GENETIC DIVERSITY OF RHIZOBIA NODULATING COMMON BEANS (*Phaseolus vulgaris* L) FROM KELLEM WOLLEGA, ETHIOPIA

YOSEF HAMBA TOLA

MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY

PAN AFRICAN UNIVERSITY

INSTITUTE OF BASIC SCIENCES, TECHNOLOGY AND INNOVATION

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Genetic Diversity of Rhizobia Nodulating *Phaseolus vulgaris* L. from Kellem Wollega, Ethiopia

YOSEF HAMBA TOLA

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DECLARATION

This thesis is my original work and has not been presented for a degree award in any other University.

Signature …….Date ……..……

Yosef Hamba Tola

This thesis report has been submitted for examination with our approval as University supervisors;

Signature ……..Date ……..……

Losenge Turoop (Prof.), JKUAT

Pathologist

Signature …….....Date ……

Moses Thuita (Dr.), IITA

Soil Scientist

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DEDICATION

THIS THESIS DEDICATED TO MY FATHER HAMBA TOLA AND MY MOTHER

AYANTU HAMBACHA

ABSTRACT

This study was conducted to understand the genetic diversity of common bean (*Phaseolus vulgaris L*.) nodulating rizobia and efficacy of rhizobial isolates from the soil of various agro-ecological regions of Kellem Wollega. Soil samples were collected from six bean fields in Manjoso Jiru, Amahara Kucho, Bile, Foge, Minko, and Humbi karo using random sampling technique. The isolate were characterized using Molecular techniques through PCR-Restriction Fragment Length Polymorphism and partial sequences of 16S rDNA. From the characterization of RFLP 8IGS profiles obtained. Phylogenetic tree were created using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6), BioEdit and Multiple Sequence Comparison by Log-Expectation (MUSCLE). Then seven isolates were classified as *Rhizobium leguminosarum and* one isolate as *Rhizobium vallis.* The distributions of these strains are not the same in all study sites. For instance; *R. leguminosarum* is more abundant than *R. vallis* in all study sites. *Rhizobiz vallis* was only found in Humbi karo. Kellem Wollega soils harbor highly diverse rhizobial populations. The rhizobium genetic diversity is attributed to the agro-ecological region or soil characteristics of the study sites.

KEY WORDS: PCR, RFLP, Phylogenetic tree, *R. leguminosarum*, *R. vallis*

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ACRONYMS

BNF Biological Nitrogen Fixation

RFLP Restriction Fragment Length Polymorphism

PCR Polymerase Chain Reaction

DNA Deoxy Ribonucleic Acid

TBE Tris borate EthylDiamine Tetracetic Acid

IGS Intergenic Spacer

rpm rotation(s) per minute

EDTA Ethyl diamine tetracitic acid

CTAB Cetyl trimethyl ammonium bromide

MLEE Multilocus enzyme electrophoresis

FAOSTAT Food and Agricultural Organization Statistics

CHAPTER ONE

General Introduction

1.1 Introduction

Rhizobia are gram negative bacteria that are known to induce the fixation of atmospheric nitrogen in certain leguminous plants (Gualtieri and Bisseling, 2000). In the process, the gaseous nitrogen is reduced to ammonia (Franche et al. 2008). The fixing of nitrogen in soils forms a symbiotic relationship between the plants and the Rhizobia which is the most important biological mechanism for providing nitrogen to the plants. This may be an important alternative to the use of synthetic fertilizers that are quite expensive for resource poor farmers (Freiberg et al. 1997). Currently there are more than 50 known *Rhizobia* species of economic importance in crops; these species are mainly distributed in genera: *Rhizobium; Ensifer; Mesorhizobium; Azorhizobium; and Bradyrhizobium* (Khan and Musarrat, 2010). Common bean is one of the crops that is colonized and nodulated by various rhizobia species.

Phaseolus vulgaris is nodulated by different genera and species of rhizobia, including *Rhizobium etli, Rhizobium leguminosarum, Rhizobium tropici, Rhizobium gallicum, Rhizobium giardinii, Rhizobium phaseoli, Rhizobium Vallis and sinorhizobium meliloti* (Garcia et al. 2010; Michiels et al. 1998). Recently a new species of *R. phaseoli* was identified (Ramirez-Bahena et al. 2008). Besides new species, some un expected rhizobial isolates have also been identified from common bean root nodules such as *Sinorhizobium fredii* like isolates from Spain (Herrera–Cervera et al. 1999) and *Mesorhizobium spp.* isolates from Brazil (Grange and Hungria, 2004).

Ethiopian soils are reported to harbor highly diverse rhizobia population (Endalkachew et al. 2004b, 2005). According to the study of Beyene et al. (2004) in some agro-ecological regions of Ethiopia 70% of rhizobia isolated were genetically related to *R. leguminosarum*. However, from analysis of their 16SrRNA genes, the majority was placed with *R. etli*. Transfer and recombination of the 16S rRNA gene from presumptively introduced *R. etli* to local *R. leguminosarum* was done to explain these contrasting results. However, it seems unlikely that bean rhizobia originating from Americas (or Europe) extensively colonized soils of Ethiopia because *R. tropici, R. gallicum,* and *R. giardinii* were not detected and only a single ineffective isolate of *R. etli* that originated from remote location was identified (Beyene et al. 2004).

According to the report of FAOSTAT in (2008) common bean is globally grown in nearly 28 million ha with average yield of between 493 Kg/ha in 1961 to 729 Kg/ha in 2008). *Phaseuolus vulgaris* is native to America (Chacon et al. 2005). Seeds of the common bean were imported to Europe after the discovery of Americas in 1492 and have been grown there extensively for over 500 years (Gepts and Bliss, 1988). However, the main producers of common beans are: East, Central and Southern Africa and all of Central America and Brazil (Stephen, 2006).

In Ethiopia, Common bean is widely cultivated in areas with altitude between1400-2000. The Rift valley contributes to 48% out of 163,688 ha and 55% of 1,384,216 quintals production of the country (Teshale et al. 2006). Due to limited access to external inputs by smallholder farmers, the yields of Ethiopian cultivars are extremely low, (Amare, 1987; EARO, 2000).

Even though Ethiopia is considered the center of origin for many other leguminous crops,

including pea, clover, and lentil (Raven and Polhill, 1981), the *Phaseolus vulgaris* is only grown in some parts of the Highlands of Ethiopia. However, the common bean is one of most important sources of protein to the local populace (Boudoin and Maquet, 1999; Arulbalachandran and Mullainathan, 2009) and is rich in vitamins, minerals and dietary fibre (Kelly and Scott, 1992; Ndegwa et al. 2006).

From different regions in the world, there are many reports as well on genetic uniformity and on large biodiversity of rhizobial strains nodulating on common bean (Martinez-Romero, 2003). In Ethiopia, the studies of rhizobial genetic diversity have not been documented in every parts of the country (EARO, 2000; Beyene, et al. 2004; Alemayehu, 2006). Hence, the present study was conducted with the aim to characterizing the indigenous rhizobia nodulating common bean from common bean growing area of Kellem Wollega, Western Ethiopia.

1.2 Statements of Problem

Declining soil fertility, high fertilizer costs and intensification of agriculture coupled with the reduction in farm sizes are major limitations to crop production in smallholder farms in developing countries, where much grain legume production occurs and many farmers cannot afford to use fertilizers; awareness and use of commercial microbial inoculants is limited (Maobe et al. 2000; Cheruiyot et al. 2001; Beyene, et al. 2004; and Chemining‟wa et al. 2004).

Significant environmental decline has been associated with the injudicious use of synthetic fertilizer. This includes loss of nitrates to the atmosphere, acid rain, induced leaching of soil nutrients, changes in the global N cycle, and nitrate pollution of groundwater (Kinzig and Socolow, 1994; Vitousek et al. 1997). Furthermore, synthetic fertilizers do not improve soil

physical structure or enhance soil biological activity and by themselves are, usually insufficient to maintain soil fertility (Wallace and Knausenberger, 1997). As a result cheaper sources of nitrogen need to be sought if yields are to be sustained and food security attained (Otieno et al. 2009).

