SYMBIOTIC EFFECTIVENESS OF RHIZOBIA FROM CHICKPEA (*Cicer arietinum* L.) AND, PHENOTYPIC AND SYMBIOTIC CHARACTERIZATION OF RHIZOBIA NODULATING FABA BEAN (*Vicia faba* L.) FROM SOUTHERN ETHIOPIA

M Sc THESIS

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SYMBIOTIC EFFECTIVENESS OF RHIZOBIA FROM CHICKPEA (*Cicer arietinum* L.) AND PHENOTYPIC CHARACTERSTICS OF FABA BEAN (*Vicia faba* L.) NODULATING RHIZOBIA

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Declaration

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

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DEDICATION

It is my great pleasure to dedicate this work to my lovely wife Aberash Tessema and cherished brother, Tagesse Ereso, for their invaluable helps, morally and financially during all my phase of study.

List of abbreviation

ANOVA	Analysis of varience
ASL	Above sea level
BTB	Bromthymolblue
BCP	Bromocrisolpurple
BNF	Biological nitrogen fixation
CR	Congo red
CRD	Complete randomized desine
CSP	Cold shock protein
DM	Dry mass
FAO	Food and agricultural organization
HSP	heat shock protein
ILRI	International live stock research institute
KDa	Killo dalton
MPN	Most peobable number
NDW	Nodule dry weight
NF	Nod factor
PGA	Pepton glucose agar
pH	Potential of hydrogen
RNS	Root nodule symbioses
SDW	Shoot dry weight
SE	Symbioses effectiveness
YMA	Yeast mannitol agar
YMB	Yeast mannitol broth

Table of Contents

Acknowledgments vi

DEDICATION vii

List of abbreviation viii

Abstract xiv

1. Introduction 15

  1.1. Objectives of the study 18

    1.1.1. General objective of the study 18

    1.1.2. Specific objectives of the study 18

2. Review of Related Literatures 19

  2.1. Legumes 19

    2.1.1. Chickpea 19

    2.1.2. Faba bean 20

  2.2. Nitrogen fertilizer and its role 22

  2.3. Biological nitrogen fixation in chickpea and faba bean 23

  2.4. Symbiotic organisms involved in biological nitrogen fixation 25

  2.5. Diversity of rhizobia nodulating chickpea and faba bean 25

    2.5.1. Chickpea rhizobia 25

    2.5.2. Fababean rhyizobia 26

  2.6. The nodulation process 26

  2.7. Specificity of rhizobium-legume symbiosis 27

  2.8. Inoculation experiences 28

  2.9. Different factors associated with symbionts interaction 28

    2.9.1. Soil pH as Limiting Factor for Biological Nitrogen Fixation 28

    2.9.2. Temperature and biological Nitrogen Fixation 30

    2.9.3. Soil water content and biological nitrogen fixation 31

    2.9.4. Salt Stress and Biological nitrogen fixation 33

    2.9.5. Soil fertility and biological nitrogen fixation 33

  2.10. Rhizobial nodulation and types of nodules formed 34

  2.11. Effectiveness of rhizobium inoculation 35

3. Materials and methods 37

  3.1. Description of the study areas 37

3.2. Biological materials used for study ... 38
3.3. Purification and activation of the test isolates ... 39
3.4. Screening for effectiveness of strains in modified Leonard Jars ... 39
3.5. Most probable number (MPN) for soil samples ... 41
3.6. Isolation of fababean nodulating rhizobia ... 41
3.7. Presumptive test ... 42
3.8. Gram reaction ... 42
3.9. Authentication test ... 43
3.10. Symbiotic effectiveness of the isolates ... 44
3.11. Phenotypic characterization of faba bean (*V. faba* L.) nodulating rhizobia ... 44
3.11.1. Colony morphology ... 44
3.11.2. Determination of mean generation time (MGT) ... 44
3.11.3. Acid and base production ... 44
3.11.4. Physiological characterization ... 45
3.12. Data Analysis ... 47
3.12.1. statistical analysis ... 47
3.12.2. Numerical Analysis ... 47
4. Results ... 48
4.1. Symbiotic effectiveness of rhizobia nodulating chickpea ... 48
4.1.1. Axenic sand culture experiment ... 48
4.1.2. Symbiotic effectiveness on pots filled with field sample soils ... 52
4.2. Phenotypic and symbiotic characterization of faba bean rhizobia ... 56
4.2.1. Presumptive test ... 56
4.2.2. Authentication test ... 57
4.2.3. Preliminary symbiotic effectiveness of faba bean isolates ... 57
4.3. Phenotypic characterization of fababean rhizobia ... 61
4.3.1. Mophological growth charactrristics ... 61
4.3.2. Numerical taxonomy ... 61
5. Disscusion ... 69
5.1. Chickpea strains symbiosis effectiveness test ... 69
5.2. Phenotypic and symbiotic characterization of faba bean rhizobia ... 72
6. Conclusions and recommendation ... 78

6.1. Conclusion 78
6.2. Recommendations 78
References 80
APPENDIXES 92

List of Tables

Table 1: Soil sample collection site description 37

Table 2: Description of sites from where faba bean rhizobia are isolated.................................... 37

Table 3: Comparison of chickpea nodulating rhizobia isolates (P<0.05)................................... 49

Table 4: The test strains effectiveness pearson correlation coefficient for chickpea plants grown on axenic sand culture.. 51

Table 5: Soil analyses test for pH, total N and MPN (Most Peobable Number) 52

Table 6: Mean distribution of NN, NDW, SDW, and STN at P<0.05 and SE% for three 55

Table 7: Pearson correlation coefficit for Bulgita Galbuge and Taba kebeles soil samples experiment.. 56

Table 8: Grouping of faba bean nodulating isolates based on symbioses effectiveness 58

Table 9: The effect of isolates on symbiosis effectiveness parameters and relative SE............... 59

Table 10: Pearson correlation coefficient comparison for nodule number, nodule and shoot dry weight... 60

Table 11: Physiological and Biochemical Characteristics of the Isolates within the Clusters 68

List of Figures

Figure 1: Leonard jars containing faba bea plants inoculated with persumtive isolates 43
Figure 2: The appearanceof nodules on chickpea inoculated with isolates ICRE18 and CA10... 48
Figure 3: Relative comparison of shoot phenotypes received KNO3 (N+), un-inoculated (N-) and inoculated.. 50
Figure 4: The effect of test strains on chickpea plants grown over field soil samples 52
Figure 5:Faba bean nodulating isolates test sample for PGA-BCP, YEMA-CR and YEMA-BTB 57
Figure 6: Dendrogram highlighting the phenotypic similarities among faba bean rhizobia (*Rhizobium leguminosarum*) from different parts of Ethiopia.. 62

Abstract

Chickpea (Cicer arietinum L.) and faba bean (Vicia faba L.) are the most important food legumes grown in Ethiopia. Their growth and yield are mainly affected by available soil nitrogen, while they symbiotically associate with soil bacteria known as rhizobia and fix atmospheric nitrogen to supplement their nitrogen requirements. However, effectiveness of this symbiosis depends on rhizobium strains, host legumes and other biotic and abiotic factors. seventeen rhizobial strains nodulating chickpea were evaluated for their symbiotic effectiveness to screen high nitrogen fixers and thirty-three faba bean nodulating isolates were characterized to determine their phenotypic and symbioti identity. For symbiotic effectiveness test total shoot nitrogen content was dertermined using Kejdhal method; phenotypic identity was determined following numerical taxonomic approach. There was a signinficant difference ($p<0.05$) in nodule number, nodule & shoot dry weights among rhizobia nodulating chickpea. The priliminary symbiotic effectiveness (PSE) of fababean isolates ranged from 10.65% to 130.5%.. Isolate FBM11 was found symbiotically effective than controls and reference strain 35. While in chickpea case strain CA10 was found to be the best at Damot Gale, Strain ICRE18 on the other hand was found to be the best effective at Eastbawawacho and Damotpullasa soils suggesting that inoculation is needed for these sites. Nodule dry weight significantly and strongly correlated with nodule number and shoot dry weight both in pot filled field soil samples and sterilized sand pot experiments, and plant total nitrogen was strongly correlated with shoot dry weight. Based on on the total nitrogen analysis,plants inoculated with ICRE18 EAL029 and CA10 strains were found to accumulate higher shoot nitrogen than plants inoculated with other tested strains. Faba bean isolates exhibited diverse and interesting features such as ability to grow on wide range of carbon and nitrogen sources, tolerance to higher and lower temperatures, salt concentration as high as 2.5%, pH ranging between 4.0 and 10.0 Most isolates were resistant to neomycin and novobiocin (5µg/ml). All of them failed to solublize phosphate and most were resistant to heavy metals. These showed that the tested isolates were fastidious in their physiological requirements, thus can be considered as enabling factors for isolates to grow and survive in diverse soil conditions. The numerical analysis revealed the existence of diversity among the test isolates and grouped them into three clusters (designated as Cluster I-III) and unclustered (designated as U) at 65% relative similarity. Ninteen of the isolates were clustered with reference strain 35. Taken together,the study identified diverse and better nitrogen fixing rhizobia in biobank. Based on the study findings it is recommend that further screening under field condition but also their identity using molecular methods should be determined in the future.

Keywords: Biological nitrogen fixation, Numerical taxonomy, Rhizobia, Symbiotic effectiveness

1. Introduction

Chickpea (*Cicer arietinum* L.*)* and faba bean (*Vicia faba* L.) are among the most important food legumes growing in different parts of Ethiopia. They are the most important sources of protein supplement for the majority of the population, and incorporated in various popular Ethiopian dishes (Asfaw Telaye *et al.,* 1994). They are provided as stew with steple food *Injera* and as snak (roasted) served during coeffee cermonies. Furthermore, they are used in infant food formulation and the crop byproducts are also used as a feed and fooder for livestocks.

Ethiopia is one of the largest chickpea and faba bean producing countries in Africa (FAOstat, 2004) with average production of 195,800 tons of chickpea; and 476,026 mt.of faba bean (FAOstat, 2010). Though it is a leading producer in Africa, majority of the production comes from smallholder farmers, who follows subsistent farming system (Smaling 1993; Voisin et al., 2007) with limited production capacity. In addition to this, many Ethiopian soils are deficient of major soil nutrient like nitrogen (N) (Wondewosen Tena *et al*., 2016). N deficiency can be corrected by application of nitrogen-fertilizer; however, excessive use of nitrogen fertilizer is potentially harmful to the environment (Chemining wa et al., 2012). Moreover, the cost of nitrogen fertilizer is not affordable to those smallholder farmers to increase crop yield on small plot of farm land.

Nitrogen, a vitally important plant nutrient (Frank *et al*., 2003), is the main factor that limits chickpea and faba bean production in Ethiopia. However, these legumes form an effective symbiotic association with a soil dewelling bacteria known as rhizobia and fix atmospheric nitrogen via biological nitrogen fixation (BNF) process in a plant root structure called nodules. The nitrogen fixed via BNF is mostly used for the plant growth and development. This accounts

to about 60% of the global nitrogen budget and is eco-friendly and cost effective (Zahran, 1999). The symbiotic association that forms BNF is affected by various factors (Zahran, 1999) such as the rhizobium strains, host legumes, biotic and abiotic soil factors.

The rhizobium strains, target of this study, are diverse in nature with symbiotic nitrogen fixation potential, distribution in soil and preferences to host plant. Ethiopia shares different agro-ecological locations accommodating growth of diversified legume plants (Smaling 1993; Voisin *et al*, 2007). This diversified legumes harbor a huge diversity of rhizobia that associate with them. Variations in legume distribution and diversity becomes a source of variation in diversity and distribution of rhizobia, which in turn impart a significant variation to their root nodule formation (nodulation) and nitrogen fixation potential. Several studies at molecular level showed that Ethiopian soils harbor diversity of Rhizobia (Endalkachew Wolde-Meskel *et al*., 2005; Tulu Degefu *et al*., 2013; Aregu Amsalu *et al*., 2013;). Similarily, different studies were conducted on rhizobial phenotypic characterization and symbiotic effectiveness (Desta Beyene and Angaw Tsige, 1986, 1988; Mamo and Asgelil Debabe, 1994; Zerihun Belay, 2006, Musa Adal, 2009; Muhammed Abdulbasit 2013 and Getahun Negash, 2015). The phenotypic and molecular diversity study as well as symbiotic effectiveness of rhizobia is important to screen potential nitrogen fixing strains for inoculum development, besides adding knowledge to the biology of rhizobia.

