybean that N_2 -fixation was more sensitive to drought than was carbon assimilation. In a similar study on 24 soybean line Serraj and Sinclair (1997), found that, in almost all soybean cultivars tested N accumulation was more sensitive to soil dehydration than was biomass accumulation.

Fast-growing *rhizobia* are sensitive to soil dehydration as compared to slow-growing strains (Sprent, 1971). On the other handZahran (1999) showed that exposing rhizobia to osmotic stress bring about alteration of bacterial membrane lipopolysaccharides, which are involved in the *Rhizobium*-host plant recognition process (Kamst *et al*., 1998).

3. MATERIALS AND METHODS

3.1. Experimental sites

The experiment was conducted in Hawassa University, college of Agriculture. Hawassa is found 275 Km south of, Addis Ababa, the capital city of Ethiopia. The latitudinal and longitudinal location of Hawassa are; 07^0 03’ 19.1’’ to 07^0 04’ 00.2’’ N and 38^0 31’ 08’’ to 38^0 31’ 01.8 E respectively. It receives mean annual rainfall of about 948 mm. Its mean annual maximum and minimum temperatures are 27.3^0C and 12.6^0 C, respectively and its average monthly relative humidity is 61% (Abay Ayalew, 2011).

3.2. Isolation of rhizobia from root nodule

During the late flowering and early pod setting stages root nodules samples were collected in vials containing silica gel plugged with cotton from young and healthy seedling of Faba bean (*Vicia faba*) from farmer’s field (10 kebeles that ranged within 7 Woredas) of Wello. These were brought in laboratory without any delay. According to Bala *et al*., (2011) the vials containing nodule sample were preserved in refrigerator at Hawassa University, College of agriculture, in soil microbiology laboratory.

Isolation of rhizobia from root nodules was done following the method of Somasegaran and Hoben (1994).Individual nodules were picked up and washed thoroughly with sterile distilled water. After being washed with several times with distilled sterilized water, nodules were surface sterilized in 95% alcohol for 10 seconds and subsequently they were submerged in 4% sodium hypochlorite for 4 min and washed in sterile distilled water with six changes. Each nodule was crushed under aseptic conditions and streaked onto a Yeast-Mannitol Agar plate (YMA) using a sterile loop and incubated at28 ± 2˚C for 3 to 5 days (Vincent, 1970). YEMA medium was prepared according to (Vincent, 1970) containing Mannitol=10gm, K_2HP0_4=0.5gm, $MgS0_4.7H_20$=0.2gm, NaCl=0.1gm, Yeast extract=0.5gm, Distilled water=1L and Agar agar=15gm, and pH was adjusted at 6.8 before addition of agar-agar. After incubation for 3-5 days at 28 ± 2˚C, single colonies were selected and restreaked on YEM agar until single pure colonies were obtained.

3.3. Presumptive test

The purity of the cultures was confirmed based on morphological parameters of different colonies, Gram staining technique (Subba Rao, 1983).The putative pure isolates that shown mucoid, watery and whitish characteristics' on YEMA media were further characterized on YEMA media which comprised of Congo red (YEMA-CR) as indicator to check if there was any contaminants in it.The working CR solution was prepared by dissolving 0.25gm of CR in 100ml of distilled water. YEMA-CR media was prepared by dispensing 10ml of CR stock solution in one litre of YEMA medium. The pure isolates were inoculated on YEMA-CR medium and the plates were wrapped with, aluminum foil, to provide a dark condition and incubated to at 28±2˚C. After 4 days of incubation the CR absorption characteristic of each isolate was scored (Somasegaran and Hoben, 1994).

The production of acid or alkaline was determined by incorporating Bromthymol blue (BTB) as reaction indicator of change in pH in yeast extract manitol agar (YEMA). YEMA- BTB media was prepared by dispensing 5ml of BTB stock solution in YEMA media. The pH of the media was adjusted at 6.8 using 0.1N NaOH and HCl. Change in media color due to acid or base production was observedin 3-5 days of incubation and the results were recorded as fast growers or slow growers depending on color change of the media(Jordan, 1984). The working BTB solution was prepared by dissolving 0.25gm of BTB in 100ml of 95% Ethanol. From working solution 5ml was added to 1L of YEMB and the pH was adjusted to 6.8 with buffer solution (0.1N NaOH) (Somasegaran and Hoben, 1994).

3.3.1. Peptone glucose agar (PGA) growth test

Peptone-glucose-agar (PGA) medium comprising 5g glucose, 10g peptone and 15g agar in a liter of distilled water (Subba Rao, 1983) was also used for similar presumptive test of the *Rhizobium.* The working solution of PGA-BCP was prepared by dissolving 10µgm of BCP stock powder in 100ml of distilled water. From the working solution 10ml was added to 1L distilled water which contains peptone and glucose. The pH of medium was adjusted to 6.8 using buffer solution 0.1N NaOH or HCl.

3.3.2. Gram's staining reaction test

Gram's stain reaction of the isolates was done to check if the purity of the culture of the test strain is Gram-negative or Gram-positive. It was carried out by using a loopful of pure culture grown on YEM broth (yeast extract mannitol broth) and stained as per the standard Gram's procedure (Somasegaran and Hoben 1994).The isolates which retained primary stain (crystal violet), blue in color, were recorded as gram positive and whereas the isolates which retained counter stain (Safranine), red in color, were recorded as gram negative. The isolate which retained primary stain was discarded realizing rhizobia are not gram positive bacteria, whereas the isolates which retained counter stain and which had rod shape under oil immersion microscopy were considered as rhizobia (Subba Rao, 1983).

3.3.3. Yeast mannitol broth (YEMB) culture

YEMB culture media was prepared similar toYEMA medium, but by leaving out agar. a representative pure isolates of the presumptive rhizobia: which failed or weaklyabsorbed CR in dark condition;turned pH of BTB mediafrom neutral to acidic (green to yellow); stained as gram negative rods for Gram's staining reaction; and unable to grow or poorly or moderately grown on PGA, were selected and duplicated in 10ml YEM-broth culture (Vincent, 1970). Ten milliliter (10ml) of YEMB media solution was dispensed in to screw-cup test tubes. Each screw cup test tubes with 10ml of YEMB media solution was autoclaved at temperature of 121˚C at steam pressure of 15lbs for 15 minutes before being inoculated with selected isolates.The incubation temperature and revolution of broth culture were 28˚C and 130 rpm, respectively. After maximum turbidity was formed; which was confirmed by measuring Optical density of the isolates, the turbid broth were dispensed with three copies in vials containing 50% glycerol in the ratio of (300µl glycerol: 700µl sample broth)and preserved in deep freeze (-21˚C).The inoculation of culture media and dispensing of the isolates in to vials was done under a laminar flow hood to prevent contamination. The collected and purified rhizobial cultures were preserved in vials and coded as: FBW1, FBW2, FBW3, FBW4; … and FBW165.The viability of preserved isolate was checked by inoculation in YEMB media after one week of preservation.

3.4. Authentication of the Isolates

The microsymbiont recovered from faba bean nodules were inoculated onto the newly emerged seedlings of faba bean CS298DK variety following aseptic laboratory procedure to verify whether the new isolates were nodulating once or non-nodulating nodule endophytes. To do so, modified Leonard jars with cotton wick were used to grow plants (fig.1). These were prepared from plastic cups filled with thoroughly washed, sun dried and autoclave sterilized river sand at (121˚C temperature for 15minnutes using steam and high pressure).

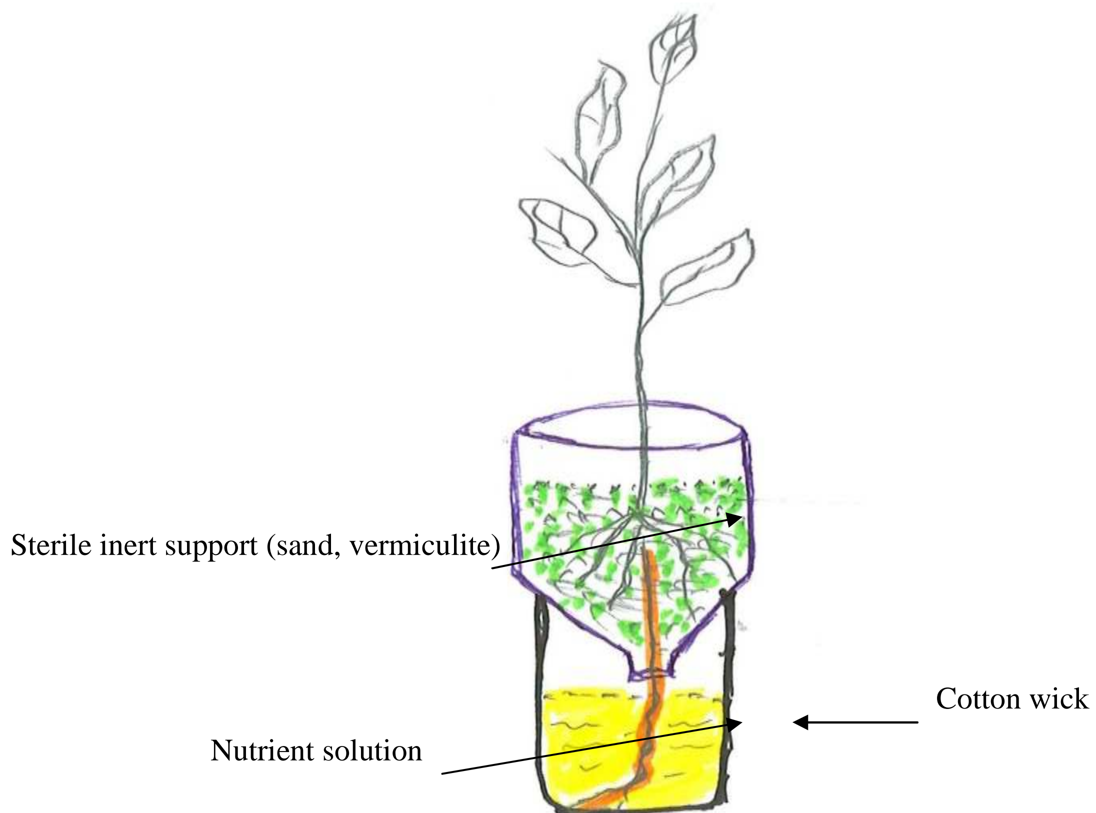


Figure 2: Schematic representation of modified Leonard Jar

Seeds of faba bean (CD298DK variety) were obtained from Hawassa Agricultural Research Center.A healthy intact seeds of faba bean (CD298DK variety), uniform in size and in color,and their phylogenic affiliation studied previously were surface treated with 95% ethanol for 10 seconds and then 3% (w/v) sodium hypochlorite solution for 3 minutes. Then seeds were further rinsed six times in sterile water. After imbibing (4hr in sterile water), seeds

were transferred aseptically to 1% water agar plates and allowed to germinate for at room temperature in the dark (Rashid *et al*., 2012).The pre-germinated faba bean seeds with similar size and radicle selected; aseptically transferred using sterile forceps. Five seedlings were initially transplanted per pot and were later on (one week after emergence) thinned down to three and were individually treated with1ml of broth culture approximately (10^8 to 10^9cells/ ml).

The experiment was laid out in complete randomized design (CRD) and replicated three times with two controls--- the negative control (without N and *Rhizobium*) and the positive control (with N and without *Rhizobium*).The pots were supplied with 100ml of quarter strength Broughton and Dilworth N-free nutrient solution at the beginning of transplantation and for further five weeks once a week and Distilled sterile water was added whenever required (Somasegaran and Hoben, 1994). For the positive controls was given as 0.05% KNO_3 (w/v) by applying 120 ml during inoculation and 21 days later (Singleton and Tavares, 1986).Stock nitrogen-free nutrient solution for plants was prepared according to Broughton and Dilworth(Somasegaran and Hoben, 1994)as indicated in table (1).

Table 1: nitrogen free plant nutrients solution (Broughton and Dilworth, 1970)

Stock solutions	**Chemicals**	**g/liter**
1	$CaCl_2.2H_2O$	294
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4 (trace elements)	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2. 2H_2O$	0.048

Source (Somasegaran and Hoben, 1994).

After the stock solutions were prepared, 10 liters of nitrogen-free plant nutrient solution was prepared by mixing 5 ml of each stock solution with 5 liters of distilled water and further diluting it to 10 liters by adding another 5 liters of distilled water. The pH of the solution was adjusted to 6.8 with 1N NaOH and HCl.

After five weeks of planting, the crops were checked for nodulation both in the inoculated and uninoculated modified Leonard jars to detect the presence or absence of nodules from each pot (+) and (-) sign were designated for nodule formation and absence, respectively (Beck *et al*., 1993).The greenness of plants were qualitatively scored by comparing the plant color with the color of the uninoculated plants (N-) and the positive controls (N+) which was assigned with scores 1 and 3,respectively, with a color in between the controls assigned as 2. nodule number per plant were scored as; 1:rare (<5 nodules), 2:few (5-10) nodules, 3:moderate (11-20 nodules), 4:abundant (>20 nodules) and 5:super nodulated (>50), while internal color were scored as;1,white,2,green,3,pink,and,4,red (Bala *et al*., 2011).

The test isolates were scored as ineffective if the total sum of plant color and nodule internal color were between 1 and 3,moderately effective if between 4 and5 and very effective if between 6 and 7 (Musiyiwa *et al*., 2005).

3.5. Symbiotic Effectiveness of test Isolates

Seventy isolates which effectively nodulated the faba bean host plant in the authentication experiment, as revealed by functional nodules (pink internally) were selected for symbiotic effectiveness. The same variety of faba bean seeds used for authentication test was also used for symbiotic effectiveness.

For this purpose, river sand was intensively water washed and filled into clean autoclavable modified Leonard jars and was steam sterilized (autoclaved). Faba bean seeds were surface sterilized as described earlier and were pre-germinated on sterilized petri dishes dispensed with sterilized water agar after which three seedling were transplanted per pot. Culture suspension of each of authenticated (effective) rhizobial isolate culture were prepared by growing in YEMB and adjusted to 10^9cells/ml (Somasegaran and Hoben, 1994). Then 1ml of the culture suspension of the isolates was taken and dispensed onto each seedling.

All the pots were irrigated as required with distilled water and fertilized with quarter strength of Broughton and Dilworth N-free nutrient solution $week^{-1}$ as described by Somasegaran and Hoben (1994). Both positive and negative controls were included as treatments. The positive control pots received 120 ml of 0.05% (w/v) of KNO_3 during inoculation and after 21 days as nitrogen source, whereas the negative control pots were devoid of both nitrogen sources and rhizobial isolates. Three Commercial isolates namely; EAL-110, Debre-sina and Butajira were included as standard control.

After forty five days of transplanting, the plants were uprooted and their root systems thoroughly rinsed with distilled water. Nodules were carefully detached and rinsed with distilled water. Symbiotic effectiveness parameters; nodule internal colour, nodule number, shoot fresh weight, shoots dry weight (gm $plant^{-1}$), root fresh weight, root dry weight and symbiotic effectiveness (%) were determined. Shoot and root dry weights were recorded after drying for 24hr at 70°C. The percent symbiotic effectiveness (SE) of the isolates was computed using the formula:

SE (%) = (shoot dry weight of inoculated plant/shoot dry weight of N supplied plant) ×100

Finally, the symbiotic effectiveness (SE) values of the isolates were rated as highly effective (> 80%), effective (50-80%), poorly effective (36-49%) and ineffective for SE < 35% (Beck *et al*., 1993).

3.6. Determination of N-content of the Plant

After the shoots dry weight of each growth unit determined, the shoots were finely grinded using pistil and mortar then were sieved with 0.5mm size.Each treatment was represented by three replicates. The total nitrogen content of the shoots was determined by modified semi-micro Kjeldahl method according to Sahlemedhin Sertsu and Taye Bekele (2000). The kjeldahl procedure is based on the principle that by treating plant material with concentrated sulfuric acid to oxidize the nitrogen in to ammonium sulphate. The ammonium liberated in the distillation process with NaOH is trapped by acid. The ammonium is absorbed in the form of NH_4^+ in boric acid and back titrated with standard H_2SO_4 (Sahlemedhin Sertsu and Taye Bekele, 2000). The percentage of total nitrogen was calculated as follows.