Successful nitrogen fixation depends on the interaction between legume genotype, rhizobium strain and environment (Giller, 2001). Little is known about the genetic diversity of indigenous rhizobia in Ethiopia soils which can be efficiently exploited to biologically fix nitrogen for improved agricultural productivity.

1.3 Justification

Rhizobium symbiosis with legume is known to contribute signifcanlty to the total global nitrogen producing 50% of 175 million tons of total biological species N_2 fixation annually worldwide (Sarioglu et al. 1993). Inoculation with efficient strains of Rhizobium may increase yields in areas where the bacteria are not already present (Giller, 2001). Most desirable species will have a very difficult time competing with weeds without the slowly released nutrients provided by bio-fertilizers.

Rhizobia are soil bacteria that can engage in a symbiosis with leguminous plants that produces biological nitrogen fixing root nodules and based on specific recognition of signal molecules, which are produced by both the bacterial and plant partners (Spaink, 2000). Additionally, their application in soil improves soil biota and minimizes the sole use of chemical fertilizers (Venkatashwarlu, 2008). Therefore a need to characterize the genetic diversity of indigenous rhizobia in Ethiopian soils which in turn can be efficiently exploited in their ability to

biologically fix nitrogen for improved agricultural productivity. Finally, this study was provided indigeneus rhizobia nodulating common beans in Kellem Wollega with their phylogenetic relationship among the isolates and reference strains.

1.4 Research Objectives

1.4.1 General Objective

The study aimed at determining the genetic diversity of rhizobia nodulating *Phaseolus vulgaris* L. from Kellem Wollega,

1.4.2 Specific objectives

1. To characterize rhizobia from root nodules of *Phaseolus vulgaris* growing in the Kellem Wollega Zone using the Molecular tools.
2. To evaluate the genetic diversity within and among populations of the rhizobial isolates

CHAPTER TWO

Literature Review

2.1 Rhizobia

Leguminuous plants obtain organic nitrogenous compounds such as glutamine or ureides from the Rhizobium an endosymbiotic nitrogen fixing association with roots of legumes (Biomate, 2008). Hence, the plant provides the bacteria organic compounds made by photosynthesis. They are mostly rhizospheric microorganisms, despite its ability to live in the soil for long period of time (Gonzalez et al. 2005). The presences of Rhizobia in soils usually depend on where the appropriate host plants are grown (Shridhar, 2012).

Rhizobia can exist either as free-living saprophytic heterotrophy or as legume-host-specific nitrogen fixing symbionts (Gonzalez et al. 2005). As the result of this dual mode rhizobia had several distinct advantages with respect to survival and persistence over most other soil bacteria. According to the study in the American Midwest soil the number of rhizobia in bulk soil that surrounds legumes often approach to 10^6 cells g^{-1} (Ellis et al. 1984) and sometimes up to 10^8 cells g^{-1} (Bottomley, 1992). The growth of rhizobia in the rhizosphere was stimulated by plant root exudates (Van Egeraat, 1975) and Phillips et al. (1999) investigated that rhizobia can also stimulate growth and respiration of leguminous plants. Therefore, these rhizobial species have ability to improve crop yields and potential in environmental application (Shridhar, 2012)

The findings of new rhizobial species through the time make complexity in the classification of

rhizobia (Somasegaran and Hoben, 1985). From the study of Jordan (1984), all bean rhizobia were classified as *R. leguminosarum*. Then *Rhizobium etli* was suggested as a species separate from *R. leguminosarum* based on results of variation in chromosomal markers determined by multilocus gel electrophoresis and 16S rRNA gene sequences (Segovia et al. 1993 and van Berkum et al. 1996). Correspondingly, *Rhizobium tropici* was recommended as a bean-nodulating species based on variation in chromosomal markers (Martinez et al. 1991) before Willems and Collins (1993) reported the 16S rRNA gene sequence.

The 16S rRNA sequences of *R. leguminosarum* and *R. etli* or *R. tropici* are very similar and vary only by 1 and 2%, respectively (Van Berkum et al. 1996). Since van Berkum and Eardly (1998) 16S rRNA gene sequence variation is inconclusive evidence to separate *R. etli* and *R.* leguminosarum and agreed that phylogenetic relationships based on these sequences are untrustworthy evidence for resolving species below the genus level. Amarger et al. (1997) has been reported that bean forms nitrogen fixing symbiotic relationships with five different rhizobial species.

Studies on other crops such as haricot bean, and woody legumes have been undertaken (Fassil and Kleiner, 1997; 1998; Beyene et al. 2004; Endalkachew et al. 2004a). The study on Ethiopian collections showed that Ethiopian soils harbor highly diverse rhizobial population (Endalkachew et al. 2004b, 2005). As the result the molecular characterization and evaluation of the genetic diversity within and among populations of the rhizobial isolates using PCR-RFLP of the 16S RNA genes were studied.

2.2 Significance of Biological Nitrogen Fixation

Biological Nitrogen Fixation (BNF) is an efficient source of nitrogen (Biomate, 2008). It is the process whereby atmospheric nitrogen (N=N) is reduced to ammonia in the presence of nitrogenase (Franche et al. 2008). The symbiotic relationship between leguminous plants and rhizobia help to fix atmospheric nitrogen which can be utilized by plants (Shridhar, 2012; Vessy et al. 2005). Nature of BNF is the process of *nitrogenase* catalyzes reaction which -split triple-bond inert atmospheric nitrogen (N2) into organic ammonia molecules (Cheng, 2008). This relationship is based on specific recognition of signal molecules, which are produced by both the bacterial and plant partners (Spaink, 2000). About 96% of the N taken up by the crop has been measured as nitrogen derived from the atmosphere (Lopez-Bellido et al. 2006).

According to the study of Rascio and Rocca (2008), it has been estimated that the 80-90% of the N available to plants in natural ecosystem derives from BNF. The total annual terrestrial inputs of N from BNF range from 139 million to 175 million tonnes of N, with symbiotic associations growing in arable land accounting for 25 to 30% (35 million to 44 million tons of N) and permanent pasture accounting for another 30% (45 million tons of N) (Burns and Hardy, 1975; Paul, 1988, Sarioglu et al. 1993).

Increased plant protein levels and reduced depletion of soil N reserves are obvious outcomes of legume N fixation. Lack in mineral nitrogen often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Freiberg, 1997).

Rhizobium-legume symbioses are the main source of fixed nitrogen in land based systems and can grant well over half of the biological source of fixed nitrogen (Tate 1995). Likewise, Walley et al. (2007) indicated that atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for sustainable agriculture and helps to reduce chemical fertilizer of N requirements. Also 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 (Zahran, 1999).

2.3 Mechanisms of Biological nitrogen fixation

Conversion of molecular nitrogen to ammonia is catalyzed by nitrogenase, and oxygen labile enzyme complex highly conserved in free-living and symbiotic diazotrophs (Franche et al. 2008). Metallo-proteins, MoFe protein and Fe protein are main composition of *Nitrogenase* (Yan et al. 2010). Nitrogen reduction is a very complex mechanism not as yet fully clarified. The result of net reduction molecular nitrogen to ammonia is generally accounted for by the following equation (Arnold et al. 1988, Franche et al. 2008):

$$N_2 + 8^+ + 8e^+ + 16Mg\ ATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

The *nitrogenase* enzymes are irreversibly inactivated by oxygen, and the process of nitrogen fixation uses a large amount of energy (Dixon and Wheeler, 1986; Postgate, 1982). The symbiotic association between the roots of legumes and rhizobia accounts for the development of a specific organ, the symbiotic root-nodule, whose primary function is nitrogen fixation (Carvalho et al. 2011).

Root nodules make a crucial contribution to the nitrogen content of the soil playing a key role in

agricultural practices (Alla et al. 2010). A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (nod, nol, noe, nif and fix genes) (Perret et al. 2000; Spaink, 2000). These genes are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be easily lost or gained (MacLean et al. 2007). The nod, nol and noe gene products are involved in production of a nodulation signal, the Nod factor, which is a lipo-chitolipochitooligosaccharide. Initiation of nodule formation on compatible host plants results from a molecular dialogue between the host and the bacteria (Dénarié et al. 1993; Schultze and Kondorosi, 1998).

