DILLA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

ISOLATION, CHARACTERIZATION OF PHENOTYPIC AND SYMBIOTIC PROPERTIES OF RHIZOBIA NODULATING FABA BEAN (*Vicia faba* L.) COLLECTED FROM URAGA WOREDA, GUJI ZONE, SOUTHEASTERN ETHIOPIA

M.Sc. THESIS

BY

AMANUEL UDESSA BIDIRE

AUGUST, 2021

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AMANUEL UDESSA BIDIRE

A THESIS SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY (SPECIALIZATION: PLANT BIOTECHNOLOGY)

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DEDICATION

It is such a great honor and pleasure to dedicate this work to my father, Udessa Bidire, and mother, Lotu Decho, for their invaluable support, for always keeping me in their prayer and helping me financially during all my phase of study.

DECLARATION

I, the undersigned, declare that this thesis, entitled **Isolation, Characterization of Phenotypic and Symbiotic properties of Rhizobia Nodulating Faba Bean (*Vicia faba* L.) Collected from Uraga Woreda, Guji Zone, Southeastern Ethiopia**, is my original work, that it has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate, that I followed all ethical and technical principles of scholarship in the data collection, data analysis and preparation of this the report, and that all the sources that I have used or quoted have been indicated and acknowledged.

Name: ____

Signature: ____________________

Date: ______________________

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CRD	Completely Randomized Design
CSA	Central Statistical Agency
DM	Dry Matter
EIAR	Ethiopian Institute of Agricultural Research
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
HARC	Holeta Agricultural Research Centre
HCl	Hydrochloric acid
IAA	Indole Acetic Acid
ICARDA	International Center for Agricultural Research in Dry Areas
LSD	Least Significant Difference
masl	meter above sea level
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PGA-BCP	Peptone Glucose Agar incorporated with Bromocresol Purple
SAS	Statistical Analysis Software
YEMA	Yeast Extract Mannitol Agar
YEMA-BTB	Yeast Extract Mannitol Agar with Bromothymol Blue
YEMA-CR	Yeast Extract Mannitol Agar incorporated with Congo red
YMB	Yeast Mannitol Broth

ABSTRACT

Faba bean (Vicia faba L.) is one of the most widely cultivated leguminous crops grown in the Highlands of Ethiopia. The symbiosis formed between leguminous crops and Rhizobia have paramount importance to enhance the productivity of the legume crops. The ability of indigenous rhizobia to nodulate a legume crop effectively was critical to successful establishment and growth of legumes. This study was aimed to isolate, characterize and evaluate the symbiotic effectiveness of rhizobia nodulating faba bean from Uraga Woreda, Guji Zone, and Southeastern Ethiopia. For this matter, a total of twenty five isolates of Rhizobium were isolated from thirty sampling sites of Uraga Woreda using plant infection method in Hawassa University, Soil Microbiology Laboratory. The rhizobial isolates were characterized morphologically and physiologically and tested on acid treated river sand to evaluate their symbiotic effectiveness. Results indicates that culturally almost all of them displayed large colonies with diameters of 1.5 to 5.0 mm, turned color of YEMA-BTB media into yellow, negative rod shaped and showed characteristics of fast growing rhizobia. The symbiotic effectiveness results on sand culture indicated that, the isolates showed shoot dry matter ranging from 0.35 (FBTB-2) to 1.43 g/plant (FBYT-2), with negative control of 0.44 g/plant and positive control of 1.26 g/plant. All the tested isolates were able to grow well within the ranges of 5.0-9.0, 0.5-2.0% and 15^0C*-*35^0C *for pH, salt and temperature, respectively. The highest and lowest nodule number score was 67.00 (FBTHW-4) and 23.33 (FBDHB-5), respectively. Shoot dry weight was found to be strongly positively correlated with symbiotic effectiveness (r= 0.9597, P <0.001). The preliminary screening of the authenticated isolates for symbiotic effectiveness on sand culture showed 55.56% of the isolates were found to be effective, while 22.22% of the isolates viz. FBDHB-2, FBGG-3, FBTHW-4 and FBYT-2 were rated highly effective, of these FBGG-3 and FBYT-2 outcompeted* KNO_3 *treated positive controls. Generally, the present work shows the physiological and symbiotic diversity of the isolates in the traditional agricultural areas of the study site and the potential of these rhizobia to be used as effective commercial inoculants in areas where the indigenous rhizobia fail to do so.*

Keywords: *Biological Nitrogen Fixation, Faba Bean, Nodulation, Rhizobia, Symbiotic effectiveness, Uraga*

CHAPTER ONE: INTRODUCTION

1.1. Background of the Study

Faba bean (*Vicia faba* L.) is globally the fourth most important food legume after garden pea, chickpea and lentil with great potential to alleviate malnutrition for the resource-poor farmers. It is cultivated in the temperate and subtropical regions of the world (Zohary and Hopf, 2000; Torres *et al.,* 2006). The world's leading producing countries for faba bean are China, Ethiopia, Australia, France and the United Kingdom (FAOSTAT, 2014). Ethiopia is the second largest producer of Faba bean in the world next to China (Temesgen *et al.,* 2015).

It is grown in the highlands of Ethiopia between 1800 and 3000 m.a.s.l. (Agegnehu *et al.,* 2006; Wondwosen *et al.,* 2016). The crop is grown in several regions of the country where annual rainfall ranges 700-1000 mm (ICARDA, 2006). Production in Ethiopia is totally rain-fed on nitosols and cambisol type of soils (Gemechu and Mussa, 2002). It makes a significant contribution to soil fertility restoration as a suitable rotation crop that fixes atmospheric nitrogen and reduce the dependence on external fertilizer inputs and also an important source of income for farmers and generates foreign currency for the country (Agegnehu and Fessehaie, 2006). An estimate of 240-325 kg of N fixed per ha by Faba bean with Faba bean-rhizobial symbioses (Somasegaran and Hoben, 1994). It is used both as food and feed due to its high protein content (Caracuta *et al.,* 2015).

Nitrogen (N) is the element most absorbed in soil by plants growing under normal conditions. Its high mobility in soil makes it more deficient for most crops all over the world (Coraspe-Leon *et al.,* 2009). The optimum N for normal plant growth varies between 2% and 5% of the dry weight of the plant (Castano *et al.,* 2008). It intensifies the green color of the leaves, and is a constituent of essential cellular components such as amino acids, proteins and nucleic acids. N also promotes photosynthesis because the N increases the amount of chlorophyll (Aroiee and Omidbaigi, 2004).

Ethiopia has a high nutrient depletion rate in Nitrogen (N), phosphorus (P) and potassium (K) in different farming systems. Nitrogen (N) reserves of agricultural soils must therefore be replenished periodically in order to maintain an adequate level for crop production through mineral N or biological N fixation (BNF) systems (EIAR, 2014).

Biological Nitrogen Fixation is a relatively low-cost source of N for small-holder farmers in developing countries where chemical N input is neither available nor affordable (Amanuel *et al.*, 2000). Biological N fixation (BNF) is all about the non-symbiotic and symbiotic association of soil microorganisms to reduce molecular N to NH_3 which is one alternative to nitrogen fertilizer. It is carried out by prokaryotes using an enzyme complex called nitrogenase and results in atmospheric N_2 being reduced into a form of nitrogen by diastrophic organisms and plants are able to use ammonia. In this way, nitrogen fixation assumes significant importance in agriculture because good crop yields depend on an adequate supply of fixed nitrogen by which the biological process supplies contributes about 65% of the total annual yield of fixed nitrogen (Fisher and Newton, 2002).

The *Rhizobium*-legume association can be manipulated, through inoculation under N limiting field conditions, to enhance crop production easily and inexpensive. Where soils do not contain the specific rhizobia needed to establish an effective association, inoculation is essential, ensuring that a large and effective rhizobial population is available in the rhizosphere of the plant (Berkum *et al.*, 1995). Currently rhizobial inoculants are widely used in various parts of the world. They are the solution to dwindling soil fertility, inexpensive, environment friendly, and easy to use with no side effects in most cases (Wondwosen Tena *et al.*, 2016). The technology, therefore, is good for Ethiopian soils where 85% are reported to have low levels of Nitrogen (EIAR, 2014). Several field demonstrations have confirmed that leguminous crops show remarkable growth and yield response to rhizobia inoculations in different agro-ecologies in Ethiopia. As a result, the use of rhizobia inoculants has shown spectacular growth in Ethiopia (EIAR, 2014).

Rhizobia are diverse group of unicellular soil bacteria which have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen (Glick, 2012). It is able to form nitrogen-fixing root nodules as result of symbiosis with legumes and this permit plant growth in the absence of exogenous N_2 fertilizers (Hatice *et al.*, 2008), due to that *Rhizobium* strains secrete growth hormones like indole acetic acid (IAA), which shows positive influence on plant growth and also plays an important role in the formation and development of root nodules (Lambrecht *et al.*, 2000).

Many research works done by varying Scholars in recent years revealed 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 (Abere Mnalku *et al.*, 2009; Assefa Keneni *et al.,* 2010; Zerihun Belay and Fassil Assefa, 2011; Anteneh Argaw, 2012; Girmaye Kenasa *et al.*, 2014; Wendesen Melak *et al.,* 2018; Alemayehu Workalemahu, 2009; Gemechu Keneni *et al.*, 2015). All the listed scholars have performed vast research on legume-rhizobia symbiosis and revealed the potential benefit of the symbiosis. Their study paves a way for many interested researchers to work on related topics and recommend potent rhizobial strains for inoculants production for commercial biotechnology. In view of the presence of heterogeneous agro-ecologies found in the country, more diversity and effective strains nodulating faba bean are expected. Most importantly, unaddressed areas of the country remain key areas of study.

Therefore, this study was initiated with the objectives of isolating, characterizing, and identifying symbiotically effective faba bean nodulating rhizobia from unaddressed areas of Uraga Woreda, Guji Zone, Southeastern Ethiopia. Rhizobia isolates effect as N nutrient source was studied in order to suggest supplementary means of N nutrient supply that is environmentally friendly and economically viable for smallholder farmers like the study area and for Ethiopian smallholder farmers at large.

1.2. Statement of the Problem

Faba bean is second most widely grown crop in Uraga Woreda, Guji Zone next to maize (Uraga Woreda Office of Agriculture and Natural Resources, 2019). The district is found in elevation range that is favorable for faba bean production i.e. 1800-2650 masl (Uraga Woreda Office of Agriculture and Natural Resources, 2019).

Due to poor utilization of fertilizer i.e. both organic and inorganic fertilizers and poor disease management of faba bean smallholder farmers of the study area are not obtaining the desired yield. Main reason for this is the ever increasing cost of chemical fertilizers and pesticides which is unaffordable by resource poor farmers of the study area (Personal communication with farmers, 2020). Mostly inorganic chemical fertilizer is unaffordable by resource poor farmers of the study area.

The mutualistic relationship between legumes and Rhizobia forms the basis for the ecological importance for legumes in natural and agricultural ecosystems in promoting increased crop yields. This symbiotic association is by far the most important contributor to the world's supply of biologically fixed N to agriculture (Hacquard *et al.,* 2017). The microsymbionts are distributed among major bacterial genera of the alpha-proteobacteria such as *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium, Ensifer (Sinorhizobium), Neorhizobium, Pararhizobium,* and *Allorhizobium,* collectively termed "rhizobia", which can form symbiotic associations with diverse legumes. Similarly, species of the beta-rhizobia *Cupriavidus, Paraburkholderia,* and *Trinickia* can also form symbiotic relationships with members of the *Papilionoideae* and *Caesalpinioideae* (Sprent *et al.,* 2017). Inoculation of plants with *Rhizobium* can improve soil fertility and reduce the production cost of next crop through reduced input in the form of nitrogen fertilizers and which also minimize hazardous effects of chemical fertilizers on human health, soil and environment (Mia and Shamseldin, 2010).

Currently there are no attempts made on the isolation and characterization of Rhizobia nodulating faba bean roots in Uraga Woreda, Guji Zone and their symbiotic effectiveness not studied yet. Therefore, the aim of this study was to isolate, characterize and evaluate symbiotic effectiveness of Rhizobial strains from faba bean root nodules and suggest potential Rhizobia isolates for inoculants production which in turn used as an alternative N nutrient supplier. The result will contribute for future endeavor of utilizing biological nitrogen fixing system of faba bean in order to increase productivity into low-input agriculture of the region and in chance the country at large.

1.3. Objectives of the Study

1.3.1. General Objectives

To isolate, characterize and investigate symbiotic effectiveness of Rhizobia nodulating faba bean (*vicia faba* L.) collected from Uraga Woreda, Guji Zone, and Southeastern Ethiopia

1.3.2. Specific Objectives

1. To isolate and characterize Rhizobia from root nodules of faba bean collected from the study area

2. To evaluate symbiotic effectiveness of Rhizobia isolates in sand pot experiment under greenhouse condition

1.4. Hypotheses

Ho: The soil of study area does not harbor effective rhizobia strains

Ho: There is no significant difference between Rhizobia isolates inoculated and uninoculated faba bean

HA: The soil of study area harbors effective rhizobia strains

HA: There is significant difference between Rhizobia isolates inoculated and uninoculated faba bean

1.5. Significance of the Study

The findings of this study would provide information about the benefits of Legume-Rhizobia symbioses which provides plants with essential nutrient N through biological nitrogen fixation. Since the aim of this study was to isolate and characterize elite isolate from unaddressed areas of Uraga Woreda, Guji Zone, Southeastern Ethiopia, it would help small farmers of the area by providing alternative means of nitrogen supply through biological nitrogen fixation. This study would raise awareness of the surrounding community about using crop rotation in order to benefit from the nitrogen fixed from the previous legume crops. Faba bean is well known for its large amount of nitrogen fixed per hectare which is about 240-325kg. Once effective rhizobia colonized the rhizosphere it would benefit both leguminous and non-leguminous crops grown around that area. Since, faba bean is major pulse crop for smallholder farmers in terms of use and economic reward, the sustainable production of this crop will be supported by indicating the alternative (supplementary) means of supplying nitrogen to sustain the productivity of Faba bean.

Thus, results of this study will help smallholder farmers at large upon recommending cost effective, non-toxic and environmentally friendly growth promoting bioinoculants. The results of the study will provide scientific information for those interested to develop biofertilizer for improving faba bean yield through N nutrient supply. Besides, findings of the study will serve as a base for interested researchers to carry on a detailed research on related topic. Results from this study will also provide information for government policy makers to look for optional environmental friendly and non-toxic N nutrient supply mechanisms to sustain agricultural productivity.

1.6. Limitations of the Study

- The study was supposed to include the soil pH values of the sampling sites in order to correlate the morphological and physiological results of laboratory test with that of the soil sampling sites. However, due to financial limitations soil chemical properties test was not conducted.
- Due to time and money consuming nature of the research work, the study was restricted to laboratory and greenhouse excluding field research.

CHAPTER TWO: LITERATURE REVIEW

2.1. Legumes

Legumes (*Fabaceae*) are the most diverse and vast families of the flowering plants. They belong to the family *leguminoseae* which is classified into three major botanical subfamilies; *Ceasalpinioideae*, *Mimosoideae*, and *Papilionoidae* which contains the majority of the most important legumes (Subba Rao, 1999). There are nearly 750 genera and 16,000-19,000 species of leguminous plants (Giller, 2001). About 20% of the leguminous species has been so far examined for nodulation; of which 23% of sub family *Ceasalpinioideae*, 90% of *Mimosoideae* and the 97% of *Papilionoidae* are known for their ability to form N fixing root nodules with soil bacteria. Legumes such as soybean (*Glycine max* L.), field pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), common bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* L.) and chickpea (*Cicer arietinum* L.) form symbiotic association with rhizobia and variation have been reported in the amount of fixed N (Subba Rao, 1999).

Legumes are an essential part of African farming systems, covering usually large parts of the farmlands in the region. For example, in the years 2006-2008 in Sub- Saharan Africa more than 20 million hectares of the farmlands (which represent about 28% of the global pulse areas) were used for food legume crop production (Akibode, 2011). Ethiopia has a long tradition of cultivation of food legumes and the country is considered as a center of genetic diversity for several cool season legume crops, such as field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum* L.), fenugreek (*Trigonella foenum-graecum* L.) and grass pea (*Lathyrus sativus*) (Tilaye *et al.*, 1994). These food legumes are extensively grown in the cooler highlands of the country, whereas the warm season pulse crops, such as common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogaea*) are common in warmer and lowland parts of Ethiopia.