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

Where, a= ml of H_2SO_4 titration of sample, b= ml of H_2SO_4 titration of blank

S= sample weight in mg, N= normality of sulfuric acid

0.014= meq weight of nitrogen in gm, Mcf= moisture correction factor

Total N_2 were assayed in the shoot by the Kjeldahl methods described by Eissa *et al.*, (2009).Total nitrogen content = $N_2\% \times$ shoot dry weight of plants.

3.7. Methods of Statistical data analysis

Data collected was statistically analyzed by subjecting to analysis of variance (ANOVA) using General Linear Models Procedure of SAS software version 9. Means of all treatments were calculated and the differences tested for significance using the least significant differences (LSD) test at 0.05 probability (p) level. Correlation coefficients were calculated to study the associative relations among the measurement traits using Pearson correlations.

3.8. Characterization of the isolates

All of the selected seventy isolates which effectively nodulated the host (faba bean) while tested for authentication test along with commercial strains were further characterized by their morphological and phenotypic features. All inoculations were standardized with an inoculum size of approximately 10^9cells/ml and were carried out in triplicates on YEMA. Except for cultures used to determine the minimum and maximum growth temperature, all inoculated plates were incubated at 28± 2˚C. Results were recorded after 4 days. Result for growth tests was determined qualitatively and presented as '+' for growth and '-'for no growth.

3.8.1. Morphological Growth characteristics of the isolates

A loop full of each of the selected isolate from authentication result was streaked on YEMA plates and incubated at 28˚C and growth was checked 4 days later. Data on colony characteristics such as; size, shape and colony texture was recorded. All tests, except

carbohydrate and N-source utilization, were carried out on YEMA plates(Hashem *et al*., 1998).

3.8.2. Biochemical and physiological characteristics of the isolates

Tolerance to extreme pH

The ability of the rhizobial isolates to grow in basic and acidic media was tested by streaking them on YEMA media for which the pHs were adjusted to 4,4.5, 5.0, 5.5, 8.5, 9.5 and 10.0by HCl or NaOH (Assefa Keneni *et al*., 2010). YEMA medium with pH of 6.8 was used as control. The isolates were incubated at 28°C and the growth was evaluated qualitatively after 3-5 days of incubation (Jordan, 1984).Isolates were considered tolerant to extreme pH when growth was similar to the growth in the control plates.

Tolerance to extreme salt concentration

The ability of the Rhizobial isolates to grow in various concentration of NaCl was tested by plating them on YEM agar Petri dishes. YEMA was prepared with different concentrations of NaCl containing 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5%(W/V) NaCl (Sadowsky *et al*., 1983;Alemayehu Workalemahu, 2010; Hewedy *et al*., 2014; Keneni *et al*., 2010). All the plates were incubated at 28°C for three days in triplicate in addition to control.

Tolerance to extreme temperature

Difference in the range of growth temperature were investigated by incubation of bacterial cultures on YEM agar at 5°C, 10°C, 15°C, 37°C, 40°C, and 45°C (Zerihun and Fassil, 2010; Alemayehu Workalemahu, 2010). Control plates were incubated at 28°C (Lupwayi and Haque, 1994). Isolates were considered extreme temperature tolerant when growth was similar to the growth in the control plates (plates incubated at 28°C).

Carbohydrate utilization test

Carbohydrate utilization of strains was determined following the method of Somasegaran and Hoben (1994) on teen carbohydrates prepared as 10% (w/v) solution in distilled water or 1gm of each carbohydrate was dissolved 10ml of distilled water. Heat labile carbohydrates; galactose, sorbitol, mannose, dextrin, trehalose and L-(+)-raffinose were filter sterilized using

0.2µm pore size sterile disposable membrane filter and 10ml hypodermic syringe.Filter sterilized carbohydrate solutions were stored in refrigerator and added to autoclaved carbohydrate free basal YEA-BTB medium aseptically in the laminar flow hood environment. Whereas heat-stable carbohydrates; Sucrose, D (+)-Glucose adydrous,D (-)-Fructose dihydrate and Starch, were added in basal YEA-BTBand autoclaved together with basal medium. YEMA-BTB basal medium was prepared similar to YEMA-BTB medium but with some modification; Mannitol was not added and yeast extract was reduced to 0.05gm per 1000ml(Somasegaran and Hoben 1994). 90ml of Carbohydrate free basal medium was dispensed in 250ml capacity Erlenmeyer flasks and 1.5gm of agar was added to each flask.

Utilization of amino acids as sole source of nitrogen

The amino acids were added at a concentration of 0.5 g/l to a similar media from which ammonium sulfate was omitted and mannitol was added at a concentration of 1 g/liter (Amarger *et al.*, 1997) and the nitrogen source (yeast extract) was replaced by one of the following amino acids as a source of nitrogen: Filter sterilized; L-histidine, L-valine, L-asparagine, L-aspartic acid, L-phenylalanine, L-arginine-alanine, isoleucine and Glycine were used as sole nitrogen source for isolates. Inoculated plates were incubated at 28°C and results were observed after 3-5 days.

Intrinsic antibiotic resistance (IAR)

Intrinsic antibiotics resistance(IAR) study was undertaken in laboratory by preparing stock solutions (10mg/ml) of antibiotics in distilled water, except erythromycin which was prepared as (5mg erythromycin in 1ml of ethanol). The stock solutions were filter sterilized and stored at 0˚C. The YEMA medium was prepared by dispensing 100ml in Erlenmeyer flasks(250ml capacity) and autoclaved at 121˚C and 15lbs for 15 minutes. The medium was cooled in water bath adjusted to 50˚C and appropriate concentration of antibiotic was delivered. A micropipette with sterilized disposable tips was used to deliver appropriate volume of the stock antibiotics solutions from storage vials. To assure uniform distribution of antibiotics in the YEMA media the medium was swilled continuously. Intrinsic antibiotics resistance (IAR) of isolates to Kanamycin sulfate, Streptomycin sulfate, Spectinomycin sulfate salt, Chloramphenicol, Neomycin trisulfate and Erythromycin with concentration of (µg/ml) 5 and

10 was tested. The melted medium with respective concentration of different antibiotics poured into four previously oven sterilized petri-dishes for each concentration and allowed to solidify by overnight incubation. Pure culture of each isolates which grown in YEMB medium were streaked on the antibiotics plates and plates without antibiotic used as control. The plates inoculated with isolates were incubated for 4 days at 28˚C. The growth was recorded as positive (+) for the presences of growth while negative (-) for absence of growth when compared with the positive control.

3.9. Numerical Taxonomy

Bacterial diversity may be high both in genetic and phenotypic (symbiotic, cultural, morphological and physiological) traits and it is thus necessary to define the level of diversity which is appropriate to characterize particular genera and species (Swift and Gignell, 2001). Phenotypic similarities among faba bean nodulating rhizobia were numerically analyzed based on their 6 phenotypic characteristics, such as pH tolerance, temperature tolerance, salt tolerance, intrinsic antibiotic resistance, carbohydrate utilization and amino acid utilization. The data were coded for bacterial growth characteristics as follows: 0 for an absence of growth, 1 for presence of growth. The final matrix contained 70 isolates plus three commercial isolates and 55 traits. A computer cluster analysis of 55 phenotypic variables was carried out using similarity coefficient by the Unweighted Pair Group Mean with the Average (UPGMA) clustering method with NTSYSpc21 program.

4.RESULTSAND DISCUSSION

4.1. Presumptive tests of the test strains

In the present study, a total of 132 isolates were isolated from root nodules of faba bean species (*vicia faba*) collected from some part of Wello, Northern, Ethiopia. All isolates were identified as gram negative rods and with the exception of FBW (19, 118,119, 120, 121, 123, 124 and 152) almost all isolatesdid not absorb congo-red at dark condition when incubated at 28˚C.These exceptional isolatesabsorbed the congo red and formed pink colony on the medium, which contradicts with the results from faba bean rhizobia of Tahtay Koraro, Northwestern Zone Of Tigray Regional State, Ethiopia by (Solomon Legesse and Fassil Assefa, 2014) where none of their isolates were found to absorb congo red. On the other hand our result was in harmony to the finding of Abere Manalku *et al.*, (2009) where 12.25% of the isolates produced dark red colony on Congo red YEMA medium. Zerihun Belay (2006) and Getaneh Tesfaye (2008) also observed one and two faba bean rhizobia isolates taking up Congo red under dark condition, respectively. All of our test strains showed growth in three days and turned the yeast mannitol agar media containing Bromthymol blue to yellow color showing that they were fast growers and acid producers (Somasegaran and Hoben, 1994). Similarly, a study made by Ayneabeba Adamu *et al.* (2001) on faba bean rhizobia isolated fromNorthern Shoa confirmed that all faba bean nodulating rhizobia were acid producing.

Growth in peptone glucose agar as reported by Vincent (1970) indicates that most of the Rhizobium isolates grow either poorly or moderately in this medium. Isolates FBW (10, 12,20, 84, 110, 133 and 164)showed poor growth on the medium. Indeed, isolatesFBW (15, 61, 123, 128, and 146) showedmoderate growth on the medium. However, the rest isolates did not grow at all in this medium. The growth of some isolates on PGA media in our experiments contradicts with the finding of Abere Manalku *et al.*, 2009 and Subba Rao, 1983 where none of the isolates was found to grow on PGA medium. This result also contradicts with the description given by(Somasegaran and Hoben, 1994) that states PGA does not allow the growth of rhizobia but other contaminants. On the other hand this finding was in line with the finding of (Anteneh Argaw, 2012) where out of 60 isolates eleven isolates were grown well on PGA media.

The results obtained from Gram staining reaction preliminarily confirm the standard culture and morphological characteristics of rhizobium species as described by (Somasegaran and Hoben, 1994). Although Congo red absorption in dark condition is generally considered a contradiction of rhizobium as described by (Somasegaran and Hoben, 1994), but augmenting idea were reported earlier by (Knee and Larue, 1983; Hahn, 1966). They reported that Congo red absorption not only depends on incubation condition but also composition of the media.On nitrogen free or synthetic nitrate-containing medium supplemented with 0.0025% Congo red, rhizobia reportedly produce white colonies which can be differentiated from colored colonies of other soil bacteria (Knee and Larue, 1983; Hahn, 1966). On the other hand on nitrogen-rich yeast extract-mannitol agar containing Congo red (CR-YMA), rhizobia cannot be easily distinguished from other organisms. Indeed, the uptake of Congo red dye varies among strains of *R.Leguminosarum*. From their conclusion Congo red absorption does not distinguish rhizobia from other bacteria, but may be useful as strain marker. Generally, results for the current study indicate that all isolates were fast-growing as reported by Jordan (1984).

4.2. Authentication and pre-screening the symbiotic effectiveness of isolates

In subsequent experiments, all isolates (132) were assessed for their ineffectiveness and symbiotic effectiveness, of which, only 70 isolates and one commercial strain (D/Sina) were authenticated as nodule forming bacteria of faba bean and the remaining isolates (62) and two commercial strains (EAL-110 and B/Jira) failed to nodulate the host. Regarding on nodule internal color out of the 70 isolates that induced nodule formation, 56(80%) were found to form effective nodules (pink in color internally) whereas the14 (20%) isolates were found to form ineffective nodules (green and white in color internally) (Annex-1). Somasegaran and Hoben (1994) suggested that nodules with a pink color indicate an effective nodule, whereas white and greenish infer ineffective symbiosis. Uninoculated plants (N- controls) and rest of the isolates did not show nodule formation and the plants started to show chlorosis and wilting after the first three weeks of the experiment. After authentication test, 70 isolates that nodulate host plant were confirmed to be the true symbiont of faba bean, and these were taken for further research undertakings.

The nodulating isolates showed differences in nodule number (Annex-1), accordingly highest nodule number (82) was recorded from the isolates FBW58 and FBW158 followed by

isolates, FBW (165, 163, 160, 162, 123, 95, 140 and 155) with nodule number of 69, 67, 65, 65, 63, 50, 50 and 50 nodules/plant, respectively. On other hand the lowest nodule numbers were recorded for isolate FBW114 (1 nodule per plant). Preliminary identification of the effectiveness of the isolates has been determined by nodule index analysis and plant leaf color following (Musiyiwa *et al*., 2005). Accordingly, 40 isolates were found to be effective, 27 isolates were found to be moderately effective and the rest 3 and commercial strain (D/Sina) were found to be ineffective (Annex-1). The typical nitrogen deficiency symptom in plant is yellowing (chlorosis) of the lower leaves (Woomer, 2010). Under extreme deficiency, leaves are pale or yellowish, fall prematurely, affected plants are stunted and yields are extremely low (Woomer, 2010). The yellow plant leaf color characteristic was observed in the negative controls of our experiment, which presupposes that the plant was in a shortage of nitrogen supply while the other essential elements were given with a nitrogen free nutrient (fig.3).

Figure 3: leaf color difference among negative control, positive control and inoculated plants

Results shown in (Table 2) suggest that bacterial inoculation significantly ($p<0.05$) influence all parameters investigated compared with control (neither inoculated nor fertilized with N). Moreover, measured parameters show significant variability among rhizobium treated plants

at $p<0.05$.The ANOVA (LSD test) declared that the mean comparison were shown to be significantly different, which basically did not necessarily mean that all means were different; but mean comparison showed several overlapping groups for all measured parameters.

The mean nodule number plant^{-1} record ranged from 3±1.73 for plant inoculated with isolate FBW120 to 78.667±3 for plant inoculated with test isolate FBW58. The mean nodule number produced in this study (25 nodules plant^{-1}) was much less than124, 87, 167.8 and 128 nodules per plant obtained by Zerihun Belay (2006); Getaneh Tesfaye (2008); Anteneh Argaw (2012) and Abere Mnalku *et al*., 2009) respectively. The mean nodule number scored from D/sina commercial inoculant was 4. This study indicated that lower nodule number could either be due to the variations in host-rhizobium genotypes or it could be a matter of survival where the plant strategically form few nodules with effective strains in order not to waste much energy while forming nodules, which actually need much amount of ATP (Somasegaran and Hoben, 1994). In fact the highest nodule obtained from this study (82 nodules plant^{-1}) almost comparable with the highest nodule number (96 nodules plant^{-1}) recorded by Alemayehu Workalemahu (2009) on faba bean rhizobia from Ofla, southern Tigray, Ethiopia.

Despite the small number of nodules plant^{-1}, recorded from plants inoculated with isolates FBW (46, 66, 72, 94, 95, 103, 140, 141, 142, 144 and 145), these isolates have showed high symbiotic effectiveness (as measured and expressed in %) as shown in (Annex-1). Somasegaran and Hoben (1994) suggested that nodule number is a less reliable indicator of strain effectiveness. This implies that the presence of few effective nodules on plant roots may be enough to fix N for maximum benefit to the host plant.

The mean root fresh weight when measured as g per plant, were found to range between 1.06 (for FBW42) and 5.07(for FBW72). The mean root fresh weight of plant inoculated with strain FBW72 exceed the mean root fresh weight of uninoculated negative control with averages up to 3-fold. Maximum and minimum mean root dry weight were (0.66 g/plant and 0.1 g/plant)scored by FBW145 and FBW42, respectively (Table-2), which was greater than that previously reported by (Cordovilla*et al*., 1996).On the other hand the current results were in agreement with those obtained by (Belal *et al*., 2013; Hamouda and Farfour, 2013) who

observed that inoculation of *R. leguminosarum bv. Viciae* to faba bean significantly increased the root fresh and dry weight over the uninoculated control (N-).