Chickpea and faba bean nodulating rhizobia are very diverse in Ethiopia as shown in several studies (Asgelil Debabe, 2000; Amanuel Gorfu *et al*., 2000; Ayneabeba Adamu *et al.,* 2001; Zerihun Belay, 2006; Muhammed Abdulbasit, 2013; Getahun Negash, 2015; and Wondiwosen Tena, 2016). Chickpea commonly associates with *Mesorhizobium species*: *M. cicer, M. mediterraneum, M. amorphae, M. haukuii, M. tianshenese, M. temperatum* (Alexandre *et al.,*

2009; Laranjo *et al.,* 2012; Maatallah *et al*., 2002), while faba bean associates with *Rhizobium leguminosarum symbiovar viceae* (Somasegaran and Hoben,1994). The symbiotic effectiveness of these strains vary in different soils and among the strains themselves due to their genetic make up. The diversity and symbiotic effectiveness study conducted on chickpea and faba bean is not well documented, properly screened for their efficiency and poorly applied in Ethiopian agriculture.

Very recently, N_2Africa-Ethiopia project strated widely introducing rhizobial inoculants to smallholder farms in Ethiopia with a production model of G_L(Legume Genotypes) x G_R (Rhizobium Genotypes) x E (Environment) x M (Agronomic Management) in legumes. The project focuses on chickpea and faba bean among other target legumes. However, for successful implementation of the the project it faced limitations in rhizobium genotypes or effective rhizobial elite strains. As part of this project, screening rhizobial strains and selecting elites among the pool of collections is a priotiy work. Moreover inoculating germinated chickpea and faba bean plats with effective rhizobial isolates and growing results in increased total plant biomass and soot total nitrogen was the proposed hypothesis.

Therefore, in collaboration with N2Africa-Ethiopia Project, this study was intended to screen and evaluate symbiotic effectiveness of rhizobial strains nodulating chickpea; and phenotypic and symbiotic characterization of newly isolated rhizobia nodulating faba bean.

1.1. Objectives of the study

1.1.1. General objective of the study

The general objective of the study was to evaluate symbiotic effectiveness of rhizobia nodulating chickpea and phenotypic and symbiotic characterization of rhizobia nodulating faba bean grown in Ethiopia.

1.1.2. Specific objectives of the study

Specific objectives of the study included:

1. Screening of symbiotically effective rhizobia nodulating chickpea
2. Evaluating effectiveness of the screened rhizobial strains by inoculating chickpea plants grown on soil samples from different sites..
3. Numerical taxonomy and symbiotically characterizing faba bean nodulating rhizobial isolates

2. Review of Related Literatures

2.1. Legumes

The *Fabaceae* (*Leguminosae*) *is* one of the largest and diverse families of plants with approximately 750 genera and 19400 species. It is divided into three closely related sub families, namely*: Papilionoideae, Caesalpinioideae* and *Mimosoideae*. The subfamily *Papilionoideae* is the most dominant sub family containing 12,000 species, including peas, beans, peanuts, chick peas, soybeans, clover, alfalfa, sweet pea, broom, and lupine (Menna *et al*., 2006).

Based on geographic distribution and climatic condition of the world food legumes (pulses) are categorized in to two groups, called cool season and warm season food legumes (Jayasundara et al,. 1988). The cool-season food legumes belong to three tribes, the Vicieae (*Lens, Pisum*, *Vicia faba, Lathyrus*), the recently separated tribe Cicereae (*Cicer)* and Genisteae (*Lupinus*) (Summerfield and Bunting, 1980; Hawtin and Hebblethwaite, 1983).

2.1.1. Chickpea

Chickpea (*Cicer arietinum* L.) belongs to genus *Cicer,* tribe Cicereae, family Fabaceae, and subfamily Papilionaceae. It originated in southeastern Turkey (Ladizinsky 1975). The name *Cicer* is of Latin origin, derived from the Greek word 'kikus' meaning force or strength. The word *arietinum* is also Latin, translated from the Greek *'krios',* another name for both ram and chickpea, an allusion to the shape of the seed which resembles the head of a ram (Aries) (Vander Maesen 1987).

Chickpea has been grown since ancient times and nowadays it is cultivated in the Mediterranean area, Middle East, in west Asia, Ethiopia and Mexico, among other countries. Despite being the third most widely grown crop legume in the world, there are only a few studies on chickpea

symbionts. Chickpea has traditionally been considered as a restrictive host for nodulation (Broughton & Perret, 1999). Chickpea *(Cicer arietinum L.)* is one of the most important food legumes grown in Ethiopia. The country is considered as one of the centers of secondary diversity for chickpea (Vander Maesen, 1987). Some wild species like *C. cuneatum*, known to grow in numerous regions (Dawit Taddesse *et al.*, 1994). Chickpea is largely cultivated between 1400 and 2300 meter above sea level where annual rainfall ranges from 700 to 2000 mm/yr. It is usually planted on heavy black clay soils with pH ranging from 6.4 to 7.9 (Geletu Bejiga, 1996). Chickpea seeds germinate at an optimum temperature (28-33°C) and moisture level in about 5-6 days.

Chickpea production manual for Souhtern Nations, Nationalities and Peoples region of Ethiopia shown from 1995-2010 report of central static office, that chickpea production area, productivity and production in Ethiopia increased by 60%, 100% and 200% respectively. The national average yield of chickpea was 1.7 t ha^{-1} which far below from potential yield of 4.5 t ha^{-1} (CSA, 2013).

Chickpea is rich in protein, complex carbohydrate, and fiber, while low in fat and cholesterol. Chickpea is valued for its nutritive seeds with high protein content, 25.3-28.9 %, (Hulse, 1991). Most of the chickpea production is used for domestic consumption. However, there has been a substantial export of chickpea by Ethiopia during the past five years, with the highest of 48,549 tons accounting to value of US$14.7 million during 2002 (FAOstat, 2004).

2.1.2. Faba bean

Faba bean (Vicia faba) is an ancient crop. Plant traces of this species have been associated with early crop domestication in archaeological deposits of the Stone, Bronze and Iron Ages in the

Near East and Mediterranean basin: in Spain, Italy, France, Switzerland and Germany. The name faba originates from one of the forms of the Greek verb “wagev” “to eat” – which highlights its use for food and feed by the ancient Greeks and Romans (Muratova, 1931). Despite numerous studies, little is known of the origin and domestication of faba bean (Maxted et al., 1991). Cubero (1973, 1974) postulated the near East as its center of origin, with four different routes radiating from this center: (1) to Europe; (2) along the north African coast to Spain; (3) along the Nile to Ethiopia; (4) from Mesopotamia to India. Secondary centers of diversity are postulated to have occurred in Afghanistan and Ethiopia. However, Ladizinsky (1975) reported the origin to be in central Asia. According to Muratova (1931) and Maxted (1995) the center of origin for the genus Vicia is southeastern Europe and southwestern Asia. Fababean was introduced to Ethiopia soon after domestication around the 5th millennium B.C (Bond, 1976). Hence the country together with Afghanistan is considered as secondary center of diversity (Bond, 1976). The oldest seeds of faba bean were found in Jericho at 6250 B.C. (McVicar et al., 2005). Favabean is grown in different agro ecology of Ethiopia that covers temperate or warm area (‘Woynadega’) and cold area (‘Dega’), with an altitude ranging from 1800-2200 asl and 2200-3000 asl; and 700-900mm and 800-1100mm annual rainfall (Biruk Bereda, 2009).

Faba bean (*Vicia faba*) is belonging to tribe Vicieae. Herbs with pinnate leaves and grows upright, ranging from 1-1.5 meters tall (Rendle, 1979). It is an annual legume with one or more strong, hollow and erect stems. Faba bean has a strong taproot, compound leaves, and large, white flowers with dark purple marking. A flower cluster may produce 1-4 pods. The pods are large (up to 10 cm long and 1-2 cm wide) and green, turning dark at maturity. Three to four oblong/oval seeds are contained within each pod. Flowering occurs in 45-60 days and requires110-130 days to mature (McVicar *et al.,* 2005).

Faba bean contain high protein and appreciable amount of minerals and vitamins (Senayt and Asrat, 1994). China is the largest faba bean producer (40.36%) with an average dry grain production (2005–2009) of 1,720,000 metric tonnes (mt) from 945,400 hectares; followed by Ethiopia (476,026 mt), France (331,122 mt), Egypt (274,040 mt) and Australia (196,800 mt) (FAO stst: 2010). Ethiopia is the world's secod largest producer next to china, about 40.5% within Africa and 6.96% in the world (Winch, 2006). Its coverage in the country is 0.54 million hactars and the total production 696 million kilograms (MoARD, 2009). Faba bean productivity in Ethiopa is 15.2 quintals ha^{-1}(CSA, 2011).The average yield of faba bean under small-holder farmers is not more than1.6 t ha^{-1} (CSA, 2013). It is an important source of protein supplement for the majority of the population, and incorporated in various popular Ethiopia dishes (Asfaw Telaye *et al.,*1994*).* Moreover, it provides large cash for producers and foreign exchange for the country (Desta Beyene, 1988).

2.2. Nitrogen fertilizer and its role

Nitrogen fertilizer is available in both organic (manure) and inorganic forms. The amount of nitrogen in organic sources varies with source material and its state of decomposition. However, for commercial crop production, the following inorganic fertilizers are primarily used: ammonium nitrate (33.5%N), potassium nitrate (13%N), Sodium nitrate (16%N), calcium nitrate (15.5%N), urea (46%N), mono-ammoniumphosphate (18%N), di-ammonium phosphate (46%N) and liquid nitrogen (30%N). Legume crops require little or no nitrogen fertilizer. Beneficial bacteria that live in the roots of these plants capture nitrogen from the atmosphere. This nitrogen is available for use by the plant (Tilman, 1986).

2.3. Biological nitrogen fixation in chickpea and faba bean

Biological nitrogen fixation is the conversion of atmospheric N_2 gas, to ammonia (NH_3) that plants can use to make essential organic molecules such as amino acids and nucleotides (Moat and Foster, 1988). The capability of biological fixation of atmospheric nitrogen (N_2) is restricted to organisms with prokaryotic cell structure namely: bacteria and Archae. Some species in 11 of the 47 bacterial families are diazotrophs, i.e. capable of N_2 fixation (Peoples and Craswell, 1992).

The biological nitrogen fixation (BNF) is a natural process of rhizobial inoculants production. The rhizobial inoculant is a cheap and non polluting source of bacteria that can provide high rates of nitrogen to the legume, and the use of improved inoculants is a strategy to increase BNF rates in agro-ecosystems (Deaker et al., 2004; Araujo *et al*., 2011).The atmosphere contains about 10^{15} tones of N_2 gas, and the nitrogen cycle involves the transformation of $3x10^9$ tones of N_2 per year on global bases (Posgate, 1982). However, N- fixation is not exclusively biological, lightning probably accounts for about 10% of the world's supply of fixed nitrogen (Sprent and Sprent, 1990). FAO, (1990) reported that, world production of fixed nitrogen in the form of chemical fertilizers accounts for about 25% of the earth's newly fixed nitrogen, and biological processes accounts for about 60%.

Rhizobial nitrogen fixation is not uniform for all host symbionants, due to host specificity, rhizobial strains, and other abiotic factors. According to Somasegaran and Hoven (1994), the amount of nitrogen fixed (Kg ha^{-1}) by fava bean have been 240-325. Rizk (1966), shown in his study that, the estimated average amount of nitrogen fixed by chickpea in Egyptian condition is about 97 kg ha^{-1}, while it is 135kg $ha^{-1}$for faba bean. Chickpea and *Rhizobium leguminosarum*

sub sp.ciceri association annually produce up to 176 kg N ha^{-1}depending on cultivar, bacterial strain, and environmental factors (Rupela and Saxena, 1987; Beck *et al.,* 1991).