They occupy 12-15% of the earth's arable land and account for a third of human dietary protein needs and for up to 2/3 of subsistence livelihood (Graham and Vance, 2003). Legume plants show a vast diversity in morphology, habitat and ecology. However, all legume species can be distinguished from non-leguminous plants as they produce pods as a common feature. With regard to economic and agricultural significance the family *Leguminosae* is second next to

Poaceae (grasses). Legumes are grown for production of food, oil, fiber, fuel, timber, medicines, forages, biodiesel fuel, and chemicals

2.2. Faba Bean

Faba bean (*Vicia faba* L.) is a legume member belonging to the family Fabaceae. Species in genus *vicia* are genetically separated from each other according to differences in some of the seed characters such as weight, shape and size (Hawtin and Hebblethwaite, 1993). Genetic variability of faba bean is quite large. The great variability may be due to the presence of intermediate crossing system between autogamy and allogamy (Hanelt and Mettin, 1989). In fact, *Vicia faba* is partially pollinated by insects, so the pollinators can carry out both self-pollinations by the tripping process when they trip the flower and out crossing and when they visit other plants flowers (Nadal *et al.,* 2003).

2.2.1. Taxonomy, Biology and Ecology of Faba Bean

Taxonomically, faba bean belongs to Kingdom: Plantae, Division: Magnoliophyta, Class: Magnoliopsida, Order: Fabales, Family: Fabaceae, Subfamily: Faboideae, Tribe: Vicieae, Genus: Vicia, Species: V. faba, Binomial name: *Vicia faba* L., Synonyms Faba sativa Moench (Anonymous, 2011). *Vicia faba* has a diploid (2n) chromosome number of 12 (Terekhina, 2009).

The phenotypic description of faba bean is cross-pollinated, erect, simple stemmed, annual, normally 20-50cm in height with one or more basal branches. Leaves are alternate and pinnate with two or more oval leaflets. Short axillary racemes form at flowering nodes, and seeds are produced in pods that vary in size with the number and size of seeds in the pods (Dennis, 1991). *Vicia faba* is an annual legume with one or more rigid, hollow and erect stems (McVicar *et al.*, 2009). Faba bean is 0.5-1.7m tall with a square cross-section stem, and has a strong tap root. The leaves are 10-25cm long, pinnate with 2-7 leaflets, and do not have tendrils for climbing over other vegetation. The flowers are 1-2.5cm long with five petals such as one standard petal white, two wing petals white with a black spot (true black, not deep purple or blue), and two keel petals white (Mussa *et al.,* 2008). A flower cluster may produce one to four pods. The pods are large and green, turning dark at maturity (McVicar *et al.,* 2009). Three to four oblong/oval seeds are contained within each pod. Faba bean is grown in temperate regions, subtropics, and tropics. It is not suited to the lowland tropics, where it may flower well but usually does not produce pods. It grow in climates ranging from temperate to semi-arid, using different cultivars and crop management practices (López-Bellido *et al.*, 2005). They are generally sown in the spring in northern latitudes, in the winter in warm-temperate and subtropical areas with specific cultivars for each region (Duc, 1997). They are grown predominately in areas with more than 400mm average annual rainfall but in drier regions, they are commonly irrigated (Agung and McDonald, 1998). They are sensitive to water stress, and irrigation is needed to improve yield and yield stability (Husain *et al.,* 1988). Where water is not limiting, temperature has a major effect on germination and initial growth of faba bean. The optimal temperature for plant growth is 15-20^{o}C and flowers will abort if temperature exceed 27^{o}C (Anonymous, 2009). The crop grows with rainfall of 700-1000mm per annum (Mussa *et al.*, 2008). Faba bean is best suited to well-structured soils. It prefers clay and loamy fertile soils with neutral or sub-acidic pH levels and high water-retention ability (Terekhina, 2009). Faba beans are vulnerable to soil compaction and hard pans. The common sowing dates are mid- June in mid-altitude areas and late June to early July in high-altitude areas. Faba bean is usually grown in altitude range of 1800-3000 masl.

2.2.2. Origin and Characterization of Genetic Diversity of Faba Bean

Faba bean (*Vicia faba* L), also variously known as broad bean, horse bean, tic bean and field bean, is a cultivated crop species belonging to the wild pea genus Vicia of the Leguminosae family. There are controversies on its origin: *Vicia faba* major may have originated in North Africa, and *V. faba* minor at the south of the Black Sea in Asia, but Long *et al*. (1989) believed that faba bean originated from the Far East. From there it was spread with the development of culture and commerce to: 1) Europe, 2) along the coast of North Africa to Spain, 3) along the Nile River to Ethiopia, where it had its secondary center of origin, and 4) from Mesopotamia into India. Afghanistan is also believed to be a secondary center of origin.

However, Vavilov (1936) discovered a primitive type of faba bean with small pods and seeds at the intersection of Himalaya and Hindu Kush, and proposed that faba bean originated from Central Asia and found that there was a gradual increase in the faba bean's size from Central Asia westward along the mountains to Iran, Turkey, the Mediterranean region and Spain. Because faba bean in Sicily and Spain was seven to eight times larger than that in Kabul and Afghanistan, Vavilov (1936) concluded that areas along the coast of the Mediterranean Sea and Ethiopia should be the secondary origin of faba bean. Later, some scientists (Li, 1984) believed that faba bean originated from Central Asia (30-40^{o}N), with Kabul, the capital of Afghanistan, as the Center.

The geographic origin of faba bean (*Vicia faba* L.) is accepted as the Near East (Cubero, 1974; Duc, 1997), with the Mediterranean basin as the most important centre of diversity for *Vicia faba* (Maxted and Kell, 2009). China, Afghanistan and Ethiopia have also been reported as secondary centers of diversity for the crop (Zong *et al.*, 2009). Globally, faba bean is genetically diverse with more than 38,000 accessions with approximately 37 collections (Duc *et al.*, 2010). Selection in response to diverse environments and human needs, natural inter-crossing among the different faba bean races, and genetic drift have contributed to increased faba bean diversity and wide variability (Duc, 1997; Maxted and Kell, 2009).

2.2.3. Use and Economic Importance of Faba Bean

Faba bean (*Vicia faba*) also known as broad bean, horse bean, its production ranks fourth in the world as an important food legume after garden pea, chickpea and lentil (Zohary and Hopf, 2000; Torres *et al.*, 2006). Faba bean grain is an important grain legume for human diets and animal feed for the reason that it is a major source of protein, starch, cellulose and minerals from its mature seed. Its seeds contain 27 to 34% protein with high lysine content and are free from tannins (Duc, 1997). The protein fraction could be used for animal feed and the carbohydrate-rich fraction for biofuel production, as proposed for other legumes (Lamb *et al*., 2014; Amato *et al*., 2016). A straw of faba bean is rich in protein, calcium and magnesium than cereal straws, and if properly harvested, it is useful roughage feeds for ruminant animals (Kossila, 1984). Generally, pulse straws contain 10-15% crude protein (CP) in DM and their energy content is higher compared to the respective cereals by-products and sugar cane, with satisfactory palatability (Kossila, 1984). Another major feature of the faba bean is its symbiotic nitrogen (N) fixing capability by forming symbiotic relationship with *Rhizobium* (Root nodulating soil bacteria), enabling it to produce substantial yields without the addition of synthetic N fertilizer, thus making it an attractive break-crop in an arable rotation (Schwenke *et al.*, 1998).

Various literatures confirmed that faba bean is rich in protein and other nutritional contents (Duranti and Cristina, 1997). That is why it is called ‘meat of the poor’. In many developing countries, it is the major source of protein in their feeding culture. Muehlbauer and Abebe (1997) stated that the whole 100g dried seeds contain 344 calories, 10.1% moisture, 1.3g fat, 59.4g total carbohydrate, and various macronutrients and trace minerals.

2.2.4. Faba Bean Production in Ethiopia

The world's leading producing countries for faba bean are China, Ethiopia, Egypt and the United Kingdom (FAOSTAT, 2014). The average productivity of faba bean since 1999 to 2008 is 1.7 metric t ha-1 and 1.2 metric t ha-1 in China and Ethiopia respectively. Ethiopia is the leading producer of faba bean in Africa accounting for 56% of the production (FAOSTAT, 2014).

Ethiopia is one of the largest faba bean-producing countries in the world and stands second to China (Hawitin and Hebblewaite, 1993). The country is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean (Asfaw *et al*., 1994). Faba bean takes the largest share of the area and production of the pulses grown in Ethiopia (CSA, 2018). In Ethiopia, faba bean is grown in the highlands (1800-3000 m.a.s.l) where the need for cold temperature is met (Yohannes, 2000). It is believed that the crop was introduced to Ethiopia from the Middle East via Egypt around 5000 B.C., immediately after domestication (Asfaw *et al.,* 1994). Ethiopia is now considered as one of the centers of secondary diversity for faba bean (Asfaw *et al*., 1994; Yohannes, 2000; Torres *et al.,* 2006). Currently the crop is grown in several regions of the country receiving annual rainfall of 700-1000 mm (ICARDA, 2006). Production in Ethiopia is totally rain-fed on nitosols and cambisol type of soils (Gemechu *et al.,* 2002). Currently, in Ethiopia the area devoted to faba bean production is 427,696.8ha and from which 8,780,108.79qt yield has been encountered (CSA, 2018).

2.2.5. Production Constraints of Faba Bean

There is a reduction in the cultivated area and productivity of faba bean in many countries. Several adverse factors have been reported to this decline of which; diseases, weeds, and insect pests are the main biotic yield limiting factors in its production (Torres *et al*., 2006). From the biotic category, diseases are important factors limiting the production of food-legume crops as a whole and faba bean specifically in Ethiopia (Nigussie *et al.,* 2008). More diseases are affecting faba bean, but only a few of them have either major or intermediate economic significance. Among these, fungi are the largest and perhaps the most important groups affecting all parts of the plant at all stages of growth great importance to faba bean (Nigussie *et al.*, 2008). Diseases such as chocolate spot (*Botrytis fabae* Sard.), rust (*Uromyces Vicia fabae*), black root rot (*Fusarium solani*), and foot rot (*Fusarium avenaceum*) are among fungal groups that contributes to the low productivity of the crop (Nigussie *et al.*, 2008). Among the biotic factors, the most damaging parasitic weed of faba bean is *Orobanche crenata* which germinates in response to chemicals released by faba bean (Joel *et al*., 2007). It is widespread in Mediterranean basin especially in northern Africa, Asia and southern and eastern Europe, attacking dicotyledonous crops, and losses of 50 to 80% have been reported in faba bean fields (Gressel *et al*., 2004). Frost is one of the abiotic stresses contributing for its low production. For example, in Ethiopian highlands 100% yield losses can be experienced especially with late planting as the plants are exposed to frost damage (Mola, 1996).

2.3. Rhizobia

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran and Hoben, 1994) and they are able to bring out nodule formation on legumes. They are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes until the point where nodulation becomes possible. Rhizobia are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile, rod shaped (approximately 0.5-0.9 µm in width and 1.2-3.0 µm in length) and heterotrophic (Somasegaran and Hoben, 1994). Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7. *Rhizobium* growth normally occurs under aerobic conditions. When fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase and hence, *Rhizobium* is able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

Rhizobia are of great importance for nitrogen acquisition through symbiotic nitrogen fixation in a wide variety of leguminous plants. These bacteria differ from most of other soil microorganisms by taking dual forms, i.e., a free-living form in soils and a symbiotic form inside of host legumes. Therefore, they should have a versatile strategy for survival, whether inhabiting soils or root nodules formed through rhizobia-legume interactions.

2.3.1. Definition and Taxonomy of Rhizobia: An Overview

The term ‘’rhizobia’’ in the strictest sense, refers to members of the genus *Rhizobium* but later becomes a repository for all bacteria capable of nodulation and nitrogen fixation with legumes (Willems, 2006; Rivas *et al*., 2009). Giller (2001) defined rhizobia as ‘’a group of diverse’’ bacterial genera that are able to induce and infect nodules on roots or stems of plants in the family Leguminosae or Fabaceae, irrespective of their ability to fix nitrogen. Rhizobia are a group of bacteria that have the capacity to form nodules on legume roots (occasionally on stems) that can fix atmospheric nitrogen to partially or fully meet the nitrogen requirements of the host plant. To describe bacteria from root nodules (Frank, 1889) proposed name “rhizobia”, and after this proposal all nodule forming bacteria have been known as rhizobia. It represents the genera *Rhizobium, Bradyrhizobium, Ensifer (Sinorhizobium), Mesorhizobium, Allorhizobium,* and *Azorhizobium.* The interactions between rhizobia and legume roots result in formation of root nodules, in which rhizobia use energy from the host plant to fix atmospheric N_2 into plant-available forms of nitrogen. Biological nitrogen fixation (BNF: atmospheric nitrogen fixation through different members of prokaryotes, specifically by diazotrophs) contributes approximately 16% of total nitrogen input in crop land (Ollivier *et al*., 2011). The amount of nitrogen fixed by a legume crop varies widely because it depends on the legume genotype, rhizobia strain and the soil environment (Lupwayi *et al*., 2011).

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), this 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).

Rhizobia promote the growth of legumes through the formation of nitrogen-fixing nodules. Rhizobia can also associate with roots of non-legumes, without forming true nodules, and can promote their growth by using one or more of the direct or indirect mechanisms of actions. Phytohormone production (Noel *et al*., 1996), secretion of other chemicals like lipo-chitooligosaccharides (Miransari and Smith, 2009) and lumichrome, solubilization of precipitated phosphorus (Chabot *et al.,* 1996; Yanni *et al.,* 2001; Peix *et al*., 2001) and mineralization of organic P (Afzal and Bano, 2008), improvement in uptake of plant nutrients by altering root morphology, production of siderophores (Antoun *et al.,* 1998; Arora *et al*., 2001; Chabot *et al.,* 1996) to meet the iron requirements of the plant under iron-stressed conditions and lowering of ethylene level through ACC deaminase enzyme, are some examples of the rhizobial mechanisms with direct positive effects on non-leguminous plant growth. Indirectly, rhizobia improve the growth of plants through bio-control of pathogens via antibiosis, parasitism or competition with pathogens for nutrients and space, by inducing systemic resistance in the host plant and through increasing root adhering soil by releasing exopolysaccarides which regulate the water movement and facilitate the root growth. Some *Rhizobium* spp. have shown antimicrobial activities towards *Pseudomonas savastanoi* (Kacem *et al.,* 2009), *Aspergillus niger* (Yuttavanichakul *et al*., 2012), *Rhizoctonia solani, Fusarium oxysporum* and *F. solani* (El-Batanony *et al.,* 2007), *Pythium* sp (Bardin *et al.,* 2004; Huang and Erickson, 2007), *Phytophthora cinnamomi* (Malajczuk *et al.,* 1984), *Fusarium solani* f. sp. *Phaseoli* (Buonassisi *et al.,* 1986), *Fusarium oxysporum* f. sp. *Lentis* (Essalmani and Lahlou, 2003) with varying degree of growth inhibition. Studies on numerous plant microbe interactions have shown that such antagonistic rhizobacteria could function by competition and antibiosis i.e. by producing antimicrobial compounds like bacteriocin (Rodelas *et al*., 1998; Joseph *et al.,* 1983) but also indirectly by induction of systemic resistance against plant diseases. The potential use of *Rhizobium* spp. due to their multifaceted beneficial activities is likely to play an important role in modern high intensive agricultural practices.

The symbiotic association between leguminous plant and the rhizobia is beneficial for both the host plant and the rhizobia. The rhizobia not only provide nitrogen source to the host plant but also promote the growth of the plant by producing plant growth hormone like IAA, inhibition of plant pathogen, making phosphate available to the plant, etc.

In the soil the bacteria are free-living and motile, feeding on the remains of dead organisms. Free living rhizobia cannot fix nitrogen and they have a different shape from the bacteria found in root nodules. They are regular in structure, appearing as straight rods; in root nodules the nitrogen-fixing form exists as irregular cells called bacteroids which are often club and Y-shaped.

2.3.1.1. Rhizobial Taxonomy

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants where they undertake symbiotic nitrogen fixation (Sahgal and Johri, 2003). It is the common name given to a group of small, rod-shaped, Gram-negative bacteria that collectively have the ability to produce nodules on the roots of leguminous plants and belong to the family Rhizobiaceae, which are part of the α-proteobacteria. The Proteobacteria represents the second largest group of bacteria and consist of a diverse range of organisms including purple phototrophic, nitrifying and enteric bacteria as well as symbiotic and free-living nitrogen-fixing bacteria (Jordan, 1984).