Similarly, all tested isolates of faba bean rhizobia exhibited high diversity in their shoot dry matter accumulation. In this experiment, noticeable shoot dry matter accumulation differences were also apparently observed between the controls and inoculated treatments. The maximum mean shoot dry weight value scored was 1.0gm plant^{-1} for isolate FBW145 while the minimum mean was 0.16gm plant^{-1} for the isolate FBW42 (Table 2). The mean shoot dry weight scored by commercial inoculant (D/Sina) was 0.49. Plant inoculated with strain FBW145 exceeded in shoot dry matter accumulation over negative control and commercial inoculant (D/Sina) with up to five and two fold, respectively. Besides this, plant inoculated with isolates FBW145 and FBW140 accumulated higher shoot dry matter even surpassed the positive control. As compared to negative control plant inoculated with strains FBW(145 and 140) exceed in shoot dry matter accumulation 0.79g (79%) and 0.76g (76), respectively. While, plants inoculated with two strains; FBW (145 and 140) had a shoot dry weight of (0.15g) 15% and (0.3g) 3% over the positive control, respectively. Though plant inoculated with isolate FBW140 accumulated higher shoot dry matter than N+ control, ANOVA result declared that there was no significance difference between plant inoculated with isolate FBW140 and N+ control (Table 2). In other respect plant inoculated with isolate FBW145 significantly accumulated higher shoot dry matter than N+ control plant. There was no significant difference at ($p<0.05$) in shoot dry weight between the faba bean plant inoculated with FBW144 strain and the nitrogen fertilized control plant.

In general, ANOVA result as shown in Annex-2 declared that inoculation sustained significantly ($P<0.05$) higher shoot dry weight than the negative control. In fact, plants inoculated with isolates FBW138 and FBW136, accumulated significantly lower SDW than negative control (uninoculated negative control). This might be due to extraneous source of errors. More than 54% of the current study isolates were significantly ($p<0.05$) increased shoot dry matter of the plants over commercial inoculant (D/Sina).

The mean shoot dry weight of plants inoculated with isolates in this study (0.5 g plant^{-1}) (appendix- was less than the average value (1.63g plant-1) obtained by Zerihun Belay (2006), (1.21g plant-1), Getaneh Tesfaye (2008) and much less than the result obtained by Assefa

Keneni *et al*. (2010) Somasegaran and Hoben (1994) and Peoples *et al*. (2002) explained that shoot dry matter is a good indicator of relative isolate effectiveness. In the current study, all strains ere better as compared to the negative (uninoculated) control in terms of biomass accumulation in the tissue even more than commercial inoculant strain (D/Sina).

Based on the shoot dry matter values of inoculated plants in relation to the nitrogen fertilized control plants, the symbiotic effectiveness of each isolate was determined (Annex-3). Accordingly, only 12.9% of the isolates were found to be highly effective with shoot dry mass accumulation ranging between 80 and117.6 percent over the nitrogen fertilized control plant. These isolates were FBW (72, 94, 95, 107, 140, 142, 144, 145 and 160). Two isolates FBW (140 and 145) showed better performance in SE% exceeding 100% over N treated plants.Similar reports were obtained with rhizobia of faba bean from Eastern and western Harerghe highlands, and central Ethiopia (Abere Mnalku *et al*., 2009; Anteneh Argaw, 2012). Similarly, recent study by (Solomon Legesse and Fassil Assefa, 2014) also reported the same where one of their isolate showed better performances over N treated plant. While nitrogen fixation could be the possible, plant growth promoting hormones produced by some rhizobial could also be another reason as reported by (Pongsilp and Nimnoi, 2009).

Besides, 54.3% of the isolates were found to effective with shoot dry matter accumulation ranging between 50 and 80% as compared to nitrogen fertilized control plants.This was contrary to the report of (Desta Beyene and Angaw Tsigie, 1988) that showed only 23 symbiotically effective isolates (21%) among the 108 isolates. In the other respect 21.4% of the current strains were found to be poorly effective with shoot dry mass accumulation of 36-49% and whereas 11.4% were found to be ineffective with shoot dry mass accumulation of <35% relative to nitrogen fertilized control plants.

As to correlation coefficients among investigated parameter, nodulation show positive correlation with all measured parameters (Annex-2). Nodules number was found to be correlated with shoot dry weight ($r=0.18$, $p<0.01$) and SE% ($r=0.17$, $p<0.01$) and strongly correlated with root fresh weight ($r=0.34$, $p<0.0001$); with root dry weight ($r=0.31$, $p<0.0001$) and with shoot fresh weight ($r=0.25$, $p<0.0001$).In our study a correlation between nodules number and shoot dry weight, and nodules number and SE% was found to be weak as

compared to other parameters. Indeed, Mulissa Jida and Fassil Assefa (2011) have also reported that there was no positive correlation between the increase of shoot dry matter and the nodules number in their previous work on rhizobia of Lentil. On contrast much of the research output indicated that nodulation status positively correlated with plant tissue shoot biomass (Abere Mnalku *et al*., 2009).

Root fresh weight was found to be strongly correlated with root dry weight ($r=0.56$, $p<0.0001$); shoot fresh weight ($r=0.55$, $p<0.0001$); shoot dry weight ($r=0.49$, $p<0.0001$) and SE% ($r=0.48$, $p<0.0001$). Root dry weigh was also found to be strongly correlated with shoot fresh weight ($r=0.53$, $p<0.0001$); shoot dry weight ($r=0.55$, $p<0.0001$) and with SE% ($r=0.55$, $p<0.0001$). Shoot fresh weight was found to be strongly correlated with shoot dry weight ($r=0.69$, $p<0.0001$) and SE% ($r=0.67$, $p<0.0001$).Correlation study revealed the positive and highly significant ($r=0.99$, $P< 0.001$) association between shoot dry weight and SE%. Somasegaran and Hoben (1994) suggested that shoot dry weight is a good estimator of symbiotic effectiveness efficiency.

Table 2:The effect of faba bean nodulating rhizobiaon different symbiotic effectiveness parametersand relative symbiotic efficiency tested on ''CD298DK'' variety of faba bean using modified Leonard jar experiment.

	Means ± SE					
Isolates	NN per plant	RFW	RDW	SFW	SDW	SE (%)
FBW9	$4.7\pm2.517^{-w-yx}$	2.27 ± 0.19^{e}	0.21 ± 0.01^{zy}	$3.1\pm0.15^{x-zw}$	$0.42\pm0.03^{x-v}$	49.3
FB10	$25\pm5^{p-n}$	$3.20\pm0.3^{lnk-m}$	$0.31\pm0.01^{q-n-r}$	$4.3\pm0.4^{l-k}$	$0.40\pm0.04^{xz-v}$	46.9
FB11	$39.3\pm2.08^{h-gi}$	4.33 ± 0.09^{cd}	$0.38\pm0.02^{gi-h}$	$4\pm0.2^{n-p}$	$0.38\pm0.02^{x-ya}$	44.9
FB12	$27.3\pm3.79^{km-n}$	$2.83\pm0.26^{t-u}$	$0.32\pm0.07^{q-r}$	4.5 ± 0.35^{ihg}	$0.39\pm0.05^{x-wy}$	46.2
FB13	$37.67\pm2.52^{h-i}$	$4.03\pm0.86^{e-f}$	$0.32\pm0.03^{q-r}$	$4.1\pm0.24^{lno-p}$	$0.5\pm0.01^{m-r}$	59
FB19	49 ± 6.24^{ed}	4.18 ± 0.13^{ed}	0.40 ± 0.04^{gf}	$4.3\pm0.09^{l-k}$	$0.46\pm0.02^{s-t-r}$	54.7
FB20	$27.3\pm3.06^{k-n}$	$2.34\pm0.44^{c-edb}$	$0.30\pm0.04^{q-t-or}$	3.4 ± 0.03^{vtu}	$0.31\pm0.03^{d-e}$	36.1
FBW23	$15\pm2.65^{u-s}$	$2.65\pm0.13^{avz-b}$	$0.28\pm0.02^{q-vr}$	$3\pm0.21^{xa-b}$	$0.39\pm0.02^{x-wy}$	46.5

FBW36	16.7±6.43^{p-s}	1.51±0.32^{-ih}	0.26±0.05^{x-tv}	2.3±0.3^{egf}	0.40±0.02^{x-yv}	47.5
FBW38	53.7±3.21^{cd}	2.65±0.33^{avz-b}	0.29±0.03^{qu-r}	3.1±0.12^{xa-w}	0.47±0.06^{s-t-r}	55.1
FBW39	27.7±3.79^{km-n}	1.49±0.29^{-ih}	0.17±0.03^{z-ab}	1.6±0.15^{h}	0.3±0.09^{df-e}	35.6
FBW40	40.3±6.51^{h-g-f}	2.65±0.25^{avz-b}	0.18±0.03^{za}	2.8±0.34^{c-db}	0.44±0.04^{s-w-v}	52
FBW42	4.7±1.53^{-w-yx}	1.06±0.15^{j}	0.10±0.02^{e}	1.7±0.05^{h}	0.16±0.03^{j}	18.4
FBW43	13.7±3.51^{u-s}	3.47±0.03^{hji}	0.34±0.03^{jmi-k}	3.4±0.27^{vtu}	0.59±0.01^{jk-l}	68.3
FBW45	21.7±4.73^{p-n}	2.77±0.15^{tvz-u}	0.23±0.01^{xy}	3±0.05^{c-y-b}	0.56±0.03^{jk-l}	66.3
FBW46	11.7±2.89^{u-s}	2.41±0.1^{ac-b}	0.13±0.01^{edc}	3.6±0.02^{r-us}	0.65±0.04^{gf}	76.4
FBW51	10±2^{u-x}	1.87±0.09^{f}	0.38±0.03^{j-fh}	3.6±0.22^{r-s}	0.48±0.03^{s-qr}	56.1
FBW52	48.7±4.73^{edf}	3.38±0.15^{l-i}	0.38±0.03^{gi-h}	5.1±0.15^{ced}	0.66±0.02^{f}	72.8
FBW56	25±3^{p-n}	2.29±0.06^{-ed}	0.27±0.03^{x-vr}	3.1±0.10^{x-zw}	0.57±0.02^{jk-l}	66.9
FBW58	78.7±3^{a}	2.32±0.1^{-ed}	0.18±0.02^{z-ab}	4.2±0.19^{ln-k}	0.46±0.02^{s-t-r}	54.7
FBW60	31.3±4.16^{k-i}	4.29±0.23^{cb}	0.3±0.01^{q-t-or}	2.9±0.05^{ca-b}	0.45±0.03^{su-v}	53.8
FBW61	35.7±1.53^{k-i}	3.40±0.19^{l-i}	0.17±0.02^{z-ab}	3.2±0.24^{x-y-w}	0.29±0.02^{dfe}	34.7
FBW65	4.3±2.08^{-w-yx}	3.42±0.03-$^{h-i}$	0.32±0.02^{q-k}	3.8±0.31^{r-qp}	0.50±0.04^{m-q}	59.3
FBW66	4.3±4.16^{-w-yx}	2.96±0.05^{t-u}	0.13±0.02^{e-b}	4±0.09^{n-p}	0.67±0.02^{ef}	79.5
FBW72	42.0±2^{e-g-f}	5.01±0.12^{a}	0.29±0.05^{q-t-or}	3.4±0.16^{v-w}	0.82±0.03^{c}	96.8
FBW73	11±3^{u-x}	2.33±0.15^{c-ed}	0.15±0.03^{d-ab}	4.1±0.15^{lno-p}	0.25±0.09^{hfg}	29.9
FBW77	4±1^{-w-yx}	2.49±0.05^{acz-b}	0.25±0.02^{x-yv}	4.3±0.28^{l-k}	0.65±0.03^{gf}	76.3
FBW79	7.3±3.51^{uw-t}	1.84±0.04^{gf}	0.17±0.01^{z-ab}	0.2±0.02^{i}	0.32±0.06^{dbc}	37.78
FBW84	10.3 ±2.52^{u-x}	1.87±0.08^{f}	0.18±0.02^{zab}	2.2±0.07^{gf}	0.26±0.02^{fge}	30.6
FBW88	9±2.65^{u-x}	2.77±0.03^{tvs-u}	0.18±0.01^{za}	3.4±0.09^{u-w}	0.47±0.02^{s-t-r}	55.1
FBW90	51.0±2.65^{d}	4.7±0.24^{b}	0.39±0.02^{g-h}	4.5±0.22^{i-g}	0.64±0.06	75.6
FBW94	39.7 ±8.33^{h-gi}	3.79±0.08^{-gf}	0.31±0.01^{q-s-r}	4.2±0.02^{l-o-k}	0.68±0.01^{ef}	80.2
FBW95	51.67 ±1.53^{cd}	3.00±0.21^{t-u}	0.41±0.02^{f}	5.3±0.33^{cb}	0.74±0.04^{d}	87.4
FBW101	8 ±2^{uw-x}	2.64±0.16^{acz-b}	0.20±0.03^{zy}	3.3±0.14^{x-uw}	0.41±0.01^{x-wv}	48.3
FBW103	8±1.73^{uw-x}	3.47±0.07^{h-ji}	0.38±0.02^{gi-h}	3.9±0.15^{r-qp}	0.59±0.04^{jk-h}	69.7
FBW106	8.3 ±2.52^{u-x}	2.83±0.03^{tvs-u}	0.24±0.04^{xw-v}	3.6±0.4^{r-s}	0.45±0.04^{su-r}	53.2
FBW107	7±3.61^{u-x}	3.00±0.11^{t-u}	0.3±0.03^{q-t-or}	4.7±0.16^{fhg}	0.72±0.03^{ed}	85.4
FBW114	5.7±306^{-w-x}	1.72±0.02^{ghf}	0.23±0.01^{xwv}	2.5±0.25^{edf}	0.35±0.05^{z-ca}	41.4
FBW116	6±4^{-w-x}	4.21±0.16^{d}	0.47±0.02^{ed}	4.4±0.25^{i-k}	0.50±0.03^{m-q}	59.3
FBW120	3±1.73^{-yx}	2.59±0.18^{acz-b}	0.28±0.03^{u-r}	3.4±0.12^{v-w}	0.47±0.03^{s-t-r}	55.5
FBW123	53.3 ±2.52^{cd}	3.36±0.1^{lhk-m}	0.30±0.01^{q-t-r}	3.4±0.02^{vtu}	0.53±0.03^{mo-l}	62.1