The host plants produce flavonoids (secondary metabolites) in the rhizosphere (Long, 2001). These signals can be perceived by a specific bacterial receptor, Nod D, acts as a transcriptional activator and Nod factor acts as an elicitor of root nodule formation (Geurts and Bisseling, 2002; Gage, 2004). It is an important host specificity determinant (Spaink, 2000).

Maintenance of *nitrogenase* activity is subject to a delicate equilibrium in the nodule and a high rate of oxygen respiration is necessary to supply the energy demands of the nitrogen reduction process (Sanchez et al. 2011), but oxygen also irreversibly inactivates the *nitrogenase* complex. These conflicting demands are resigned by control of oxygen flux through a diffusion barrier in the nodule cortex and by the plant oxygen carrier, leg haemoglobin, which is present exclusively in the nodule (Minchin et al. 2008).

2.4 Biological Nitrogen fixation in *Phaseolus vulgaris* L

Common bean is a short-season crop with most varieties maturing in a range of 65 to 110 days from emergence to physiological maturing (Buruchara, 2007).and maturity period can continue

up to 200 days after planting amongst climbers that are used in cooler upland elevations (Gomez, 2004). It shows variation in growth habits from determinate bush to indeterminate, extreme climbing types. Bushy type bean is the most predominant type grown in Africa (Buruchara, 2007). It is 20-60 cm tall with most of the pods held above the ground while climbers may grow 2-3 m tall if they have support (Cobley and Steele 1976). Common bean is a warm-season crop that does not tolerate frost and usually high temperatures do not affect it if adequate soil water is present (Wortmann et al. 1998).

Cultivation of common bean in Africa is widespread, but approximately 80 percent of African bean production is concentrated in 10 countries. Kenya is the leading producer of common bean in Africa followed by Uganda and then Tanzania in terms of area. Malawi and Ethiopia rank eighth and ninth, respectively according to FAO statistics (FAO, 2008). However, in terms of production, Kenya comes second after Uganda, with Tanzania keeping its third position. Common bean yields are higher in Uganda than in Kenya because of a relatively favorable biophysical environment in Uganda compared to Kenya. In the latest figures from FAO for 2007, however, the production in Kenya has moved above 500,000 tones.

Common bean in Ethiopia is produced in almost all the regional states with varying intensity (Legesse et al. 2006). Production is concentrated in two regions: Oromia and the Southern National Nationality Peoples region (SNNPR), which account for about 85 percent of the total national production. The remaining 25 percent comes from Afar, Amhara, Tigray, Somali, Gambella and Benishangul-Gumuz (Ferris and Kaganzi, 2008).

Common bean is considered a poor nitrogen fixing pulse in comparison with other grain legumes (La Rue and Patterson, 1981). For instance, faba bean (*Vicia faba*), lupin (*Lupinus spp*.) and pigeon peas (*Cajanus cajan*) have been found to be very efficient; soybean (*Glycine max*), groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) to be average; and common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) rather poor in fixing atmospheric nitrogen (Hardarson, 1993).

2.5 Factors affecting biological nitrogen fixation

Symbiotic Nitrogen fixation and nodulation can be affected by environmental factors in some cases reducing rhizobial survival and diversity in important factors is: acidity, temperature, mineral nutrition, salinity and alkalinity (Graham, 1998). Phosphorous (P) fertilization is the major mineral nutrient yield determinant among legume crops (Chaudhary et al. 2008). According to the study of Karasu and Dogan, in 2011, the ineffectiveness of bacteria inoculation and nitrogen doses on yield may be dependent upon a lot of factors. Because seed yield is a quantitative character and is affected by genotype and environmental factors.

2.5.1 Soil acidity

Soil acidity is one of the main environmental factors that interrupt the symbiotic association between *Phaseolus* and *Rhizobium* (Graham, 1981). The low pH of soil influences all stages of the legume-*Rhizobium* symbiosis, including strain survival in the soil, root hair infection, nodule initiation, and nitrogen fixation (Graham et al. 1982). The early steps during pre-infection are the more acid sensitive events as a result of negative influence of the low pH on the bacterial

attachment to roots as reported by Caetano Anolle and Favelukes, (1986). Additionally, the failure of nodulation was reported in legumes especially in the acid soil below pH 5 due to the inability of *Rhizobium strain* to survive under these conditions (Graham et al.1982).

Poor nodulation of bean roots was commonly seen in the tropics due to soil acidity (Ssahi 1981). Soil acidity also causes Aluminum and Manganese toxicity on the rhizobial symbiosis and reduction of phosphorus (Franco and Munns, 1982; Sanchez and Vehara, 1980 which is the characteristics of some soils in Eastern Africa (Le Mare, 1984). Management of soil acidity for nitrogen fixation depends on the selection of acid-tolerant legume cultivars and compatible rhizobia (Howieson et al. 1995).

According to the study of Anyango et al. (1995) in Kenya, the dominant types of *Phaseolus* nodulating rhizobia differ between an acidic soil and a high-pH soil, with *Rhizobium tropici* dominating in the acidic soil. It is tempting to assume that *R. tropici* might generally be better adapted to acidic soils than other species of *Phaseolus* nodulating rhizobia. *R. tropicis* the most acid-tolerant *Rhizobium* species described to date (Graham et al. 1994) a potential to replace other less tolerant bean rhizobia. Therefore, soil acidity is affecting the diversity of rhizobia.

In view of the fact that, pH values between 5.5 and 6.5 have been reported to be optimum for bean growth identification of both acid-tolerant host and rhizobial endosymbionts are priority areas to increase bean production are prone areas (Munns and Fox, 1979). However, if Soil pH is less than 5 or more than 8 it affects the amount of nitrogen fixed. For example, in a very acidic soil (pH 4.4), Nitrogen fixation can be reduced up to 30% (Abendroth et al. 2005). Moreover,

amendment of acid soils with lime provides nutrients (Ca and Mg) and formulates better conditions for growth of bacterial cells in the short time by altering soil pH and increasing the availability of phosphorus and molybdenum(Andrade et al. 2002).

2.5.2 Soil temperature

Temperatures in tropical soils are limiting the response of inoculation with introduced or indigenous rhizobial strains (Michiels et al. 1994). Several stages of symbiosis such as root hair infection, bacteroid differentiation, nodule structure, and functioning can be affected by temperatures (Roughley and Dart, 1970). For instance high soil temperature is associated with delaying or restricting nodulation in the subsurface region (Graham, 1992).

Optimal temperature for nodule functioning in common bean is between 25 and 30 °C, but is hampered by root temperatures between 30 and 33 °C (Piha and Munns, 1987). Michiels et al. (1994) reported that the acetylene reduction activity of common bean plants was strongly diminished at 35 °C when plants were inoculated by heat-sensitive or heat tolerant strains. In other way, continually cool root zone temp can significantly delay the onset of nitrogen fixation compared to an optimum soil temperature (Abendroth et al. 2006). However, Surange et al. (1997) isolated highly temperature tolerant strains (50°C) of *Rhizobium* nodulating leguminous trees from tropical soils. The remedy is administration of inoculum in deeper soils and application of surface mulch to reduce soil temperature (Roughley, 1980).

2.5.3 Soil Nutrient

The existence of native *Rhizobium* population and high rates of soil nitrogen in soil reduce the efficiency of *Rhizobium* strains that are covered to the ambient via inoculation and therefore lead to an insufficient response to inoculation by bean (Sparrow and Ham, 1983). Heavy soil with sufficient nitrogen and poor aeration probably affected the nodule formation, since nodule formation is affected by physical components of soil as aeration, soil reaction (pH),soil nutrients, temperature, light, humidity and (Azkan, 2002).

Phosphorus insufficiency is a main constraint of effective nitrogen fixation because phosphorus is an essential nutrient in the process of nodulation and nitrogen fixation (World Bank, 2006). Since the high requirement for P in legumes is consistent with the involvement of P in the high rates of energy transfer that must take place in the nodule; under P shortage conditions, legumes may lose the distinct advantage of unlimited source of symbiotic N (Sulieman et al. 2008).