In all N_2 fixing microorganisms the principle and steps of this reaction are the same. The key enzyme complex, referred to as *Nitrogenase*, is unique to N_2-fixing microorganisms (Thornely, 1992) Nitrogenase consists of two proteins in the ratio of 2:1 azoferredoxin and molybdoferredoxin. A common property of all nitrogenase preparation is their sensitivity towards oxygen. The enzyme is irreversibly inactivated by oxygen so that nitrogen fixation can be regarded as a strictly anaerobic process (Smith, 1999).

Biological reduction of N2 to NH3 is also a highly endergonic process with a minimum energy requirement of Ca.960 KJ mol-1 N- fixed (Sprent and Raven, 1985). For the nitrogenase reaction, energy in the form of ATP and reducing equivalents (electrons) are required, supplied by respiration (ATP) and electron carriers, usually ferrodoxin. Nitrogenase catalyzes the reduction of several substrates, including H+, N2, and C_2H_2. The principal reaction for dinitrogen reaction is as follows:

$$N_2+8H+8E-+16Mg.ATP \longrightarrow 2NH_3+H_2+16\ Mg.ADP+16Pi$$

The N derived from the biological reaction is obtained in the form of ammonia. The predominant assimilation pathway for ammonia is a two-step process. Glutamine synthetase (GS) adds ammonium to glutamic acid to form glutamine with concomitant hydrolysis of ATP. This glutamine is then used by glutamine-2-oxoglutamate-amino-transfrase (glutamate synthetase or GOGAT) to aminate two molecules of 2-oxaloglutaric acids, with the production of two molecules of glutamate (Lea *et al.,* 1990).

2.4. Symbiotic organisms involved in biological nitrogen fixation

Organisms live together by association to common benefit or benefit of either in symbiosis. This mutual association is common in rhizobial bacteria and leguminous plants. Tulu *et al*., (2013), have discussed abut different rhizobia genera together with study of novel *Mesorhizobium* species and showed different rhizobial species found in Ethiopia and neighboring African countries, like Kenya and Sudan. Until the early 1980s, all symbiotic nitrogen fixing bacteria from leguminous plants were classified in the single genus *Rhizobium.* Six species were identified in to *R.leguminosarum, R. meleloti, R. trifolii, R. phaseoli, R. lupine* and *R. japonicum* based on their cross-inoculation groups with pea, alfalfa, clover, bean, lotus, and soybean, respectivelsy (Zerihun, 2006). *Rhizobia* are nitrogen fixing bacteria that form root nodules on legume plants. Most of these bacterial species are in *rhizobiacae* family in the *alpha proteobacteria* and are in either, the *Rhizobium, Mesorhizobium, Ensifier, or Bradyrhizobium* (Ramire-Bahena *et al*., 2008).

2.5. Diversity of rhizobia nodulating chickpea and faba bean

2.5.1. Chickpea rhizobia

Rhizobial legume symbiotic association is not uniform due to rhizobial species, type of legume and other factors related to soil and climatic condition. Legumes are able to establish nitrogen-fixing symbioses with bacterial microsymbionts (rhizobia), thus reducing the need for chemical fertilizers. This association further provides a nitrogen supplement for the subsequent crops (Zahran, 1999). Chickpea (*Cicer arietinum* L.) is the third most important legume crop worldwide, after dry bean and pea (FAO. 2003). Rhizobia that nodulate chickpea was described for the first time by Cadahía and colleagues (1986). Later, Jarvis and coworkers (1997) included them in the genus *Mesorhizobium*. Two species were first identified as specific chickpea

microsymbionts, these are called *Mesorhizobium ciceri* (Nour, *et al.,.1994*) and *Mesorhizobium mediterraneum* (Nour, *et al., 1995*). The promiscuity of a given legume is related to the number of Nod factors it can interact with, rather than the diversity of rhizobia, which are able to nodulate such legume (Downie, 1998).

Recent studies have shown that chickpea is able to establish symbioses with several species of mesorhizobium, namely *M. amorphe*, *M. loti*, and *M. tianshanense*; however, which may lead to the production of similar Nod-factors (Laranjo, *et al.,* (2002), Rivas*, et al.,* (2007). Moreover in the current taxonomic perspectives of rhizobia shown that diversiy of rhizobia nodulating chickpea include *M. loti, M. ciceri, M. muleiense* and *M.mediterranum* (Berrada and Fikri-Benbrahim, 2014).

2.5.2. Fababean rhyizobia

According to Spaink *et al., (*1989) *R. leguminosarum* bv. *viciae* have a very broad host range such as peas, vetches (*Vicia* and *Lathyrus*) and lentils. Faba bean is a legume capable of fixing nitrogen in an endo-symbiotic association with root nodule bacteria: *Rhizobium leguminosarum symbio* var. *viceae.* It is the most efficient nitrogen fixer of the pulse crops grown (McVicar *et al.*, 2005). Acording to Berrada and Fikri-Benbrahim, (2014), rhizobia nodulating faba bean include *R. fabae* that nodulate faba bean and *R.leguminosarum* Symbiobar viciae nodulating Pisum, Lens, Viciae and Lathyrus.

2.6. The nodulation process

The rhizobial symbiosis with legumes is generally a specialized affair, both partners having narrow host ranges. Exceptions include Rhizobium sp. NGR234, which nodulates over 110

genera of legumes (Pueppke & Broughton, 1999) and *Phaseolus vulgaris* L. or the common bean plant, which is nodulated by at least 20 species of rhizobia (Michiels et al., 1998).

The outcome of the interaction is dependent on an elaborate signal exchange that continues throughout the entire symbiotic process and has been likened to matching locks and keys (Broughton et al., 2000), with only the correct combination giving rise to efficient symbiosis. A type of flavonoids and lectins secreted from the host plant to rhizosphere, and different rhizobial secretions enable their interaction. The first secreted rhizobial protein for which a role in symbiosis could be shown was (*Rhizobium leguminosarum* Symbiovar. viciae) NodO (de Maagd et al., 1989b).

2.7. Specificity of rhizobium-legume symbiosis

The specific genes on the bacterial chromosomes that codes for proteins involved in recognition and uptake of specific signal molecules present in root exudates governs specificity of Rhizobium-Legume symbioses. These root exudates contain chemical attractants such as flavonoids and betains, secreted by the roots (Barbour *et al*., 1991; Krishanan and Pueppke, 1993).

These compounds stimulate the expression of a set of *Rhizobium* genes; the nod genes (Heidstra and Bisseling, 1996). The nod genes encode approximately 25 proteins required for bacterial synthesis and export of nod factor. Nod factor is a lipooligosaccharide signal consisting of a chitin backbone, four to five N-acetyl-glucosamine units in length, with a lipid attached to non reducing end and host specific modification on the back bone (Gage, 2004). In response to nod factors, many of the developmental changes seen in the host plant early in the nodulation process including root hair deformation to establish a meristem and nodule prmordeium. Rhizobia

continue to differentiate inside the nodule and synthesize proteins required for nitrogen fixation and for the maintenance of the mutualistic partnership (Gage, 2004).

2.8. Inoculation experiences

Some of the chickpea inoculants in use at national level in Ethiopia includes strain Cp41, CPEAL029 and CpNSTC (Wodewosen Tena, *et al., 2016*). On the other hand FB1535 from National Soil Testing Centre (NSTS) andstrain 35 (comertial strain) from Holota Agricultural Research Institute are avilable commertial inoculants of fababean in use. The use of Cp41 for chickpea inoculation improved grain yield from 1.2 t ha^{-1} to 2.5 t ha^{-1} at Choroko and 1.8 t ha^{-1} to2.5 t ha^{-1} at Taba (Wondewosen Tena *et al*., 2016).

2.9. Different factors associated with symbionts interaction

.The existence of competent micro symbiont and appropriate host cannot lead to effective biological nitrogen fixation, because there are other factors limiting such activities. Fluctuations in pH, nutrient availability, and temperature and water status, among other factors greatly influence the growth, survival, and metabolic activity of nitrogen fixing bacteria and plants, and their ability to enter into symbiotic interactions (Werner and Newton, 2005).

2.9.1. Soil pH as Limiting Factor for Biological Nitrogen Fixation

The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan 1984), and relatively few rhizobia grow well at pH Less than 5.0. The fast growing strains of rhizobia have generally been considered less tolerant to acid pH than have slow-growing strains of *Bradyrhizobium,* but low pH tolerant strains exist in many species(Graham *et al.,* 1994). Although slow growing bacteria are in general more tolerant to low soil P^H than fast-growers, strain to strain difference exists. *Rhizobium meliloti* is particularly sensitive to acid conditions.

Some slow growing rhizobia native to acid soils are acid requiring and grow at approximately P^H 4.5 (Eaglesham and Aynaba 1984).

In general, low soil pH is often associated with increased Al and Mn toxicity, reduced calcium (Ca) supply, and phosphorus (P) and molybdenum (Mo) deficiencies (Hungria and Vargas, 2000). These additional stresses affect the growth of rhizobia, of the host legume and symbiosis. The effect on symbiosis is evident from the fact that, nodulated legumes are more sensitive to Al and Mn toxicity than plants receiving mineral N (Hungaria and Vargas, 2000). The number of nodules,the nitrogenase activity, the nodule ultra-structure and the fresh and dry weights of nodules are affected to a greater extent at a low medium pH (< 4.5) (Vassileva *et al.,* 1997).
Tolerance to high alkalinity has also been observed for rhizobial strain isolates with tolerance up to pH 9.5 for rhizobiaceae (Jordan, 1984). Nour *et al*. (1994) found the pH tolerance of rhizobial strains isolated from chickpea (*Cicer arietinum*) to be 10.0. Rhizobia appear to be more tolerant to alkalinity than do their legume plants (Zahran, 1999). Rao and Sharama (1995) reported that while germination of pigeon pea was decreased at pH values of > 8.8, growth of rhizobia was unaffected up to pH 11.5.
Rhizobia with a higher tolerance to acidity have been identified (Graham *et al*., 1982). Aarons and Graham (1991), reported high cytoplasmic potassium and glutamate levels in acid-stressed cells of *Rhizobium leguminosarum* are linked with low pH tolerance. Furthermore, differences in LPS composition, proton exclusion and extrusion (Chen *et al*.; 1993), accumulation of cellular polyamines (Fujihara and Yoneyama, 1993), and synthesis of acid shock proteins (Hickey and Hirshfield, 1990) have been associated with the growth of cells at acid pH.

The composition and structure of outer membrane could also be a factor in pH tolerance (Graham *et al*., 1994). At least two loci of either megaplasmid or chromosomal location for pH genes are necessary for the growth of rhizobia at low pH (Chen *et al*., 1993). The expressions of

one membrane proteins of 49.5 KDa and three soluble proteins of 66.0, 85.0, and 44.0 KDa increased when the cells of acid-tolerant strains were grown at pH 4.0 (Correa and Barneix,1997). Soil acidity can be corrected by different practicalmeasures. Two strategies that have been adopted to solve the problem of soil acidity are liming the acidic soil to ameliorate the effects of acidic conditions and selecting and isolating tolerant strains of rhizobia and plant cultivars to increase plant production on acidic soils (Graham, 1992; Bordeleau and Prevost, 1994; Zahran, 1999).

2.9.2. Temperature and biological Nitrogen Fixation

Among factors equivalently necessary to rhizobium growth is temperature, and it has a marked influence on survival and persistence of rhizobial strains in soils. Rhizobia are mesophiles and most have a poor growth at temperature below 10^0C or above 37^0C (Graham, 1992). Although response to temperature is strains dependent, rhizobia are found to tolerate from 4 - 42.4 ^{0}C. However, growth at 4^0C is rare, and only *R. meliloti* can grow at 42.5^0C. *R. leguminosarum* isolates from lentil plants in Southern Nile Valley of Egypt were tolerant to 35 to 40^0C inducing less effective symbiosis with their legume host (Bordeleau and Prevost, 1994). In arid regions, high soil temperature affects lives of both free and symbiotic rhizobia (Zahran, 1999).