FBB126	39.7±6.51^{h-gi}	3.3±0.04^{lnk-m}	0.2±0.04^{xw-v}	2.7±0.69^{cdb}	0.55±0.07^{m-kl}	63.8
FBB128	37.3±1.15^{h-i}	2.7±0.04^{-vz-u}	0.4±0.04^{j-hk}	3.9±0.06^{n-qp}	0.55±0.03^{m-l}	64.6
FBW133	9.3±5.13^{u-x}	2.7±0.09^{avz-u}	0.2±0.03^{xwv}	2.6±0.03^{ced}	0.56±0.02^{jk-l}	65.7
FBW135	23±2.64^{p-n}	3.3±0.06^{lnk-m}	0.2±0.01^{xy}	3±0.11^{ca-b}	0.36±0.02^{zb-a}	42.0
FBW136	6.7 ±4.16^{u-x}	1.3±0.02^{ji}	0.1±0.01^{ed}	1.5±0.2^{h}	0.19±0.01^{ji}	21.9
FBW138	13.3±6.43^{u-s}	1.7±0.44^{ghf}	0.1±0.02^{e-ab}	1.7±0.05^{h}	0.22±0.01^{hgi}	26.4
FBW140	49.33 ±3.06^{ed}	2.5±0.06^{acz-b}	0.2±0.03^{xwy}	5.4±0.17^{b}	0.88±0.02^{b}	103.4
FBW141	29.7±4.51^{k-n}	4.2±0.09^{d}	0.3±0.01^{q-n-r}	3.8±0.03^{rqp}	0.65±0.01^{gf}	76.3
FBW142	10.7±6.11^{u-x}	2.2±0.06^{e}	0.4±0.01^{gi-h}	3.9±0.15^{n-qp}	0.75±0.02^{d}	88.4
FBW143	11.7±3.06^{u-s}	3.3±0.02^{l-m}	0.2±0.01^{xy}	3.6±0.06^{r-s}	0.36±0.02^{zb-a}	42
FBW144	5.3±3.51^{-w-x}	3.5±0.05^{h-ji}	0.5±0.04^{e}	4.9±0.06^{fed}	0.83±0.02^{cb}	98.4
FBW145	23.7±13.1^{p-n}	3.1±0.03^{ln-m}	0.7±0.05^{a}	4.8±0.22^{feg}	1.0±0.02^{a}	117.6
FBW146	8.3±3.51^{u-x}	4.1±0.04^{edf}	0.3±0.01^{q-s-r}	4.20±0.09^{l-j-k}	0.5±0.01^{m-nl}	63.7
FBW147	13.3±7.02^{u-s}	3.17±0.11^{l-m}	0.3±.01^{q-t-or}	3.39±0.03^{v-w}	0.5±0.04^{m-nl}	63.8
FBW148	4±4.36^{-w-yx}	2.7±0.03^{avz-u}	0.3±0.02^{x-s-v}	2.25±0.04^{gf}	0.36±0.02^{zb-a}	42.1
FBW151	19±6.56^{p-s}	2.4±0.03^{c-edb}	0.2±0.01^{xw-v}	2.79±0.17^{c-db}	0.4±0.02^{x-ya}	44.90
FBW152	35.3±4.16^{k-i}	2.7±0.03^{avz-u}	0.35±0.01^{j-k}	4.02±0.17^{n-p}	0.5±0.04^{s-t-r}	54.8
FBW153	48.7±12.06^{e-d-f}	3.6±0.02^{hgi}	0.55±0.01^{tus}	3.49±0.01^{m-p-l}	0.52±0.02^{m-l}	61.4
FBW154	32 ±2.65^{k-i}	3.14±0.04^{l-m}	0.4±0.01^{gf}	3.34±0.14^{x-w}	0.64±0.02^{gfh}	75.1
FBW155	50.67 ±3.06^{d}	3.17±0.02^{l-m}	0.35±0.01^{j-k}	5.11±0.5^{cbd}	0.47±0.06^{str}	56
FBW157	21.3±1.54^{p-n}	2.86±0.02^{t-u}	0.2±0.04^{xw-v}	3.46±0.03^{tu}	0.59±0.02^{gih}	71.8
FBW158	76.67±6.11^{a}	2.92±0.02^{t-u}	0.3±0.02^{q-n-r}	3.81±0.37^{r-sp}	0.56±0.02^{jk-l}	65.7
FBW159	11 ±7^{u-x}	1.07±0.03^{j}	0.2±0.03^{d-ab}	2.15±0.05^{gf}	0.3±0.01^{df-e}	35.8
FBW160	63.7±3.21^{b}	4.3±0.2^{cb}	0.5±0.03^{cb}	5.04±0.14^{ced}	0.69±0.04^{ef}	81.1
FBW161	25.7±12.89^{k-n}	3.61±0.03^{hg}	0.6±0.02^{b}	3.25±0.22^{x-y-w}	0.55±0.02^{m-l}	64.9
FBW162	60.0±5^{cb}	2.95±0.07^{t-u}	0.3±0.02^{qu-r}	2.89±0.27^{ca-b}	0.50±0.03^{m-r}	58.9
FBW163	64.0±3^{b}	3.05±0.14^{t-m}	0.3±0.03^{j-k}	2.75±0.17^{cdb}	0.33±0.04^{d-a}	38.9
FBW164	14.7±9.24^{u-s}	4.18±0.1^{d}	0.3±0.01^{q-s-r}	4.37±0.19^{l-k}	0.57±0.02^{jk-l}	67.2
FBW165	49.7±17.21^{ed}	3.08±0.08^{l-m}	0.50±0.03^{cd}	3.86±0.15^{r-qp}	0.59±0.02^{gih}	69.9
D/Sina	4±5.29^{-w-yx}	1.53±0.23^{g-f}	0.3±0.03^{x-yv}	3.55±0.39^{r-us}	0.49±0.04^{so-r}	57.7
N+	-	3.87±0.57^{e-f}	0.35±0.1^{jmi-k}	6.45±0.19^{a}	0.85±0.05^{cb}	100.0

N-	-	1.85 ± 0.57^{f}	$0.2\pm0.04^{z-ab}$	2.11 ± 0.09^{g}	0.21 ± 0.01^{hgi}	24.4
LSD ($p<0.05$)	7.02	0.81	0.04	0.27	0.04	7.9
CV (%)	20.73	6.68	9.6	5.8	7.9	9.9
MSE	27.32	0.04	0.001	0.04	0.001	34.56

-, not found,Values are Mean ± SE of the replication, Means in the same column followed by the same letters are not significantly different at $p<0.05$ (LSD test), N+: with optimum nitrogen fertilizer, N-: neither inoculated nor nitrogen supplied

4.3. Determination of N-content of the plant

Data presented in Table 3 shows significant differences in total Nitrogen(expressed in mg / plant) at 45 days after planting. The results revealed inoculation of faba bean with FBW145, FBW140 and FBW144 isolates increased significantly the total Nitrogenmg/plant compared with the uninoculated N+ control. Beside this, plants inoculated with isolate FBW145 significantly differed in total nitrogen accumulation from all plants treated with other strains.On the other hand there was no significance difference in total nitrogen accumulation between plants irrigated with KNO_3 and inoculated with isolate FBW107. As shown in table 3 below ANOVA result declared that there were an over lapping results (no significance difference) in total nitrogen accumulation among treatment groups. Indeed, all selected 21 isolates significantly increased the total Nitrogen mg/plant when compared with uninoculated N- control. These finding was in agreement with previous work of Belal *et al*., 2013 who concluded that inoculation of faba bean with *R. leguminosarum bv. Viciae* significantly increased plant total nitrogen content over uninoculated controls.

The results of correlation coefficient between total nitrogen percentage, shoot dry weight and total nitrogen content studied were shown in annex-3. The data show that the shoot dry weight ($r=0.8$), %N ($r=0.89$) were positively and significantly associated with total nitrogen content.As shown in Table 3 the highest correlation coefficients found was plant total nitrogen percentage with total nitrogen content mg/plant. This is in line with the result of other studies (Abere Mnaluk., 2013; Anteneh Argaw, 2012). Somasegaran and Hoben (1994) stated that SDW is best estimator of total nitrogen fixed in plants over other parameters used to estimate symbiotic effectiveness.

Table 3: The effect of inoculation of faba bean with 21 selected and D/Sina commercial strains, on %N, plant total nitrogen mg/plant and shoot dry weight in faba bean after five weeks under lath house condition.

Isolates	Means			SE%
	Shoot dry weight	PTN (%)	PTC(mg/pl)	
FBW145	1^{a}	2.37^{a}	23.7^{a}	117.6
FBW140	0.88^{b}	2.29^{a}	20.1^{ba}	103.5
FBW144	0.84^{cb}	$2.03^{b\text{-}a\text{-}c}$	17^{bc}	98.8
FBW107	0.72^{ed}	$2.01^{b\text{-}a\text{-}c}$	14.5^{dc}	84.7
N+	0.85^{cb}	$1.73^{b\text{-}g\text{-}f}$	14.5^{dc}	100
FBW95	0.74^{d}	$1.92^{b\text{-}a\text{-}f}$	14.3^{dce}	87.1
FBW141	0.65^{gf}	2.16^{ba}	13.9^{dce}	76.5
FBW157	0.6^{jhi}	2.07^{bac}	12.4^{dfe}	70.6
FBW72	0.82^{c}	$1.47^{i\text{-}f}$	$12.1^{d\text{-}fe}$	96.5
FBW94	0.69^{ef}	$1.76^{b\text{-}cf}$	$12.1^{d\text{-}f\text{-}e}$	81.2
FBW52	0.66^{gf}	$1.69^{d\text{-}cf}$	$11.2^{h\text{-}g\text{-}e}$	77.6
FBW165	0.59^{jki}	$1.74^{b\text{-}cf}$	$10.3^{h\text{-}g\text{-}e}$	69.4
FBW90	0.64^{gh}	$1.56^{i\text{-}f}$	$10.1^{h\text{-}fe}$	75.3
FBW160	0.69^{ef}	$1.46^{i\text{-}f}$	$10.1^{h\text{-}fe}$	81.2
FBW154	0.48^{ghi}	$1.48^{i\text{-}f}$	9.4^{hgf}	56.5
FBW46	0.65^{gf}	1.41^{igh}	9.1^{hgf}	76.5
FBW164	0.55^{lk}	$1.64^{d\text{-}cf}$	9^{hgf}	64.7
FBW146	0.54^{lm}	$1.56^{di\text{-}f}$	8.4^{hgf}	63.5
FBW65	$0.5^{di\text{-}f}$	$1.65^{d\text{-}cf}$	8.3^{hgf}	58.8
FBW77	0.65^{gf}	1.24^{ih}	8^{hg}	76.5
FBW43	0.59^{jk}	1.34^{ih}	7.9^{hg}	69.4
FBW162	0.5^{m}	$1.54^{i\text{-}f}$	7.7^{h}	58.8
D/Sina	0.5^{m}	$1.54^{i\text{-}f}$	7.7^{h}	58.8
N-	0.21^{n}	1.2^{i}	2.5^{i}	24.7
LSD	0.04	0.42	0.08	
MSE	0.001	0.09	0.07	
CV (%)	4.2	17.36	22.3	

Data are means of three replicates, Means within a column of the same factor followed by the same letter(s) are not significant at $p<0.05$. PTN (%) = plant total nitrogen in %; PTC (mg/pl) = plant total nitrogen content in mg/plant, SE% Symbiotic efficiency, SDW=Shoot dry weight

4.4. Morphological characterization of the isolates

All colonies of the 70 isolates and three reference strains were similar in appearance with large mucoid texture, milky colored and raised or convex shape on YEMA medium. The isolates also exhibited regular and circular margin with diameters ranging between 2.5 and 5.2mm. The largest diameter (> 4mm) was shown by FBW11 (5.2mm) and FBW20 (4.2mm) and the smallest (2.5 mm) by FBW12 (2.5mm) and FBW19 (3.5mm).With exception of FBW13, which was opaque, all isolates were transparent(data not shown).

These results were in line with the findings of Getaneh Tesfaye (2008), who reported that 70% of faba bean rhizobial isolates showed large mucoid colonies with diameter lying between 2 and 5.5 mm. Similar study by Abere Mnaluk *et al*., (2009) on faba bean rhizobia collected from Easternand Western Hararghe highlands of Ethiopia showed that faba bean rhizobia as having a diameter ranging between 2 and 5mm with mucoid in texture, milky in color, and raised or convex in shape. Moreover, Zerihun Belay (2006) also reported that colony diameter of faba bean rhizobia were in the range of 2-5 mm with milky color, large mucoid and raised colony characteristics. Cultural and infective properties have been used to identify species of Rhizobium (Fred *et al*., 1932). Thus, the presence of large colonies in 3-5 days on YEMA media also substantiates the typical characteristics of fast growing rhizobia of faba bean (Jordan, 1984).

4.5. Physiological and Biochemical characters

4.5.1. Stress tolerance test

Salt stress tolerance of the isolates

Bacterial isolates varied in their response to salt stress, which range from 0.5-5% NaCl. The most sensitive strains which did not grow on all tested NaCl concentrations were FBW73 and FBW144.This finding is contrary to previous reports that, fast growing Rhizobium in general, grew well at NaCl concentration between 3 and 5% (Abdel-wahab and Zahran, 1979). Although few stains failed to show growth as NaCl concentration increases but majority of our isolates (63%) were tolerant to NaCl up to 5% (annex-7).The result was in harmony with previous work of (Zerihun Belay and Fassile Assefa, 2011; Mulissa Jida and Fassil Assefa,

2012;Girmaye Kenasa *et al*., 2014).The native rhizobial strains isolated from Northern parts of Ethiopia tolerated a higher salt concentration (5% NaCl) than exotic rhizobial strains (Tall 1402 and Tall 1397) as described by Assefa Keneni *et al.* (2010) which also was in concord with ours finding. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. In present study most of the isolates were persisted under salt concentrations of 5.0%. Hence, these isolates may be the candidates for applications in the saline influenced soil.

Tolerance to extreme pH

The pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow best at neutral pH, i.e., 7 (Singh *et al*., 2008). The majority of our isolates were able to grow in a pH range of 4.5 to 10.0(Table 4 and annex-8), which coincided with the findings of Drouin *et al*. (1996) whose isolates grew in pH range of 5.0 and 9.0.Among our isolates; FBW (40, 84, 88, 116, 128, 140, 158 and 159) were found to be sensitive to the pH values of 9.0 and above, of which eleven percent were sensitive to the upper pH test value of 10.0, whereas, 56% of the isolates were sensitive to the lower pH test value of 4.0. The commercial stains (EAL-110, D/S and B/J) used in this study and 57% of our isolates were tolerant to pH at 3.5. 60% of the isolates in our investigation were shown to growth on a wide range of pH (3.5-10). In contrast to our findings Zerihun Belay and Fassil Assefa (2011) observed very poor growth of their isolates at a pH of 4.0 and 4.5. Unlike our findings, Assefa Keneni *et al.* (2010) reported that none of the faba bean isolates from Northern Wello region grew at pH values lower than 4.0 and 4.5. Our findings were coincided with Girmaye Kenasa *et al*. (2014) who reported that rhizobial isolates were tolerant to pH ranges 4.5-9. The ability of our isolates to grow at a wide pH range would make them practically significant to use them as inoculant under a wide range of soil pH conditions.The best thing of this finding is that the isolates that had survived at low pH could help us to develop inoculants that can be used in the fields with acidic soil. Therefore, it is necessary to verify those isolates under field experiments and develop them into inoculants, particularly for acidic soil.

Temperaturestress tolerance of the isolates

Although the optimum temperature for rhizobia on culture is between 27-39˚C (Munevar and Wollum, 1981), some of our isolates such as FBW (10, 23, 36, 42, 51, 52, 88, 133,138, 143, 147, 154, 155 and 163) were tolerant to low temperature to the level of 5˚C (annex- 9).This was in harmony with Drouin *et al*. (1996) who reported that isolates from *Lathyrus*species ,were capable to grow at lower temperatures. Low temperature tolerance of some isolates in laboratory could grant for inoculant production for the highland pulse crop particularly faba bean. On the other hand, FBW (40, 42, 45, 106, 107, 133, 138, 151, 153 and 158) were tolerant up to 45˚C (annex-9).Four isolates: FBW (42, 106, 133 and 138) showed growth in wide range of temperature (5-45˚C). It could be concluded that the isolates that had survived the extreme temperature could help us to develop inoculants that can be used in the fields with harsh temperature conditions.