The more the supply of phosphorus, the more abundant are the nodules (Gowarikeer et al. 2009) and it is a key for structural, metabolism, and functional element in the plants: Thus, the decrease in phosphorus requirement mainly reflects on reducing the leaf areas, shoot dry matter and phosphorus content in shoot and root. However, root growth has inverse relations with P as under deficiency root growth is stimulated as a strategy to improve the phosphorus nutrition (Ahmed, 2007).

2.5.4 Soil salinity

Tropical and Mediterranean regions (Surange et al. 1997; Zahran, 1999) are included under the

10% of the world's land surface which endangered by salinity. Legumes such as *Phaseolus vulgaris, Vicia faba* and *Glycin max* are more salt tolerant than *Pisum sativum* (Cordovilla et al. 1995). Likewise, the legume plants are more sensitive to salt or osmotic stress than the rhizobia (Elshinnawi et al. 1989; Zahran and Sprent, 1986). Also Amarger et al. (1997) distinguished that tolerance to salinity, acidity and alkality is more strain-specific than species specific. Graham and parker, (1964) illustrated that strains of fast growing acid-producing rhizobia such as *R. etli* are generally more salt tolerant than slow growing alkali producing strains.

Moreover there are considerable positive correlation between salt tolerance and adaptation of rhizobial strains in alkaline condition (Kulkarni et al. 2000; Abdelaal Shamseldin, 2005). Rhizobia nodulating common beans isolated from Morocco were able to accept a sodium chloride concentration up to 4% NaCl (680 mM NaCl) in liquid culture (Priefer et al. 2001).There are some evidence that rhizobia strains isolated from alkaline soils are rather tolerant to high temperature, pH, and salt stress (Surange et al. 1997).

According to the study of Islam and Ghoulam (1981) salinity affected root exudates, this altered chemotaxis ratios of the rhizobia, resulting in poor nodulation. Failure of nodulation has also been accredited to damaged root hairs (Elsheikh and Wood 1990a). Reduction in photosynthetic activity might also affect N_2 fixation by chickpea under salt stress (Soussi et al. 1999; Garg and Singla, 2004). Also saline stress led to the yellowing of leaves, and chlorophyll measurements as well indicated that leaf chlorophyll content was reduced significantly under salinity conditions.
Leguminous plants have long been recognized to be extremely salt sensitive (Wang et al. 2005). Salinity causes various forms of stress, such as an osmotic effect and ionic toxicity, and affects

the overall metabolic activities. Symbiosis response to this constraint includes morphological modification, such as the change of nodule cortex structure and biochemical adaptations, such as the modulation of antioxidant enzyme expression in nodules (Abdelly et al. 2008, Yadav et al. 2010)

High salt concentration was reported to reduce nodulation and amounts of N_2 fixed (Bekki et al. 1987; Miller and Wood, 1996). Cordovilla et al. (1999) found a reduction in nodule number and nodule weight in faba bean by 45% and 59% respectively, at 0.45% NaCl. Consequently, the success of *Rhizobium*-legume symbiosis under salt stress requires a good selection for both salt tolerant rhizobial strains (Zahran, 1991) and host plants (Saadallah et al. 2001).

2.5.5 Drought stress

Drought, related to a decrease in soil water content (Katerji et al. 2011) and symbiotic fixation of atmospheric nitrogen (N2) is sensitive to even modest soil water deficits (Sinclair et al. 2007). Drought related inhibition of nitrogen fixation (NF) seriously limits legume yield in many arid and semi-arid region of the world. Three major factors have been proposed to be involved in drought effects on NF: Oxygen limitation, carbon shortage, and regulation by nitrogen metabolism (Ladrera et al. 2007). Decline of N_2 fixation with soil drying causes yield reduction due to inadequate N for protein production, which is the critical seed product (Sinclair et al. 2007).

Several studies have shown that nitrogen fixation at pod filling stage were found to have higher yield under water stress than those having low nitrogen fixation (Patterson and Hudak, 1996; Pimratch et al. 2008). This suggested that maintaining high N_2 fixation under drought stress

could be a means for a legume genotype to achieve high yield under water limited conditions.

Establishment and activity of the legume-*Rhizobium* symbiosis have been found to be extremely sensitive to drought stress. In a two year field experiment with Soya bean, Sinclair et al. (1987) found that nodule number and dry weight decrease after a severe drought. Compared to host plants, *rhizobial* strains are quite resistant to soil desiccation and can survive in water films surrounding soil particles (Williams and De Mallorca, 1984). Fast-growing rhizobia, however, are sensitive to soil dehydration as compared to slow-growing strains (Sprent, 1971). Evidence indicated that the decline of soyabean nitrogen fixation under drought was associated with a decline in photosynthesis (Huang et al. 1975). Zahran et al. (1994) showed that exposing rhizobia to osmotic stress bring about alteration of bacterial membrane lipo-polysaccharides, which are involved in the *Rhizobium*-host plant recognition process.

2.6 Genetic diversity of Rhizobium by use of molecular methods

Traditionally, variation has been determined using morphological features such as growth rate and colony morphology (size, shape, color, texture and general appearance) and antibiotic resistance methods (Graham et al. 1991). However, these methods are not sufficiently discriminative to account for all the variation exhibited in the target species. They cannot delineate sources of observed phenotypic variation into its components that may be due to environmental factors or underlying genetic factors.

An array of methods based on PCR have been developed to characterize *Rhizobium species* and strains, and to examine genetic relationships in the *Rhizobium* group (Laguerre et al. 1996). These include restriction fragment length polymorphism PCR-RFLP analysis of the 16S- 23

rDNA intergenic region and sequence analysis of the 16S RNA gene are important tools in clustering genetically related rhizobia. These have been frequently used in microbial taxonomy to determine inter and intra specific relationships (Abaidoo et al. 2000; Doignon Bourcier et al. 2000; Sarr et al. 2005). In these methods, the generated PCR fingerprints are unique to each isolate and are used to group them at strain level.

CHAPTER THREE

Materials and Methods

3.1 Study Site Description

Been roots and soils were sampled at the Ethiopian eastern highlands of Kellem. The study sites are located between 8°10’58”N - 9° 21’53”N latitude and 34°07’37”E-35°26’53”E longitude with a mean annual temperature of 15°C to 25°C. The mean annual rainfall of the eastern highlands range from 1800-2000mm, while in the central plateaus range between 1600-1800mm while in the remaining parts of the zone ranges between 1200-1600mm and less than 1200mm in the south western parts of the zone. The soils of Kellem Wollega zone are Dystric Nitosols (MoA, 2000).