All Proteobacteria are Gram-negative and motile by means of flagella. Based on analyses of the highly conserved small subunit 16S ribosomal RNA (16S rRNA) gene sequence, the Proteobacteria are divided into the five groups α-, β-, γ-, δ- and ε- Proteobacteria (Zakhia and de Lajudie, 2001). Nodulation and nitrogen fixation is restricted to the α-Proteobacteria and β-Proteobacteria. The terms α- and β- rhizobia were proposed to distinguish rhizobia of α-Proteobacteria and β- Proteobacteria (Chen *et al*., 2003a). The most recent taxonomy of rhizobia consists of 62 species found in 12 genera of nodule forming diazotrophic bacteria, nine of which are α- rhizobia (*Azorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium, Rhizobium* and *Sinorhizobium*) and three β- rhizobia (*Burkholderia, Cupriavidus* and *Herbaspirillum*) (Wolde-Meskel *et al*., 2005; Weir, 2006).

2.4. Symbiotic Association of Legumes with Rhizobia

The term symbiosis refers “the living together of differently named organisms”. The word was used as synonymous to mutualism, in which both interacting organisms live together for mutual benefit in contrast to commensalism, where one organism benefits and the other partner is unaffected or parasitism, in cases when one of the interacting organism profits at the expense of the other partner (Martin and Schwab, 2013). The confusion, disagreements and turbulence of the use of the term “living together” have been continued among the biologists for more than 130 years (Martin and Schwab, 2013). Nevertheless, the “living together” usage has been accepted as a suitable concept to define the term “symbiosis” and recently the broader definition of symbiosis (i.e. mutualism, commensalism, and parasitism) is increasingly used in the textbooks (Martin and Schwab, 2013).

The nitrogen-fixing bacteria that form symbiotic associations with leguminous plants are commonly known as rhizobia. The rhizobia-legume symbiotic interaction induces specialized organs known as nodules on roots or stems of host legumes. Inside nodules rhizobia reduce atmospheric N_2 to NH_3. The symbioses between rhizobia and legume plants are mainly a mutualistic interaction (Lindström and Mousavi, 2010; Saffo, 2001). However, it seems that there are cases where these partnerships can also be considered as parasitic. The rhizobia can have two lifestyles: as endo-symbiont inside nodules or as free-living saprophytes in the soil or rhizosphere. As endo-symbiont, the rhizobia promote growth of the host plant by supplying fixed nitrogen and in turn rhizobia get carbohydrates and energy from the host legumes. This phenomenon indicates the existence of mutualism between the two organisms. But the rhizobia can also be considered as parasitic when they form ineffective symbiosis with legumes, in which the rhizobia get a continuous nutrient supply while they fix little or do not fix nitrogen for the host plant. These types of situations may occur when multiple rhizobial strains compete for the same plant and when the strains infect non-specific hosts promiscuously. These rhizobia can also form effective symbioses (mutualistic) when they interact with their own specific host legumes (Denison and Kiers, 2004).

2.5. Biological Nitrogen Fixation

Biological N fixation represents the major source of N input in agricultural soils. Biological N fixation (BNF) is a complex biochemical reaction whereby inert atmospheric N is enzymatically reduced into a form utilizable by plants through nitrogenase enzyme complex or it is a process by which atmospheric nitrogen (N_2) is converted into ammonia (NH_3) and subsequently available for plants (Bruijn, 2015). The major N 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 effect of BNF 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).

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).

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).

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

Three major types of BNF can be differentiated in terrestrial ecosystems; these are symbiotic, associative and free living N fixing systems. These systems differ in energy source and fixation capability. The associative N fixers present in rhizosphere, rhizoplane or grow endophytically for their energy source to the energy intensive N fixing process. They make a modest contribution of fixed N to agriculture and forestry as compared to the free living diazotrophs. Some important non symbiotic N fixing bacteria are *Acetobacter*, *Alcallgenes, Anhrobacteria*, *Azomonas, Bacillus, Clostridium* and *Pseudomonas* (Paul, 2007). Symbiotic N fixers include two groups of N fixing bacteria: Rhizobia and frankia. Frankia non-legume symbiosis is a major contributor of N inputs in forests, wetlands and disturbed sites of the temperate and tropical regions. What makes Rhizobia and frankia species different from associative N fixing microbes is that most of the N they fix is transferred to and assimilated by the plant for the plant growth (Coyne, 1999).

According to (Mona *et al.*, 2016), the faba bean has an important role in improving soil fertility by fixing nitrogen from the atmosphere in association with bacteria, and it is used in crop rotation with cereal crops. The crop can be grown for green manure, silage, cover crop, and animal forage. Production in Ethiopia is entirely rain fed on nitosols and cambisol soil types. In addition faba bean is the highest nitrogen fixing annual legume making it an excellent rotational crop. Faba bean can fix upwards of 90 percent of their own nitrogen requirements. It is one of the excellent suppliers of soil nitrogen to the subsequent crops; the estimated average amount of N_2-fixed by faba bean is 340 Kg ha^{-1}. There has been some anecdotal evidence that cereal crops following faba bean can see a yield increase of 10 to 15 percent. This could be a result of the nitrogen that is slowly released from the faba bean stubble.

2.5.1. Factors Affecting Biological Nitrogen Fixation

Environmental stress affecting Biological Nitrogen Fixation (BNF) may be either abiotic factors such as drought, salinity, water-logging, temperature, soil acidity and inadequate mineral nutrients or biotic factors; insects, pests and diseases (Zahran, 1999). Various factors can interfere the infection process and nodulation can influence the activity of nitrogen fixation during the symbiosis (Kinkema *et al.*, 2006) and environment contain conditions hostile for survival of Rhizobia (Tortora *et al.,* 2013).

2.5.1.1. Salt and Osmotic Stress

Salt is one of the major factors threatening agriculture in arid and semi-arid areas. Nearly 40% of the world’s land surface can be categorized as having a potential salinity problem (Niste *et al.*, 2013). Salinity is concentration of dissolved mineral salts comprising cations and anions present in the soil and in water. The principal cations in solution consist of Sodium, Calcium, Magnesium and Potassium and the major anions are Chlorine, Sulphate, Bicarbonate, Carbonate and Nitrate (Aggarwal *et al.,* 2012). The responses to saline stress varies among free Rhizobia for which the growth inhibited at 100mM NaCl, and symbiotic Rhizobia is to be tolerant to NaCl concentration ranging from 300 to 700 mM (Zahran., 2001). Rhizobial strains differ in their ability to tolerate osmotic stress and can use different adaptation mechanisms such as intracellular accumulation of low molecular weight organic solutes (Adil *et al.,* 2012), including amino acids such as glutamate, sugar and polyamines or the accumulation of ions such as potassium. Rhizobia subjects to salt stress may undergo morphological alterations leading to change in cell morphology and size in the pattern of extracellular polysaccharides and lipo-polysaccharides (Ventorino *et al.,* 2012). Salinity may act as a water stress, which affects the photosynthetic rate, or may affect nodule metabolism directly. Saline soils are generally deficient in nutrients and microbial activities and population is low. Increases in the salinity of soils or water supplies used for irrigation result in decreased productivity of most crop plants and lead to marked changes in the growth pattern of plants. Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Sobati *et al.,* 2015).

2.5.1.2. Temperature Stress

High soil temperature is one of critical factor which can prevent the development of nitrogen fixing association between the two symbiotic partners' especially arid and semi-arid regions. The survival of Rhizobia in soil is more affected by high temperature than by low temperature because it can be deleterious (Niste *et al.,* 2013). In arid regions high soil temperature affects lives of both free and symbiotic Rhizobia (Werner and Newton, 2005). The optimum temperatures for growth in culture varies among strains and species, values between 27 - 39°C have been noted. The maximum temperatures are generally 35 - 39°C, but proliferation may take place up to 42°C (Al-Falih, 2002). Temperature can influence not only the survival of free Rhizobia but also exchange of molecular signals between the symbiotic partners (Sadowsky, 2005). High temperature can induce an inhibiting effect on bacterial adherence to root hairs, on bacteriodes differentiation, on nodule structure and on legume root nodule functioning (Alexandre and Oliveira, 2013).

2.5.1.3. Heavy Metals

Heavy metals are known as the most important pollutants which persist in the soil over long periods and have ecotoxicological effects on plants and soil micro-organisms. Some metals such as Ni, Zn, Cu and Cr are essential for growth of both Rhizobia and their host plants, whereas others such as Cd, Hg and Pb seems to be not beneficial and could be toxic even at relatively low concentrations (Gradd, 1992). When exposed to moderate heavy metal conditions, soil micro-organisms where found to be very sensitive (Giller *et al.,* 1998). Rhizobial response to different types of heavy metals depends on the applied concentrations (El-Hilali, 2006). Hence, Cadmium even at considerably low concentration was found toxic for micro symbionts inhibited the nitrogenase activity and adversely affected the metabolic activities such as legume's photosynthesis (Ahmed *et al.,* 2012). In contrast nickel can induce the significant increase in the activity of hydrogenase in bacteriodes; Nodulation delayed and reduced plant growth (El-Hilali, 2006).

2.5.1.4. pH Stress

Either alkaline or acidic agricultural soil has a great influence on the survival or multiplication of Rhizobia and can affects both the symbiosis partners. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic nitrogen fixation (Lin *et al*., 2012). Rhizobia are sensitive to low pH (acid soils) (Tortora *et al*., 2013) and the effect of which is low productivity in legumes though indirect and affect *Rhizobium* population (Getachew and Angaw, 2006). The optimum pH for Rhizobial growth is considered to be between 6.0 and 7.0 (Hungria and Vargas, 2000).

2.5.1.5. Drought Stress

Drought stress can hampers plant growth and induces leaf senescence leading to substantial losses in agricultural productivity (Staudinger *et al.,* 2016). Under drought conditions the formation of certain molecules particularly hydroxyl and peroxyl radicals can induce lipid per oxidation, protein denaturation and nucleic acid damage. Reducing sugars may covalently react with amino side chain of amino acid residues via non enzymatic browning or Maillard reaction causing protein damage (Casteriano, 2014). Severe water stress reduces the number and size of nodules formed on roots of legumes as well as soil aeration status, the nature and amount of soluble materials, the osmotic pressure, and the pH of the soil solution (Powell and Klironomos, 2007)

2.5.1.6. Soil Fertility

Soil fertility can also affect the biological nitrogen fixation in *rhizobium*-legume symbiosis. Deficiencies of nutrients essential for the growth of bacteria or plants can cause reductions in the numbers and size of nodules formed and in the amount of N_2 fixed (Giller, 2001). In fact, an excess of nitrates may cause an inhibitory action on nodulation and Nitrogen fixation activity. The process of this inhibition is not fully understood, although several hypotheses have been proposed. Some studies have concluded that legume plantation in soils containing a significant quantity of nitrates can have negative effect on the symbiosis induced by Rhizobia (Lucinski *et al.*, 2002) and can inhibit nodulation and nitrogen fixation (Brockwell *et al.*, 2005). Previous studies showed that the presence of nitrate ions reacts negatively on root infection, nodule development and nitrogenase activity in legume plants because of the accumulation of nitrite (Lucinski *et al.*, 2002). In the same context it is demonstrated that the addition of nitrate to plant seedling growth medium reduced significantly the number of Rhizobial cells adhering to the plant seedling roots (Zahran, 1999).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Description of the Study Area

The soil sampling area is located in Southeastern part of Ethiopia, Guji Zone, and Uraga Woreda (Figure 1). It is located at latitude 5^{o}52’18”N to 6^{o}14’40”N and at longitude 38^{o}19’45”E to 38^{o}45’10”E with altitude range of 1820 - 2650masl. The area has mean rainfall of 1334mm/annum with temperature range of 8^{O}C - 24^{O}C. The district is located at a distance of 435km and 202km from Ethiopia’s capital Addis Ababa and Zonal capital Negelle, respectively.

The study area is well known for its production of Arabica Coffee. It also mainly produces crops like Maize, Field pea, Harricot bean, Barley, and Enset. Farmers living in the study area generate an income for their living mainly from agricultural activities.

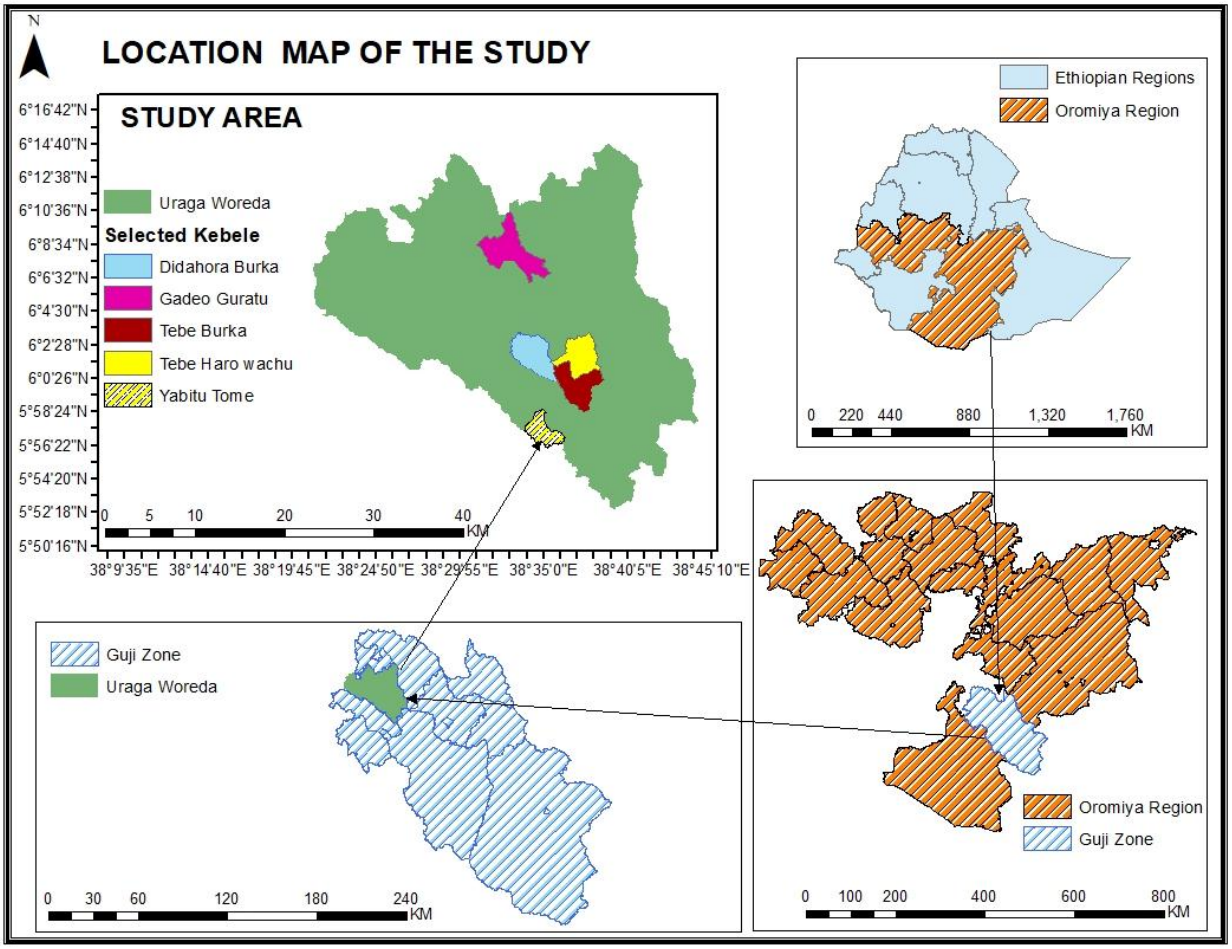

Figure 1: Map of soil sampling area

The geographic coordinates and growing history of the soil sampled fields was also recorded (Table 1).