Table 4:Tolerance of the isolates and commercial inoculant to extreme pH, Temperature and NaCl on YEMA medium

Physiological test values		No. of isolates	Percentage
	3.5	43	58.9
	4.0	48	65.7
	4.5	52	71.2
pH	5.0	56	76.7
	5.5	68	93.1
	8.0	73	100
	8.5	73	100
	9.0	70	95.9
	9.5	68	93.1
	10	67	91.8

	0.5	69	98.6
	1	69	98.6
	1.5	68	97.1
	2	66	94.3
	2.5	66	94.3
NaCl (%)	3	63	90
	3.5	63	90
	4	62	88.6
	4.5	59	83.1
	5	50	71.4
	5	13	18.6
	10	22	31.4
	15	35	50
Temp (˚C)	20-35	70	100
	37	55	78.6
	40	13	18.6
	45	7	10

4.5.2. Substrate utilization test and IAR patterns

Carbon source utilization

The majority our isolates were capable of utilizing most of the carbohydrates provided as their sole carbon sources (Table 5; annex-10).Although the majority of isolates were found to utilize 72.6 to 100% of the tested monosaccharides, disaccharides and polysaccharide, while many failed to metabolize dextrin, fructose and galactose. This in agreement with the finding of Drouin *et al*. (1996) who reported that all of their isolates were able to use 14 carbon sources while none were utilized dextrin and fructose. In contrast to this, Assefa Keneni *et al*. (2010) isolated faba bean nodulating rhizobia that were capable of utilizing fructose as their sole carbon source, which is quite similar to our faba bean rhizobial isolates. The failure of our test strain to utilize galactose is in agreement with the finding of Girmaye Kenasa *et al*., (2014) who reported that one of their faba bean isolate was not capable of utilizing this particular C source. Only one carbon source (trehalose) was found to be utilized by all our isolates and sucrose, raffinose, mannose and glucose were utilized by more than 90% of our isolates. This finding corroborated with, other study (Sadowsky *et al*., 1983; Mulissa Jida and Fassil Assefa, 2011) who observed that disaccharides such as, maltose, trehalose and sucrose, and trisaccharide such as raffinose were catabolized by fast-growing strains of rhizobia. On the other hand 20(28.6%) of the test isolates were failed to utilize at least 3 ormore carbon sources. Among the 70 the test isolates; FBW (46 and 128) were found to be least metabolically active, thus metabolize less than 40% of tested carbon sources. These particular isolates may be unable to secrete corresponding carbohydrate degrading enzymes (Robledo *et al*., 2011).

Utilization of amino acids as sole source of nitrogen;

Among the N sources tested, the lowest utilization percentage (47.9%) was found from Aspartic acid (annex-11).It reasonably seems that the acidic nature of this nitrogen source limited the growth of the isolates on the medium. The isolate FBW73 was found to utilize only six out of ten nitrogen sources, whereas 37.1% of our isolates and commercial strains (D/S, B/J and EAL-110) were able to metabolize all of the given nitrogen sources.In general more than 77% of tested nitrogen sources were utilized by most of our isolates. This result is

found to be similar with the previous work on faba bean by (Girmaye Kenasa *et al*., 2014) in which 67-100% of tested nitrogen sources were utilized by their isolates. The capabilities of the isolates to utilize different carbon and nitrogen sources would definitely enable them to survive under a limited nutrient availability in a soil condition. This supported the fact that rhizobia can survive in a nutrient deficient and marginalized lands and form symbiosis with legume plants under such conditions. Therefore, it is very practical to screen these types of isolates and test their effectiveness under field conditions and develop them in to inoculants for the marginalized lands.

Intrinsic antibiotic resistance patterns

Rhizobial isolates of the faba bean plant were shown different pattern of resistances to various intrinsic antibiotics (Table 4; Annex12). Accordingly, all our isolates were sensitive to kanamycin at all levels of concentration tested except isolate 146 which resisted kanamycin at 5µg/ml. Similar results were reported by (Zerihun Belay and Fassil Assefa, 2011; Girmaye Kenasa, 2014) for *Vicia faba* rhizobia in which more than 70% of their isolates were found to be sensitive to kanamycin. ninetythree percent of the tested isolates were resistant to Erythromycinat a concentration of 5µgm/ml. This is in accordance with the previous work on faba bean by (Zerihun Belay and Fassil Assefa, 2011) who reported that ninety five percent of their isolates were resistant to erythromycin at (5µg/ml). On contrast to our finding, isolates from Wollega by Girmaye Kenasa (2009) were more sensitive to erythromycin(5µg/ml). 58.9% of the isolates were resistant to neomycin at 5µg/ml and but none of the isolates were able to grow at 10µg/mlNeomycin. The commercial inoculant strains included in our investigation were tolerant to erythromycin at two levels of concentrations (5 and 10µg/ml) tested and while resistant to neomycin at 10µg/ml. on the other hand this reference strains were sensitive to kanamycin, chloramphenicol and streptomycin at two level of concentration. FBW51 was the most resistant isolate to antibiotics as compared to the others. In contrast this 20% of the isolates were sensitive to 80% antibiotics.

Table 5:Carbon andnitrogen source utilizationof faba bean nodulating rhizobial isolates.

Isolates	C-sources % utilized	N-sources % utilized

FBW9	90	88.9
FBW10	100	100
FBW11	100	100
FBW12	100	100
FBW13	100	100
FBW19	100	100
FBW20	100	100
FBW23	100	88.9
FBW36	70	100
FBW38	100	100
FBW39	100	88.9
FBW40	100	100
FBW42	60	88.9
FBW43	100	100
FBW45	80	88.9
FBW46	30	77.8
FBW51	100	100
FBW52	90	77.8
FBW56	100	100
FBW58	80	88.9
FBW60	100	100
FBW61	90	88.9
FBW65	80	88.9
FBW66	70	77.8
FBW72	80	88.9
FBW73	80	66.7
FBW77	60	100
FBW79	90	88.9
FBW84	90	88.9
FBW88	90	77.8
FBW90	100	88.9
FBW94	90	100
FBW95	90	88.9
FBW101	100	88.9
FBW103	100	88.9
FBW106	70	88.9
FBW107	80	100
FBW114	80	100
FBW116	70	88.9
FBW120	100	100
FBW123	80	88.9
FBW126	90	100
FBW128	40	88.9
FBW133	80	88.9
FBW135	70	88.9

FBW136	60	77.8
FBW138	40	100
FBW140	90	88.9
FBW141	60	88.9
FBW142	100	100
FBW143	70	77.8
FBW144	60	88.9
FBW145	100	88.9
FBW146	70	77.8
FBW147	70	100
FBW148	100	88.9
FBW151	90	88.9
FBW152	70	100
FBW153	70	77.8
FBW154	80	77.8
FBW155	80	77.8
FBW157	40	77.8
FBW158	60	88.9
FBW159	80	88.9
FBW160	90	100
FBW161	100	100
FBW162	100	88.9
FBW163	90	100
FBW164	100	88.9
FBW165	90	88.9
D/Sina	80	100
EAL-110	90	100
B/Jira	90	100

Table 6: Antibiotic resistance patterns of faba bean nodulatingrhizobia and commercial strains

Antibiotics	Conc. (µg/ml)	No resistant isolates	%
Chloramphenicol	5	20	28.6
	10	15	21.4
Erythromycin	5	55	78.6
	10	49	70
Streptomycin	5	12	17.1
	10	4	5.7
Neomycin	5	35	50
	10	19	27.1
Kanamycin	5	0	0
	10	0	0

4.6. Numerical taxonomy

Cluster analysis of an adequate number of traits and comparison with rhizobial reference species permit the identification and grouping of large populations (L'taief *et al*., 2007). Thus, the numerical multivariate cluster analysis of 55 traits tested on 70 rhizobial type isolates and 3 commercialinoculant species produced three different clusters including the reference strains at 63% of similarity cut point (Fig. 4). The clusters contained different number of isolates. The majority of the isolates 65 (89%) were grouped in cluster I with the reference strains B/Jira, D/sina and EAL-110 (*R. leguminosarum*), which was thought to nodulate faba bean. Cluster II contained only one strain (FBW143), which did not show any affinity to other strains. Cluster III contains 7 strains FBW (73, 51, 152, 107, 88, 114 and 144). The current faba bean isolates included within theses 3 clusters were originated from different faba bean producing sites of Wello.

Cluster I had almost common physiological characteristics; these isolates were, highly salt-tolerant and moderately acidic pH tolerant. All of the isolates under this cluster were able to grow up to the salt concentration of 0.5 to 4%. But, as the concentration of the salt increased, the number of surviving isolateswas slightly decreased. Thus, 7.7% of the isolates were notsurvived at the salt concentration of 4.5% and 16.5% of the isolates under this cluster were

unable to survive at the salt concentration of 5%. Again isolates in this cluster were characterized by their ability to survive at the lower pH (3.5). In fact, 20(30.7%) of the isolates clustered under this group were sensitive to low pH and 7 (10.8%) isolates sensitive to high pH. Ten isolates of this cluster were able to survive at the lower temperature test value (5^0C) and eight isolates were capable to survive the higher temperature (40^0C and 45^0C).
Regarding the substrate utilization patterns of the isolates in cluster I, 64.4% of the isolates were capable of metabolizing fructose, sorbitol and galactose as their sole carbon sources. More than 75% of the isolates were able to metabolize all the rest carbon sources except dextrose and (35.4%) and starch (67.7%). In this cluster, most the isolates were not capable of metabolizing aspartic acid as their sole nitrogen sources. With this respect, this cluster shared similarity with cluster III but differed from cluster II. 100% of the isolates utilized asparagine, glycine, histidine and L-isoleucine while 98.5%, 96.9% and 89% of the isolates utilized L-alanine, Valine and Arginine, respectively. With this respect, cluster I shared similarity with cluster II and clusterIII.In addition, isolates grouped in cluster I were highly sensitive to Kanamycin 5 and 10µg/ml, and Neomycin 10µg/ml, but moderately resistant to Neomycin 5µg/ml. In contrast, about 80% of the isolates were resistant to erythromycin 5µg/ml.

The second cluster (cluster II) had no sub-clusters as in the dendrogram presented in (Fig.3). Isolates clustered under group II (FBW143) had physiological characteristics such as sensitive to low pH, high salt concentration, low and high temperature. As to the C and N utilization of patterns, isolate FBW143 (cluster II) were able to use less efficiently the tested carbon sources and moderately metabolized the tested nitrogen sources. It was observed that most of the isolates in all clusters were failed to metabolize aspartic acid. Cluster II isolate was found to be resistant to all tested antibiotics at both concentrations except to Kanamycin.

The third cluster (clusterIII) had two sub-clusters in the dendrogram presented in (Fig. 3).Isolates clustered under group III had unique physiological characters. 57% of the isolates under this cluster were unable to grow at the pH of 4.All of the isolates in cluster III were able to grow at pH of neutral to upper (basic) pH. All the isolates in this cluster were resistant to 2.5% of the salt concentration. Two isolates, FBW73 and FBW144 were not tolerant to all salt concentration tested in this investigation. Of all the test isolates in cluster III, only two isolates

FBW51 and FBW88 were able to grow at lower temperature (5˚C) and as opposed to these isolates only isolate FBW152 was able to grow above optimum temperature, at 40˚C. As to the C and N utilization patterns, only 28.6% of the isolates utilized fructose as sole source of carbon. On the other hand isolates FBW144 and FBW152 were found to be less efficient in carbon source utilization as compared to the rest of the isolates in this cluster. More than 70% of carbon sources were utilized by all isolates grouped under this cluster.

Intrinsic antibiotic resistance to erythromycin (5µg/ml and 10µg/ml), was noted for all of the isolates in cluster III except for isolate FBW73.71.1% of the isolates in this cluster were resistant to chloramphenicol (5µg/ml). It was noted that in all clusters all isolates were sensitive to kanamycin at two level of concentration.

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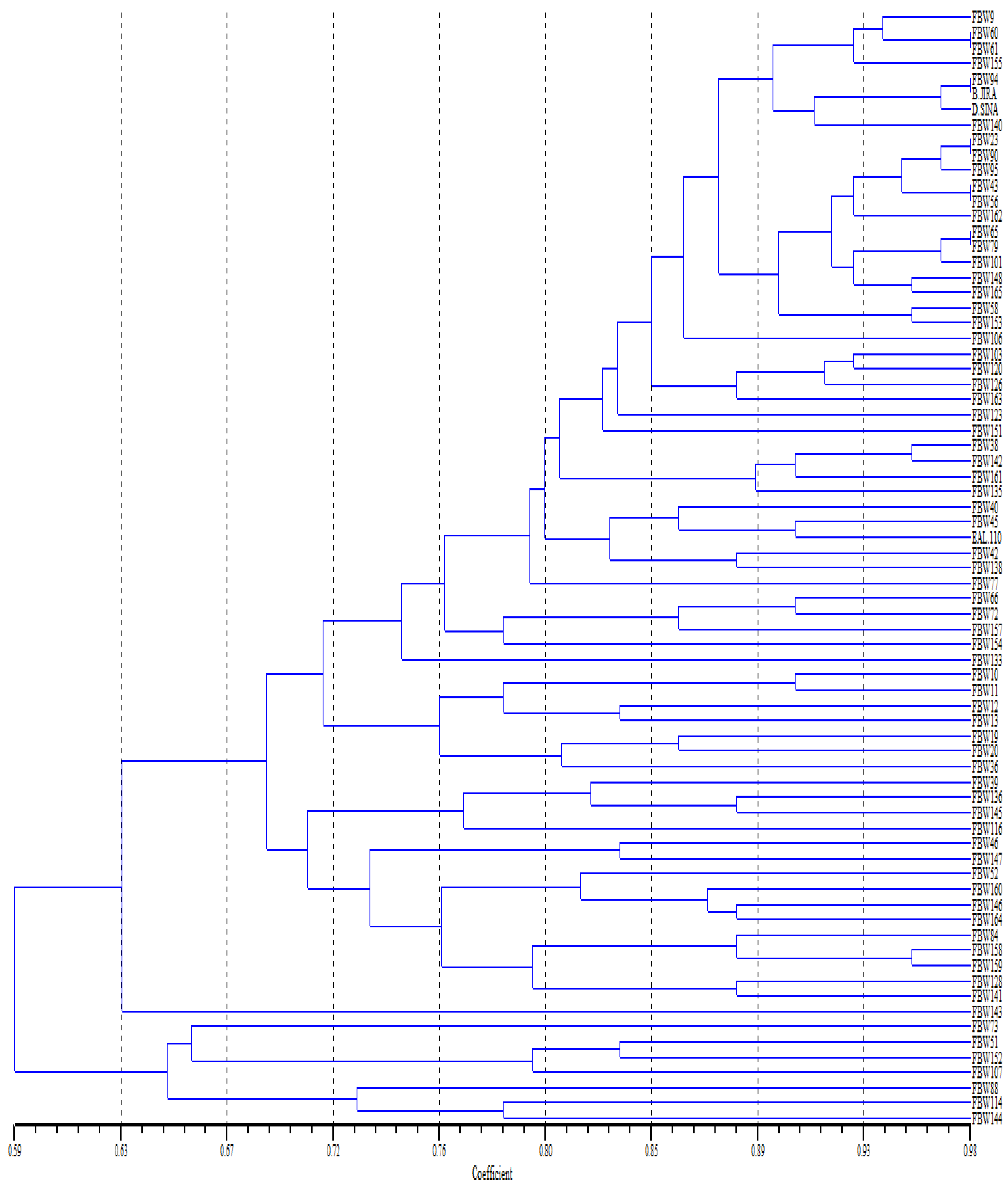

Figure 4: Dendrogram highlighting the phenotypic similarities among faba bean nodulating rhizobia from different areas of Wello, Northern Ethiopia

5. CONCLUSION AND RECOMMENDATIONS

From the study, it could be observed that absence Congo red absorption on YEMA at dark condition incubation is not distinctive attribute of rhizobia. Presence in growth on PGA medium also is not absolute feature for other bacteria's rather than rhizobia also can grow on PGA medium. Authentication test result proved that only 70 isolates in this study were true faba bean *rhizobium species*.Percentage of nitrogen fixation is higher for indigenous rhizobial isolates with these isolates found to be superior to the commercial inoculant (D/Sina) ones in, nodule number, root fresh weight, root dry weight, shoot fresh weight and shoot dry matter yield, of faba bean in sterile sand culture. 53% of the isolates were found to be effective even better than commercial inoculant (D/Sina) indicating the sampling sites of Wello harbor effective rhizobia. This strain possesses different characteristics such as tolerance for abiotic stress (salinity, growth at wide range from pH, higher and lower temperature), which can make it candidate for multipurpose inoculants production for faba bean production system.