Most rhizobia have an optimum growth temperature at 28-31°C and many of them are unable to grow at 38°C (Graham, 1992). Sudden temperature change cause formation of heat shock protein (HSP) (Yura et al., 2000), and cold shock protein (CSP) (Cloutier et al., 1992) that have positive

impact on resistance of higher and lower temperature in order to grow. These opened the way for the appearance of novel strains, because of special proteins formation through temperature change stress.

Soil temperature may increase at seeding time, during the period of plant growth and following the harvest; in each instance, the high temperature may be deleterious. The period after harvest should not be ignored, because survival of the bacteria at that time is important. In tropical and sub tropical regions, the temperature near the soil surface is often above 40°c and sometimes may reach 60°c (Alexander, 1985). On the other hand the raise in soil temperature results in increase of root temperature. The infection of the root hairs, the rate of nodule appearance, and the number of nodules formed, the amount and rate of nitrogen fixation, and the distribution of fixed nitrogen from the nodules are affected by root temperature (Gibson, 1975). Root temperature effects on legume nodulation and N_2 fixation are subject matter under consideration to choice of time of sowing of winter-grown legumes. Optimum temperatures for nodulation are often higher than for N_2 fixation (Gibson, 1971). Furthermore, high temperature accelerates nodule senescence (Hungria and Franco, 1993). The remedy is administration of inoculum in deeper soils and application of surface mulch to reduce soil temperature.. Likewise, it was also found that low temperature decrease nodulation and nitrogen fixation (Waughman, 1977).

2.9.3. Soil water content and biological nitrogen fixation

As one moves from the equator, the seasonal variation in temperature as well as the length and severity of the dry season are greater (Eaglesham and Aynaba, 1984). Distribution of the rain during the wet season can sometimes be uneven resulting in the surface layers of the soil becoming desiccated during crop growth. On the other hand soil water influences the growth of soil microorganisms through the processes of diffusion, mass flow and nutrient concentration

(Mohammadi *et al.,* 2012). Water stress reduces both N2 fixation and respiration of nodules by influencing legumes to produce short and stubby roots which are not suitable for infection by rhizobia(Zahran *et al*., 1994). The effect of water stress on vegetative growth is more detrimental to nodulation and nitrogen fixation than during reproduction stage (Zahran, 1999). Immediate morphological change manifests due to water stress (low water potential), and such a rhizobial cell modification will eventually lead to a reduction in infection and nodulation of legumes (Zahran *et al*., 1994).

Rhizobia have evolved a variety of mechanisms for adapting to osmotic stress, mostly by the intracellular accumulation of inorganic and organic solutes. For example, *R. meliloti* overcomes osmotic stress-induced growth inhibition by accumulating compatible solutes, such as K+, glutamate, proline, glycine betaine, proline betaine, trehalose, and the di-peptide, N-acetyl glutaminyl glutamine amide (Zahran, 1999).

As stated by Sprent (1971), fast-growing *rhizobia* are sensitive to soil dehydration as compared to slow-growing strains. In a silt loam soil two distinct phases of decline were observed with seven diverse types of slow and fast growing rhizobia (Sprent, 1971). The first phase occurred during the loss of water from the soil, when bacterial fell rapidly and extensively at exponential rate. The second phase is a much slower and linear decline in numbers after the soil had reached a constant moisture level or the dry state (Sprent, 1971). From these points of view one should give attention for the level of water content in the soil before applying inoculants as a nitrogen sources in agricultural process.

2.9.4. Salt Stress and Biological nitrogen fixation

Salt stress affects the symbiotic process established between rhizobia and legume (Zahran, 1999). Optimal amount of salt is required by symbotic and free living bacteria which are able to fix nitrogen for their normal osmotic activity. Salinity 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 (Cordovilla *et al.,* 1994). Raising salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate, 1995).

Rhizobial cells have various mechanisms for adapting high salt-stress conditions. Rhizobial adaptations to salt-stress are attributed to the accumulation of intracellular low molecular weight organic solvents (Osmolytes) such as glutamate, proline, glycinebetaine, glycine amino acids, ectoine, amide, polyoles called osmotically active solutes (Zahran, 1999).

Successful *Rhizobium*-legume symbiosis under salt stress requires the selection of salt tolerant rhizobia from the indigenous population (Zahran, 1991). It was also suggested that a need to select plant genotype that is tolerant to salt stress and then match them with the salt tolerant and effective strain of rhizobia (Cordovilla *et al*., 1995).

2.9.5. Soil fertility and biological nitrogen fixation

Soil is taken as the natural store for accumulation of different naturally available nutrients, which are formed through synthesis and decomposition reaction and other additives, such as industrial fertilizers. Excess or lower accumulation of different nutrient in the soil has its effect on biological nitrogen fixation. The legume-rhizobia symbiosis imposes additional nutritional requirements apart from minerals need for plant growth, as whole. Nutrient that affect symbiotic

nitrogen fixation include; $NO3^-$, N, P, B, Zn, S, Molybdenum (Mo) and cobalt (Serraj and Adu-Gyamfi, 2004).

2.10. Rhizobial nodulation and types of nodules formed

Nodulation is a process of complex interactions between the partners. First of all the molecular mechanisms of regulations between the host plants and the bacteria symbionants are considered as a form of cell-to-cell inter-organismal communication. A precise exchange of molecular signals between the host plant and rhizobia over space and time is essential for the development of effective root nodules. The first appearance of exchange of signals is involved in secretion of phenolic and/or isoflavinoid, by host plants (Subramanian *et al*. 2006). The specific plant exudates activate the nod gene expression of rhizobia mediated by the nod D regulatory gene product (Peters et al., 1996).

Rhizobia use the molybdenum-nitrogenase for nitrogen fixation, however the number of *nif* genes found in different species is variable. For example, the *Azorhizobium caulinodans* genome comprises a total 15 different *nif* genes, while *Rhizobium leguminosarum* bv. *viciae* holds the lowest number of *nif* genes found in rhizobia (a total of 8 *nif* genes) (Yang *et al.*, 2007).

Two major morphological types of nodules exist in legumes: determinate and indeterminate and type of nodule is determined by the host plant. Ferguson (2010), shown nodulation as: Emerging root hairs exude flavonoid compounds, which attract compatible rhizobia and stimulate them to produce nod factors (NF). The root hair deforms and forms a pocket, in which the rhizobia become entrapped. Infection thread structures initiate in the pocket enabling the rhizobia to enter the plant. Cell divisions are first observed in the inner cortex for indeterminate nodules or the

sub-epidermal cell layer for determinate nodules. Additional cell layers later divide leading to the formation of the nodule primordium.

The infection threads progress towards this primordium and release the rhizobia into infection droplets, in which they differentiate into nitrogen-fixing bacteroids. At the top of the primordium of indeterminate nodules, a meristem develops that continually gives rise to new cells. As these new cells mature, many subsequently become infected, leading to successive zones of rhizobia invasion and differentiation within the nodule. At maturity, indeterminate nodules contain a heterogeneous population of nitrogen-fixing bacteroidsdue to continued cell division activity, giving rise to a gradientof developmental states as the nodule continues to elongate. These nodules also have a different, less branched vascular system than determinate nodules. In contrast, determinate nodules do not develop a persistent meristem and hence their invaded cells are all at a similar developmental phase.

2.11. Effectiveness of rhizobium inoculation

According to Howieson *et al.* (2000), one of the current techniques used to select appropriate strain of root nodule bacteria require screening for effective nitrogen fixation. Rhizbial nodulation efficiency results in development of nodules with sufficient Leg- haemoglobin as well as increased number of shoot dry weight and high shoot nitrogen content. For example inoculation of different chickpea cultivars with highly efficient strains seems to result in nodules with leg-haemoglobin and high shoot dry weight (Ben Romdhane *et al.*, 2008). In addition, inoculation with multiple strains may further increase the total nitrogen content (Içgen *et al.*, 2002). Even for the biocontrol of plant parasites using plant growth promoting bacteria, rhizobia inoculation represents an advantage for chickpea crops (Siddiqui & Akhtar, 2009).

Nodulation effectiveness is not sol result of rhizobibium strain's nitrogen fixing ability. There are other factors included to nodulating activity. For instance rhizobia inoculations together with phosphorus solubilizing bacteria, such as *Bacillus subtilis* or *Bacillus megaterium*, could be a good alternative to both nitrogen (N) and phosphorus (P) fertilizers in chickpea crops (Rudresh *et al.*, 2005, Elkoca *et al.*, 2008). On the other hand, rhizobia inoculation seems to activate chickpea genes involved with the production of phenolic compounds/phytoalexins and higher levels of these compounds benefice the plant by restricting disease development (Arfaoui *et al.*, 2007). This rhizobial protection was previously reported as effective on chickpea for the deleterious effects of Fusarium wilt (Arfaoui *et al.*, 2006).

3. Materials and methods

3.1. Description of the study areas

Damotpulasa, Damotgale and East Badawacho Woredas growing chickpea in SNNPR, Ethiopia were selected for soil sample collections. The agronomic description of each sites is given below in Table 1.

Bule, Dalle, East Badawacho, Hangacha woredas and Ada'aa (Dhibaayu Kebele) sites were selected for faba bean rhizobia isolation and their agronomic description is given in table 2. The experiments were conducted at Hawassa University, Soil Microbiology Laboratory.

Table 1: Soil sample collection site description

Zone	Woreda	Longitude	Latitude	Altitude	History of inoculation
Hadiya	East Badawacho KebeleBulgita	07^0 11' 62.4"N	37^0 93' 85.5"E	1923m	no
Wolayta	Damotpulasa KebeleGalebuge	07^0 05' 15.5"N	037^0 55' 41.8"E	1892m	no
Wolayta	Damotgale KebeleTaba	07^0 01' 40.2"N	037^0 90' 75.2"E	1905m	no

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

The soil samples collected for chickpea effectiveness test were three samples for chosen study site, each about 21kg from boarder linked cultivated lands at rainy season with no inoculation history. The soil samples were collected from depth of 20cm as indicated in Somasegaran and Hoben, (1994).

Table 2: Description of sites from where faba bean rhizobia are isolated

Zone	Woreda	Longitude	Latitude	Altitude	Isolate code
Kambatatambaro	Hangacha	07^0 20' 51.2"N	037^0 51'52.0"E	2280m	FBH

Kambatatambaro	Kacchabira	$07^0$15'52"N	037^0 44'35"E	2324m	KB1,KB2,KB3
Hadiya	East Badawacho	$07^0$11'29.8"N	037^0 57'28.1"E	2010m	FBM and FBA
Gedeo	Bule	06' 15.604	038'23.620	2842.57m	FBGE
Sidama	Dale	06'41.342	038'28.934	2096.1m	FBSD
Wolayta	Kindokoysha	6^0 53'44"N	$037^0$52'10"E	2043.8m	KK
East Showa	Ada'aa	$08^0$44'0" N	$38^0$58'0" E	2320m	FBD

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

3.2. Biological materials used for study

Fifteen rhizobial strains nodulating chickpea such as CA10, Cp1, Cp26, Cp67, Cp68, Cp98, Cp99, Cp129, Cp130, Cp151, ICRE18, ICRE25b, ICNRE03, M4b and M7 were obtained from Hawassa University, College of Agriculture, Soil Microbiology Laboratory and EAL029 and CPM20b chickpea strains were taken from International Livestock Research Institute (IlRI) at Addis Ababa as reference strains.

Faba bean nodules were collected from faba bean grown on farmers plot of study area. During nodules collection a healthy faba bean plants were chosen and dug up at the radius of approximately 15cm radius and 20cm depth. The soil removed by caring all roots and nodules. Pink coloured nodules (nodules with leghemoglobin) stored in vials containing silicagel as recommended in Somsegaran and Hoben, (1994). moreover faba bea nodulating strain 35 was included as a reference to compare new isolates characteristics.

Chickpea cultivar "*Natoli*" was obtained from Debrezeit agricultural research centre in order to test strains effectiveness as standard productive variety. Faba bean cultivar, "*Tumsa*" from Holota Agricultural Research Institute was obtained as highland avilable seed variety to test isolates nodulation response during reinfection.