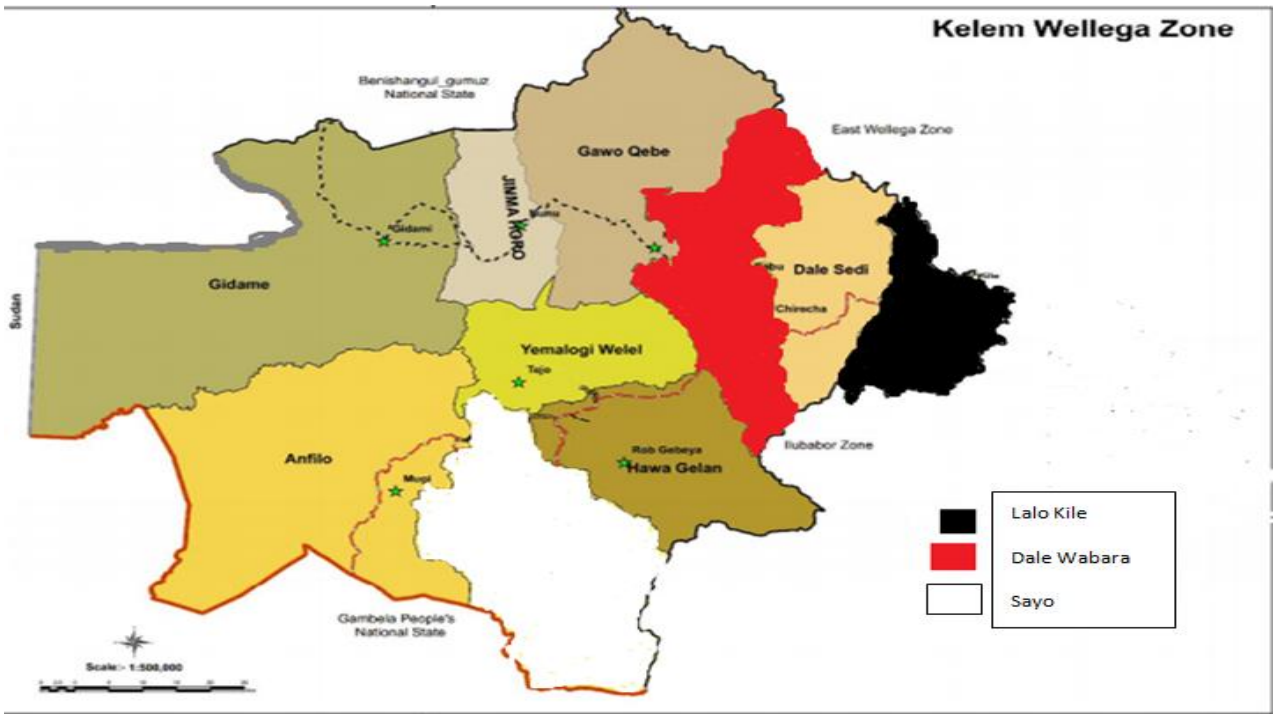

Figure 3.1: Map of Sample Location, Lali kile (Manjoso Jiru and Amahara Kucho), Dale wabara (Bile and Foge) and Sayo (Minko and Humbi karo).Source: Etefa and Dibaba, 2011

3.2 Sample Collection procedures

A clean spade was used to dig approximately 15 cm to either side of the bean plant stalk to a depth of at least 20 cm. The clump of soil and roots lifted out carefully, placed in clean plastic bags and kept in an ice box (Berrada et al. 2012). Then samples were taken to IITA lab and Horticulture lab of JKUAT.

3.3 Soil Analysis

Soil samples from six sites were analyzed at Jomo Kenyatta University of Agriculture and technology laboratory in Nairobi, Kenya according to the procedure described by Ghosh, *et al*. (1983). Soil texture, pH, Ec, N, P, K, Na, Mg, Ca, S, Fe, Mn, and Cu were assessed.

3.4 Trapping Indigenous rhizobia from different soils nodulating *Phaseolus vulgaris*

The objective of this greenhouse trial was to trap Rhizobia capable of nodulating *P. vulgaris* from the soils of Kellem Wollega Zone. Sand that was washed and autoclaved was used as the growth media for this experiment. One kilogram of the dry soil was mixed with sand in equal weights. Two liter (2L) perforated PVC pots were filled with the potting mixture and placed on 400cm diameter plastic saucers. Seeds were surface sterilized by soaking in 3.3% sodium hypochlorite (NaClO) solution for 3 minutes and then thoroughly rinsed and pre-germination was done at 28^{o} C for 48 hrs. Three pre-selected healthy seeds of uniform size were planted per pot and thinned to one plant per pot of comparable height and vigor between 1-2 weeks after planting the seeds were sown and grown under greenhouse conditions for 35 days.

Macronutrients N, P, K, Mg, Ca, and S, and micronutrients Mn, Zn, Cu, B, Mo, and Co were supplied through the irrigation water. Nutrients were applied as solutions of KH_2PO_4 (136.1g/L), $CaCl_2$ (294g/L), $MgSO_4$ (123.3g/L), K_2SO_4 (87g/L), $MnSO_4$ (0.338g/L), $ZnSO_4$ (0.288g/L), $CuSO_4$ (0.1g/L), $CoSO_4$ (0.056g/L), H_2BO_3 (0.247g/L), Fe-citrate (6.7g/L) and NA_2MoO_4 (0.048g/L) (Somasegaran and Hoben, 1985).

Plants were watered daily using distilled water or twice daily depending on the weather condition. At the later growth stages plants were frequently watered to avoid water stress. Harvesting was done at 50% podding. Plants were harvested by removing and washing off the sand and all the nodules collected and preserved.

After the nodules are collected from the plant, they were thoroughly cleaned through sieves and stored at 4^oC awaiting sterilization for long term storage. McCartney bottles were filled with 10 ml of glycerol. McCartney bottles were autoclaved for 20 min at 121^oC. Five bowls and distilled water were prepared. NaOCl (3.3%) solution and 70% ethanol were prepared. The work station was arranged under the hood and the bowls were arranged in this order: the first bowl was put with sterile distilled water; the next with NaClO the last three bowls with sterile distilled water. The nodules were poured onto the sieve and the sieve put onto the bowl of sterile distilled water to rinse off any dirt on the nodules. Stones, leaves, or any other waste were removed. The sieve was put with the nodules onto the first bowl with calcium hypochlorite for 1 min and transferred to the next bowl for another 1 min. Finally the nodules waere transferred in the last two bowls of sterile distilled water so as to rinse the nodules. Nodules were transferred to storage bottles using sterilized forceps.and kept at -20^oC.

3.5 DNA Extraction from the nodules

DNA extraction was carried out from the bean nodules according to the Krasova-Wade et al. (2003) protocol. The nodules were surface sterilized as follow:

The nodules were put in petri plates containing 70% Ethanol for 30 seconds and thereafter transferred using sterilized forceps to the 3.3% NaOCl solution for 2 minutes. Hereafter, they were transferred to the subsequent plates of distilled water and rinsed three times. A single nodule was transferred to a 1.5 ml Eppendorf tube under aseptic conditions. Then 200 µl of sterile distilled water was added and the nodule crushed by using a sterile plastic pestle.Then150 µl of 2 X CTAB/PVPP buffer was added to the nodule suspension and the mixture incubated in a water bath at 65^{o}C for 60 minutes and shaken at 15 minutes intervals. The solution was centrifuged for 15 minutes at 13000 rpm at room temperature and the upper phase (supernatant) was transferred to clean sterile Eppendorf tube. The upper phase was cleared under the hood with 250 µl of phenol: chloroform: iso-amylalcohol (25:24:1 v/v/v) and centrifuged for 5 min at 13000 rpm at room temperature. The supernatant was transferred to a clean sterile Eppendorf tube. Then150µl of chloroform: isoamylalcohol (24:1 v/v) was added and centrifuged for 5 minutes at 13000 rpm at room temperature. The upper phase (supernatant) was transferred in a clean sterile Eppendorf tube. For the purpose of DNA precipitation 100 µl of ice-cold iso-propanol was added and placed at -20^{o}C overnight.

The samples were then centrifuged for 5 minutes at 13000 rpm at 4^{o}C, and the supernatant was poured off and the pellet washed by 100 µl of 70% ethanol. It was centrifuged for 15 minutes at 13000 rpm at room temperature. Ethanol was poured off and the pellet was air dried. The pellet was re-suspended with 50 µl of sterile distilled water and stored at -20^{o}C for further analysis.