This study was only focused on the efficiency of the indigenous rhizobial isolates under a controlled condition. But a success in lath house experiment may not necessarily assure a success in the field; hence these isolates must be tested on field conditions prior to proceeding to utilize them as inoculants. After in field experiments, the isolates FBW (52, 94, 95, 107, 140, 144, 145 and 160) and particularly FBW(107, 140, 144 and 145) can be recommended for field trail and ecological competitiveness studies under different Ethiopian soil and climatic conditions.During this study methods used for characterizing and distinguishing rhizobial strains were morphological, physiological, numerical and symbiotic. However, these traditional methods of rhizobial characterization frequently fail to identify strains within a species. Thus, the isolates should also be molecularly characterized to species level so as to understand their nature and competitiveness in terms of nodule occupancy.

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7. ANNEXES

Annex 1:The effect of faba bean rhizobia onLeaf color, nodule number and nodule internal colorof faba bean‘’*CD298DK* variety’’

Isolates	Leaf color	NIC	Nodules mean replication	Efficiency rate
FBW9	3	1	4.7	4=e
FBW10	3	3	25	6=E
FBW11	3	2	39.3	5=e
FBW12	3	2	27.3	5=e
FBW13	3	3	37.7	6=E
FBW19	2	2	49	4=e
FBW20	2	2	27.3	4=e
FBW23	3	3	15	6=E
FBW36	2	1	16.7	3=I
FBW38	3	4	53.7	7=E
FBW39	3	3	27.7	6=E
FBW40	2	3	40.3	5=e
FBW42	3	1	4.7	4=e
FBW43	3	3	13.7	6=E
FBW45	3	3	21.7	6=E
FBW46	3	3	11.7	6=E
FBW51	3	1	10	4=e
FBW52	3	2	48.7	5=e
FBW56	2	2	25	4=e
FBW58	2	3	78.7	5=e
FBW60	2	3	31.3	5=e
FBW61	3	3	35.7	6=E
FBW65	2	3	4.3	5=e
FBW66	3	3	4.3	6=E
FBW72	3	3	42.0	6=E
FBW73	3	3	11	6=E
FBW77	3	3	4	6=E
FBW79	2	3	7.3	3=e
FBW84	3	1	10.3	6=E
FBW88	3	3	9	6=E
FBW90	3	3	51.0	6=E
FBW94	3	3	39.7	6=E
FBW95	3	4	51.7	7=E
FBW101	3	3	8	6=E
FBW103	3	3	8	6=E
FBW106	3	3	8.3	6=E
FBW107	3	3	7	6=E
FBW114	1	3	5.7	4=I
FBW116	2	3	6	5=e
FBW120	2	3	3	5=e
FBW123	3	3	53.3	6=E
FBW126	2	1	39.7	3=I
FBW128	3	4	37.3	7=E

FBW133	3	1	9.3	4=e
FBW135	3	3	23	6=E
FBW136	2	3	6.7	5=e
FBW138	3	3	13.3	6=E
FBW140	3	4	49.3	7=E
FBW141	3	4	29.7	7=E
FBW142	3	4	10.7	7=E
FBW143	3	1	11.7	4=e
FBW144	3	3	5.3	6=E
FBW145	2	4	23.7	6=E
FBW146	3	1	8.3	4=e
FBW147	3	1	13.3	4=e
FBW148	2	2	4	4=e
FBW151	2	3	19	5=e
FBW152	3	3	35.3	6=E
FBW153	3	4	48.7	7=E
FBW154	2	4	32	6=E
FBW155	3	4	50.7	7=E
FBW157	2	3	21.3	5=e
FBW158	2	3	76.7	5=e
FBW159	3	1	11	4=e
FBW160	3	4	63.7	7=E
FBW161	2	3	25.7	5=e
FBW162	3	4	60.0	7=E
FBW163	3	4	64	7=E
FBW164	3	3	14.7	6=E
FBW165	2	4	49.7	6=E
D-sina	2	1	4	3=I

*NN, nodule number, NIC, nodule internal color, e=moderately effective, E=effective, I=ineffective, D/S, Debre-sina commercial strain, Leaf color scores; 1=similar with negative control, 2= between positive and negative control, 3=similar with positive control, NIC scores, 1=white, 2=green, 3=pink, 4=red

Annex 2: Correlation coefficients among investigated parameter in faba bean, nodule number, root fresh and dry weight, shoot fresh and dry weight and symbiotic effectiveness efficiency.

	NN	RFW	RDW	SFW	SDW	SE%
NN	1					
RFW	0.34***	1				
RDW	0.31***	0.56***	1			
SFW	0.25***	0.55***	0.53***	1		
SDW	0.18**	0.49***	0.55***	0.69***	1	
SE%	0.17**	0.48***	0.55***	0.68***	0.99***	1

*, ** and *** = Significant at p<0.05, p<0.01 and p<0.001, respectively; NN=nodule number, RFW= root fresh weight, RDW= root dry weight, SFW=shoot fresh weight, SDW= shoot dry weight and SE (%)= symbiotic effectiveness efficiency

Annex 3: Correlation coefficient among shoot dry weight, N% and total nitrogen of faba bean inoculated with 21 strains selected for nitrogen analysis.

	SDW	PTN%	PTC
SDW	1		
PTN%	0.5***	1	
PTC	0.8***	0.89***	1

*** = Significant at p<0.001; SDW= shoot dry weight and SE (%) = symbiotic effectiveness efficiency, PTN (%) = plant total nitrogen in %; PTC (mg/pl) = plant total nitrogen content in mg/plant, SDW=Shoot dry weight.

Annex 4: Mean squares for faba bean (CS298DK) inoculated with different 70 rhizobium isolates including commercial strain (D/sina commercial strain).

Source of variation	DF	Mean square					
		NN	RFW	RDW	SFW	SDW	SE%
Strains	72	1198.26	2.31	0.04	3.17	0.09	1204.66
error	146	27.32	0.04	0.001	0.04	0.001	34.56
LSD (p<0.05)		7.02	0.81	0.04	0.27	0.04	7.9
CV (%)		20.73	6.68	9.6	5.8	6.51	9.9

Annex 5: Mean squares for faba bean inoculated with selected 21 isolates for nitrogen analysis

Source of variation	Mean Squares			
	df	SDW	PTN (%)	PTC
Isolates	22	0.08	0.31	0.61
SE	46	0.01	0.09	0.07
LSD (P<0.05)		0.04	0.042	0.08
CV (%)		4.2	17.36	22.3

Annex 6: ANOVA of nodule number, root fresh and dry weight, shoot fresh and dry weight, and Symbiotic effectiveness of faba bean inoculated with 70 isolates of faba bean and one commercial strain; including uninoculated positive and negative control.

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
rep	219	2	0.81837	438	1	3
NN	219	25.2	20.3482	5522	0	82
RFW	219	2.9	0.88753	641.11	0.9	5.2
RDW	219	0.29	0.11245	64.25	0.09	0.7
SFW	219	3.5	1.03634	770.63	0.19	6.57
SDW	219	0.5	0.1717	110.32	0.13	1.01
SE	219	59.4	20.5	13002	15.3	126.3

Annex 7: Salt stress tolerance of faba bean nodulating rhizobia, on YEMA medium contained0.5 to 5 % (W/V) NaCl

Isolates	0.5%	1%	1.5%	2%	2.5%	3%	3.5%	4%	4.5%	5%
FBW9	+	+	+	+	+	+	+	+	+	+
FBW10	+	+	+	+	-	-	-	-	-	-
FBW11	+	+	+	+	-	-	-	-	-	-
FBW12	+	+	+	+	+	+	+	-	-	-
FBW13	+	+	+	+	+	+	-	-	-	-
FBW19	+	+	+	+	+	+	+	+	+	-
FBW20	+	+	+	+	+	+	+	-	-	-
FBW23	+	+	+	+	+	+	+	+	+	-
FBW36	+	+	+	+	+	+	+	+	-	-
FBW38	+	+	+	+	+	+	+	+	+	+
FBW39	+	+	+	+	+	+	+	+	-	-
FBW40	+	+	+	+	+	+	+	+	+	+

FBW42	+	+	+	+	+	+	+	+	+	+
FBW43	+	+	+	+	+	+	+	+	+	+
FBW45	+	+	+	+	+	+	+	+	+	+
FBW46	+	+	+	+	+	+	+	+	+	-
FBW51	+	+	+	-	-	-	-	-	-	-
FBW52	+	+	+	+	+	+	+	+	+	+
FBW56	+	+	+	+	+	+	+	+	+	+
FBW58	+	+	+	+	+	+	+	+	+	+
FBW60	+	+	+	+	+	+	+	+	+	+
FBW61	+	+	+	+	+	+	+	+	+	+
FBW65	+	+	+	+	+	+	+	+	+	+
FBW66	+	+	+	+	+	+	+	+	+	+
FBW72	+	+	+	+	+	+	+	+	+	+
FBW73	-	-	-	-	-	-	-	-	-	-
FBW77	+	+	+	+	+	+	+	+	+	+
FBW79	+	+	+	+	+	+	+	+	+	+
FBW84	+	+	+	+	+	+	+	+	+	+
FBW88	+	+	+	-	-	-	-	-	-	-
FBW90	+	+	+	+	+	+	+	+	+	+
FBW94	+	+	+	+	+	+	+	+	-	-
FBW95	+	+	+	+	+	+	+	+	+	+
FBW101	+	+	+	+	+	+	+	+	+	+
FBW103	+	+	+	+	+	+	+	+	+	+
FBW106	+	+	+	+	+	+	+	+	+	+
FBW107	+	+	+	+	+	-	-	-	-	-
FBW114	+	+	+	+	-	-	-	-	-	-
FBW116	+	+	+	+	+	+	+	+	+	-
FBW120	+	+	+	+	+	+	+	+	+	+
FBW123	+	+	+	+	+	+	+	+	+	+
FBW126	+	+	+	+	+	+	+	+	+	+
FBW128	+	+	+	+	+	+	+	+	+	+
FBW133	+	+	+	+	+	+	+	+	+	+
FBW135	+	+	+	+	+	-	-	-	-	-
FBW136	+	+	+	+	+	+	+	+	-	-
FBW138	+	+	+	+	+	+	+	+	+	+
FBW140	+	+	+	+	+	+	+	+	+	+
FBW141	+	+	+	+	+	+	+	+	+	+
FBW142	+	+	+	+	+	+	+	+	+	+
FBW143	+	+	+	+	+	+	+	-	-	-

FBW144	-	-	-	-	-	-	-	-	-	-
FBW145	+	+	+	+	+	+	+	+	-	-
FBW146	+	+	+	+	+	+	+	+	+	+
FBW147	+	+	+	+	+	+	+	+	+	+
FBW148	+	+	+	+	+	+	+	+	+	+
FBW151	+	+	+	+	+	+	+	+	+	+
FBW152	+	+	-	-	-	-	-	-	-	-
FBW153	+	+	+	+	+	+	+	+	+	+
FBW154	+	+	+	+	+	+	+	+	+	+
FBW155	+	+	+	+	+	+	+	+	+	+
FBW157	+	+	+	+	+	+	+	+	+	+
FBW158	+	+	+	+	+	+	+	+	+	+
FBW159	+	+	+	+	+	+	+	+	+	-
FBW160	+	+	+	+	+	+	+	+	+	-
FBW161	+	+	+	+	+	+	+	+	+	+
FBW162	+	+	+	+	+	+	+	+	+	-
FBW163	+	+	+	+	+	+	+	+	+	+
FBW164	+	+	+	+	+	+	+	+	+	-
FBW165	+	+	+	+	+	+	+	+	+	+
D/Sina	+	+	+	+	+	+	+	+	+	+
B/Jira	+	+	+	+	+	+	+	+	+	+
EAL-110	+	+	+	+	+	+	+	+	+	+
Total growth	71	71	70	68	65	63	62	59	54	46
Growth %	97.3	97.3	95.9	93.1	89	86.3	84.9	80.8	73.9	63

Annex 8: Acid/basestress tolerance of faba bean nodulating rhizobia;on YEMA medium adjusted at different pH values

Isolates	3.5	4	4.5	5	5.5	6.8	8	8.5	9	9.5	10
FBW9	+	+	+	+	+	+	+	+	+	+	+
FBW10	+	+	+	+	+	+	+	+	+	+	+
FBW11	-	+	+	+	+	+	+	+	+	+	+
FBW12	-	+	+	+	+	+	+	+	+	+	+
FBW13	-	+	+	+	+	+	+	+	+	+	+
FBW19	+	+	+	+	+	+	+	+	+	+	+
FBW20	+	+	+	+	+	+	+	+	+	+	+

FBW23	+	+	+	+	+	+	+	+	+	+	+
FBW36	-	+	+	+	+	+	+	+	+	+	+
FBW38	+	+	+	+	+	+	+	+	+	+	+
FBW39	-	-	-	-	-	+	+	+	+	+	+
FBW40	+	+	+	+	+	+	+	+	+	+	-
FBW42	+	+	+	+	+	+	+	+	+	+	+
FBW43	+	+	+	+	+	+	+	+	+	+	+
FBW45	+	+	+	+	+	+	+	+	+	+	+
FBW46	-	-	-	+	+	+	+	+	+	+	+
FBW51	-	-	-	-	+	+	+	+	+	+	+
FBW52	+	+	+	+	+	+	+	+	+	+	+
FBW56	+	+	+	+	+	+	+	+	+	+	+
FBW58	+	+	+	+	+	+	+	+	+	+	+
FBW60	+	+	+	+	+	+	+	+	+	+	+
FBW61	+	+	+	+	+	+	+	+	+	+	+
FBW65	+	+	+	+	+	+	+	+	+	+	+
FBW66	-	-	-	-	+	+	+	+	+	+	+
FBW72	-	-	-	-	-	+	+	+	+	+	+
FBW73	-	-	+	+	+	+	+	+	+	+	+
FBW77	+	+	+	+	+	+	+	+	+	+	+
FBW79	+	+	+	+	+	+	+	+	+	+	+
FBW84	-	-	-	-	+	+	+	+	-	-	-
FBW88	+	+	+	+	+	+	+	+	+	+	+
FBW90	+	+	+	+	+	+	+	+	+	+	+
FBW94	+	+	+	+	+	+	+	+	+	+	+
FBW95	+	+	+	+	+	+	+	+	+	+	+
FBW101	+	+	+	+	+	+	+	+	+	+	+
FBW103	+	+	+	+	+	+	+	+	+	+	+
FBW106	+	+	+	+	+	+	+	+	+	+	+
FBW107	-	-	+	+	+	+	+	+	+	+	+
FBW114	+	+	+	+	+	+	+	+	+	+	+
FBW116	-	-	-	-	-	+	+	+	+	-	-
FBW120	-	+	+	+	+	+	+	+	+	+	+
FBW123	+	+	+	+	+	+	+	+	+	+	+
FBW126	-	-	+	+	+	+	+	+	+	+	+
FBW128	-	-	-	+	+	+	+	+	+	+	+
FBW133	+	+	+	+	+	+	+	+	+	+	+
FBW135	+	+	+	+	+	+	+	+	+	+	+
FBW136	-	-	+	+	+	+	+	+	+	+	+