3.3. Purification and activation of the test isolates

Yeast Extract Mannitol Agar (YEMA) containing 10g D-Mannitol, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 0.5g yeast extract and 15g Agar powder in a liter of distilled water was prepared. pH of the solution was adjusted to 6.8 before autoclaving at 121^0c for 15 min. at 15 lb as described elsewhere (Vincent, 1970; Somersagan and Hoben, 1994). Then strains from -20% grlycerol stock were thawed and serially streaked on the medium to activate them.

3.4. Screening for effectiveness of strains in modified Leonard Jars

Modified Leonard Jars were prepared according to Somarsegran and Hoven (1994). These were prepared from plastic cups filled with: acid (H_2SO_4 or HCl) soaked and thoroughly washed with tap water, sun dried and autoclave sterilized river sand at (121°C temperature for 15minnutes using steam and high pressure). Chickpea and faba bean seeds were filled 25% of 250ml sterilized Erlenmeyer flask and surface sterilized using and 96% ethanol for 10 seconds and 3.5% Sodium hypochlorite for 4 minutes, and rinsed in six changes of sterile water. The surface sterilized seeds were germinated on 1% water agar as shown in Somasegaran and Hobben (1994). The germinated seeds were aseptically transplanted into Leonard Jars. 1ml of the strains broth culture at their logarismic growth phase (10^9cells ml^{-1}) or turbed (milky broth) was inoculated to the base of the seedlings growing in the jars in triplicates (Somesegran and Hobben, 1994). The positive controls and negative controls were included.

All the inoculated seedlings and the controls were weekly supported with Jensons N-free nutrient medium. The negative controls were the seedlings that were not inoculated with any rhizobial strain and only supplied with Jensons N-free nutrient medium consisting (gl^{-1}) of water $CaHPO_4$ 1.0g, K_2HPO_4 0.2g, $MgSO_4$ 0.2g, NaCl 0.2g, 1ml trace elements stock solution and $FeCl_3$ 0.1gwith adjusted pH value 6.8 and autoclaved at 121°c for 20min) (Roughley 1984). while

positive controls were the seedlings that were not inoculated with rhizobial strains, but received both 0.05% KNO_3^- and Jensons N-free nutrient medium. The Leonard jars were arranged in complete randomized design (CRD) with three replications.

After growing the seedlings in greenhouse for 45days, the seedlings were carefully up rooted and investigated for nodulation and nitrogen fixation, and NN, NFW, SFW and SFW were recoded carefully. Shoot and nodule dry weightes were obtained by oven drying them at 70°c for 24hrs. Nitrogen fixation during nodulation was checked by observing presence of leghemoglobin inside of the nodules by split cut. Nodulation assessment and symbiotic effectiveness of the strains were screened for final pot experiment by using symbioses effectiveness equation proposed by Date *et al.* (1993) as cited in Purcino *et al.* (2000).

$$\mathrm{SE} = \frac{\text{100x inoculated plant shoot DM}}{\text{N} - \text{fertilized plant shoot DM}} \text{x100}$$

Where SE is symbiotic effectiveness, N is nitrate and DM is dry matter

The strains screened as effective were further tested on potted soil samples collected from chickpea growing areas of Southern Ethiopia (Table 1). Culture preparation, seed germination and inoculation were similar with that of strile sand experiment. Except non inoculated positive controls which were supplied with 0.05% KNO_3, all strain inoculated and non inoculated negative controls were supplied with only water as required by plant. At similar days of growth as in sand experiment plants were carefully uprooted, assessed for nodulation, nitrogen fixation and shoot nitrogen content was analyzed following “Wet” Kejldhal method according to Sahlemedhin Sertsu and Taye Bekele (2000).

3.5. Most probable number (MPN) for soil samples

The indigenous soil rhizobial background population was estimated by most probable number (MPN) according to Somasegaran and Hoben (1994). Modified Leonard Jars were used to estimate the population of rhizobia by most probable number (MPN). 1g of each soil sample (Table1) was serially diluted in sterile distilled water upto 10^{-6}. 1ml from each dilution was inoculated to the base of chickpea seedlings growing in the jars as shown in Somasegaran and Hoben (1994). The seedlings were transferred to lath house and nursed with Jenson's nitrogen-free nutrient solution weekly. After 45 days of growth the plants were uprooted and scored as '+' for nodulation and '-' for the absence of nodulation. Finally MPN of cell per gram of soil was calculated following procedures described in Woomer (1994) and the equation:

MPN = Fxd

Where d= last dilution before negative nodulation and F=factor from the table given.

3.6. Isolation of fababean nodulating rhizobia

Fresh nodules were collected from field standing faba bean plants and preserved in plastic vials containing silca jell. The nodules were collected from farmers plot by asking permission and by randomly selecting fava bean plant with better growth, healthy appearance and after uprooting the nodules were large enough and the interior was pink when splitted. Ten nodules per plants were collected using standard collecting procedure. Desiccated nodules were rehydrated by imbibing in tap water and left in refrigerator for over night (Somasegaran and Hobben, 1994). Fresh and rehydrated nodules were separately (as a source they brought); surface sterilized by soaking in 95% ethanol for 8 seconds to break surface tension and then in 3% sodium

hypochlorite (NaOCl) for 4 minutes. They were rinsed in six changes of sterile distilled water using alcohol saocked and flame sterilized forceps.

Surface sterilized nodules were crushed in drops of sterilized water. The suspension of each nodule was streaked on YEMA in a laminar flowing hood and incubated at 28^0C. After three to five days of incubation, the appearance of colonies was checked and a single colony was re-streaked on YEMA until a pure isolate was obtained (Somasegaran & Hobben, 1994).

3.7. Presumptive test

Presumptive test was conducted by growing the isolates on YEMA containing Congo Red (CR), and Bromothymol blue (BTB) and on peptone glucose agar (PGA) containing Bromocresol purple (BCP) dye. The PGA-BCP medium contained 5g Glucose, 10g Peptone and 15g Agar per liter of distilled water to which 10ml BCP stock solution (1g BCP dissolved in 100ml of ethanol) was added to achieve a BCP concentration of 100µg ml^{-1} per liter. The pH of the solution was adjusted to 6.8 and autoclaved at 121^0C for 15min. 10ml stock solution of CR (0.25g mixed in 100ml distilled water) were added to YEMA to achieve 25µg ml^{-1} CR per liter. YEMA-BTB was prepared by adding 5ml Bromothymol Blue indicator stock solution (0.5g of BTB dissolved in 100ml ethanol) to YEMA to achieve a final concentration of 25µg ml^{-1}. PGA-BCP, YEMA-CR and YEMA-BTB were used to test the growth, CR absorbance and acid-alkaline reaction of the isolates, respectively (Vincent, 1970). Copies of the isolates pure culture were kept in 20% of glycerol (V/V) at 4°C for further study tests and -20°C for future study works.

3.8. Gram reaction

Gram's stain reaction of the isolates was done to check whether the isolates were Gram positive or Gram negative. It was conducted in Gram's stain solutions I to IV as indicated by Vincent

(1970). The test was conducted by using loopful of pure culture grown on YEMA and stained as per standard Gram's test procedure (Somasgaran and Hobben, 1994). The isolates which retained primary stain (Crystal Violet), blue in color, were recorded as Gram positive; whereas the isolates which retained counter stain (Safranine), red or pink in color, were recorded as Gram negative. The isolates which retained primary stain was discarded realizing rhizobia are not gram negative bacteria, whereas the isolates which retained counter stain and which had rod shape under oil immersion lens were considered as rhizobia (Rao, 1983).

3.9. Authentication test

Presumptive isolates were authenticated by re-infecting the host plant variety, "*Tumsa*". This was conducted on modified Leonard Jars according to Somasegaran & Hoben (1994). Seedlings growing in jars (fig.1) were inoculated with 1ml of the fully turbed (milky) isolates culture at approxmate logarithimic growth phase (10^9 cells ml^{-1}) (Vincent 1970). Positive and negative controls were also included as described above.

Figure 1: Leonard jars containing faba bea plants inoculated with persumtive isolates

3.10. Symbiotic effectiveness of the isolates

Isolates symbiosis effectiveness was calculated as described 3.4 above. Nitrogen fixing relative effectiveness classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

3.11. Phenotypic characterization of faba bean (*V. faba* L.) nodulating rhizobia

3.11.1. Colony morphology

Colony morphology of the isolates were examined on YEMA (Smasegaran & Hobben, 1994) by colony color (watery translucent, white translucent, dull glistening and milky), shape (convex, dome or flat), size (mm), capacity to produce extra cellular polysaccharide (low, moderate or copious amount) and colony texture (elastic or buttery).

3.11.2. Determination of mean generation time (MGT)

Bacterial growth was assessed on YEM broth in Erlenmeyer flask incubated in a Gyratory shaker at 130 rev. min^{-1}. Optical density was measured using Spectrophotometer (Jenway 6405 UV/vis.) at 600nm every 6hrs for 24hrs. Mean generation time or doubling time was calculated from the logarithmic phase (Somasegaran and Hoben, 1994).

3.11.3. Acid and base production

Colony of the isolates were streaked on YEMA-BTB, incubated at 28^0C and the change of medium from green to yellow or blue was assessed (Vincent, 1970). Isolates that changed the medium to yellow color was considered as acid producers and fast growers.

3.11.4. Physiological characterization

3.11.4.1. Salt tolerance

Tolerace to salt of the isolates were cheeked by growing them on YEMA plates containing various concentration of salt. YEMA was prepared with different concentrations of NaCl containing 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5% salt (Gao et al., 1994).

3.11.4.2. Growth at different pH test

The pH tolerance of the isolates were determined on YEMA at different pH values of 4, 4.5, 5, 5.5, 6, 8, 8.5, 9, 9.5 and 10 (Gao et al.,1994). 100ml YMA medium solution was prepared for each pH test and the pH of each was adjusted using0.1N HCl or NaOH. Then 1.5g agar mixed with 100ml YEMA solution, autoclaved at 121°C for 15 min and YMA plates were prepared with different pH concentration (Somasegaran and Hoben,1994)

3.11.4.3. Temperature tolerance test

YEMA was used to investigate the growth response of isolates to temperature by incubating the media at 4, 10, 15, 20, 35, 40, and 45^{0}C (Hungaria et al., 2000). The isolates were plated over YEMA medium and incubated in a given changes of temperatures by adjusting incubator for desired study temperatures.

3.11.4.4. Intrinsic heavy metal resistance

The intrinsic heavy metal resistance of the isolates were tested on TYA plates consisting 5. 0 g Tryptone, 3.0 g Yeast-extract, 0.87g $CaCl_2$, 1000 distilled water and 12 g Agar according to Zhang *et al*., (1991). The stock solution of metals (µg ml^{-1}) Cobalt ($CoSO_4.7H_2O$ 100µg ml^{-1}), Zinc ($ZnCl_2$ 500µg ml^{-1}), Lead $Pb(CH_3COO)_2$ 500µg ml^{-1}), Manganese ($MnSO_4.H_2O$ 500µg ml^{-1}) and Aluminum ($AlCl_3$ 500µg ml^{-1}) were filter sterilized using 0.2 µm mesh sized sterile filter

papers with a Luer-Lock system and added to the medium after the medium was autoclaved and cooled to approximately 50^0C (Zhang *et al*., 1991).

3.11.4.5. Antibiotic Resistance

The intrinsic antibiotic resistance of the isolates were tested on YEMA plates consisting different concentration of the following antibiotics (μg ml^{-1}), Streptomycin (40&80), Neomycine (5&10), Novobiocin (5), Kanamycine (15), Chloromaphinicol (5&20), Erytheromycin (10&20) and Spectinomycine (250&500). The antibiotic solutions were filter sterilized using 0.2 µm mesh sized sterile filter papers with a Luer-Lock system and added to the medium after the medium was autoclaved and cooled to approximately 50^0C (Somasegaran and Hoben, 1994).