Table 1: The site names, altitude, geographic points and previous growing history of the farms from where the soil samples were collected for rhizobia induction

SN	Site Name (kebele)	District	Altitude (masl)	Geographic points		Previous growing history
				Latitude (N)	Longitude (E)	
1	Gadeo Guratu	Uraga	2546	06^{0} 09’38.9”	038^{0} 32’53.2”	Faba bean
2	Gadeo Guratu		2306	06^{0} 08’07.0”	038^{0} 32’40.4”	Faba bean
3	Gadeo Guratu		2405	06^{0} 08’16.2”	038^{0} 32’00.6”	Faba bean
4	Gadeo Guratu		2499	06^{0} 08’10.4”	038^{0} 33’42.0”	Faba bean
5	Gadeo Guratu		2447	06^{0} 08’47.5”	038^{0} 31’59.7”	Faba bean
6	Gadeo Guratu		2490	06^{0} 09’13.8”	038^{0} 33’24.2”	Faba bean
7	Tabe HW		2410	06^{0} 01’32.6”	038^{0} 33’23.8”	Faba bean
8	Tabe HW		2396	06^{0} 02’12.7”	038^{0} 34’06.3”	Faba bean
9	Tabe HW		2385	06^{0} 02’37.2”	038^{0} 34’52.6”	Faba bean
10	Tabe HW		2326	06^{0} 01’18.5”	038^{0} 33’00.8”	Faba bean
11	Tabe HW		2294	06^{0} 01’02.4”	038^{0} 33’26.3”	Faba bean
12	Tabe HW		2286	06^{0} 01’49.7”	038^{0} 34’09.2”	Faba bean
13	Yabitu Tome		2341	05^{0} 58’14.6”	038^{0} 32’00.7”	Faba bean
14	Yabitu Tome		2359	05^{0} 57’37.4”	038^{0} 32’18.3”	Faba bean
15	Yabitu Tome		2310	05^{0} 57’56.1”	038^{0} 33’16.2”	Faba bean
16	Yabitu Tome		2265	05^{0} 58’47.3”	038^{0} 32’43.8”	Faba bean
17	Yabitu Tome		2275	05^{0} 58’08.2”	038^{0} 32’07.5”	Faba bean
18	Yabitu Tome		2246	05^{0} 57’26.4”	038^{0} 32’41.7”	Faba bean
19	Dida HB		2290	06^{0} 01’07.3”	038^{0} 31’12.8”	Field pea
20	Dida HB		2265	06^{0} 01’28.3”	038^{0} 31’51.7”	Faba bean
21	Dida HB		2316	06^{0} 02’14.2”	038^{0} 32’00.6”	Faba bean
22	Dida HB		2237	06^{0} 02’37.6”	038^{0} 30’46.1”	Faba bean
23	Dida HB		2189	06^{0} 01’52.8”	038^{0} 30’23.7”	Faba bean
24	Dida HB		2176	06^{0} 01’02.4”	038^{0} 31’35.3”	Faba bean
25	Tabe Burka		2135	06^{0} 00’12.7”	038^{0} 33’04.1”	Faba bean
26	Tabe Burka		2162	06^{0} 00’38.6”	038^{0} 33’27.5”	Faba bean
27	Tabe Burka		2184	05^{0} 59’00.4”	038^{0} 32’56.8”	Faba bean
28	Tabe Burka		2151	05^{0} 59’31.7”	038^{0} 33’47.4”	Faba bean
29	Tabe Burka		2173	06^{0} 00’58.2”	038^{0} 32’28.2”	Faba bean
30	Tabe Burka		2195	06^{0} 00’34.8”	038^{0} 33’18.6”	Faba bean

NB. The coordinates and altitudes were directly measured using Handheld GPS model Garmin etrex 10 at the time of samples collection.

3.2. Description of the Experimental Design and Procedure

3.2.1. Soil Sampling

Soil samples were collected from 5 Kebeles in Uraga Woreda, Guji Zone *viz.* Gadeo Guratu, Tabe Burka, Yabitu Tome, Dida Hora Burka and Tabe Haro Wachu based on their productivity of faba bean in the last few years. Soil samples were collected from farmers' field in which faba bean has been grown with no history of inoculation with Rhizobia. The method used to collect the soil sample was composite sampling (Walworth, 2006). From each Kebele, representative 6 farmer fields were selected deliberately based on their previous history of cultivating faba bean crop. The soil was sampled across and diagonally from 5 points in each field at a depth of 0 - 20cm and collected using an Auger. About 3kg of soil samples were collected per fields and a total of 30 soil samples were collected using sterilized polyethylene bags. Each composite soil samples were packed independently and transported to the experimental site for nodule induction.

Both laboratory and pot experiments were conducted at **Hawassa University, College of Agriculture, Soil Microbiology Laboratory.**

3.2.2. Study Design

The experiment was laid out in Completely Randomized Design (CRD) with three replications for each treatment and control.

3.2.3. Root Nodule Induction

Faba bean variety namely 'Hachalu' were used in this study. The seed of the cultivar were obtained from Holeta Agricultural Research Center, Holeta, Ethiopia. Polyethylene pots with diameter of 25cm which have a capacity of carrying 3kg soil sample were used for sowing the seed.

The pots were surface sterilized with 95% ethanol by swabbing and filled with each of the homogenized soil of 3kg. Faba bean seeds of 'Hachalu' variety were briefly surface sterilized by soaking them in 95% ethanol for 10 seconds and were rinsed in 3% Sodium Hypochlorite (NaOCl) for 4 min. They were rinsed in 5 changes of sterilized distilled water to remove sterilizing chemicals and allowed to germinate on sterile water agar plates (7.5g of agar in 1L water) for 2 days. Five pre-germinated seeds were transplanted to each pot, which were, then, thinned down to 3 after a week. All pots were kept in greenhouse and watered every three days for 45 days.

Then after 45 days, the plants were gently uprooted from the pots and immersed several times in a container containing 10L of water to remove soil particles. Clean, healthy and pinkish color nodules were collected randomly and stored in vial containing silica gel covered with cotton.

3.2.4. Isolation of Rhizobia from Root Nodules

Dehydrated or desiccated root nodules were immersed in sterile distilled water for overnight in labeled sets of Petri-dishes (Vincent, 1970) and were then surface sterilized according to Somasegaran and Hoben (1994). The nodules were first subjected to 95% ethanol for 10 sec to break surface tension and to remove air bubbles from nodule tissues. The nodules were then transferred to a 3% (v/v) solution of sodium hypochlorite (NaOCl) for 4 min. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals.

The surface sterilized nodules were transferred into sterile Petri-dishes one by one and were crushed with alcohol flamed sterile glass rod in a drop (1ml) of normal saline solution (0.85% NaCl) inside a laminar air flow hood. One loopful of the nodule suspension (crushed nodule saps) were streaked across the surface of Yeast Extract Mannitol Agar (YEMA) containing 25mg of Congo red per liter to obtain a pure isolated colony and were incubated at 28±2ºC for 3-5 days. Daily observations were made for the appearance of typical colonies of Rhizobia and single colonies were picked up and periodically purified by re-streaking on new YEMA plates.

3.2.5. Purification and Preservation of the Isolates

Colonies were picked with sterile inoculating loop and streaked repeatedly on sterile YEMA plates and incubated at 28 ± 2^0C. A total of 25 pure rhizobial isolates from each soil sampled nodule induced were obtained and were preserved on YEMA slant tubes containing 0.3% (W/V) $CaCO_3$ at 4^0C (Somasegaran and Hoben, 1994) for further use.

3.2.6. Designation of Rhizobial Isolates

The pure isolates were designated as FB (the host Faba bean), first letter of the name of the sampling sites i.e. GG, THW, TB, YT, DHB representing Gadeo Guratu, Tabe Haro Wachu, Tabe Burka, Yabitu Tome, Dida Hora Burka, respectively and followed by the different serial numbers representing each isolate.

3.2.7. Presumptive Test for Root Nodule Bacteria

The purity of the cultures was confirmed based on morphological parameters of different colonies and a Gram staining technique (Subba Rao, 1983). Pure isolates that shows mucoid, watery and whitish color colony characteristics on YEMA media were further characterized on YEMA-CR, YEMA-BTB and PGA- BCP media to test whether the isolates colony are Rhizobia or not.

3.2.7.1. YEMA-CR (Congo red Absorption) Test

Colonies were tested for Congo-red absorption on Congo-Red incorporated YEMA media (YEMA-CR) (Somasegaran and Hoben, 1994). Stock solution of Congo Red (CR) was prepared by dissolving 0.25gm of CR in 100ml sterile distilled water from which, 10ml was added to one liter of YEMA. Culture suspensions were inoculated into YEMA-CR medium, and the plates were wrapped with aluminum foil, to provide a dark condition and incubated at 28±2 ^{0}C for 3-5 days. After 4 days of incubation the CR absorption characteristic of each isolate was scored qualitatively (Somasegaran and Hoben, 1994).

Rhizobia colonies on Congo red YEMA medium are white, translucent, glistening, elevated and small. Most Rhizobia, except *Sinorhizobium,* lack the ability to absorb Congo red, added at a final concentration of 0.025g/l or 25µg/ml from YEMA medium (Ondieki *et al.,* 2017) while a common contaminants Agrobacterium colonies on YEMA media absorbs Congo red readily and becomes pink initially and then turns deep black (Fentahun *et al*., 2013).

3.2.7.2. YEMA-BTB Reaction (Acid - Base Production)

The production of acid or alkaline were determined by preparing YEMA-BTB media incorporated with bromothymol blue dye (BTB) as an indicator of pH change by dispensing 5ml of BTB stock solution in one liter of YEMA media. To prepare stock solution of BTB 0.5%

(w/v) of Bromothymol blue dye was used. The ability of the rhizobial isolates to produce color changes of the medium to acid or alkaline were observed in 3-5 days of incubation at 28^0C and the results were recorded as fast growers (acid producers) or slow growers (base producers) depending on color change of the media (Jordan, 1984). Fast growers (acid producers) change the color of the media into yellow, whereas slow growers (alkali producers) change the color of the media into blue.

3.2.7.3. Glucose Peptone Agar – Bromocresol Purple (GPA - BCP) Test

Glucose peptone agar medium is widely used for distinguishing pure colonies of Rhizobial isolates from other contaminants usually Agrobacterium. Most rhizobia show no growth or very poor growth on glucose peptone agar medium (Singh *et al.*, 2013).

According to the procedure of Lupwayi and Haque (1994) Peptone Glucose Test (PGT) were prepared by dissolving 10g of peptone, 5g of glucose, 15g of agar and 10ml of bromocresol purple (BCP) in a liter of distilled water and the pH were adjusted to 6.8 with 1N NaOH and HCl. Stock solution of BCP was prepared by dissolving 1g of BCP in 100ml of ethanol. The medium were streaked with three days old rhizobial isolates and were incubated at 28^0C for 48 hrs and were observed for any growth after 24 hrs (Singh *et al.*, 2013). Observation of heavy bacterial growth is indicative of contamination.

3.2.7.4. Gram Staining Reaction Test

Gram staining was conducted for all isolates as a means for rapid identification of gram-positive contaminants as the method indicated by Lupwayi and Haque (1994).

Gram staining was done to study gram reaction of rhizobial isolates and staining was carried out according to standard Gram's procedure (Lupwayi and Haque, 1994). It was carried out to confirm that all isolates were gram negative and do not contain any gram positive bacteria or contaminants (Lupwayi and Haque, 1994). All previously purified isolates were tested for this presumptive purity test using gram staining. A loop full of each isolate from broth culture was spread on a microscopic slide and thin smear was prepared. Then the smear was gram stained following all the steps beginning at first Stains with crystal violet for 1 min, and then it was washed lightly with water and was dried immediately. The dried smear was flooded with iodine solution for 1 min and was dried. Having doing this it was washed lightly with water and was decolorized with 95% alcohol for 5-15 seconds for thin smear and up to 30 seconds for thick smear lastly was washed with water and was blot dried carefully counter stain with safranin for 1 min and washed with water and then air dried. The shapes and gram reaction of rhizobial isolates were observed microscopically in order to confirm the identity of the isolates as indicated by Lupwayi and Haque (1994).

3.2.8. Morphological Characterization

3.2.8.1. Colony Characteristics

The colony characteristics (i.e. shape, size, color, elevation and margin of the bacterial colony) were determined by observing the colonies of rhizobial isolates from 48hrs old grown cultures in YMB (Vincent, 1970) and were spread on YEMA plates and incubated at 28±2°C for 3-5 days. After 48hrs the color, shape, size in diameter, margin, colony texture, elevation of the colonies were observed and recorded as indicated in (Martinez-Romero *et al.*, 1991).

3.2.9. Biochemical and Physiological Characterization

For each biochemical and physiological test, inoculation of a loopful of 48hrs old broth culture was streaked on to the YEMA medium. The inoculated YEMA plates were incubated at 28 ± $2^{O}C$ for 3-5 days (Somasegaran and Hoben, 1994). For each experiment, three replicates and controls were used per test suggested by (Maatallah *et al.*, 2002). Ultimately, the growth of each rhizobial isolate was determined qualitatively as, (+) for growth and (-) for no growth.

3.2.9.1. Salt Tolerance Test

The isolates were tested for their tolerance to the salinity on YEMA medium supplemented with 10 level of NaCl at concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5% (w/v) (Lupwayi and Haque, 1994). Control plates with 0.2% NaCl were used as control for each isolate. Growth was evaluated qualitatively as (+) for growth and (-) for no growth after 3-5 days of incubation at 28 ± $2^{O}C$ (Lupiwayi and Haque, 1994).

3.2.9.2. Temperature Tolerance Test

The ability of all isolates (presumptive rhizobial isolates) to grow at varying temperatures was assessed on YEMA plates incubated at the temperatures of $5^{0}C$, $10^{0}C$, $15^{0}C$, $20^{0}C$, $35^{0}C$, $40^{0}C$ and $45^{0}C$ according to (Lupwayi and Haque, 1994). YEMA medium with temperature of $28^{o}C$

was used as control (Lupwayi and Haque, 1994). Growth was qualitatively recorded as (+) for growth and (-) for no growth. Isolates were considered tolerant to extreme temperature when growth was similar to the growth in the control plates.

3.2.9.3. pH Tolerance Test

The ability of the isolates to grow on acidic and alkaline media was determined by inoculating each isolate on YEMA medium adjusted with pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.8, 8.0, 8.5, 9.0, 9.5 and 10.0, adjusted using 1N NaOH and HCl (Amarger *et al.*, 1997; Bernal and Graham, 2001). YEMA medium with pH of 6.8 was used as control. The results were recorded qualitatively as + for presence or – for absence of growth after 3-5 days of incubation at 28 ± 2^0 C. Isolates were considered tolerant to extreme pH when growth was similar to the growth in the control plates.

3.2.9.4. Intrinsic Antibiotic Resistance

The resistance of isolates to antibiotics was tested by streaking them on solid YEMA medium containing freshly prepared filter sterilized antibiotics using 0.22 µm sized membrane filters: Kanamycin, Streptomycin, Penicillin, Chloramphenicol, Ampicillin and Nalidixic acid and three levels of concentration (2.5, 5 and 10µg/ml). The stock solution of each antibiotic was first prepared by dissolving 2g of each antibiotic in 100ml of water as described in Lupwayi and Haque (1994). Nalidixic acid was dissolved in 1M NaOH, whereas the other was dissolved in sterilized distilled water. Each filter sterilized antibiotic solution was added to sterile YEMA cooled to 50^0 C and mixed thoroughly. The isolates were then streaked on the plates and incubated at 28 ± 2^0 C for 3-5 days. The result was recorded qualitatively either as +/- for growth and no growth, respectively.

3.2.9.5. Carbon Source Utilization Test

All the isolates were tested on ten carbohydrates as a sole source of carbon i.e. Glucose, Maltose, Fructose, Xylose, Sucrose, Lactose, Sorbitol, Mannitol, Dextrose and Arabinose (Somasegaran and Hoben, 1994). 10ml of 10% carbohydrate solutions (1g of carbohydrate in 10 ml of distilled water) were prepared in sterilized plastic vials. The heat stable carbon sources (Fructose, Glucose, Sucrose and Mannitol) were added into 90ml carbohydrate-free basal medium in which yeast-extract is reduced to 0.05g/liter dispensed in 250ml capacity Erlenmeyer flasks and 15g of agar was added to each flask at a final concentration of 1g/liter and autoclaved together with the medium. The rest heat labile carbon sources were filter sterilized using 0.2μm pore size sterile disposable membrane filter and Micropipettes. Filter sterilized carbohydrate solutions were stored in refrigerator and added to autoclaved carbohydrate free basal medium at least at 55^0C in a laminar hood flow. Then the solutions were aseptically dispended in to sterile Petri dishes and allowed to solidify in the hood. Finally, 48hrs old rhizobial suspensions were inoculated in to these basal media and were incubated at 28 ± 2^0C for 3-5 days and were examined for bacterial growth.