3.6 **Polymerase Chain Reaction (PCR) and Restriction**

Genetic diversity was determined by **P**olymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) amplification and restriction of the 16S-23S rDNA intergenic spacer region (Diouf et al. 2000). A 930–1100 bp intergenic region between the 16S and 23S rDNA was amplified by PCR using rhizobia specific primers derived from the 3′ end of the 16S rDNA (FGPS 1490-72; 5′-TGCGGCTGGATCCCCTCCTT-3′) (Navarro et al. 1992) and from the 5′ end of the 23S rDNA (FGPL 132-38; 5′-CCGGGTTTCCCCATTCGG-3′) (Ponsonnet and Nesme, 1994). PCR amplification was carried out in a 25 µl reaction volume containing 2 µl of total DNA extract, 10 pmol of each primer and one freeze-dried bead (puReTaq Ready-To-Go PCR beads, GE Healthcare UK Ltd) containing 2.5 U of Taq DNA polymerase, 200 µM in 10 mM Tris-HCl (pH 9 at Room Temperature) of each dNTP, 50 mM KCl and 1.5 mM $MgCl_2$. PCR amplification was performed in a Bio-Rad iCycler^{TM} thermal cycler adjusted to the following program: initial denaturation for 5 min at 94°C, 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C) and extension (30 s at 72°C) and a final extension (7 min at 72° C). PCR products were visualized by electrophoresis of 3 µl of the amplified DNA on 2% horizontal agarose gel in TBE buffer (1.1% Tris-HCl, 0.1% $Na_2EDTA.2H_2O$ and 0.55% boric acid), pre-stained with 0.033 mg ml^{-1} of Ethidium Bromide. The gel was photographed under UV illumination with Gel Doc (BIO-RAD) Software (USA). Aliquots (10 µl) of PCR products were digested with the restriction endonucleases *Msp*I and *Hae*III (5 U) in a total volume of 15 µl for 2 h at 37° C. The restriction fragments were separated by horizontal electrophoresis in 1X TBE buffer with 3% agarose gel pre-stained with 0.033 mg ml^{-1} of Ethidium Bromide. The gels were run at 100 V for 3 hours and photographed under UV illumination with Gel Doc (BIO-RAD, USA) software. Strains with identical restriction fragment profiles (in individual fragment size

and number) were classified into the same intergenic spacer (IGS) group.

3.7 Partial 16S rDNA gene sequencing

To examine the taxonomic status of the isolates in more detail, the 8 IGS profiles obtained after PCR-RFLP representing a diversity of isolates in the 16SrDNA genotype groups, were selected for partial sequence analysis. PCR Products were purified using the high pure PCR product kit version 15 (Roche 2010). After purification the PCR products were again subjected to electrophoresis to check for purity and molecular size. Purified PCR amplified 16S rDNA fragments were sequenced at the Segolip unit od the BeCa hub, ILRI. Primers used for sequencing were the following: 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'TACGGCTACCTTGTTACGACTT 3') (Lane 1991).

3.8 Data Analysis

Sequence results were edited using BioEdit sequence alignment editor, *BioEdit v7.2.* (Hall, 1999) Multiple sequence alignments of the consensus sequences were performed using MUSCLE (Edgar, 2004). The maximum-likelihood (ML) phylogenetic analysis of multiple aligned sequences with bootstrap values for 1000 bootstrap replicates was performed using Phylogentic maximum- likelihood, version 3.5 (Guindon and Gascuel, 2003). Similarly, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013)

CHAPTER FOUR

Results

4.1 Soil Analysis

Soil pH was slightly in acidic range particularly Foge soil has most acidic when we compared with other sites and soil textures of the sites are sand except Bile and Foge are loam sand. According to analysis the Na and Cu is trace. Additionally, P is highly available in Manjoso Jiru and low available in Foge.

Table 4.1: Soil Analysis from study sites Study Sites

Study Sites	% Sand	% Clay	% Silt	Texture Class	pH	Ec	N	P	K	Na	Mg	Ca	S	Fe	Mn	Cu
Manjoso Jiru	94.1	2.5	3.4	Sand	5.22	0.14	0.07	0.16	1.23	Trace	1.21	3.25	0.94	13.3	4.6	Trace
Amahara Kucho	91.6	5	3.4	Sand	6.31	0.28	0.2	0.14	2.78	Trace	1.10	6.75	0.52	36.1	13.7	Trace
Bile	86.6	7.5	5.9	Loam sand	5.16	0.20	0.05	0.11	1.03	Trace	1.63	5.65	0.65	16.9	12	Trace
Foge	84.1	5	10.9	Loam sand	4.99	0.19	0.06	0.09	1.08	Trace	1.54	5.50	0.32	28.9	14.1	Trace
Minko	91.6	2.5	5.9	Sand	5.22	0.17	0.03	0.15	0.62	Trace	2.23	6.4	0.28	42.2	21.7	Trace
Humbi Karo	91.6	2.5	5.9	Sand	5.67	0.13	0.09	0.11	2.15	Trace	1.69	6.55	0.93	28.9	15.3	Trace

4.2 Green house analysis

Rhizobial trap was done in greenhouse from the soil of six sites in Kellem Wollega Zone. The, nodules were harvested from study sites soil.

4.3 PCR-RFLP Analysis of the 16S rDNA gene

The PCR products with high DNA template, as indicated by the intensity of the band on the gel after electrophoresis, were restricted with restriction enzymes (*Msp*I and *Hae*III) that targets the 16S intergenic spacer region (Figure 1). Common bean rhizobia isolates showed 8 IGS different profiles (I, II, IV, V, VII, VIII, IX and X). The numbers of Profiles were categorized depending on the soil of study sites. Soil of Manjoso Jiru (I), Amahara Kucho (II), Humbi Karo (IV and V), Minko (VII and VIII), and Bile (IX and X) (Table 2). Each of the soils has two profiles except manjoso Jiru and Amahara Kucho soils. In contrary, IGS profile is not obtained from Foge soil.

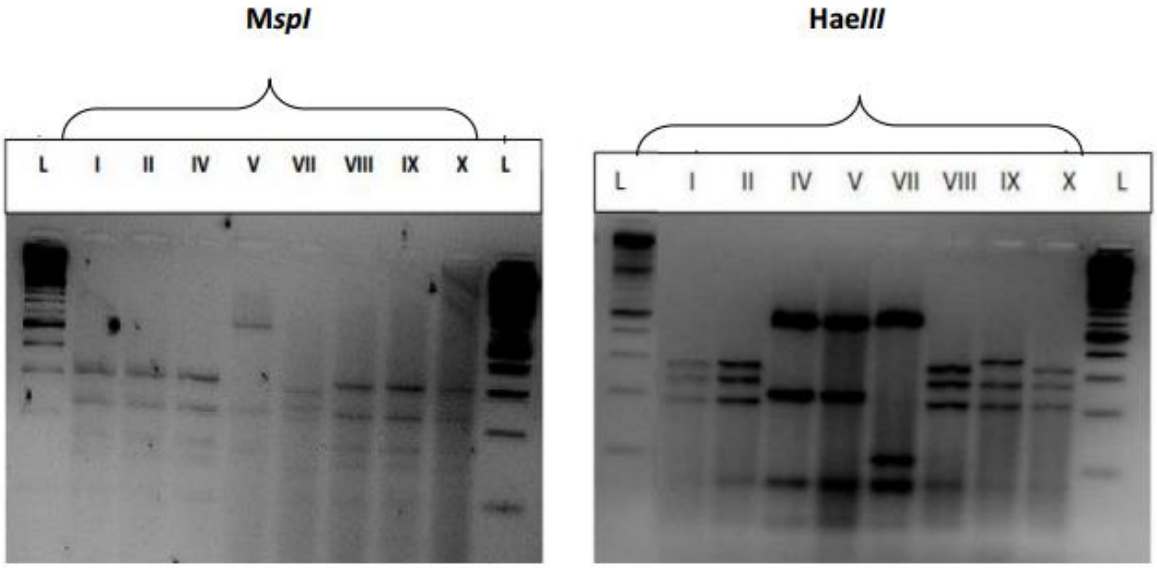

Figure 4.1: 16S rDNA PCR-RFLP patterns derived from digestions with restriction enzymes MspI and HaeIII. DNA ladder was used to estimate the sizes of digested nucleotide bands.