3.11.4.6. Utilization of amino acid as sole nitrogen source

The isolates abilities to utilize amino acids as sole source of nitrogen were tested on basal medium (Amarger et al.1997). Eight amino acids including, L.proline, L.valine, L.arganine, L.asparatic acid, Giycine, L.histadine, Lasparagine and isolucine were used. The stock solution was added at concentration of $0.5gl^{-1}$ to basal medium containing (per liter) 1g K_2HPO_4, 1g KH_2PO_4, 0.01g $FeCl_3.6H_2O$, 0.2g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 1g mannitol and 15g agar. All the substances were filter sterilized as in antibiotic resistance test before they were added to basal medium..

3.11.4.7. Carbon source utilization test

Utilization of various carbon sources was tested on the basal medium as indicated in Somasegaran and Hoben (1994). Eleven carbon sources including starch, glucose, L.arabinose, D.galactose, rhaminose, trehalose, fructose, xylose, sucrose, mannose and mannitol as a referece, were used in the study. The stock solution was prepared as 10% (w/v) solution in distilled water.

Each filer sterilized heat labile carbon source solutions was mixed to basal medium (prepared in a procedure of YMA except yeat extract was reduced to $0.05gl^{-1}$) cooled to 50^0C after autoclaved. Heat stable ones mixed with basal medium before autoclaving. The carbon source media were poured to plates aseptically in laminar hood flow, allowed to solidify and stored for 24 hrs to check contamination.

3.11.4.8. Phosphate solublizing test

Phosphate solublizing ability was checked by inoculating each of them on Picovaskaya Agar medium containing (g/l): Glucose (10), Tricalcium phosphate (5), Ammonium Sulphate (0.5), Yeast extract (0.5), Magnesium Sulphate (0.1), Sodium Chloride (0.2), Manganese Sulphate (0.002), and Agar(15). The pH of the medium was adjusted to 7.0. The medium was supplemented with 2.5 g/lt of $Ca_3(PO_4)_2$ (TCP) as P source. The growth and clear zone formation around the colonies was considered for phosphate solublization (Somasgaran and Hobben, 1994).

3.12. Data Analysis

3.12.1. statistical analysis

The data generated from experiments were subjected to statistical analysis to determine the mean variations between the treatments. The analysis of variance (ANOVA) and list significant difference (LSD) at $p < 0.05$ were determined using SAS statistical package version 9.3.

3.12.2. Numerical Analysis

A computer cluster analysis of phenotypic variables of the isolates was carried out using a similarity coefficient and phenogram was constructed by the unweighted pair group method with the average (UPGMA) clustering method, using NTSYS Version 21 software

4. Results

4.1. Symbiotic effectiveness of rhizobia nodulating chickpea

4.1.1. Axenic sand culture experiment

Fifteen experimental chickpea rhizobial isolates and two reference strains were uesd as study inputs to check effectiveness cosistency on an axenic sand culture. Strains screening on axenic sand culture showed a significant variation ($p < 0.05$) for mean distributions of nodule number, nodule dry weight and shoot dry weight (Table 3). Nodule internal color of efficient test strains was pink due to presence of leghemoglobin and larger in size (figure 2).

Figure 2: The appearanceof nodules on chickpea inoculated with isolates ICRE18 and CA10

The highest nodule number (51 nodules plant^{-1}) was scored from strain M4b and the smallest number from strain CP26 (20 nodules plant^{-1}). Regarding mean nodule dry weight, M4b and ICRE18 produced higher nodule mean dry weight 0.15g and 0.13g, respectively; and those inoculated with ICRE25b (0.05g) and ICNRE03 (0.06g) produced nodules with least mean dry weight.

The data also showed variations among the strains with regard to shoot dry matter accumulation. The highest shoot dry matter (1.24g plant^{-1}) was scored for N-fertilized plant followed by plant inoculated with CA10 (1.17g plant^{-1}) and M4b (0.95g plant^{-1}). The least mean shoot dry matter

accumulation (0.38g plant^{-1}) was obtained from plant inoculated with CP68, which is still higher than the value obtained from negative control (0.26g plant^{-1}). Shoot dry weight was higher for plants inoculated with effective strains such as CA10, M4b and ICRE18. Plnts fertilized with nitrate solution were score relatively higher SDW compared to isolate inoculated ones and those neither N-fertilized nor inoculated were accumulated least SDW (Table 3).

Table 3: Comparison of chickpea nodulating rhizobia isolates (P<0.05)

Strain	**NN**	**NDW (gm)**	**SDW (gm)**	**SE %**
CA10	33.0^{a-c}	0.13^{a-c}	1.17^{a}	94.35
CP1	36.67^{a-c}	0.17^{a-c}	0.43^{cd}	34.7
CP129	31.0^{a-c}	0.07^{bc}	0.45^{cd}	36.3
CP130	47.67^{ab}	0.16^{a-c}	0.40^{cd}	32.26
CP151	47.33^{ab}	0.16^{a-c}	0.44^{cd}	35.5
CP26	20.33^{c}	0.07^{bc}	0.42^{cd}	33.9
CP67	49.3^{ab}	0.16^{a-c}	0.42^{cd}	33.9
CP68	28.0^{bc}	0.06^{bc}	0.38^{cd}	30.65
CP98	47.67^{ab}	0.09^{bc}	0.43^{cd}	34.7
CP99	40.67^{ab}	0.07^{bc}	0.52^{cd}	42
CPM20b	45.67^{a}	0.11^{a-c}	0.57^{c}	46
EAL029	38.33^{ab}	0.14a-c	0.56^{c}	45.2
ICNRE03	44.0^{ab}	0.27a	0.44^{cd}	35.5
ICRE18	38.33^{a-c}	0.18^{ab}	0.82^{b}	66.11
ICRE25b	40.33^{a-c}	0.14^{a-c}	0.45^{cd}	36.3
M4b	51.33^{a}	0.26^{a}	0.95^{b}	77
M7	37.00^{a-c}	0.18^{ab}	0.46^{cd}	37.1
N+	0.00^{d}	0.00^{c}	1.24^{a}	100

N-	0.00^d	0.00^c	0.26^d	20.9
CV%	31.51	51.45	15.98	

NN=nodule number, NDW=Nodule dry weight, SDW=Shoot dry weight, CV=Coefficient of varience, and SE=Symbioses effectiveness.(Means with the same letters within the Colum are not significantly different).

There were a considerable difference in soot growth and color among positive control (N-fertilized but not inoculated with strain), negative control (none N-fertilized) and Strain inoculated but not N-fertilized plant (Fig.3). The inoculated plants and the plants fertilized with nitrogen were more or less similarity in shoot color (deep green) indicating that the strain was effectively fixed nitrogen.

Figure 3: Relative comparison of shoot phenotypes received KNO3 (N+), un-inoculated (N-) and inoculated

The results generated, based on symbiotic effectiveness test indicated the test isolate, CA10 was found to be highly effective with SE value of 94.35%. Furthermore, M4b (SE=77%), ICRE18 (66.11%) were effective, while, M20b (SE=46%), EAL-029 (SE=45.2%), CP99 (SE=42%), M7

(SE=37.1%), ICNRE25b (SE=36.3%), CP151 (SE=35.5%) and ICNRE 03 (SE=35.5%) were moderately effective. On the other hand, the remaining test isolates including CP1 and CP98 (each with SE=34.7%), CP26 and CP67 (each with SE=33.9%), CP130 (SE=32.26%) and CP68 (SE=30.65%) were found to be least effective. Nevertheless, they still produced higher SE when compared with the negative control (SE=20.9%). It is to be noted that none of the test isolates were found to produce SE% value of higher than the positive control.

Based on the relatively high shoot dry matter accumulated by plants, strains CA10, M4b and ICRE18 were screened for further symbiosis effectiveness study on pot filled with soils collected fromchickpea growing areas.

Pearson correlation coefficients comparison (Table 4) showed that at $p < 0.05$, nodule number was significantly and positively correlated with nodule dry weight (0.67^{***}) and NN was negatively correlated with SDW (-0.05). The other parameters had none-significant correlations.

Table 4: The test strains effectiveness pearson correlation coefficient for chickpea plants grown on axenic sand culture

	REP	**NN**	**NDW**	**SDW**
REP	1.00			
NN	0.002	1.00		
NDW	0.06	0.67***	1.00	
SDW	-0.08	-0.05	0.16	1.00

REP=replicates, NN=nodule number, NDW=nodule dry weight, SDW=shoot dry weight, **= indicator for significance ***= highly significant

4.1.2. Symbiotic effectiveness on pots filled with field sample soils

Soil pH, total nitrogen and MPN of background rhizobial population that infect the host is presented in Table 5. Soil pH in the three districts was found to range between 6.41 and 7.22, The soil rhizobial background population determined as MPN (cells/gsoil) indicated that the highest number of rhizobia per gram of soil ($1.09x10^6$) was recorded at Bulgita followed by $2.92x10^5$ at Taba and the least was $2.76x10^3$ at Galebuge. The soil total nitrogen was highest for Taba, medium for Galebuge and least for Bulgia.

Table 5: Soil analyses test for pH, total N and MPN (Most Peobable Number)

Study site	Soil pH	Soil total N(%)	MPN of cells/g soil
Eastbadawacho (Bulgita)	6.41	0.135	$1.09x10^6$
Damotpullasa (Galebuge)	6.57	0.188	$2.76x10^3$
Damotgale (Taba)	7.22	0.245	$2.92x10^5$

There was a deep green shoot growth of chickpea plants inoculated and grown on soil samples from Galebuge when compared to Taba and Bulgita (fig.4).

Figure 4: The effect of test strains on chickpea plants grown over field soil samples

Marked variation among the strains in mean nodule number, nodule dry weight, shoot dry weight and total nitrogen content for three sites at $P < 0.05$were obtained (Table 6).

The effect of test strains on chickpea plants grown on soil samples from Bulgita kebele (Table 6) showed significant variations in NN, NDW, SDW, STN (shoot total nitrogen) and SE. In the case of NN, the highest NN was scored for chickpea plant inoculated with ICRE18 71.33 plant^{-1} followed by EAL029 and M4b 66.33 plant^{-1} and 51.33 plant^{-1}, respectively. Whereas, the smallest NN was recorded by plants treated with CA10 (38.33 plant^{-1}) and CPM20b (37.33 plant^{-1}). With regard to NDW, the higest value was scored for plants inoculated with ICRE18 (0.37g) followed by EAL029 (0.29g). But the least NDW was recorded from plant inoculated with M4b (0.17g), N-treated plant (0.17g) and CA10 (0.14g).

The data also revealed variation among strains with regard to SDW. The highest SDW accumulation was scored for treatment ICRE18 (3.95g) followed by N-fertilized plant (3.80g). The least SDW was scored for plant treated with CA10 (1.2g). In the case of STN, 3.76% and 3.52 were scored for plant inoculated with ICRE18 and N-fertilized respectively. Likewise the relative SE data showed that, the highest SE was scored by plant inoculated with ICRE18 (106.5%) followed by N-fertilized which was 2.52%. The least STN and SE was scored for plant treated with CA10. Moreover, the correlation coefficients comparison (Table 7) showed that, NDW was strongly and positively correlated with NN ($r=0.83^{***}$) at $P<0.0001$; and SDW was positively correlated with NDW ($r=0.59^{*}$) and STN ($r=0.56^{*}$) at $P<0.05$.

Regarding strains efficiency over soil sample from Galebuge (Table 7), chickpea plants inoculated with CPM20b, (reference strai), scored highest NN (38.67 plant^{-1}) followed by IRE18 (38.0 plant^{-1}); whereas the lowest NN was recorded for strain EAL029 (25.3 plant^{-1}) which was also reference strain. The data also showed that the highest and least NDW were scored for

plants inoculated with ICRE18 (0.27g) and EAL029 (0.06g), respectively. The SDW result revealed that plants treated with nitrate accumulated highest SDW (4.39g) and next to this was plant inoculated with strain ICRE18 (4.36g). The data highest STN was scored for ICRE18 (4.17%) followed by plant treated with nitrate which was 4.13%. The least STN was accumulated for CPM20b (3.56%). Highest effectiveness (100%) value was obtained from plant treated with N followed by plants inoculated with ICRE18 and M4b which were 99.3% and 91.1% respectively. Plant treated with CA10 scored the least SE which was 79.5%.