3.2.9.6. Nitrogen Source Utilization Test

The following amino acids were tested as sole N-sources for the Faba bean rhizobial isolates according to (Amarger *et al.,* 1997). Alanine, Arginine, Asparatic acid, Glycine, Valine, Aspargine, Isoleucine, and Histidine were included to a basal medium at the final concentration of 0.5g per liter in order to determine rhizobial isolates' ability to utilize them (Somasegaran and Hoben, 1994). The basal medium was adjusted using the same basal media to be used to test carbon source utilization but lack ammonium sulfate and supplemented with 1g/l of mannitol.

The amino acids which were kept filter sterilized by filter membrane of size 0.2μm were added into autoclaved basal medium immediately after cooling at least to a temperature of 55^0C in a laminar hood. Finally, 48hrs old rhizobial cultures were inoculated in to these medium and incubated at 28 ± 2^0C for 3-5 days and were examined for bacterial growth.

3.2.10. Authentication and Evaluation of Symbiotic Effectiveness of the Isolates

In order to test the definitive purity of all rhizobial isolates, nodulation test was carried out for each of the purified isolates. Accordingly, the infectiveness and effectiveness of the Rhizobial isolates were tested. They were inoculated into the host plant potted into 3 kg capacity plastic pots containing sterilized and nitrogen free sand (Somasegaren and Hoben, 1994). The sand was thoroughly washed with sulfuric acid whereas the pots were surface sterilized with 95% ethanol. Faba bean seeds of uniform size and color were surface sterilized briefly with 95% ethanol for 10 seconds and 3% NaOCl for 3 minutes. Then the seeds were rinsed in six changes of sterile distilled water (Vincent, 1970). They were transferred aseptically with forceps to the surface of a 2% water agar Petri dishes containing sterile tissue paper and were incubated at 25°C until the development of a radicle of 0.5 to 1cm long (Woomer *et al.,* 2011). Five pre-germinated Faba bean seedlings were then aseptically transferred into the pots. The seedlings were thinned to 3 per pot after one week growth. Each seedling was inoculated with 1ml of presumptive isolates taken by micropipette from pure broth cultures at their logarithmic growth phases (10^9 cells ml^{-1}) and then the pots were arranged in a completely randomized design in a greenhouse with 12hrs photoperiod, 28°C and 15°C day and night temperatures, respectively. All seedlings were supplied with distilled water every two days, and fertilized once a week with 100 ml of quarter strength of Broughton and Dilworth N-free nutrient solution (Broughton and Dilworth, 1970).

Control pots were included for unfertilized and uninoculated negative control and uninoculated but nitrogen fertilized (0.05% KNO_3/week) positive control. For positive control seedlings that were not inoculated with the isolates, 70mg/liter of N applied as potassium nitrate solution (0.05% KNO_3 (w/v)) every week (Somasegaran and Hoben, 1994). Negative controls which were neither inoculated with the isolates nor treated with chemical N-fertilizer were given nitrogen-free nutrient solution.

All the treatments were conducted in triplicates for 45 days from sowing. Then, seedlings were examined for nodulation and preliminary effectiveness of the isolates was qualitatively examined looking at the seedlings. Forty five days after planting (DAP), plants were uprooted to record nodule number, nodule dry weight and shoot dry weight. Root and shoot fractions were separated, and nodules were collected from the roots and washed carefully with stream water and were counted. Root and shoot fractions were oven dried at 70^0C for 48 hrs according to (Prevost and Antoun, 2008) and were weighed; thereby obtained root and shoot dry weight.

The effectiveness of isolates in accumulating plant shoot dry matter was calculated as described in (Somasegaren and Hoben, 1984) and (Mulongoy, 2004) as follows:

$$SE(\%) = \frac{Inoculated\ plant\ DM}{Nfertilized\ plant\ DM} * 100$$

Where, DM = dry matter SE = symbiotic effectiveness N = Nitrogen

The rate of nitrogen-fixing effectiveness was evaluated as: Highly effective, > 80%; Effective, 50 - 80%; Lowly effective, 35 - 50% and Ineffective, <35% (Purcino *et al.*, 2000).

3.2.11. Data Analysis

Data collected was statistically analyzed by subjecting to analysis of variance (ANOVA) (one-way ANOVA) using General Linear Models Procedure of SAS software version 9. The effect of independent variable i.e. rhizobial isolates on dependent or respondent variables like nodule number, nodule fresh weight, nodule dry weight, shoot fresh weight, shoot dry weight, and shoot length were analyzed using one-way ANOVA. 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.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. Root Nodule Induction

All the 30 soil samples induced nodulation and clean, intact and pinkish color root nodules were randomly collected from each pot after 45 days of growth using a vial containing desiccant silica gel with cotton plug to prevent contact between nodules and the desiccant and brought to laboratory for further isolation.

Figure 2: Nodulation of Faba Bean induced through plant infection method

4.2. Isolation of Rhizobia

A total of twenty five Faba bean nodulating rhizobia were isolated from soil sampled across five kebeles of Uraga Woreda, Guji Zone, Southeastern Ethiopia viz. Gadeo Guratu, Yabitu Tome, Tabe Haro Wachu, Dida Hora Burka, and Tabe Burka 5, 6, 5, 5, and 4, respectively. From all nodule samples, rhizobia were isolated on YEMA media incorporated with Congo red dye by incubating 3-5 days at 28℃ in order to assist easy identification of pure rhizobial isolates.

4.3. Presumptive Test of the Rhizobial Isolates

The present study work produced a total of 25 rhizobial isolates from root nodules of Faba Bean (*Vicia faba* L.) collected from some kebeles of Uraga District, Guji Zone, and Southeastern Ethiopia.

Accordingly, Congo red absorption test revealed that none of the isolates absorbed the dye after 3-5 days of incubation at 28℃ in dark condition (Appendix A: Table 5). The result is concurrent with previous works of (Solomon Legesse and Fassil Assefa, 2014; Bilal *et al*, 2013; Amha Gebremariam and Fassil Assefa, 2018) where none of their isolates were found to absorb Congo red.

Observation on reaction to acidity and alkalinity (YEMA-BTB test) showed that all the isolates turned the color of the YEMA media into yellow after 3-5 days of incubation at 28℃ (Appendix A: Table 5) showing that they are acid producers and fast growers. This finding was similar with the previous work of (Abere Mnalku *et al*., 2009; Zerihun Belay and Fassil Assefa, 2011; Getahun Negash, 2015; Ondieki *et al*., 2017) who reported that when all isolates were streaked on YEMA media incorporated with Bromothymol blue dye turned the media to yellow.

Regarding PGA-BCP growth test none of the isolates showed growth on this media. This preliminary test indicates the tested isolates are rhizobia which do not show growth on the PGA-BCP media.

All the 25 isolates were Gram negative and rod shaped as revealed by Gram’s staining technique (Appendix A: Table 5).

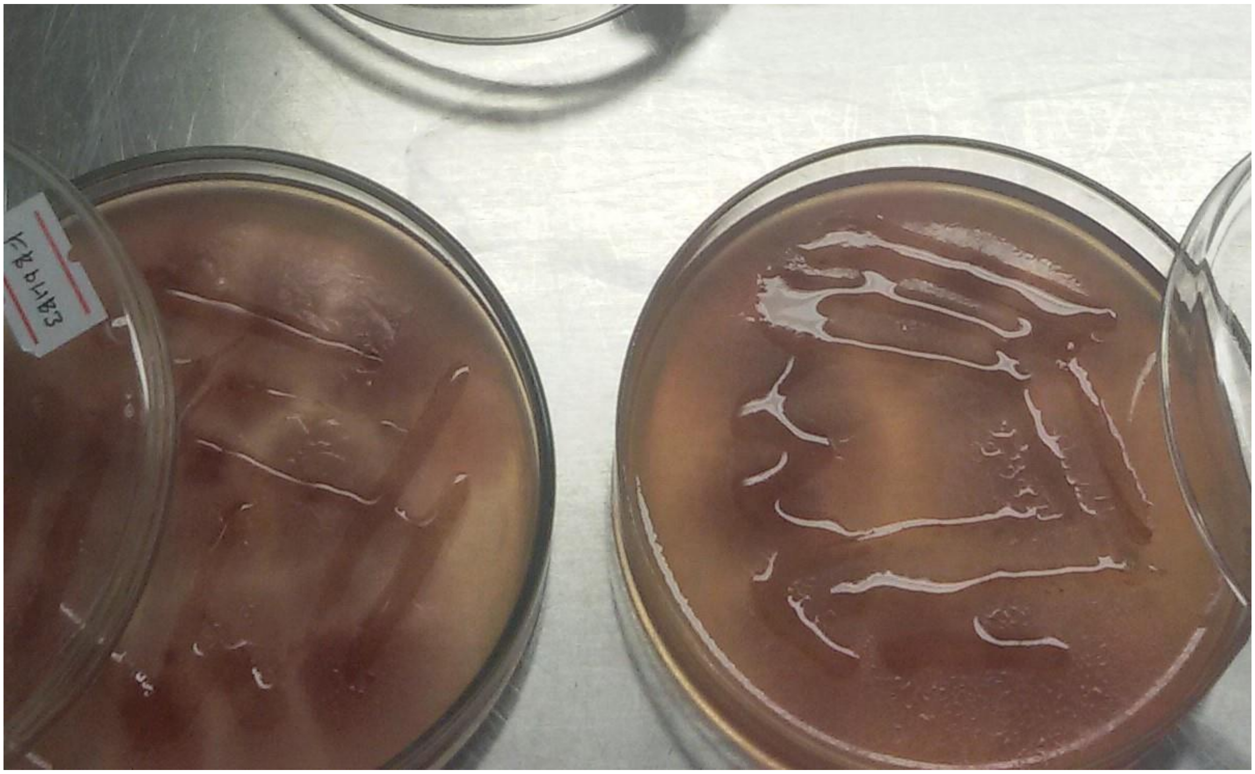

Figure 3: Faba bean nodulating rhizobial isolate presumptive test sample; Growth on YEMA-CR

4.4. Morphological Characterization of the Rhizobial Isolates

All colonies of the 25 presumptive isolates 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 1.5 and 5mm. The largest colony diameter (5.0mm) was shown by FBGG-1, FBTHW-6 and FBDHB-5 from Gadeo Guratu, Tabe Haro Wachu and Dida Hora Burka, respectively. Whereas, the smallest colony diameter (1.5mm) was displayed by FBYT-5, FBDHB-2 and FBTHW-3 from Yabitu Tome, Dida Hora Burka and Tabe Haro Wachu, respectively (Appendix A: Table 6). This finding is in line with (Zerihun, 2006; Solomon and Fassil, 2014; Zewdu and Samuel, 2018; Getahun Negash, 2015) who reported that *Rhizobium* isolates attain colony sizes ranging from 2 to 5 mm; 1.5 to 4.5 mm; 1.5 to 4.5 mm and 2 to 5.5 mm, respectively.

With regard to colony texture 76% showed large mucoid colonies (LM) with exopolysaccharide production and 24% of the isolates were characterized as large watery (LW) texture on YEMA media (Appendix A: Table 6).

4.5. Biochemical and Physiological Characterization of the Isolates

4.5.1. Salt Tolerance Test

Almost all of the isolates were grown in medium having 0.5 – 2.0% concentration of NaCl (Appendix A: Table 7). Few of the isolates showed disparity in tolerance to different concentrations of salt (Appendix A: Table 7). On increasing the concentration of the salts the growth of the isolates were decreased. Out of 25 rhizobial isolates 22 (8%) and 16(64%) isolates were able to grow at 3.0% and 4.0% NaCl concentrations, respectively. As the concentration of the salt increases it assures the number of tolerant isolates decreased yielding only 7 (28%) isolates (FBYT-5, FBGG-2, FBGG-6, FBDHB-3, FBDHB-5, FBTHW-4 and FBTB-2) that tolerated 5.0% NaCl concentration (Appendix A: Table 7). These observations might highlight the concomitant *Rhizobium*-host efficiency which is hampered by high levels of salinity which decreases the Ca^{2+} content of *Rhizobium* cells, and the outer membrane structure of the *Rhizobium* cells was greatly distorted (Zahran, 1999). In that regard Giller (2001) mentioned that salt stress limits legume growth, especially when the crop relies on symbiotically fixed nitrogen. However, this study reported that most isolates are tolerant to high and low salt concentrations and have the potential to improve acquiesce of legumes. This finding is in line with the report of (Girmaye *et al*., 2014; Solomon and Fassil, 2014; Assefa Keneni *et al*., 2010; Amha Gebremariam and Fassil Assefa, 2018) and Belal *et al*. (2013) who found thus rhizobial isolates were not completely inhibited by low and high levels of salinity concentration. Thus, *Rhizobium* differs in their capability of salt tolerance as some strains may grow at high salt concentrations; others may not grow even at low salt concentration (Kucuk and Kivanc, 2008). From this fact that the development of new nodules, the activity of nodules and the formation of the nitrogenase enzyme are reduced by salinity (Ahmed and Elsheikh, 1998) so the observed diversity in tolerance for these isolates has paramount importance. Result indicated above can suggest that rhizobial isolates that tolerated high salt concentration *in vitro* could be used to develop inoculants for saline soils though further field test is needed.

4.5.2. pH Tolerance Test

Differences in pH tolerances of the isolates were observed among isolates (Appendix A: Table 8). All the isolates showed growth on a wide range of pH 5.0 to 9.0. Out of the tested isolates 7 (28%), 14 (56%), 18 (72%), and 5(20%) isolates showed growth at pH 4.0, 4.5, 9.5 and 10.0, respectively (Appendix A: Table 8). Thus study showed that isolates were grown at wider range of pH level even at low pH. This result is in contrast to the work of Bilal *et al.,* (2013) and Assefa Keneni *et al*. (2010) who reported that strains showed sensitivity to low pH and no *Rhizobium* isolates were able to grown at pH of medium adjusted to 4 and 4.5. However, concurrent with the previous works of Amha Gebremarium and Fassil Assefa (2018) who obtained 33.33% and 66.66%; Getahun Negash (2015) recorded 65.7% and 71.2% and Wendesen Melak *et al*. (2018) recorded 76% and 86.7% of isolates were grown well at pH 4 and pH 4.5, respectively. Also Hajjam *et al*., (2016) recorded 71.87% of isolates were grown well at pH of 4.0. Strains of rhizobia differed markedly in their tolerance to acidic pH (Ballen *et al*., 1998; Zahran, 1999). Even within the same species, strains differed considerably in their tolerance to acidity in culture media (Glenn and Dilworth, 1994). Girmaye Kenasa *et al*. (2014) emphasized strains resistant to different soil stresses such as pH have potential to improvement in the production of legumes grown on the area and extend the ranges of soils upon which legumes adapted to grow. In addition, this diverse opportunity can be further utilized in future production of biofertilizers from which extreme condition tolerant strains may be tried and used. We can conclude that rhizobial isolates that had good growth on acidic media can be used to develop bioinoculants for acidic soils and would enable to maintain legumes productivity regardless of acidic nature of the soil.

4.5.3. Temperature Tolerance Test

All isolates of Rhizobia from all soil sites displayed varying temperature tolerance ranging between 5 – 45^{o}C (Appendix A: Table 9). All the 25 presumptive isolates were able to grow at 15 to 35°C and none of them grow at 45°C (Appendix A: Table 9). Out of 25 tested isolates 16 (64%) isolates with the exception of (FBYT-2, FBYT-4, FBGG-1, FBGG-3, FBDHB-1, FBDHB-6, FBTHW-2, FBTHW-3 and FBTB-2) were found to be tolerant to 5°C temperature, 21 (84%) isolates with the exception of (FBYT-2, FBGG-3, FBDHB-1 and FBTHW-2) were grown at 10°C, however, only 6 (24%) isolates (FBYT-5, FBGG-2, FBDHB-3, FBTHW-1, FBTHW-4 and FBTB-2 were found to be tolerant to 40°C (Appendix A: Table 9). Surprisingly, 5 isolates FBYT-5, FBGG-2, FBDHB-3, FBTHW-1 and FBTHW-4 showed growth in a wide range of temperature (5-40˚C) (Appendix A: Table 9). These results are in harmony with previously reported works of (Wendesen Melak, 2018; Amha Gebremarium and Fassil Assefa, 2018; Solomon and Fassil, 2014) indicating that Some *Rhizobium* strains were able to grow on higher temperature and some were able to grow on low temperature. The upper temperature limit for rhizobial growth ranges between 32 and 47^0C though tolerance varies among species and strains (Mc Vicar *et al*, 2005). Physiological and genetic modifications in bacteria such as plasmid deletion and genomic rearrangements may occur in soils subject to high temperature tolerance (Hungria and Vargas, 2000). Moreover, temperature range depends highly on the strains of *Rhizobium.* Despite the fact that some strain may survive in high temperature but it does not represent that, they are proficient in nitrogen fixation. It could be concluded that rhizobial isolates that showed good growth on the media exposed to extreme temperatures could help us to develop inoculants that can be used in the fields with harsh temperature conditions.