FBW138	+	+	+	+	+	+	+	+	+	+	+
FBW140	+	+	+	+	+	+	+	+	+	-	-
FBW141	-	-	-	-	-	+	+	+	+	+	+
FBW142	-	-	-	+	+	+	+	+	+	+	+
FBW143	-	-	-	-	+	+	+	+	+	+	+
FBW144	+	+	+	+	+	+	+	+	+	+	+
FBW145	-	-	-	-	+	+	+	+	+	+	+
FBW146	-	-	-	+	+	+	+	+	+	+	+
FBW147	-	-	-	-	+	+	+	+	+	+	+
FBW148	+	+	+	+	+	+	+	+	+	+	+
FBW151	+	+	+	+	+	+	+	+	+	+	+
FBW152	-	-	-	-	+	+	+	+	+	+	+
FBW153	+	+	+	+	+	+	+	+	+	+	+
FBW154	-	-	-	-	+	+	+	+	+	+	+
FBW155	+	+	+	+	+	+	+	+	+	+	+
FBW157	-	-	-	-	-	+	+	+	+	+	+
FBW158	-	-	-	-	+	+	+	+	-	-	-
FBW159	-	-	-	-	+	+	+	+	-	-	-
FBW160	-	-	-	-	+	+	+	+	+	+	+
FBW161	+	+	+	+	+	+	+	+	+	+	+
FBW162	+	+	+	+	+	+	+	+	+	+	+
FBW163	+	+	+	+	+	+	+	+	+	+	+
FBW164	-	-	-	-	+	+	+	+	+	+	+
FBW165	+	+	+	+	+	+	+	+	+	+	+
D/Sina	+	+	+	+	+	+	+	+	+	+	+
B/Jira	+	+	+	+	+	+	+	+	+	+	+
EAL-110	+	+	+	+	+	+	+	+	+	+	+
Total growth	**43**	**48**	**52**	**56**	**68**	**73**	**73**	**73**	**70**	**68**	**67**
Growth (%)	**58.9**	**65.7**	**71.2**	**76.7**	**93.1**	**100**	**100**	**100**	**95.9**	**93.1**	**91.8**

Annex 9:Different incubation temperature tolerance of faba bean nodulating rhizobia; inoculated on YEMA medium

Isolates	5°C	10°C	15°	20°-30°C	37°C	40°C	45°C
FBWW9	-	+	+	+	-	-	-
FBW10	+	+	+	+	+	+	-
FBW11	-	-	+	+	+	-	-

FBW12	-	-	+	+	+	-	-
FBW13	-	+	+	+	+	-	-
FBW19	-	-	+	+	+	-	-
FBW20	-	-	+	+	+	-	-
FBW23	+	+	+	+	+	-	-
FBW36	+	+	+	+	+	-	-
FBW38	-	+	+	+	+	-	-
FBW39	-	-	+	+	+	-	-
FBW40	-	+	+	+	+	+	+
FBW42	+	+	+	+	+	+	+
FBW43	-	-	+	+	+	-	-
FBW45	-	-	+	+	+	+	+
FBW46	-	+	+	+	-	-	-
FBW51	+	+	+	+	+	-	-
FBW52	+	+	+	+	+	-	-
FBW56	-	-	+	+	+	-	-
FBW58	-	-	+	+	+	-	-
FBW60	-	+	+	+	+	-	-
FBW61	-	-	+	+	+	-	-
FBW65	-	+	+	+	+	-	-
FBW66	-	+	+	+	+	-	-
FBW72	-	+	+	+	+	-	-
FBW73	-	-	+	+	-	-	-
FBW77	-	+	+	+	+	-	-
FBW79	-	+	+	+	+	-	-
FBW84	-	+	+	+	+	-	-
FBW88	+	+	+	+	+	-	-
FBW90	-	+	+	+	+	-	-
FBW94	-	+	+	+	+	+	-
FBW95	-	+	+	+	+	-	-
FBW101	-	+	+	+	+	-	-
FBW103	-	+	+	+	+	-	-
FBW106	+	+	+	+	+	+	+
FBW107	+	+	+	+	+	+	+
FBW114	-	+	+	+	+	-	-
FBW116	-	+	+	+	+	-	-
FBW120	-	+	+	+	+	-	-
FBW123	-	+	+	+	+	-	
FBW126	-	+	+	+	+	-	-

FBW128	-	+	+	+	-	-	-
FBW133	+	+	+	+	+	+	+
FBW135	-	+	+	+	-	-	-
FBW136	-	+	+	+	+	-	-
FBW138	+	+	+	+	+	+	+
FBW140	-	+	+	+	+	-	-
FBW141	-	+	+	+	-	-	-
FBW142	-	+	+	+	+	-	-
FBW143	+	+	+	+	+	-	-
FBW144	-	+	+	+	+	-	-
FBW145	-	+	+	+	+	-	-
FBW146	-	+	+	+	-	-	-
FBW147	+	+	+	+	-	-	-
FBW148	-	+	+	+	+	-	-
FBW151	-	+	+	+	+	+	+
FBW152	-	+	+	+	+	+	-
FBW153	-	+	+	+	+	-	-
FBW154	+	+	+	+	-	-	-
FBW155	+	+	+	+	-	-	-
FBW157	-	-	+	+	+	-	-
FBW158	-	+	+	+	+	+	+
FBW159	-	+	+	+	+	-	-
FBW160	-	+	+	+	+	-	-
FBW161	+	+	+	+	+	-	-
FBW162	-	-	+	+	+	-	-
FBW163	+	+	+	+	+	-	-
FBW164	-	+	+	+	+	-	-
FBW165	-	+	+	+	+	-	-
D/Sina	-	+	+	+	+	+	-
B/Jira	-	+	+	+	+	+	-
EAL-110	-	+	+	+	+	+	-
Total growth	**17**	**60**	**73**	**73**	**63**	**15**	**9**
Growth %	**23.3**	**82.2**	**100**	**100**	**86.3**	**20.5**	**12.3**

Annex 10: Growth of isolates on different carbon sources

Isolates	glucose	fructose	sucrose	starch	sorbitol	Dextrin	mannose	Raffinose	Galactose	trehalose
FBW9	+	+	+	+	+	-	+	+	+	+
FBW10	+	+	+	+	+	+	+	+	+	+
FBW11	+	+	+	+	+	+	+	+	+	+
FBW12	+	+	+	+	+	+	+	+	+	+
FBW13	+	+	+	+	+	+	+	+	+	+
FBW19	+	+	+	+	+	+	+	+	+	+
FBW20	+	+	+	+	+	+	+	+	+	+
FBW23	+	+	+	+	+	+	+	+	+	+
FBW36	+	-	-	+	-	+	+	+	+	+
FBW38	+	+	+	+	+	+	+	+	+	+
FBW39	+	-	+	+	+	-	-	+	-	+
FBW40	+	+	+	+	+	+	+	+	+	+
FBW42	-	+	+	+	-	-	+	+	-	+
FBW43	+	+	+	+	+	+	+	+	+	+
FBW45	+	+	+	+	+	-	+	+	-	+
FBW46	-	-	-	-	-	+	+	-	-	+
FBW51	+	-	+	+	+	+	+	+	+	+
FBW52	+	+	+	+	+	-	+	+	+	+
FBW56	+	+	+	+	+	+	+	+	+	+
FBW58	+	-	+	+	+	-	+	+	+	+
FBW60	+	+	+	+	+	+	+	+	+	+
FBW61	+	+	+	+	+	-	+	+	+	+
FBW65	+	-	+	+	+	+	+	+	-	+
FBW66	+	+	+	-	-	-	+	+	+	+
FBW72	+	+	+	+	-	-	+	+	+	+
FBW73	+	+	+	-	+	-	+	+	+	+
FBW77	+	-	+	-	-	+	+	-	+	+
FBW79	+	+	+	+	+	+	+	+	-	+
FBW84	+	+	+	+	+	-	+	+	+	+
FBW88	+	+	+	-	+	+	+	+	+	+
FBW90	+	+	+	+	+	+	+	+	+	+
FBW94	+	+	+	+	+	-	+	+	+	+
FBW95	+	+	+	+	+	-	+	+	+	+
FBW101	+	+	+	+	+	+	+	+	+	+

FBW103	+	+	+	+	+	+	+	+	+	+
FBW106	+	+	+	-	+	-	+	+	-	+
FBW107	+	-	+	+	+	-	+	+	+	+
FBW114	+	-	+	+	+	+	+	+	-	+
FBW116	+	-	+	-	+	+	-	+	+	+
FBW120	+	+	+	+	+	+	+	+	+	+
FBW123	+	+	+	-	+	-	+	+	-	+
FBW126	+	+	+	+	+	+	+	+	+	+
FBW128	-	-	+	+	-	-	-	+	-	+
FBW133	+	-	+	+	+	-	+	+	+	+
FBW135	+	+	+	+	-	-	+	+	-	+
FBW136	+	-	+	+	+	-	+	-	-	+
FBW138	-	+	-	+	-	-	+	+	-	+
FBW140	+	+	+	+	+	-	+	+	+	+
FBW141	+	-	+	+	+	-	+	-	-	+
FBW142	+	+	+	+	+	+	+	+	+	+
FBW143	-	-	+	+	-	+	+	+	+	+
FBW144	+	-	+	+	+	-	-	+	-	+
FBW145	+	+	+	+	+	+	+	+	+	+
FBW146	+	+	+	+	-	-	+	+	-	+
FBW147	-	+	+	+	-	+	+	+	-	+
FBW148	+	+	+	+	+	+	+	+	+	+
FBW151	+	+	+	+	+	+	+	+	-	+
FBW152	+	-	+	-	-	+	+	+	+	+
FBW153	+	-	+	+	+	-	+	+	-	+
FBW154	+	+	+	+	-	+	+	+	-	+
FBW155	+	+	+	+	-	-	+	+	+	+
FBW157	-	-	-	+	-	-	+	+	+	+
FBW158	+	-	+	+	+	-	+	+	+	+
FBW159	+	+	+	+	-	-	+	+	+	+
FBW160	+	+	+	+	+	-	+	+	+	+
FBW161	+	+	+	+	+	+	+	+	+	+
FBW162	+	+	+	+	+	+	+	+	+	+
FBW163	+	+	+	+	-	+	+	+	+	+
FBW164	+	+	+	+	+	+	+	+	+	+
FBW165	+	+	+	-	+	+	+	+	+	+
D/Sina	+	+	+	+	-	-	+	+	+	+
B/Jira	+	+	+	+	+	-	+	+	+	+
EAL-	+	+	+	+	+	-	+	+	+	+

110										
Total growth	**66**	**53**	**69**	**53**	**54**	**39**	**69**	**69**	**53**	**73**
Growth %	**90.4**	**72.6**	**94.5**	**72.6**	**73.9**	**53.4**	**94.5**	**94.5**	**72.6**	**100**

Annex 11: Growth of isolates on different N sources

Isolates	alanine	glycine	Arginine	Valine	aspartic acid	Asparagine	Phenyl alanine	histidine	isoleucine
FBW9	+	+	+	+	-	+	+	+	+
FBW10	+	+	+	+	+	+	+	+	+
FBW11	+	+	+	+	+	+	+	+	+
FBW12	+	+	+	+	+	+	+	+	+
FBW13	+	+	+	+	+	+	+	+	+
FBW19	+	+	+	+	+	+	+	+	+
FBW20	+	+	+	+	+	+	+	+	+
FBW23	+	+	+	+	-	+	+	+	+
FBW36	+	+	+	+	+	+	+	+	+
FBW38	+	+	+	+	+	+	+	+	+
FBW39	+	+	-	+	+	+	+	+	+
FBW40	+	+	+	+	+	+	+	+	+
FBW42	+	+	-	+	+	+	+	+	+
FBW43	+	+	+	+	+	+	+	+	+
FBW45	+	+	+	+	-	+	+	+	+
FBW46	+	+	+	+	-	+	-	+	+
FBW51	+	+	+	+	+	+	+	+	+
FBW52	+	+	+	-	-	+	+	+	+
FBW56	+	+	+	+	+	+	+	+	+
FBW58	+	+	+	+	-	+	+	+	+
FBW60	+	+	+	+	+	+	+	+	+
FBW61	+	+	+	+	-	+	+	+	+
FBW65	+	+	+	+	-	+	+	+	+
FBW66	+	+	-	+	-	+	+	+	+
FBW72	+	+	+	+	-	+	+	+	+
FBW73	+	+	+	-	-	+	-	+	+
FBW77	+	+	+	+	+	+	+	+	+
FBW79	+	+	+	+	-	+	+	+	+

FBW84	+	+	+	+	-	+	+	+	+
FBW88	+	+	-	+	-	+	+	+	+
FBW90	+	+	+	+	-	+	+	+	+
FBW94	+	+	+	+	+	+	+	+	+
FBW95	+	+	+	+	-	+	+	+	+
FBW101	+	+	+	+	-	+	+	+	+
FBW103	+	+	+	+	+	+	+	+	+
FBW106	+	+	+	+	-	+	+	+	+
FBW107	+	+	+	+	+	+	+	+	+
FBW114	+	+	+	+	+	+	+	+	+
FBW116	+	+	+	+	-	+	+	+	+
FBW120	+	+	+	+	+	+	+	+	+
FBW123	+	+	-	+	-	+	+	+	+
FBW126	+	+	+	+	+	+	+	+	+
FBW128	+	+	+	+	-	+	+	+	+
FBW133	+	+	+	+	-	+	+	+	+
FBW135	+	+	+	+	-	+	+	+	+
FBW136	+	+	-	+	-	+	+	+	+
FBW138	+	+	+	+	+	+	+	+	+
FBW140	+	+	+	+	-	+	+	+	+
FBW141	+	+	+	+	-	+	+	+	+
FBW142	+	+	+	+	+	+	+	+	+
FBW143	+	+	+	-	-	+	+	+	+
FBW144	+	+	+	+	-	+	+	+	+
FBW145	+	+	-	+	+	+	+	+	+
FBW146	+	+	-	+	+	+	-	+	+
FBW147	+	+	+	+	+	+	+	+	+
FBW148	+	+	+	+	-	+	+	+	+
FBW151	+	+	+	+	-	+	+	+	+
FBW152	+	+	+	+	+	+	+	+	+
FBW153	+	+	-	+	-	+	+	+	+
FBW154	+	+	+	-	-	+	+	+	+
FBW155	+	+	+	+	-	+	-	+	+
FBW157	-	+	+	+	-	+	+	+	+
FBW158	+	+	+	+	-	+	+	+	+
FBW159	+	+	+	+	-	+	+	+	+
FBW160	+	+	+	+	+	+	+	+	+
FBW161	+	+	+	+	+	+	+	+	+
FBW162	+	+	+	+	-	+	+	+	+

FBW163	+	+	+	+	+	+	+	+	+
FBW164	+	+	+	+	-	+	+	+	+
FBW165	+	+	+	+	-	+	+	+	+
D/Sina	+	+	+	+	+	+	+	+	+
B/Jira	+	+	+	+	+	+	+	+	+
EAL-110	+	+	+	+	+	+	+	+	+
Total growth	**72**	**73**	**64**	**69**	**35**	**73**	**69**	**73**	**73**
Growth %	**98.6**	**100**	**87.7**	**94.5**	**47.9**	**100**	**94.5**	**100**	**100**

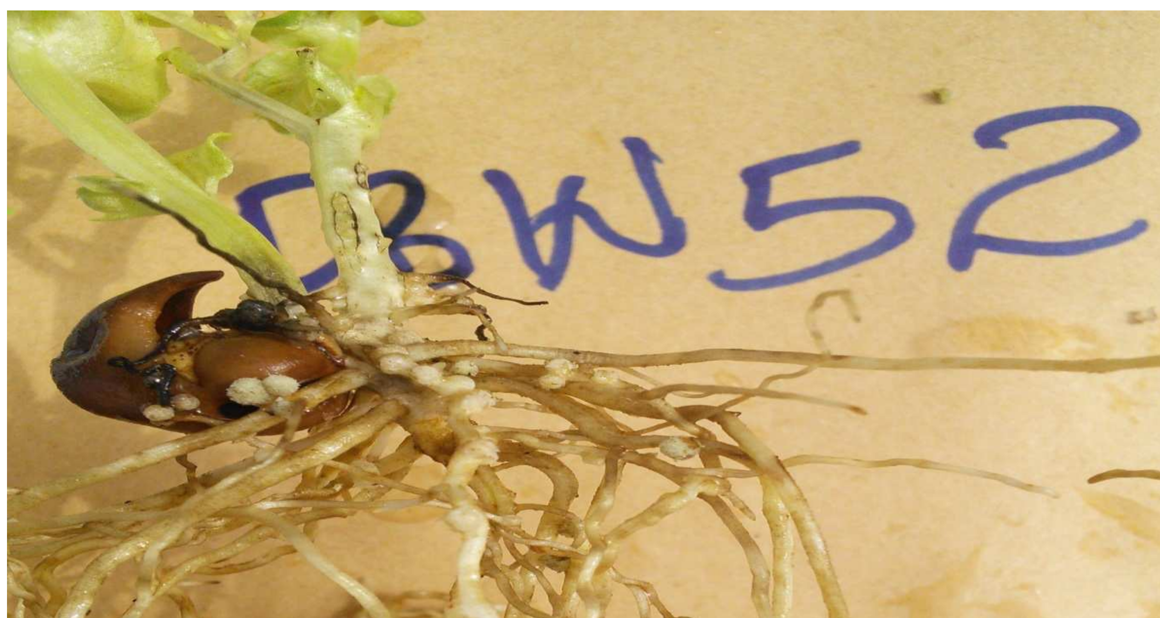
Annex 12: Intrinsic Antibiotic Resistance (IAR) patterns of the faba bean nodulating rhizobial isolates