In addition to this the correlation comparison (Table 7) at $P<0.05$ for Galebuge soil sample indicated that NDW was positively correlated with NN ($r=0.84^{***}$) at $P<0.0001$ and SDW was positively correlated to STN ($r=0.54^{**}$) at $P<0.001$..

Table 6: Mean distribution of NN, NDW, SDW, and STN at P<0.05 and SE% for three

Sites	Strain	NN	NDW (gm)	SDW (gm)	STN%	SE. %
Bulgita	CA10	38.33^{b}	0.14^{b}	1.2^{c}	2.63^{b}	32.34
	EAL029	66.33^{ab}	0.29^{ab}	3.05^{ab}	3.45^{ab}	82.2
	CPM20b	37.33^{b}	0.21^{b}	3.3^{ab}	3.34^{ab}	88.9
	Control	49.00^{ab}	0.26^{ab}	3.80^{ab}	3.52^{a}	102.4
	ICRE18	71.33^{a}	0.37^{a}	3.95^{a}	3.76^{a}	106.5
	M4b	51.33^{ab}	0.17^{b}	2.37^{bc}	3.22^{ab}	63.9
	N+	42^{b}	0.17^{b}	3.71^{ab}	3.28^{ab}	100
	CV %	29.14	36.26	24.81	12.71	
Galebuge	CA10	29.0^{ab}	0.22^{ab}	3.51^{b}	4.03^{ab}	79.95
	EAL029	25.3^{ab}	0.06^{b}	3.66^{ab}	4.06^{ab}	83.4
	CPM20b	38.67^{a}	0.22^{ab}	3.58^{b}	3.56^{b}	81.5
	Control	33.0^{ab}	0.19^{ab}	3.74^{ab}	3.95^{ab}	85.2
	ICRE18	38.0^{ab}	0.27^{a}	4.36a	4.17^{a}	99.3
	M4b	34.67^{ab}	0.17^{ab}	4.0ab	3.88^{ab}	91.1
	N+	29^{b}	0.08^{b}	4.39a	4.13^{a}	100
	CV %	37.16	56.3	10.21	6.60	
Taba	CA10	57.67^{a}	0.5^{a}	3.18^{ab}	2.83^{b}	90.6
	EAL029	42.33^{a}	0.31^{a}	2.68^{bc}	2.79^{b}	70.9
	CPM20b	47.0^{a}	0.21^{a}	2.49^{c}	2.91^{b}	70.9
	Control	50.0^{a}	0.31^{a}	3.11^{ac}	3.19^{a}	88.6
	ICRE18	51.33^{a}	0.41^{a}	2.82^{bc}	2.92^{b}	80.3
	M4b	51.67^{a}	0.26^{a}	2.77^{bc}	2.88^{b}	78.9
	N+	34.0^{a}	0.23^{a}	3.51^{a}	3.19^{a}	100
	CV %	28.94	51.96	11.8	4.16	

NN= Nodule number, NDW =Nodule dry weight, SDW=Shoot dry weight and STN=shoot total nitrogen.
SE= symbiosis effectiveness.
Means in the same column followed with the same letter are not significantly different.

In general observation through study parameters mean distribution at Taba site there were no siginificant variation in NN and NDW. But, slightly variated mean distributions were seen incase

of SDW and STN. Chickpea plants inoculated with strain CA10 grown in potted soil from Taba had the highest NN (57.6 plant^{-1}) and NDW (0.5g) excceding plants inoculated by M4b by 5.43% in NN and ICRE18 by 9.89% in NDW. Likewise, SDW of the N-fertilized plants exceeded SDW of CA10 by 4.92%. The least effective strain CPM20b inoculated plant scored 2.49g. The highest STN 3.51% was obtained from positive control followed by plant treated with ICRE18 (2.92%). Plant inoculated with EAL029 scored the least STN 2.79%. With regard to SE, CA10 scored 90.6% following 100% SE vaue for positive treatment. NDW was strongly and positively correlated with NN ($r = 0.86^{***}$) at $P < 0.0001$ and SDW correlated with NDW ($r = 0.44^{*}$) and SDW strongly correlated with STN ($r = 0.58^{***}$) at $P < 0.0001$. .

Table 7: Pearson correlation coefficit for Bulgita Galbuge and Taba kebeles soil samples experiment

Site		Rep	NN	NDW	SDW	TN
	Rep	1				
	NN	0.08	1			
	NDW	-0.06	0.83^{***}	1		
Bulgita	SDW	0.002	0.46	0.59^{*}	1	
	TN	-0.08	0.36	0.36	0.56^{*}	1
	Rep	1				
	NN	-0.16	1			
Galebuge	NDW	-0.29	0.84^{***}	1		
	SDW	-0.49	0.30	0.36	1	
	TN	-0.23	0.06	0.17	0.54^{**}	1
	Rep	1				
	NN	-0.49	1			
Taba	NDW	-0.49	0.86^{***}	1		
	SDW	-0.21	0.29	0.44^{*}	1	
	TN	-0.21	0.19	0.08	0.58^{***}	1

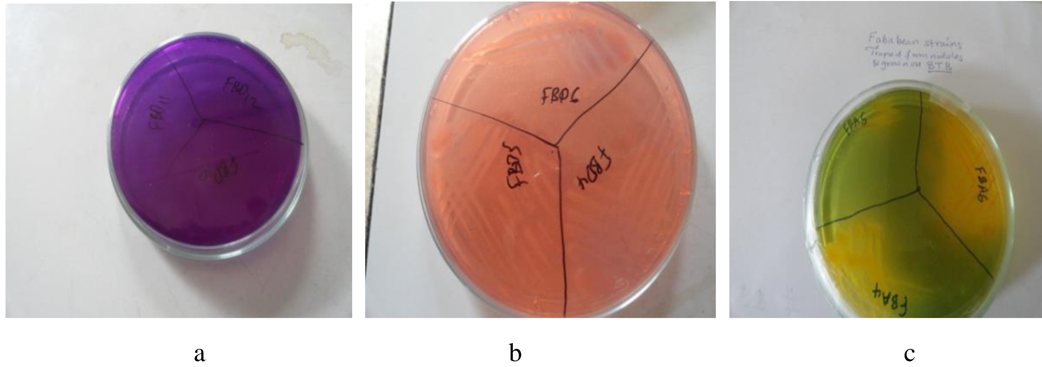
*=significant at p<0.05, **=significant at p<0.01 and ***= significance at p<0.0001 Rep=replicate, NN=nodule number, NDW=nodule dry weight, SDW= shoot dry weight, TN=total nitrogen

4.2. Phenotypic and symbiotic characterization of faba bean rhizobia

4.2.1. Presumptive test

A total of 33 bacteria with characteristic colony growth of rhizobia were isolated from root nodules of faba bean collected from different parts of Ethiopia (Table2). All of the 33 isolates were identified as Gram negative rods and did not asorbed CR (fig. 5); showed growth in three

days and turned YEMA-BTB to yellow color (fig.5c) showing that they were acid producers and fast growers; and were not showed growth in the medium PGA-BCP (Fig.5a). The growth features of isolates in the study mediums were similar to that of reference strain 35.



a b c

Figure 5: Faba bean nodulating isolates test sample for PGA-BCP, YEMA-CR and YEMA-BTB

4.2.2. Authentication test

All of the 33 presumptive faba bean rhizobial isolates and reference strain "35" were able to nodulate homologous host plant variety '***Tumsa***' on re-infection. The nodules were evenly distributed on the roots and concentrated around the bases of the roots. Almost all of the nodules were elongated in shape and few were aggregated, branched (dichotomous) and oval in shape (Data was not provided). The nodules from some isolates inoculated plants were pink in color when splited and interior was observed The result revealed that leaf color for nodulated plants infected with isolates and reference strain "35" was green and deep green compared to yellowish-green color of un-inoculated and none N-fertilized plant. .

4.2.3. Preliminary symbiotic effectiveness of faba bean isolates

Testing isolates for their ability to nodulate roots of the target legume and for their symbiosis effectiveness under sterile conditions is of prime importance in studies of biological nitrogen fixation (Rao and Sharama, 1995). Accordingly, faba bean nodulating rhizobia were analyzed for

preliminary symbiotic effectiveness. The result in Table 8 had shown that isolate FBM11 was highly effective with SE>80%; 10 isolates and reference strain "35" were effective, i. e., their symbiotic effectiveness valuewas found to range between 50% and 80%. Of the total isolates, 10 isolates were moderately effective and 12 were ineffective by the fact that their SE values were <35%. Furthermore, the result showed that the mean SDW of plant inoculated with FBM11 was greater than N-fertilized plants; and the isolate was symbiotically highly effective (SE =130.5%).

Table 8: Grouping of faba bean nodulating isolates based on symbioses effectiveness

	Zone	**Highly effective (>80%)**	**Effective (50-80%)**	**Moderately effective (35-50%)**	**Ineffective (<35%)**	Total
Adia	East showa	None	FBD1,8,9&14	FBD4,7,&11,	FBD5	8
Dalle	Sidama	None	FBSD2	FBSD7	None	2
Bule	Gedeo zone	None	FBGE1, 5 & 7	FBGE8	None	4
Hangacha	Kambata tambaro	None	FBH3	FBH2	FBH1	3
Kachabira	Kambata tambaro	None	KB2d	KB2b	KB1c,KB2e, KB3e KB2c, KB2g	7
Eastbadawacho	Hadiya	FBM11	None	FBAy,FBM8,&FBM6	FBA4, FBA11,12	7
Kindokoysha	Wolayta	None	None	None	KK1c&KK1d	2
HARI		None	35	None	None	1
Total		1	11	10	12	34
Percentage		2.94	32.35	29.41	35.29	100

The nodulation and symbiotic effectiveness of isolates showed marked variations among the isolates in NN, NDW and SDW at $p < 0.05$. The highest NN was recorded from plant inoculated with FBD9 while the least was recorded from plants inoculated with FBA1 and KB3e (Table 9).

The higher mean NDW was measured from plants inoculated with “35” and isolates FBD9, KB1c and FBD1. The lowest nodule dry weight was measured from plants inculcated with FBA11, KK1c and FBH1.In the case of SDW N-fertilized plants and plants inoculated with FBM11 and FBD1 recorded higher SDW 2.16g, 2.82g and1.58g, respectively. But, those inoculated with KB2g and KB2e had the lowest SDW 0.23 & 0.39, respectively (Table 9).