Here is below the summary of physiological characteristics of the isolates from the study area (Uraga Woreda, Guji Zone, and Southeastern Ethiopia) (Table 2).

Table 2: Summary of physiological characteristics of rhizobial isolates

No	Isolates code	NaCl (%)	pH	T(℃)
1	FBYT-1	0.5-4.0	4.5-9.5	5-35
2	FBYT-2	0.5-2.5	5-9	15-35
3	FBYT-3	0.5-4.5	4-10	5-35
4	FBYT-4	0.5-4	5-9.5	10-35
5	FBYT-5	0.5-5	4-9	5-40
6	FBYT-6	0.5-3.5	5-9.5	5-35
7	FBGG-1	0.5-2.5	5-9	10-35
8	FBGG-2	0.5-5	4-10	5-40
9	FBGG-3	0.5-4	5-9.5	15-35
10	FBGG-4	0.5-4.5	4.5-9.5	5-35
11	FBGG-6	0.5-5	4-9	5-35
12	FBDHB-1	0.5-4.5	5-9	15-35
13	FBDHB-2	0.5-2.5	4.5-9.5	5-35
14	FBDHB-3	0.5-5	4-9.5	5-40
15	FBDHB-5	0.5-5	5-10	5-35
16	FBDHB-6	0.5-3	4.5-9.5	10-35

17	FBTHW-1	0.5-3	5-9.5	5-40
18	FBTHW-2	0.5-4	4-9	15-35
19	FBTHW-3	0.5-4	5-9.5	10-35
20	FBTHW-4	0.5-5	4.5-10	5-40
21	FBTHW-6	0.5-3.5	4-9.5	5-35
22	FBTB-1	0.5-3	5-9	5-35
23	FBTB-2	0.5-5	4.5-9.5	10-40
24	FBTB-4	0.5-3.5	5-9.5	5-35
25	FBTB-5	0.5-4	4.5-9.5	5-35

4.5.4. Intrinsic Antibiotic Resistance

In the present study, it was found that Rhizobial isolates showed significant difference in their intrinsic antibiotics resistance to different types and concentrations (Appendix A: Table 10). Hundred percent of the tested isolates were resistant to Ampicillin at 2.5μg, 5.0μg and 100μg of concentrations. Similar results were also obtained that (92%) of isolates resist the antibiotics Chloramphenicol and Nalidixic Acid at all three levels of concentrations (2.5μg, 5.0μg and 10μg). Resistance to pencillin at concentrations of 2.5μg, 5.0μg and 10μg were recorde as 100%, 92% and 88%, respectively (Appendix A: Table 10). The least resistance of isolates to antibiotics was observed on Kanamycin where only 40% of the isolates viz. FBYT-2, FBGG-3, FBYT-4, FBDHB-1, FBDHB-3, FBTHW-3, FBDHB-6, FBTB-2 and FBTB-5 from Yabitu Tome (2), Dida Hora Burka (3), Gadeo Guratu (2), Tabe Burka (2) and Tabe Haro Wachu (1) were resistant to the antibiotics at all three level of concentrations (Appendix A: Table 10). On the other hand, 36% of the isolates are sensitive to Streptomycin at all three levels of concentrations i.e. 2.5μg, 5.0μg and 10μg (Appendix A: Table 10). In this study, some isolates are resistant to different concentration of antibiotics. Surprisingly, four isolates viz. FBGG-6, FBDHB-3, FBYT-4 and FBTB-2 from Gadeo Guratu, Dida Hora Burka, Yabitu Tome and Tabe Burka, respectively managed to grow on all types of antibiotics with all the three levels of concentrations. From this result, we observed that growth was affected when concentration of antibiotics increased. This finding was in line with previous reports of Assefa Keneni *et al*. (2010), Zerihun and Fassil (2011), Wendesen *et al*. (2018) and Dereje *et al*. (2015) reported that intrinsic antibiotic resistance are major factors to competition during natural invasion. Moreover, studies stated that strains to vary in their success to cope up environmental contests and grow well on diverse antibiotics (Tolera Abera *et al*., 2015).

Table 3: Isolates % of resistance to different antibiotics with varying concentrations (μg/ml)

Antibiotics	Concentration (μg/ml)	% of resistant isolates
Chloramphenicol	2.5	92
	5	92
	10	92
Ampicillin	2.5	100
	5	100
	10	100
Kanamycin	2.5	40
	5	40
	10	40
Nalidixic acid	2.5	92
	5	92
	10	92
Streptomycin	2.5	64
	5	64
	10	64
Penicillin	2.5	100
	5	92
	10	88

4.5.5. Carbohydrate Utilization Test

The Rhizobial isolates utilized 100% of Glucose, Fructose, Xylose, Sucrose, Mannitol, Dextrose and Maltose as their sole sources of carbon for their growth (Table 4). With regard to the remaining carbohydrates the rhizobial strains exhibited less diversity in their growth. Almost all strains were able to catabolize Lactose, which accounts for 88% isolates (Table 4) with the exception of FBDHB-2 from Dida Hora Burka, FBTHW-1 from Tabe Haro Wachu and FBTB-5 from Tabe Burka (Appendix A: Table 11). Eighty four percent (84%) of the isolates had utilized Arabinose as a major sources of carbon for their growth (Table 4) except FBYT-3 from Yabitu Tome, FBGG-4 from Gadeo Guratu, FBDHB-1 from Dida Hora Burka, and FBTB-1 from Tabe Burka (Appendix A: Table 11) and eighty percent (80%) of the isolates had metabolized Sorbitol for their growth (Table 4) except FBYT-1 from Yabitu Tome, FBYT-6 from Yabitu Tome, FBGG-3 from Gadeo Guratu, FBDDHB-1 from Dida Hora Burka, and FBTHW-4 from Tabe Haro Wachu (Appendix A: Table 11). In general, the isolates were found to utilize 80 to 100% of the tested carbohydrates which were served as good sources for the growth of rhizobial isolates (Table 4). This competence in nutrient utilization is useful trait in which the efficiency of symbiosis affected by the levels of the various nutrients in the soil according to Somasegaran and

Hoben (1994). The ability to utilize a wide range of carbon sources have an ecological advantage in colonizing the rhizosphere as compared to strains having a degree of specificity in their requirements (Sukrita and Chakrabarti, 1981). Similar findings have been reported on carbohydrate metabolism of *R. leguminosarum bv.viciae* by (Getahun Negash, 2015; Assefa Keneni *et al*., 2010; Zerihun, 2006; Zerihun and Fassil, 2011).

Percentage of Isolates grown on varying carbohydrates is summarized below (Table 4).

Table 4: Carbon source utilization by *Rhizobium* isolates

Carbon sources	% of isolates grown on the carbohydrate
Glucose, Fructose, Xylose, Sucrose, Maltose, Mannitol, Dextrose	100
Lactose	88
Arabinose	84
Sorbitol	80

4.5.6. Amino Acid Utilization Test

All the rhizobial isolates were tested whether they metabolize amino acids as their sole source of N for their growth. Accordingly, four (50%) of the amino acids (Asparagine, Histidine, Glycine and L-isoleucine) were catabolized 100% by all the isolates (Appendix A: Table 12). Whereas, Alanine were catabolized by 23 (92%) isolates with FBYT-6 from Yabitu Tome and FBDHB-2 from Dida Hora Burka failed to do so, Valine were catabolized by 22 (88%) of the isolates which excludes FBGG-3 from Gadeo Guratu, FBTHW-2 from Tabe Haro Wachu and FBTB-1 from Tabe Burka, and Arginine were catabolized by 21 (84%) of the isolates with the exception of FBYT-4 from Yabitu Tome, FBGG-1 from Gadeo Guratu, FBDHB-5 from Dida Hora Burka and FBTB-2 from Tabe Burka (Appendix A: Table 12). Due to acidic nature of the amino acid, asparatic acid, was catabolized by 11 (44%) isolates that included FBYT-2 and FBYT-5 from Yabitu Tome, FBGG-1, FBGG-3 and FBGG-4 from Gadeo Guratu, FBDHB-3 and FBDHB-6 from Dida Hora Burka, FBTHW-1, FBTHW-3 and FBTHW-4 from Tabe Haro Wachu and FBTB-2 from Tabe Burka (Appendix A: Table 12).

Table 5: Summary of Physiological and Symbiotic properties of Rhizobial isolates from Uraga Woreda, Guji Zone, Southeastern Ethiopia that nodulates Faba bean

Isolates type	pH tolerance	Temperature tolerance (°C)	Salt tolerance (%)	Carbon utilization (%)	Amino acid utilization (%)	Antibiotic tolerance	Symbiotic Effectiveness	Rate
FBYT-1	4.5-9.5	5-35	0.5-4.0	90	87.5	Chl, Amp, Pen, Nal, Str	79.64	E
FBYT-2	5-9	15-35	0.5-2.5	100	100	Chl, Amp, Pen, Kan, Nal	113.8	HE
FBYT-4	5-9.5	10-35	0.5-4	100	75	Chl, Amp, Pen, Kan, Nal, Str	74.39	E
FBYT-6	5-9.5	5-35	0.5-3.5	90	75	Chl, Amp, Pen, Nal	45.53	LE
FBGG-1	5-9	10-35	0.5-2.5	100	87.5	Chl, Amp, Pen, Nal, Str	73.45	E
FBGG-2	4-10	5-40	0.5-5	100	87.5	Chl, Amp, Pen, Nal, Str	59.31	E
FBGG-3	5-9.5	15-35	0.5-4	90	87.5	Chl, Amp, Pen, Kan, Str	109.0	HE
FBGG-4	4.5-9.5	5-35	0.5-4.5	90	100	Chl, Amp, Pen, Nal	44.81	LE
FBDHB-1	5-9	15-35	0.5-4.5	90	87.5	Chl, Amp, Kan, Nal, Str	65.46	E
FBDHB-2	4.5-9.5	5-35	0.5-2.5	90	75	Chl, Amp, Pen, Nal	81.92	HE
FBDHB-5	5-10	5-35	0.5-5	90	75	Amp, Pen, Nal, Str	48.32	LE
FBDHB-6	4.5-9.5	10-35	0.5-3	100	100	Chl, Amp, Pen, Kan, Nal	69.92	E
FBTHW-1	5-9.5	5-40	0.5-3	90	100	Chl, Amp, Pen, Nal, Str	66.36	E
FBTHW-2	4-9	15-35	0.5-4	100	75	Chl, Amp, Pen, Str	69.26	E
FBTHW-4	4.5-10	5-40	0.5-5	90	100	Chl, Amp, Pen, Nal, Str	83.83	HE
FBTB-2	4.5-9.5	10-40	0.5-5	100	87.5	Chl, Amp, Pen, Kan, Nal, Str	27.71	I
FBTB-4	5-9.5	5-35	0.5-3.5	100	87.5	Chl, Amp, Pen, Nal	52.19	E
FBTB-5	4.5-9.5	5-35	0.5-4	90	87.5	Chl, Amp, Pen, Kan, Nal	57.49	E

Where; Chl = Chloramphenicol, Amp = Ampicillin, Pen = Penicillin, Kan = kanamycin, Nal = Nalidixic Acid, Str = Streptomycin

4.6. Authentication and Evaluation of Symbiotic Effectiveness of the Isolates

4.6.1. Analysis of Variance

Eighteen isolates obtained from faba bean root nodules were assessed for their infectiveness and effectiveness of nitrogen fixation on Hachalu variety of faba bean on sterile and acid treated sand in a pot experiment under greenhouse (Table 6). All isolates formed nodules on the tested faba bean root authenticating as *Rhizobium*.

The results of analyses of variance showed that *Rhizobium* inoculation significantly (P<0.05) increased at all investigated parameters such as number of nodules per plant, nodule fresh weight, nodule dry weight, shoot length, shoot fresh weight, shoot dry weight and symbiotic effectiveness as compared to the control treatments (Table 6). This finding is concurrent with the work of Dereje *et al*. (2015) symbiotic characteristics of *R.leguminosarum.bv.viciae* nodulating faba bean collected from acidic soils of Ethiopia, Solomon Legesse and Fassil Assefa (2014) symbiotic and phenotypic characteristics of rhizobia nodulating faba bean (*Vicia faba*) isolated from tahtay koraro, Northwestern zone of Tigray regional state, Ethiopia and Anteneh (2012) symbiotic effectiveness of *Rhizobium leguminosarum var.viciae* nodulating faba bean isolated from central Ethiopian. In this study, all isolates are better as compared to the negative (uninoculated) control in terms of all investigated parameters.

In the present study, the Rhizobia isolate showed difference with nodule number. The minimum mean nodule number recorded per plant was 23.33 exhibited by isolate FBDHB-5 and the maximum mean number of nodules recorded was 67 by isolate FBTHW-4. Nodule number is a less reliable indicator of symbiotic effectiveness because from the result highly effective isolates had few nodules. 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 (Somasegeran and Hoben, 1994). The process of nodule formation requires a number of highly specific signaling interactions between the plant and Rhizobial partners (Ferguson, 2017). From this result the mean number of nodules was 39.083 (Table 6) comparably very low when compared to the result of other works by (Anteneh Argaw, 2012; Abere Mnalku *et al*., 2009; Zerihun Belay and Fassil Assefa, 2011) which they recorded respectively of 167, 128, and 124, nodules per plant. Whereas, it was greater than the mean nodule number for faba beans on sand culture recorded by (Dereje *et al*, 2015; Getahun Negash, 2015; Wendesen Melak *et al*., 2018) were recorded 9.5, 25 and 33.8 respectively. Studies indicated that variation in nodulation could be due to the result of low rhizobial density, inconsistence of the *Rhizobium* and edaphic factors that delay the effectiveness of the rhizobial isolates (Zahran, 1999; Slattery and Pearce, 2002; Kiros and Singh, 2006).

In this study the isolates also showed difference in nodule dry weight with Hachalu faba bean (Table 6), there was significant difference among the isolates (treatments) and the controls at (P < 0.05) level of significance. Maximum and minimum mean nodule dry weight of 0.090 and

0.008 were exhibited by FBYT-2 and FBTB-2, respectively (Table 6). The average nodule dry weight recorded in this study was 0.035g. The result is lower than the previous works reported by (Dereje *et al*, 2015; Zerihun and Fassil, 2011 and Girmaye Kenessa *et al*, 2014) which obtained mean nodule dry weight value of 0.079g p^{-1}, 0.078 g p^{-1} and 0.077 g p^{-1} , respectively, also much lower than 0.145g p^{-1} which was reported by Anteneh (2012). The preceding difference in nodule dry weight might be, attributed to the difference in efficiency of nodules in fixing N and the host species since isolates from different agro-ecologies were tested on different host varieties explained similarly by Endalkachew (2007). On the other hand, nodule fresh weight was recorded 0.017g p^{-1} for isolate (FBTB-2) to 0.161 g p^{-1} for isolate (FBYT-2). The mean nodule fresh weight in this study was 0.063g p^{-1} with Hachalu faba bean variety comparably lower when compared to previously reported in Tigray Highlands, Northern Ethiopia by Alemayehu (2009) who recorded mean nodule fresh weight of 0.88 g p^{-1} and North and South Gondar, Ethiopia by Zewdu and Samuel (2018) who recorded 0.153 g p^{-1}.