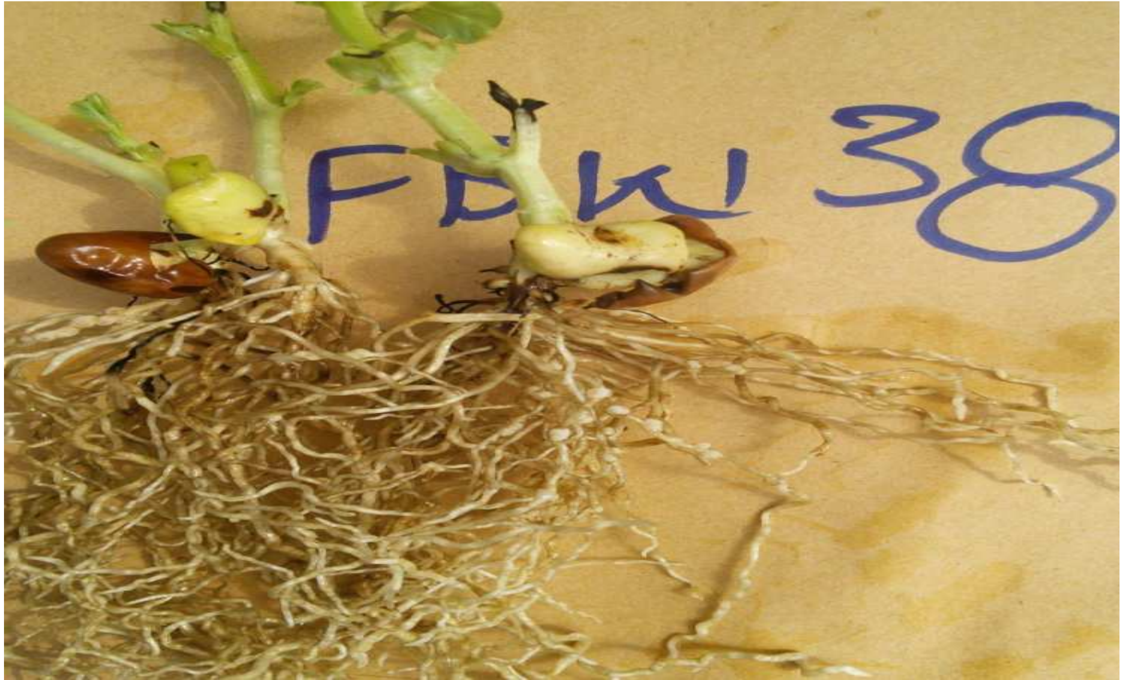
Isolates	Erythromycin		Chloramphenicol		Kanamycin		Streptomycin		Neomycin	
	5µg/ml	10µg/ml	5µg/ml	10µg/ml	5µg/ml	10µg/ml	5µg/ml	10µg/ml	5µg/ml	10µg/ml
FBW9	+	-	-	-	-	-	-	-	+	-
FBW10	+	-	+	-	-	-	-	-	-	-
FBW11	+	-	+	-	-	-	-	-	-	-
FBW12	+	-	-	-	-	-	-	-	+	-
FBW13	+	-	-	-	-	-	+	-	+	-
FBW19	+	-	+	-	-	-	-	-	-	-
FBW20	+	-	-	-	-	-	-	-	-	-
FBW23	+	-	-	-	-	-	-	-	+	-
FBW36	+	-	+	+	-	-	+	-	-	-
FBW38	+	+	+	+	-	-	+	+	+	-
FBW39	+	-	+	-	-	-	+	-	+	-
FBW40	+	-	-	-	-	-	-	-	+	-
FBW42	+	-	-	-	-	-	-	-	+	-
FBW43	+	-	-	-	-	-	-	-	+	-
FBW45	+	-	-	-	-	-	-	-	+	-
FBW46	+	+	+	-	-	-	-	-	-	-
FBW51	+	-	+	+	-	-	+	+	+	-
FBW52	+	+	+	+	-	-	+	+	+	-

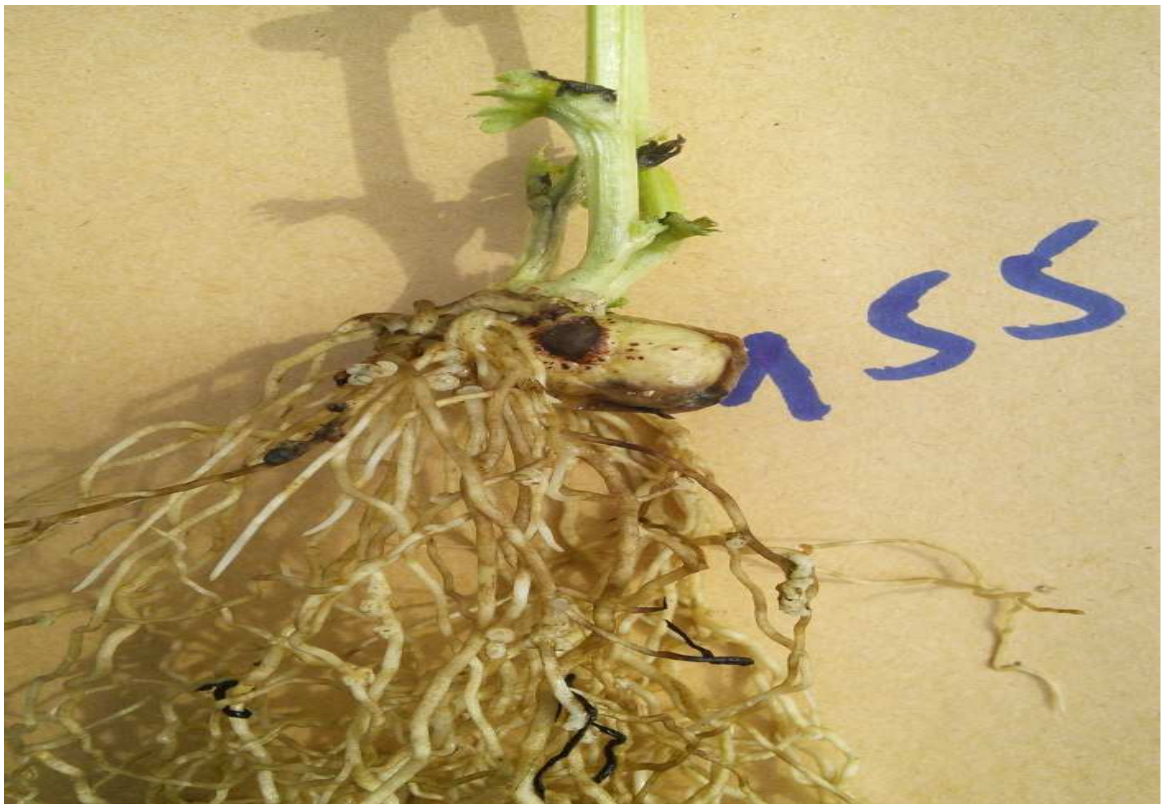
FBW56	+	-	-	-	-	-	-	-	+	-
FBW58	+	-	-	-	-	-	-	-	-	-
FBW60	+	+	-	-	-	-	-	-	+	-
FBW61	+	+	-	-	-	-	-	-	+	-
FBW65	+	-	-	-	-	-	-	-	-	-
FBW66	+	-	-	-	-	-	-	-	+	-
FBW72	+	-	-	-	-	-	-	-	-	-
FBW73	-	-	+	-	-	-	-	-	+	-
FBW77	+	+	-	-	-	-	-	-	-	-
FBW79	+	-	-	-	-	-	-	-	-	-
FBW84	+	+	+	+	-	-	-	-	-	-
FBW88	+	-	-	-	-	-	-	-	-	-
FBW90	+	-	-	-	-	-	-	-	+	-
FBW94	+	-	-	-	-	-	-	-	+	-
FBW95	+	-	-	-	-	-	-	-	+	-
FBW101	+	-	-	-	-	-	-	-	-	-
FBW103	+	-	-	-	-	-	-	-	-	-
FBW106	+	-	-	-	-	-	-	-	+	-
FBW107	+	+	+	+	-	-	+	-	+	-
FBW114	+	+	+	-	-	-	-	-	+	-
FBW116	+	-	-	-	-	-	-	-	-	-
FBW120	+	-	-	-	-	-	-	-	-	-
FBW123	+	+	-	-	-	-	-	-	+	-
FBW126	+	-	-	-	-	-	-	-	-	-
FBW128	+	+	+	+	-	-	-	-	+	-
FBW133	+	-	-	-	-	-	-	-	+	-
FBW135	+	+	-	-	-	-	-	-	+	-
FBW136	+	-	-	-	-	-	-	-	-	-
FBW138	+	-	-	-	-	-	-	-	+	-
FBW140	+	-	-	-	-	-	-	-	+	-
FBW141	+	+	+	+	-	-	+	-	+	-
FBW142	+	+	+	+	-	-	+	+	+	-
FBW143	-	-	+	+	-	-	-	-	+	-
FBW144	+	-	-	-	-	-	-	-	+	-
FBW145	+	-	-	-	-	-	+	-	-	-
FBW146	+	+	+	+	+	-	-	-	-	-
FBW147	+	+	+	+	-	-	+	+	-	-
FBW148	+	+	-	-	-	-	-	-	-	-
FBW151	+	-	-	-	-	-	-	-	-	-

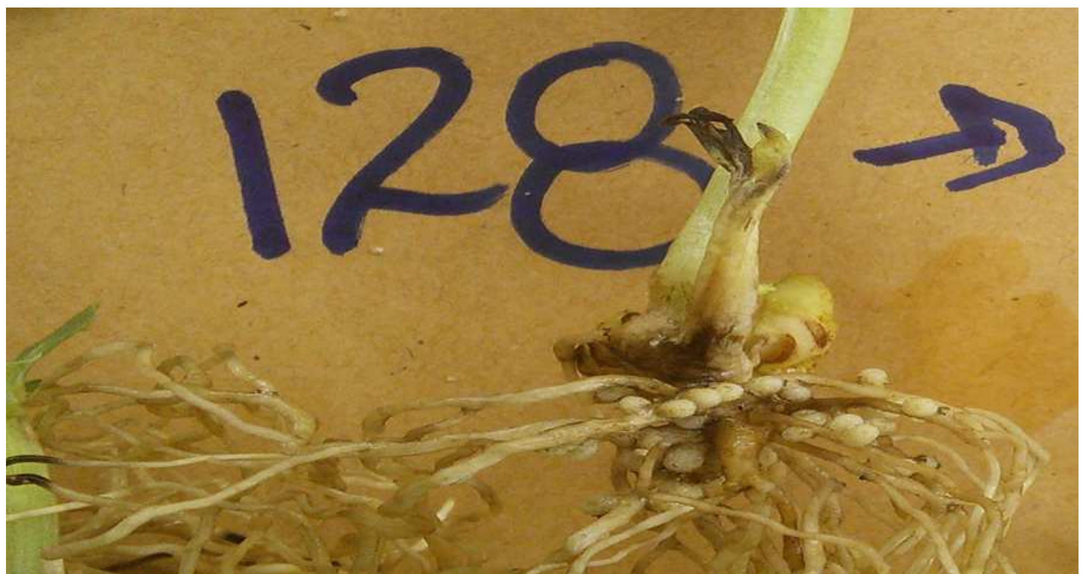
FBW152	+	+	+	+	-	-	+	+	+	-
FBW153	+	+	-	-	-	-	-	-	-	-
FBW154	+	+	-	-	-	-	+	-	+	-
FBW155	+	+	-	-	-	-	-	-	+	-
FBW157	-	-	-	-	-	-	-	-	+	-
FBW158	-	-	+	+	-	-	-	-	+	-
FBW159	-	-	+	+	-	-	-	-	+	-
FBW160	+	+	+	+	-	-	+	-	-	-
FBW161	+	+	+	-	-	-	+	-	-	-
FBW162	+	+	-	-	-	-	+	-	+	-
FBW163	+	-	-	-	-	-	-	-	-	-
FBW164	+	+	+	+	-	-	-	-	-	-
FBW165	+	-	-	-	-	-	-	-	-	-
D/Sina	+	-	-	-	-	-	-	-	+	-
B/Jira	+	-	-	-	-	-	-	-	+	-
EAL-110	+	-	+	-	-	-	-	-	+	-
Total growth	**68**	**25**	**26**	**17**	**1**	**0**	**16**	**6**	**43**	**0**
Resistant %	**93.1**	**34.2**	**35.6**	**23.3**	**1.4**	**0**	**21.9**	**8.2**	**58.9**	**0**

Annex 13: Samples showing good nodulation status of faba bean grown in lath house in sterile sandculture

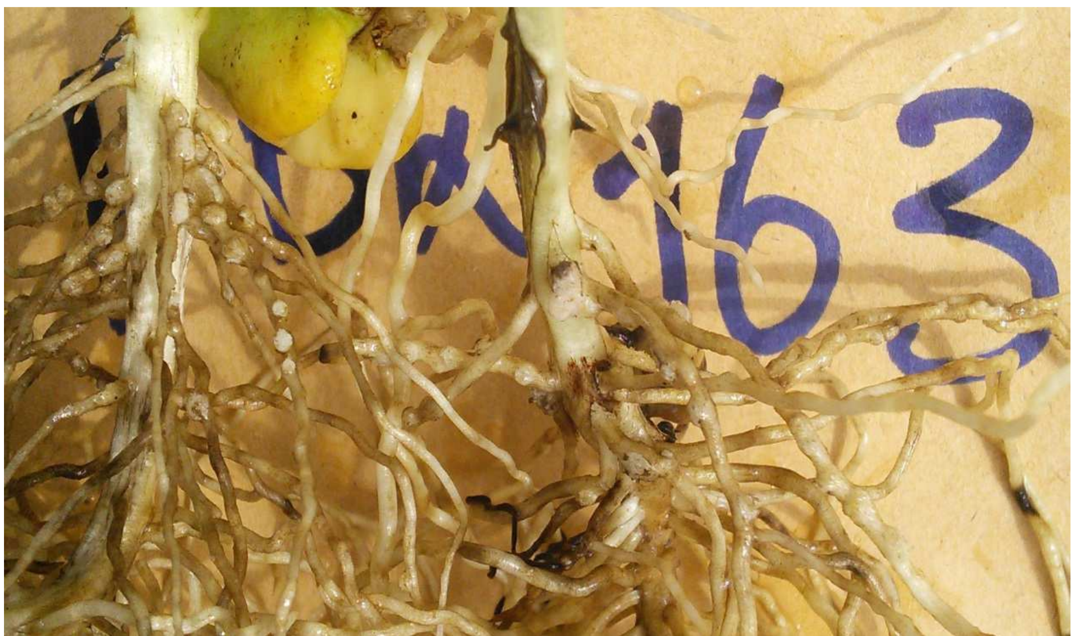





155


128

W72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