Table 4.2: PCR_RFLP profiles and sequence analysis of 16S rDNA from Kellem Wollega isolates

Isolates	Soil sites	IGS group	Sequence length	Species Affiliation	Accession Number	Identical (%)
Lalo1	Manjoso Jiru	I	1388	*Rhizobium leguminosarum bv. viciae 3841*	NR_103919.1	76
Lalo2	Amahara Kucho	II	1400	*Rhizobium leguminosarum bv. viciae 3841*	NR_103919.1	80
Dale1	Bile	IX	1413	*Rhizobium leguminosarum bv. viciae 3841*	NR_103919.1	78
Dale2	Bile	X	1401	*Rhizobium leguminosarum bv. viciae 3841*	NR_103919.1	78
Sayo1	Humbi Karo	IV	773	*Rhizobium leguminosarum bv. viciae 3841*	NR_103919.1	77
Sayo2	Humbi karo	V	645	***Rhizobium vallis strain CCBAU 65647***	NR_116835.1	93
Sayo3	Minko	VII	1393	*Rhizobium leguminosarumbv. viciae 3841*	NR_103919.1	80
Sayo4	Minko	VIII	1394	*Rhizobium leguminosarumbv. viciae 3841*	NR_103919.1	77

4.4 Phylogeny based on 16S rDNA Sequences

An analysis of the sequences of 16S rDNA genes of all isolates was performed to confirm the phylogenetic position estimated using the RFLP analysis results. From sequence analysis six isolates' with sequence length between 1300 and 1420, where as two isolates were between 600 and 800 (Table 2). Hence, phylogenetic analysis of 16S rDNA sequences of our strains and type strains confirmed the presence of common bean nodulating rhizobial species (reference strains) in Kellem Wollega soils (Figure 2). Accordingly, seven of strains (Lalo1, Lalo2, Dale1, Dale2, Sayo1, Sayo3 and Sayo4) can be classified as R. leguminosarum and Sayo2 was placed with R. vallis.

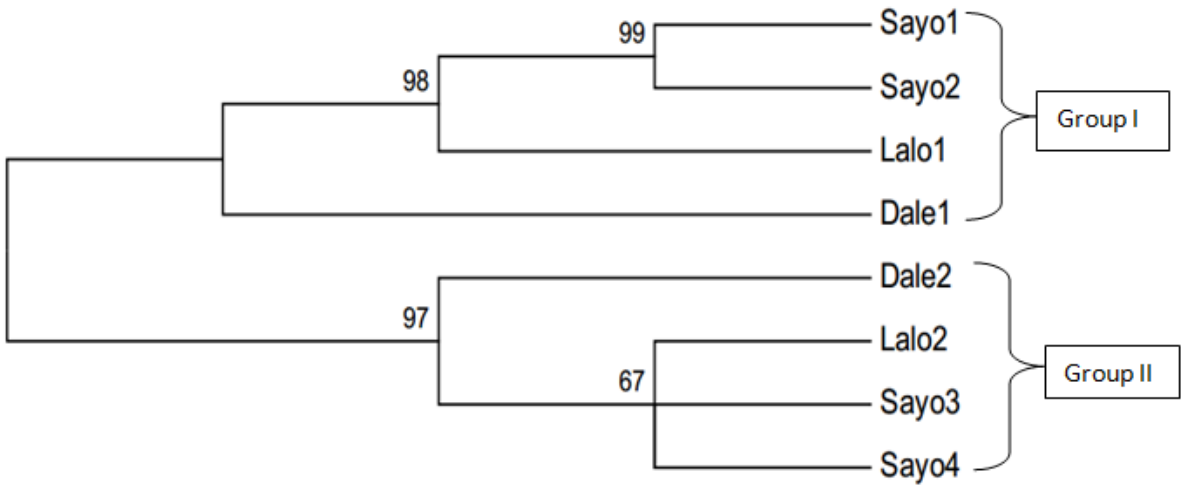

Figure 4.2: Phylogenetic relationship among eight isolates constructed from aligned 16S rDNA gene sequences from Kellem Wollaga using Nearest Neighbor-Interchange (NNI). Number indicates the bootstrap analysis with 1000 replicates and greater than 50% are indicated in the corresponding nodes. This polygenetic tree explained that the isolates are clearly separated in to two distinct groups. Group I that consisted Sayo1, Sayo2, Lalo1 and Dale1; group II includes Dale2, Lalo2, Sayo3, and Sayo4 isolates.

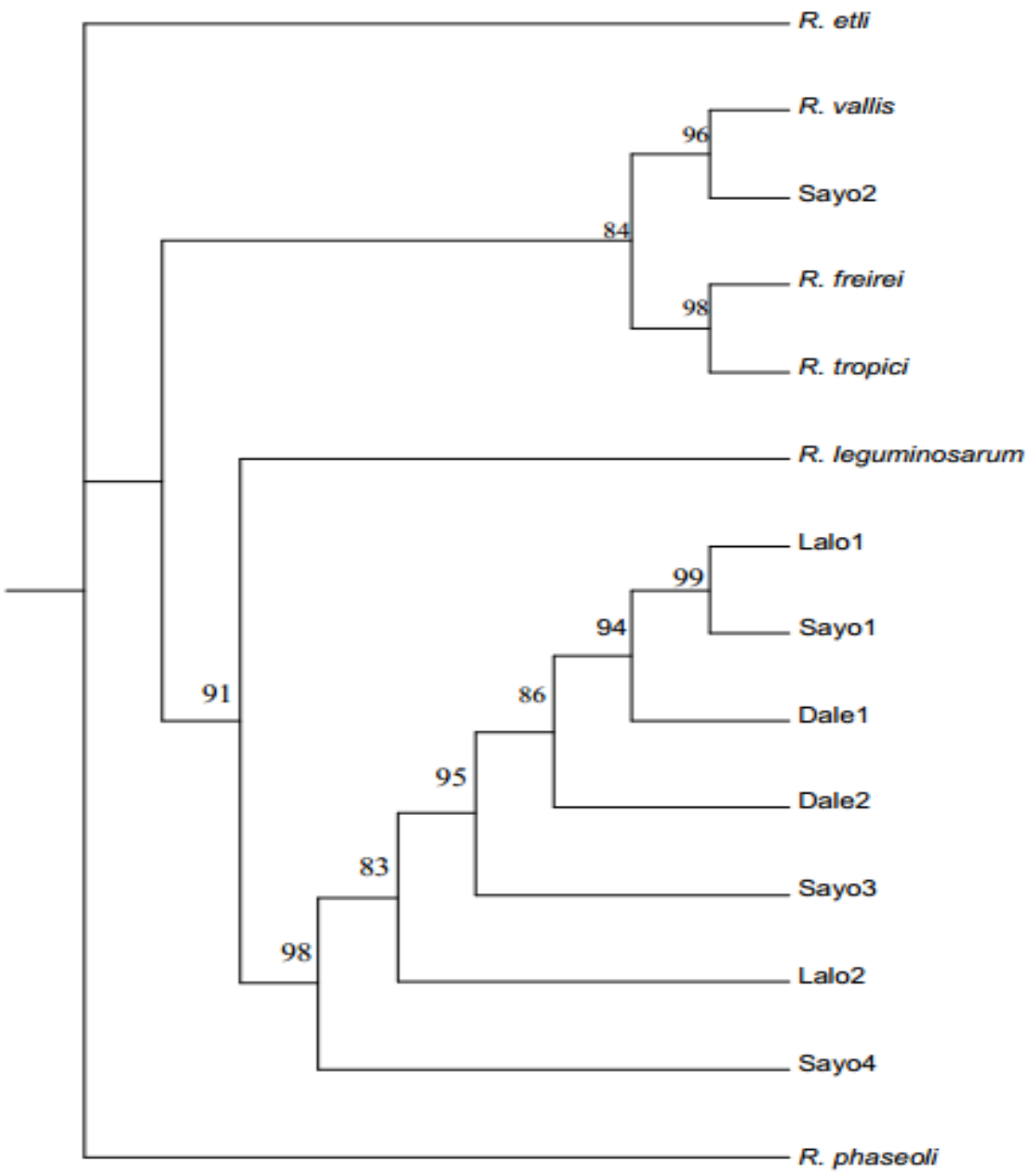

Figure 4.3: This Phylogenetic tree obtained from Common bean rhizobial strains isolated from Kellem wollega soils (Lalo1, Lalo2, Dale1, Dale2, Sayo1, Sayo2, Sayo3, and Sayo4) and reference strains from database using Nearest-Neighbor-Interchange (NNI). Number indicates the bootstrap analysis with 1000 replicates and greater than 50% are indicated in the corresponding nodes.