From this study, it was shown that inoculation induces significant improvement in plant shoot length as compared to the control treatments. The maximum mean shoot length of 49.00cm was recorded by isolates FBGG-3 and FBYT-2 (Table 6). These results were almost similar with the results of Anteneh Argaw (2012) study on faba bean inoculation with Degaga variety which was noted 49.7cm shoot height with rhizobial isolate NSFBR-48 collected from Central Ethiopia which showed pronounced improvement in shoot height 51% and 14% over negative and N treated plants, respectively. Besides, Zewdu and Samuel (2018) study on faba bean inoculation with Adet variety with 48.67cm for both isolates KD-4 and LG-1 collected from North and South Gondar, Ethiopia, was shown noticeable improvement in shoot height 54.8% over negative control and 11% nitrogen treated plants. This enhancement of shoot length could be attributed to the fact rhizobia may increase plant growth by providing products through nitrogen fixation (Kumar *et al*., 2014). In pot experiment on sand culture during authentication application of mineral nitrogen fertilizer did not induce nodulation instead it delayed and inhibited nodulation and effectiveness of nitrogen fixation potential of *Rhizobium* isolates (Chemining'wa *et al*., 2004; Crews *et al*., 2004) reported that addition of nitrogen fertilizer has a negative effect on the nodulation and nitrogen fixation of *Rhizobium* isolates. As a result the present study did not include use of starter nitrogen.

Significant variation observed among the treatment for mean shoot dry weight of inoculated plants. The maximum mean shoot dry weight was recorded 1.43 g p^{-1} with the isolates FBYT-2 and the minimum mean shoot dry weight was recorded 0.35 g p^{-1} with isolates FBTB-2. The average shoot dry weight recorded 0.852g p^{-1} is slightly greater than that previously reported by Getahun Negash (2015) who recorded mean shoot dry weight of 0.5 g p^{-1} whereas, slightly fewer than the finding of Getaneh Tesfaye (2008) who recorded mean shoot dry weight of 1.21g p^{-1} but much less than the results obtained by Assefa Keneni *et al*. (2010) and Zewdu and Samuel (2018) who explained that shoot dry weight is a good indicator of relative isolate effectiveness.

According to the percentage differences of shoot dry weight of inoculated and nitrogen-fertilized plants measure of effectiveness all inoculated plants were symbiotically effective with Hachalu faba bean varieties except one isolate i.e. FBTB-2 which was found infective. Accordingly, 4 (22.22%) of isolates were found to be highly effective of which the tested isolates attained 81-113.78% (> 80%). The other 10 (55.56%) of the tested isolates displayed SE of 52.19-79.64% and were similarly rated as effective (50-80%). The remaining 3 (16.67%) of the isolates namely FBYT-6, FBGG-4 and FBDHB-5 were found lowly effective with SE of 45.53, 44.81 and 48.32, respectively. From the tested rhizobial isolates two isolates viz. FBGG-3 and FBYT-2 outcompeted the N treated plants and scored SE above 100% which is 109.02 and 113.78, respectively. The existence of such variability in symbiotic effectiveness of *Rhizobium* across the country might be primarily accounted to the biodiversity of rhizobial resource resident in the soils of different agro-ecological zones of Ethiopia (Endalkachew, 2007). Similar reports were, obtained with rhizobia of faba bean growing in some parts of Wello, Northern Ethiopia and Central Ethiopia (Getahun Negash, 2015; Anteneh Argaw, 2012). Similarly, study by (Solomon Legesse and Fassil Assefa, 2014) also reported the same where two of their isolates, 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).

In this study, more highly effective isolates were obtained compared to other investigator reports. According to Dereje *et al*. (2015) finding 56% of the isolates were highly effective in both Degaga and Dosha varieties collected from acidic soils of Ethiopia. Girmaye *et al*. (2014) revealed that 16% of the isolates of faba bean were highly effective collected from acidic soils of

Wollega Western Ethiopia and Zerihun and Fassil (2011) result shows 23% of isolates were highly effective collected from major faba growing areas in Northern Gonder, Ethiopia. Such variability in symbiotic effectiveness of faba bean *Rhizobium* was found to be widespread in Ethiopia (Van Berkum *et al*., 1995) and in the USA (Brockman and Bezdicek, 1989). Generally, the result of this study and other studies suggest that the existence of effective naturally occurring faba bean rhizobial isolates in different agro ecological zones of Ethiopia gives paramount importance for enhancement of dinitrogen fixation to takes place in faba bean.

Table 6: Nodulation and symbiotic effectiveness of the isolates on sand culture

Isolates	NN(P^{-1})	NFW(gp^{-1})	NDW(gp^{-1})	SFW(gp^{-1})	SDW(gp^{-1})	SL(cmp^{-1})	SE (%)	Rate
FBTB-4	27.778^{gih}	0.049^{e}	0.031^{gh}	4.587^{efdg}	0.6553^{hfgi}	43.000^{bdac}	52.19	E
FBDHB-2	32.333^{gifh}	0.034^{fihg}	0.020^{ijk}	6.172^{dc}	1.0287^{dc}	46.333^{ba}	81.92	HE
FBYT-1	49.444^{ed}	0.065^{d}	0.041^{f}	6.000^{efdc}	1.0000^{dc}	48.000^{ba}	79.64	E
FBGG-3	61.667^{ba}	0.155^{a}	0.083^{b}	8.214^{a}	1.3690^{a}	49.000^{a}	109.02	HE
FBYT-6	32.000^{gifh}	0.039^{feg}	0.020^{ijk}	4.575^{efg}	0.5718^{hji}	45.667^{ba}	45.53	LE
FBGG-1	39.000^{f}	0.043^{fe}	0.024^{ij}	6.456^{bc}	0.9223^{dce}	47.000^{ba}	73.45	E
FBGG-4	50.556^{edc}	0.070^{d}	0.037^{gf}	4.501^{fg}	0.5627^{hji}	42.667^{bdc}	44.81	LE
FBYT-4	62.000^{ba}	0.101^{c}	0.057^{d}	6.539^{bc}	0.9341^{dce}	46.333^{ba}	74.39	E
FBDHB-1	25.667^{i}	0.028^{ih}	0.015^{lk}	6.576^{bc}	0.8220^{dfge}	46.333^{ba}	65.46	E
FBTB-5	41.556^{ef}	0.024^{ij}	0.013^{lm}	6.498^{bc}	0.7220^{hfge}	44.333^{bac}	57.49	E
FBDHB-5	23.333^{i}	0.042^{fe}	0.026^{ih}	4.247^{g}	0.6067^{hgi}	43.667^{bac}	48.32	LE
FBDHB-6	36.667^{gfh}	0.034^{fhg}	0.019^{ljk}	6.146^{edc}	0.8780^{dfce}	46.333^{ba}	69.92	E
FBTHW-4	67.000^{a}	0.124^{b}	0.065^{c}	7.896^{ba}	1.0527^{bc}	48.000^{ba}	83.83	HE
FBTB-2	26.667^{ih}	0.017^{j}	0.008^{m}	1.740^{h}	0.3480^{j}	39.000^{dc}	27.71	I
FBTHW-2	38.000^{gf}	0.032^{ihg}	0.019^{ljk}	6.088^{edc}	0.8697^{dfce}	46.000^{ba}	69.26	E
FBTHW-1	53.667^{bdc}	0.092^{c}	0.049^{e}	7.084^{bac}	0.8333^{dfce}	45.667^{ba}	66.36	E
FBYT-2	60.333^{bac}	0.161^{a}	0.090^{a}	8.572^{a}	1.4287^{a}	49.000^{a}	113.78	HE
FBGG-2	54.000^{bdc}	0.153^{a}	0.082^{b}	5.957^{efdc}	0.7447^{hfge}	44.000^{bac}	59.31	E
N+	0.000^{j}	0.000^{k}	0.000^{n}	7.956^{ba}	1.2557^{ba}	48.333^{ba}		
N-	0.000^{j}	0.000^{k}	0.000^{n}	3.515^{g}	0.4393^{ji}	37.000^{d}		
Mean	39.083	0.063	0.035	5.966	0.852	45.283		
CV (%)	16.111	9.890	11.063	16.106	16.092	8.414		
LSD (0.05)	10.391	0.0103	0.0064	1.5856	0.2263	6.2874		

Where; NN = nodule number, NFW = nodule fresh weight, NDW = nodule dry weight, SFW = shoot fresh weight, SDW = shoot dry weight, SL = shoot length, SE = symbiotic effectiveness, p^{-1} = per plant. N^{-} = without chemical and inoculation, N^{+} = with optimum amount of N-fertilizer, CV = Coefficient of variation, LSD = least significant difference. 0 = not found. Means within a column followed by the same letters are not significant at $p< 0.05$.

4.6.2. Correlation Analysis

Nodule number was found to be strongly positively correlated with nodule fresh weight (r = 0.8180 P <0.001), nodule dry weight (r = 0.8182 P <0.001) as reported by (Zewdu and Samuel, 2018), shoot length (r = 0.2858 P <0.05). As reported by Anteneh Argaw (2012a), and positively correlated with shoot dry weight (r = 0.4209 P <0.01) and symbiotic effectiveness (r = 0.2705 P <0.05) (Table 7). This is in agreement with previous work on Rhizobia of faba bean by Getahun Negash (2015); Wendesen *et al*. (2015).

Nodule dry weight was strongly positively correlated with nodule fresh weight (r = 0.9960 P<0.001), shoot dry weight (r = 0.5016 P<0.001) and symbiotic effectiveness (r = 0.4261 P<0.01). Moreover, there was also positive correlation with shoot length (r = 0.3106 P<0.05) as reported by (Wendesen *et al*., 2018; Anteneh Argaw, 2012a; Solomon Legesse and Fassil Assefa, 2014) (Table 7).

Shoot length was found to be strongly positively correlated with shoot dry weight (r = 0.5662 P<0.001) and symbiotic effectiveness (r = 0.5313 P<0.001) (Zewdu Teshome and Samuel Sahile, 2018; Anteneh Argaw, 2012a). Shoot length also had positive correlation with nodule fresh weight (r = 0.3040 P<0.05) (Alemayehu, 2009) (Table 7).

Shoot dry weight was also found to be strongly positively correlated with symbiotic effectiveness (r = 0.9597 P<0.001). Similar findings was done by (Getahun Negash, 2015; Somasegaran and Hoben, 1994; Anteneh Argaw, 2012) (Table 7), finding reveals that shoot dry weight was used regularly as an indicator of the determinant factor of nodulation on nitrogen fixation and efficiency of symbiosis in faba bean.

Table 7: Correlation coefficients among investigated parameters showing the strength of relationship between variables (NN, NFW, NDW, SL, SFH, SDW, and SE)

Variable	NN	NFW	NDW	SFW	SDW	SL	SE
NN	1.0000						
NFW	0.8180***	1.0000					
NDW	0.8182***	0.9960***	1.0000				
SFW	0.4865***	0.4931***	0.4923***	1.0000			
SDW	0.4209**	0.4919***	0.5016***	0.9226***	1.0000		
SL	0.2858*	0.3040*	0.3106*	0.5487***	0.5662***	1.0000	

SE	0.2705*	0.4187**	0.4261**	0.8632***	0.9597***	0.5313***	1.0000

*** = significant at $p<0.001$, ** = significant at $P < 0.01$, * = significant at $P < 0.05$ NN = nodule number, NDM (g) = nodule dry mass, NFW (g) = nodule fresh weight, SFW (g) = shoot fresh weight, SDW (g) = shoot dry weight, SL (cm) = shoot length, SE= symbiotic effectiveness.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In the present study, most of our isolates displayed abundant diversity in their response to morphological and physiological characteristics. The presumptive and authentication test results of this investigation proved that all isolates were true faba bean *Rhizobium* species. Thus, *Rhizobium* sp. was isolated from the root nodules of faba bean (*Vicia faba),* to observe their morphological, physiological, biochemical and symbiotic characteristics to determine their effects on plant growth. The growth of isolates at stressed laboratory conditions (at pH 4.0 and 10, 5% NaCl, 5 and 40^{o}C) indicated their significance in contributing biologically fixed nitrogen to stressful ecosystems. Isolates that show tolerance to both salinity and pH extremes may be potential candidates for inoculum production for saline and acidic soils, this in turn effective for nitrogen fixation in combination with the available cultivars. Several carbon sources have been utilized by isolates obtained in this study. This nutritional versatility has an ecological advantage in colonizing the soil or rhizosphere. Hence, isolation and polyphasic characterization studies were essential for the selection of strains adapted to marginal edaphic- climatic conditions that can perform better for nodulation of plant growth and provides information about their genetic diversity.

Inoculation of isolates significantly increased all investigated parameters such as number of nodules per plant, nodule fresh weight, nodule dry mass, shoot fresh weight, shoot dry weight, shoot length and symbiotic effectiveness as compared to inorganic fertilizer treated control treatment. About 4 (22.22%) of the isolates *viz.* FBDHB-2, FBGG-3, FBTHW-4 and FBYT-2 collected from major faba bean growing areas of Uraga Woreda, Guji Zone, Southeastern Ethiopia were found to be highly effective. About 18 (55.56 %) were effective, three isolates (16.67%) namely FBYT-6, FBGG-4 and FBDHB-5 from Yabitu Tome, Gadeo Guratu and Dida Hora Burka kebeles, respectively were categorized as lowly effective and only one isolate (FBTB-2) grouped as ineffective. Accordingly, isolate FBDHB-2, FBGG-3, FBTHW-4 and FBYT-2 was found to be highly effective in nitrogen fixation and found resistant to a wide range of physiological and biochemical stresses. Surprisingly, two isolates FBGG-3 and FBYT-2 from Gadeo Guratu and Yabitu Tome kebeles, respectively was found more superior and to be the best candidate for inoculants production among the isolates for most of the growth related parameters from these study areas.

Generally, from the current investigation it can be concluded that some *Rhizobium* bacteria isolated from faba bean growing areas of Uraga Woreda, Guji Zone, Southeastern Ethiopia showed better enhancement over nitrogen treated plants on sand culture using pot experiment under greenhouse condition. Therefore, we conclude that the use of inorganic fertilizers which have adverse effects on human health and the environment should be minimized and substituted with environmental friendly and affordable bioinoculants. Since, many of the study areas harbored with effective isolates, this would minimize additional expense of using commercialized bioinoculants. This practice enhances not only the productivity of the crops it also maintains and preserves the environment.

5.2. Recommendations

Based on the finding of our study, the following recommendations were suggested:

- Out of the tested Rhizobial isolates four rhizobial isolates *viz.* FBDHB-2, FBGG-3, FBTHW-4 and FBYT-2 should be used to develop inoculants as they are ecologically competant, symbiotically highly effective and nutritionally versatile
- There is a need for extensive research regarding biological nitrogen fixation by soil bacteria i.e. rhizobia in the area of our study area since this is just a start up
- Awareness creation trainings has to be conducted for the farmers about the nitrogen fixer bacteria and practices of using bioinoculants, thereby to raise habit of utilizing such environmental friendly fertilizer source
- Field research should be conducted on varying agro-ecologies and soil type in order to be convinced with the effectiveness of Rhizobial isolates that are best performing under greenhouse experiment
- Molecular characterization (Protein and DNA analysis) should be done to support the phenotypic characterization performed

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APPENDICES

Appendix A: Table 1. Yeast Extract Mannitol Agar (YEMA) composition (Vincent, 1970)

Chemicals	**Amount (g/l)**
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.5
NaCl	0.1
Mannitol	10
Yeast extract	0.5
Agar	15
Distilled water	1000ml

(Source: Vincent, 1970)

The pH of the medium was adjusted to 7±0.1 and autoclaved at $121^{O}C$ for 15’

Appendix A: Table 2. Carbon free Basal Medium Composition Ingredients and Amount (g/l) (Vincent, 1970)

Chemicals	**Amount (g/l)**
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.5
NaCl	0.1
Yeast extract	0.05
Agar	15
Distilled water	1000ml

(Source: Vincent, 1970)

Appendix A: Table 3. Amino acid free basal medium composition (Amarger, 1997):

Chemicals	**Amount (g/l)**
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.5

NaCl	0.1
$FeCl_3.6H_2O$	0.01
$CaCl_2$	0.1
Mannitol	1
Agar	15
Distilled water	1000ml

(Source: Amarger, 1997)

Appendix A: Table 4. N-free nutrient solution (Broughton and Dilworth, 1970)

Stock solutions	**Element**	**Form**	**MW**	**Amount (g/l)**
1	Ca	$CaCl_2·2H_20$	147.03	294.1
2	P	KH_2P0_4	136.09	136.1
3	Fe	Fe-Citrate	335.04	6.7
	Mg	$MgSO_4·7H_20$	246.5	123.3
	K	K_2SO_4	174.06	87.0
	Mn	$MnSO_4·H_20$	169.02	0.338
4	B	H_3BO_4	61.84	0.247
	Zn	$ZnSO_4·7H_20$	287.56	0.288
	Cu	$CuSO_4·5H_20$	249.69	0.100
	Co	$CoSO_4·7H_20$	281.12	0.056
	Mo	$Na_2MoO_4·2H_20$	241.98	0.048

Appendix A: Table 5. Presumptive tests of the Rhizobia isolated from Faba bean grown on YEMA at 28℃ for 3-5 days

Isolates	**Color in YEMA-CR**	**Color in YEMA-BTB**	**Growth on PGA-BCP**	**Gram Reaction**
FBYT-1	White	Yellow	-	-ve, rod
FBYT-2	White	Yellow	-	-ve, rod
FBYT-3	White	Yellow	-	-ve, rod

FBYT-4	White	Yellow	-	-ve, rod
FBYT-5	White	Yellow	-	-ve, rod
FBYT-6	White	Yellow	-	-ve, rod
FBGG-1	White	Yellow	-	-ve, rod
FBGG-2	White	Yellow	-	-ve, rod
FBGG-3	White	Yellow	-	-ve, rod
FBGG-4	White	Yellow	-	-ve, rod
FBGG-6	White	Yellow	-	-ve, rod
FBDHB-1	White	Yellow	-	-ve, rod
FBDHB-2	White	Yellow	-	-ve, rod
FBDHB-3	White	Yellow	-	-ve, rod
FBDHB-5	White	Yellow	-	-ve, rod
FBDHB-6	White	Yellow	-	-ve, rod
FBTHW-1	White	Yellow	-	-ve, rod
FBTHW-2	White	Yellow	-	-ve, rod
FBTHW-3	White	Yellow	-	-ve, rod
FBTHW-4	White	Yellow	-	-ve, rod
FBTHW-6	White	Yellow	-	-ve, rod
FBTB-1	White	Yellow	-	-ve, rod
FBTB-2	White	Yellow	-	-ve, rod
FBTB-4	White	Yellow	-	-ve, rod
FBTB-5	White	Yellow	-	-ve, rod

Appendix A: Table 6. Morphological and cultural characteristics of Rhizobia Isolates

No	Isolates	Size in Diameter (mm)	Appearance	Color	Shape	Colony margin
1	FBYT-1	3.5	Large mucoid	Yellowish	Domed	Circular
2	FBYT-2	2.5	Large mucoid	Yellowish	Domed	Circular
3	FBYT-3	4.0	Largel mucoid	Yellowish	Domed	Circular

4	FBYT-4	4.5	Large mucoid	Yellowish	Domed	Circular
5	FBYT-5	1.5	Large watery	Watery	Domed	Circular
6	FBYT-6	3.0	Large mucoid	Yellowish	Domed	Circular
7	FBGG-1	5.0	Large mucoid	Yellowish	Domed	Circular
8	FBGG-2	4.0	Large watery	Watery	Domed	Circular
9	FBGG-3	4.5	Large mucoid	Yellowish	Domed	Circular
10	FBGG-4	3.0	Large mucoid	Yellowish	Domed	Circular
11	FBGG-6	2.0	Large watery	Watery	Domed	Circular
12	FBDHB-1	2.5	Large mucoid	Yellowish	Domed	Circular
13	FBDHB-2	1.5	Large mucoid	Yellowish	Domed	Circular
14	FBDHB-3	3.5	Large mucoid	Yellowish	Domed	Circular
15	FBDHB-5	5.0	Large watery	Watery	Domed	Circular
16	FBDHB-6	2.0	Large mucoid	Yellowish	Domed	Circular
17	FBTHW-1	4.0	Large mucoid	Yellowish	Domed	Circular
18	FBTHW-2	3.0	Large mucoid	Yellowish	Domed	Circular
19	FBTHW-3	1.5	Large mucoid	Yellowish	Domed	Circular
20	FBTHW-4	3.5	Large mucoid	Yellowish	Domed	Circular
21	FBTHW-6	5.0	Large watery	Watery	Domed	Circular
22	FBTB-1	4.0	Large mucoid	Yellowish	Domed	Circular
23	FBTB-2	2.5	Large mucoid	Yellowish	Domed	Circular
24	FBTB-4	2.0	Large mucoid	Yellowish	Domed	Circular
25	FBTB-5	3.0	Large watery	Watery	Domed	Circular

Appendix A: Table 7. Effect of salt tolerance on *Rhizobium* isolates

Isolates	**Salt tolerance (NaCl)**									
Code	**0.5%**	**1.0%**	**1.5%**	**2.0%**	**2.5%**	**3.0%**	**3.5%**	**4.0%**	**4.5%**	**5.0%**
FBYT-1	+	+	+	+	+	+	+	+	-	-
FBYT-2	+	+	+	+	+	-	-	-	-	-
FBYT-3	+	+	+	+	+	+	+	+	+	-

FBYT-4	+	+	+	+	+	+	+	+	-	-
FBYT-5	+	+	+	+	+	+	+	+	+	+
FBYT-6	+	+	+	+	+	+	+	-	-	-
FBGG-1	+	+	+	+	+	-	-	-	-	-
FBGG-2	+	+	+	+	+	+	+	+	+	+
FBGG-3	+	+	+	+	+	+	+	+	-	-
FBGG-4	+	+	+	+	+	+	+	+	+	-
FBGG-6	+	+	+	+	+	+	+	+	+	+
FBDHB-1	+	+	+	+	+	+	+	+	+	-
FBDHB-2	+	+	+	+	+	-	-	-	-	-
FBDHB-3	+	+	+	+	+	+	+	+	+	+
FBDHB-5	+	+	+	+	+	+	+	+	+	+
FBDHB-6	+	+	+	+	+	+	-	-	-	-
FBTHW-1	+	+	+	+	+	+	-	-	-	-
FBTHW-2	+	+	+	+	+	+	+	+	-	-
FBTHW-3	+	+	+	+	+	+	+	+	-	-
FBTHW-4	+	+	+	+	+	+	+	+	+	+
FBTHW-6	+	+	+	+	+	+	+	-	-	-
FBTB-1	+	+	+	+	+	+	-	-	-	-
FBTB-2	+	+	+	+	+	+	+	+	+	+
FBTB-4	+	+	+	+	+	+	+	-	-	-
FBTB-5	+	+	+	+	+	+	+	+	-	-

+ = for growth “ –“ = for no growth

Appendix A: Table 8. Effect of pH tolerance on *Rhizobium* isolates

Isolates Code	**pH Tolerance**								
	pH= 4.0	**pH=4.5**	**pH=5.0**	**pH=5.5**	**pH=8.0**	**pH=8.5**	**pH=9.0**	**pH=9.5**	**pH=10.0**
FBYT-1	-	+	+	+	+	+	+	+	-
FBYT-2	-	-	+	+	+	+	+	-	-
FBYT-3	+	+	+	+	+	+	+	+	+

FBYT-4	-	-	+	+	+	+	+	+	-
FBYT-5	+	+	+	+	+	+	+	-	-
FBYT-6	-	-	+	+	+	+	+	+	-
FBGG-1	-	-	+	+	+	+	+	-	-
FBGG-2	+	+	+	+	+	+	+	+	+
FBGG-3	-	-	+	+	+	+	+	+	-
FBGG-4	-	+	+	+	+	+	+	+	-
FBGG-6	+	+	+	+	+	+	+	-	-
FBDHB-1	-	-	+	+	+	+	+	-	-
FBDHB-2	-	+	+	+	+	+	+	+	-
FBDHB-3	+	+	+	+	+	+	+	+	-
FBDHB-5	-	-	+	+	+	+	+	+	+
FBDHB-6	-	+	+	+	+	+	+	+	-
FBTHW-1	-	-	+	+	+	+	+	+	-
FBTHW-2	+	+	+	+	+	+	+	-	-
FBTHW-3	-	-	+	+	+	+	+	+	-
FBTHW-4	-	+	+	+	+	+	+	+	+
FBTHW-6	+	+	+	+	+	+	+	+	-
FBTB-1	-	-	+	+	+	+	+	-	-
FBTB-2	-	+	+	+	+	+	+	+	-
FBTB-4	-	-	+	+	+	+	+	+	-
FBTB-5	-	+	+	+	+	+	+	+	-

“+” = for growth “–“ = for no growth

Appendix A: Table 9. Effect of temperature tolerance on *Rhizobium* isolates

Isolates	**Temperature (℃)**						
	5℃	**10℃**	**15℃**	**20℃**	**35℃**	**40℃**	**45℃**
FBYT-1	+	+	+	+	+	-	-
FBYT-2	-	-	+	+	+	-	-
FBYT-3	+	+	+	+	+	-	-

FBYT-4	-	+	+	+	+	-	-
FBYT-5	+	+	+	+	+	+	-
FBYT-6	+	+	+	+	+	-	-
FBGG-1	-	+	+	+	+	-	-
FBGG-2	+	+	+	+	+	+	-
FBGG-3	-	-	+	+	+	-	-
FBGG-4	+	+	+	+	+	-	-
FBGG-6	+	+	+	+	+	-	-
FBDHB-1	-	-	+	+	+	-	-
FBDHB-2	+	+	+	+	+	-	-
FBDHB-3	+	+	+	+	+	+	-
FBDHB-5	+	+	+	+	+	-	-
FBDHB-6	-	+	+	+	+	-	-
FBTHW-1	+	+	+	+	+	+	-
FBTHW-2	-	-	+	+	+	-	-
FBTHW-3	-	+	+	+	+	-	-
FBTHW-4	+	+	+	+	+	+	-
FBTHW-6	+	+	+	+	+	-	-
FBTB-1	+	+	+	+	+	-	-
FBTB-2	-	+	+	+	+	+	-
FBTB-4	+	+	+	+	+	-	-
FBTB-5	+	+	+	+	+	-	-

+ = for growth - = no growth

Appendix A: Table 10. Intrinsic Antibiotic Resistance of Rhizobial Isolates on different antibiotics with varying concentrations

Isolate	**Chloramphenicol (µg/ml)**			**Ampicillin (µg/ml)**			**Penicillin (µg/ml)**			**Kanamycin (µg/ml)**			**Nalidixic acid (µg/ml)**			**Streptomycin (µg/ml)**		
	2.5	**5**	**10**	**2.5**	**5**	**10**	**2.5**	**5**	**10**	**2.5**	**5**	**10**	**2.5**	**5**	**10**	**2.5**	**5**	**10**
FBYT-1	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBYT-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBYT-3	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBYT-4	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
FBYT-5	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBYT-6	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-
FBGG-1	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBGG-2	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBGG-3	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
FBGG-4	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-
FBGG-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBDHB-1	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
FBDHB-2	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-
FBDHB-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBDHB-5	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBDHB-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBTHW-1	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBTHW-2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
FBTHW-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBTHW-4	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBTHW-6	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-
FBTB-1	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
FBTB-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBTB-4	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-
FBTB-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Isolates no	23	23	23	25	25	25	25	23	22	10	10	10	23	23	23	16	16	16
%	92	92	92	100	100	100	100	92	88	40	40	40	92	92	92	64	64	64

+ = for growth - = no growth

Appendix A: Table 11. Carbon source utilization pattern of the rhizobial isolates

Isolates Code	**Carbohydrate utilization**									
	Glucose	**Fructose**	**Arabinose**	**Xylose**	**Sucrose**	**Lactose**	**Sorbitol**	**Maltose**	**Mannitol**	**Dextrose**
FBYT-1	+	+	+	+	+	+	-	+	+	+
FBYT-2	+	+	+	+	+	+	+	+	+	+
FBYT-3	+	+	-	+	+	+	+	+	+	+
FBYT-4	+	+	+	+	+	+	+	+	+	+
FBYT-5	+	+	+	+	+	+	+	+	+	+
FBYT-6	+	+	+	+	+	+	-	+	+	+
FBGG-1	+	+	+	+	+	+	+	+	+	+
FBGG-2	+	+	+	+	+	+	+	+	+	+
FBGG-3	+	+	+	+	+	+	-	+	+	+
FBGG-4	+	+	-	+	+	+	+	+	+	+
FBGG-6	+	+	+	+	+	+	+	+	+	+
FBDHB-1	+	+	+	+	+	+	-	+	+	+
FBDHB-2	+	+	+	+	+	-	+	+	+	+
FBDHB-3	+	+	+	+	+	+	+	+	+	+
FBDHB-5	+	+	-	+	+	+	+	+	+	+
FBDHB-6	+	+	+	+	+	+	+	+	+	+
FBTHW-1	+	+	+	+	+	-	+	+	+	+
FBTHW-2	+	+	+	+	+	+	+	+	+	+
FBTHW-3	+	+	+	+	+	+	+	+	+	+
FBTHW-4	+	+	+	+	+	+	-	+	+	+
FBTHW-6	+	+	+	+	+	+	+	+	+	+
FBTB-1	+	+	-	+	+	+	+	+	+	+
FBTB-2	+	+	+	+	+	+	+	+	+	+
FBTB-4	+	+	+	+	+	+	+	+	+	+

FBTB-5	+	+	+	+	+	-	+	+	+	+
Isolates no	25	25	21	25	25	22	20	25	25	25
%	100	100	84	100	100	88	80	100	100	100

+ = for growth - = for no growth

Appendix A: Table 12. Nitrogen source utilization pattern of the isolates

Isolation	**Amino acid utilization**							
	Alanine	**Histidine**	**Arginine**	**Asparatic acid**	**Glycine**	**Valine**	**Asparagine**	**Isoleucine**
FBYT-1	+	+	+	-	+	+	+	+
FBYT-2	+	+	+	+	+	+	+	+
FBYT-3	+	+	+	-	+	+	+	+
FBYT-4	+	+	-	-	+	+	+	+
FBYT-5	+	+	+	+	+	+	+	+
FBYT-6	-	+	+	-	+	+	+	+
FBGG-1	+	+	-	+	+	+	+	+
FBGG-2	+	+	+	-	+	+	+	+
FBGG-3	+	+	+	+	+	-	+	+
FBGG-4	+	+	+	+	+	+	+	+
FBGG-6	+	+	+	-	+	+	+	+
FBDHB-1	+	+	+	-	+	+	+	+
FBDHB-2	-	+	+	-	+	+	+	+
FBDHB-3	+	+	+	+	+	+	+	+
FBDHB-5	+	+	-	-	+	+	+	+
FBDHB-6	+	+	+	+	+	+	+	+
FBTHW-1	+	+	+	+	+	+	+	+
FBTHW-2	+	+	+	-	+	-	+	+
FBTHW-3	+	+	+	+	+	+	+	+
FBTHW-4	+	+	+	+	+	+	+	+
FBTHW-6	+	+	+	-	+	+	+	+

FBTB-1	+	+	+	-	+	-	+	+
FBTB-2	+	+	-	+	+	+	+	+
FBTB-4	+	+	+	-	+	+	+	+
FBTB-5	+	+	+	-	+	+	+	+

"+" = for growth "-" = for no growth

Appendix A: Table 13. Summary of Carbon and Nitrogen source utilization of faba bean nodulating rhizobial isolates

No	**Isolates code**	**C-source % utilized (10)**	**N-source % utilized (8)**
1	FBYT-1	90	87.5
2	FBYT-2	100	100
3	FBYT-3	90	87.5
4	FBYT-4	100	75
5	FBYT-5	100	100
6	FBYT-6	90	75
7	FBGG-1	100	87.5
8	FBGG-2	100	87.5
9	FBGG-3	90	87.5
10	FBGG-4	90	100
11	FBGG-6	100	87.5
12	FBDHB-1	90	87.5
13	FBDHB-2	90	75
14	FBDHB-3	100	100
15	FBDHB-5	90	75
16	FBDHB-6	100	100
17	FBTHW-1	90	100
18	FBTHW-2	100	75
19	FBTHW-3	100	100
20	FBTHW-4	90	100
21	FBTHW-6	100	87.5

22	FBTB-1	90	75
23	FBTB-2	100	87.5
24	FBTB-4	100	87.5
25	FBTB-5	90	87.5

Appendix B. List of representative photographs taken during soil sample collection, laboratory activities and greenhouse works

Appendix B. Figure 1: Some pictures taken during soil sample collection from Faba bean grown fields in Uraga Woreda, Guji Zone, Southeastern Ethiopia for nodule induction

Appendix B. Figure 2. Photos taken during laboratory activities