CHAPTER FIVE

Discussions

These sites have their own agro ecological features with different soil characteristics and land management system. In the zone the productivity of soils are relatively good but due to various reasons the soil fertility is lost. Small holder farmers in the zones use various method of maintaining soil fertility. One of the modern methods maintaining soil fertility is applying inorganic fertilizers (Etefa and Dibaba 2011). The genetic biodiversity of Rhizobium in bean nodules is significantly affected by soil fertilization, especially by ammonia and nitrate (Caballero-Mellado and Martinez-Romero 1999). Studies have showed that continuous cultivation improves build up of rhizobia in soil and increases nodulation (Raposeiras et al. 2006); however other factor like soil pH could also have contributed to the low nodulation in this soil. Also Low Ca and available P in the soil have impact on genetic diversity of rhizobia. Sufficient calcium levels and suitable soil pH are required for good nodulation in legumes (Mohammadi et al. 2012), as acidic conditions and low calcium levels inhibit formation of nodules. High availability of phosphorus in the soil is reported to stimulate nodulation in legumes overcoming the inhibitory effects of high N on nodulation (Werner and Newton 2005, Muthini et al. 2014). Phosphorus becomes involved as an energy source when 16 molecules of adenosine triphosphate (ATP) are converted to adenosine diphosphate (ADP) as each molecule of N_2 is reduced to NH_3. The ATP is generated during the process of photosynthesis, when light energy is transformed and stored in the form of ATP for later use by the plant. Similarly, higher P availability could have contributed for the presence of rhizobial isolates in the soils of Manjoso Jiru and Minko compared to others.

Soil acidity has become a serious threat to crop production in most highlands of Ethiopia in general and in the western part of the country in particular. Currently, it is estimated that about 40% of the total arable land of Ethiopia is affected by soil acidity (Abdenna et al. 2007; Mesfin 2007). Similarly the current study was elucidated as the Kellem Wollega soils are slightly acidic. Extremes of pH affect nodulation by reducing the colonization of soil and the legume rhizosphere by rhizobia. Highly acidic soils (pH<4.0) frequently have low levels of phosphorus, calcium, and molybdenum and high concentrations of aluminum and manganese which are often toxic for both bean plant and the rhizobia (Bordeleau and Prévost 1994). Consistent with the result of his study, Evans et al. (1980) reported that nodulation was 10 times more sensitive to acidity than the growth of rhizobium or the legume root alone. In addition similar to the findings of this study, Vargas and Graham (1989) found that soil acidity is a major factor limiting nodulation and nitrogen fixation in common bean. Soil acidity adversely affects nodulation and nitrogen fixation was also reported by Bambara and Ndakidemi (2010) who observed that it drastically affected legume-rhizobium symbiosis.

Acidity has direct effect on the survival and growth of *Rhizobium* bacteria, which fixes nitrogen in association with legumes (Mensah et al. 2006). The cumulative effects of the increased concentration of Mn in the soil as the result of soil acidity as well as effects of acidity on the *Rhizobium* resulted in the observed decreases in the shoot/root dry weight and seed yield/plant recorded. From here we can say that the presence of high Mn in the soil of Minko affected the diversity of *Rhizobium*. Both acidity and high salinity reduced the growth of rate and survival of the rhizobium species and hence productivity of the host plant.

Soil pH is affected by land use, land management and vegetation type in Kellem Wollega. For example, areas of forestland tend to be more acidic than areas of grass land. In accordance to the study released from the Land Use Planning and Regulatory Department of the Ministry of Agriculture, there are three forestry land utilization types in the country: industrial, construction and fuel (Etefa and Dibaba 2011), these events caused deforestation in these areas. Also conversion of land from forestland or grassland to cropland can result in drastic pH changes after a few years. These changes are caused by a loss of organic matter, removal of soil minerals when crops are harvested, erosion of the surface layer, and effects of nitrogen and sulfur fertilizers, Etefa and Dibaba (2011), reported these events as main problem in Kellem Wollega.

Nodulation is also affected by soil texture and heavy metals (Catroux et al. 2001; Muthini et al. 2014). Interactions between the microsymbiont and the plant are complicated by edaphic, climatic, and management factors. A legume-*Rhizobium* symbiosis might perform well in a loamy soil but not in a sandy soil. These parameters might be one of the factors in these study sites according to our evidences from analyzed soil micronutrients (Fe, Ca, Mn, Mg, Cu) improving solubility and diffusion in soil and they are necessary for N_2 fixation. Also some of these are components of *nitrogenase*. *Nitrogenase* is composed of two metallo-proteins: MoFe protein and Fe protein. From soils of study sites most of these micronutrients are about good, but Cu is trace.

Kellem wollega farmers had no history of inoculation with rhizobia, but were constantly applied inorganic fertilizers, herbicides and pesticides (EECMY –WWBS 2005). However, mineral composition of the soil could have contributed to the low diversity of rhizobia capable of

nodulating common beans in the soil (Muthini et al. 2014). Therefore, all of these parameters influenced the diversity of indigenous rhizobium in soil of Kellem Wollega.

The study elucidated the genetic diversity of rhizobia nodulating *Phaseolus vulgaris* in the Kellem Wollega, Western Ethiopia. Kellem wollega has production of bush and climbing beans as a common farming activity. Bush common beans are dominantly found in various places at Kellem Wollega zone. Since it is dominantly kola climatic zone the area is very appropriate for the cultivation of common beans Reference.

The 16S rDNA was sequenced to determine the taxonomic position of these strains and the results revealed that there was a great genetic diversity among 8 rhizobial strains studied. Sequence analysis and phylogenetic tree analysis indicated that all of the strains (Lalo1, Lalo2, Dale1, Dale2, Sayo1, Sayo3 and Sayo4) were classified as *R. leguminosarum* except Sayo2 was placed with *R. valli*. Additionally, the genetic similarity among isolates was highly appeared within two distinct groups. Similarly, the presence of *R. leguminosarum* in Ethiopian soil was detected by Beyene et al. (2004).

The soil of Kellem Wollega harbor *Rhizobium* species which forms symbiotic relationships with *P. vulgaris*. The studies of (Endalkachew et al. 2004b, 2005) on the Ethiopian soils showed highly diverse rhizobial populations. Beyene et al. (2004) characterized the diversity of bean rhizobia from different agro-ecological region of Ethiopia (Adet, Deneba, Debre berhan, Akaki, Debre zeit, Kulumsa, Hollota, Ginchi, Amaresa) and determined whether they are related to *R. leguminosarum* or to the latin American species *R. etli* and *R. tropici*. Beyane's and his

colleagues work was significantly different from the study of (Diouf et al. 2000) in West Africa; because *R. leguminosarum* was predominantly detected in Ethiopian soils. However, as concluded from genetic data presented the evidence for separating *R. leguminosarum* and *R. etli* into two separate species is inconclusive.

Soils of Ethiopia and Kenya have been reported to harbor bean-nodulating rhizobia similar to *R. leguminosarum, R. tropici, R. phaseoli* and *R. etli* (Anyango et al. 1995 and Beyene et al. 2004). According to the study of Adhikari et al. (2012), R. leguminosarum was only limited to temperate climate with slightly acidic to neutral soils (soil pH 5.3 to 7.0). From this point we can generalize that the range of soil pH in Kellem Wollega is suitable for the survival of *R. leguminosarum.*

5.1 Main Finding

According to the phylogenetic analysis from the 16S rDNA sequences the rhizobial isolates from Kellem Wollega beleongs to two strains: *Rhizobium Leguminosarum* and *Rhizobium vallis*

5.2 Conclusions

The aims of this work were to investigate the genetic diversity of rhizobium isolates from nodules of Common beans of Kellem Wollega soils in Western Ethiopia. Rhizobia strains isolated from Common beans nodules in Kellem Wollega soils are genetically diverse. All of strains were belong to *R. leguminosarum* except Sayo2 which classified as *R. vallis*. Therefore, from this study two types of strains were obtained.

5.3 Recommendation

1. The result indicates that Kellem Wollega is a hot spot for exploration of the biodiversity of the micro-symbionts and further studies should be carried out to unearth the natural biodiversity resources resident in its soils.
2. Finally, soils from other locations not covered in this study should be investigated in order to provide further information about the species of rhizobia that nodulate common bean in Ethiopia.

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