Table 9: The effect of isolates on symbiosis effectiveness parameters and relative SE

Strain	NN	NDW (gm)	SDW (gm)	SE %
KB1c	21.00^{b-d}	0.09^{af}	0.72^{e-j}	33.3
KK1c	8.0^{cd}	0.004^{ef}	0.73^{e-j}	33.8
KK1d	13.5^{cd}	0.05^{a-f}	0.46^{h-j}	21.3
KB2b	8.67^{cd}	0.02^{b-f}	0.85^{d-i}	39.35
KB2c	8.5^{cd}	0.06^{a-f}	0.56^{f-j}	25.9
KB2d	12.5^{cd}	0.04^{a-f}	1.43^{cd}	66.2
KB2e	13.33^{cd}	0.05^{a-f}	0.39^{ij}	18.05
KB2g	15.5^{b-d}	0.05^{a-f}	0.23^{e-j}	10.65
KB3e	7.5^{cd}	0.045^{a-f}	0.7^{e-j}	32.4
FBD1	20^{b-d}	0.09^{a-c}	1.58^{c}	73.1
FBD4	26.33^{b-d}	0.08^{a-e}	1.07^{c-h}	49.5
FBD5	25.0^{b-d}	0.03^{a-f}	0.73^{d-j}	33.8
FBD7	23.5^{b-d}	0.02^{c-f}	0.79^{d-j}	36.6
FBD8	29.0^{b-d}	0.046^{a-f}	1.14^{c-f}	52.8
FBD9	66.00^{a}	0.10^{ab}	1.27^{c-e}	58.8
FBD11	20.5^{bc}	0.04^{a-f}	0.86^{d-j}	39.8
FBD14	13.33^{cd}	0.023^{b-f}	1.096^{c-g}	50.7
FBGE1	34.33^{bc}	0.10^{ab}	1.29^{c-e}	59.7
FBGE5	34.67^{bc}	0.05^{a-f}	1.11^{c-g}	51.4

FBGE7	44.33^{ab}	0.09^{a-c}	1.03^{c-i}	47.7
FBGE8	17.0^{b-d}	0.03^{a-f}	0.81^{d-j}	37.5
FBH1	9.33^{cd}	0.007^{d-f}	0.51^{f-j}	23.6
FBH2	34^{bc}	0.04^{a-f}	0.95^{c-i}	44.0
FBH3	29.63^{bc}	0.05^{a-f}	1.09^{c-g}	50.5
FBA4	7.5^{cb}	0.041^{a-f}	0.51^{f-j}	23.6
FBA11	9.0^{cd}	0.003^{ef}	0.61^{f-j}	28.2
FBA12	15^{cd}	0.067^{a-f}	0.71^{e-j}	32.9
FBAy	10.5^{cd}	0.025^{a-f}	0.76^{e-j}	35.2
FBSD2	30.67^{bc}	0.067^{a-f}	1.15^{c-f}	53.2
FBSD7	24^{b-d}	0.04^{a-f}	1.03^{c-i}	47.7
FBM6	13^{cd}	0.07^{a-f}	0.855^{d-j}	39.6
FBM8	18.5^{b-d}	0.035^{a-c}	0.96^{c-i}	44.4
FBM11	16^{b-d}	0.075^{a-f}	2.82^{a}	130.5
Ref-35	35.33^{bc}	0.10^{a}	1.14^{c-f}	52.8
N+	0^{d}	0^{f}	2.16^{b}	100
N-	0^{d}	0^{f}	0.43^{h-j}	20
CV %	64.49	73.32	30.09	
MSE	13.0	0.035	0.29	

NN=nodule number, NFW &NDW = nodule fresh and dry weight, SFW&SDW =shoot fresh and dry weight

As shown in the Table 10, NDW was strongly correlated with NN (r=0.73***) at $p<0.0001$ and SDW was correlated with NDW (r=0.34**) at $p<0.001$.

Table 10: Pearson correlation coefficient comparison for nodule number, nodule and shoot dry weight

	Rep	**NN**	**NDW**	**SDW**
Rep	1			
NN	0.14	1		
NDW	0.12	0.73***	1	

SDW	0.01	0.31^{**}	0.34**	1

*** = strong significance at p<0.0001, **=significance at p<0.01 and *=significance at p<0.5

4.3. Phenotypic characterization of fababean rhizobia

4.3.1. Mophological growth charactrristics

As shown in Appendix 4, faba bean nodulating rhizobia were characterized based on their colony morphology on YEMA according to Somasegaran and Hoben (1994). Texturally, 29 , 2 and 3 isolates had buttery, milky and watery or translucent colonies, respectively. Colony elevation varied from flat to raised and most of the isolates had domed colony elevation. Exo-polysaccharide was produced by KK1c, KK1d and FBA12 isolates. Their colony appearance was circular with copious mucus production. The least colony diameter was recorded for isolate KB1c (1.7mm) and FBM8 (1.8mm) and the highest colony diameter was scored for isolates KB2e (5.4mm), FBH2 (5.2mm) and FBD14 (5.0mm). The study on the isolates mean generation time showed that the isolates growth time ranged between 1.59hr and 3.65hr from least to highest (Appendx 4). The highest mean generation times were scored for 2 isolates 3.4hr and 3.65hrs; 20 isolates were scored the mean generation time in the range between 2.0hr and 2.98hrs; and 12 isolates scored <2hrs.

4.3.2. Numerical taxonomy

Cluster analysis of an adequate number of traits and comparison with rhizobial reference species permit the identification and grouping of large populations (Swift and Gignell, 2001 and L'taief *et al.,*2007). Thus, Numerical analysis based on different phenotypic characteristics (Table 11) puts the isolates in to three different clusters and 2 un-clustered isolates at 65% of similarity cut-off point (Fig.6).

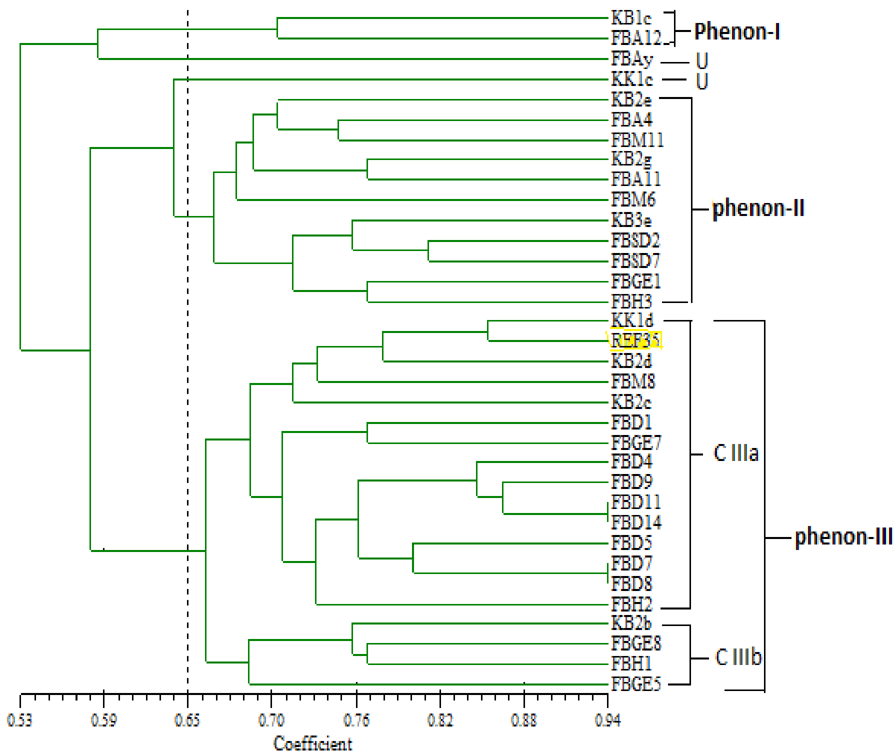


Figure 6: Dendrogram highlighting the phenotypic similarities among faba bean rhizobia (*Rhizobium leguminosarum*) from different parts of Ethiopia

2 isolates were grouped under cluster I (Phenon), 11 isolates were grouped in cluster II (Phenon II) and 19 isolates were grouped under cluster III (Phenon III) including the reference strain "35", which nodulate faba bean (Jordan, 1986; Somasegaran and Hoben, 1994; Zahran *et al.*, 1999; Wier 2006).

Isolate KB1c and FBA12 were grouped in cluster I. They were able to survive at pH range of 4.5 to 8.0; with this character they were similar with those isolates grouped in other clusters and unclustered isolates. None of them survived at higher pH values 9.5 & 10. In the case of salt stress tolerance both isolates in the cluster were able to resist salt stress at concentration range of 0.5% and 1.5% and KB1c tolerated 2.0%.They were different from isolates in cluster III because

of sensitivity to 2.5% salt cocentration. All isolates in the cluster were also able to grow at temperature range of 15^0C and 45^0C, but only KB1c was grown at temperature of 10^0C. In the case of antibiotic resistance of isolates in the cluster, KB1c was found to resist erythromycin at concentration of 10µgml^{-1}, chloromaphiicol 20µgml^{-1}, neomycin 5µgml^{-1} and 10 µgml^{-1}. Both isolates were resistant to erythromycin 20µgml^{-1} and chloromaphenicol 10µgml^{-1}. In response to heavy metals of isolates in the cluster, KB1c was resistant to Cobalt and FBA12 was resistant to Zinc. Regarding N-source utilization, FBA12 and KB1c utilized L. asparagin and L. arganine respectively. Both isolates in the cluster utilized carbon source, such as sucrose, galactose and L-arabinose; and FBA12 utilized starch and D- xylose (Table 11).

The features unique to isolates included in this cluster were isolates growth inabiliy at pH 8.5 and 9.0; growth at higher temperature 45^0C; sensitivity to heavy metals $MnSO_4.H_2O$ and $Pb(CH_3COOH)_2$, and poor utilization of N-sources (Table 11).

Isolates grouped unde clustr II were 11. Of the isolates FBM6, FBM11 and FBH3 grew at lowest pH 4.0; all of them grew at pH range of 5.0 to 9.0; about 91% of the isolates were grown at pH 9.5 and only two isolates KB2e and FBM6 were found to grow at pH 10.0.

With regard to salt tolerance, all isolates in the cluster were grown at concentration range of 0.5% and 1.5%; Ten isolates were grown at concentration of 2.0% and none of them were found to grow at concentration range of 2.5% to 5.0%. Isolates growth response to temperature changes in cluster II had shown that all of them grew in temperature range of 15^0C and 30^0C. Four of the isolates grew at temperature of 10^0C. 45^0C. 45% and Four of the isolates in the cluster were able to grow at 40^0C and 45^0C, respectively. The characteristics of cluster II matches with whole

cluster III in growth response to temperature range of 15^0C to 30^0C. Of the isolates none of them was found to solublize phosphorous in the medium containing $Ca_3(PO_4)_2$.

In case of antibiotic resistance test, most isolates in cluster II were sensitive to antibiotics and none of them were found to grow on medium containing erythromycin ($20\mu gml^{-1}$), chloromaphenicol ($10\mu gml^{-1}$ and $20\mu gml^{-1}$), novobiocin ($5\mu gml^{-1}$) and spectnomycin ($250\mu gml^{-1}$ and $500\mu gml^{-1}$). But there were some isolates in the cluster, such as KB2g and FBSD2 that resisted $10\mu gml^{-1}$ neomycin and $10\mu gml^{-1}$ erythromycin. KB3e was resistant to $40\mu gml^{-1}$ streyptomycin and $10\mu gml^{-1}$ erythromycin. Similarily, FBGE1 resisted $5\mu gml^{-1}$ neomycin and novobiocin and FBH3 was resistant to $80\mu gml^{-1}$ streyptomycin, $10\mu gml^{-1}$ erythromycin and $5\mu gml^{-1}$ neomycin (Table 11).

In case of resistance to heavy metals, among isolates in cluster II, 8, 4, 3 and 3 isolates resisted CoSO4.7H2O, $MnSO_4.H_2O$, $AlCl_3$ and $Pb(CH_3COOH)_2$, respectively. Only FBM11 resisted $ZnCl_2$. Of the isolates in the cluster, 6 were able to utilize L-asparagine and L-asparatic acid as nitrogen source, whereas the rest amino acids included in the study were utilized by less than half of the isolates. Among carbon sources, sucrose was metabolized by all isolates in the cluster. D-fructose, D-mannose and D-rhaminose were utilized by 3, 2 and 4 isolates, respectively. Others such as starch, D-xylose, glucose, D-trehalose, D-galactose and L-arabinose were utilized by isolates ranging 7 to 9 (Table 11).

Unique features of cluster II (Table 11) had less growth response to pH 4.0 and 10.0. More than 10 of the isolates had shown tolerance to 2.0% salt concentration. They also had poor resistance to most antibiotics included in the experiment. Except $CoSO_4.7H_2O$, which was resisted by 8 isolates, most isolates in the cluster weakly resisted heavy metals.

The third cluster (cluster III) comprised 55.9% of the isolates including reference strain “35” and sub-divided in to two sub-clusters and designated as CIIIa and CIIIb. Sub-cluster CIIIa contained 15 isolates including reference strin “35” and the remaining 4 were clustered in sub-cluster CIIIb. Of pH values tested, 19 isolates in the cluster III were found to grow at pH ranges 5.0 and 9.5, at pH 4.5 all isolates in CIIIa and 3 isolates in CIIIb were grown. In the lower pH 4.0, 8 isolates in CIIIa and 2 isolates in CIIIb were grown. At higher extreme pH 2 isolates of CIIIa and 1 isolate of CIIIb were grown. 0.5%, 1.0%, 1.5%, 2% and 2.5% salt concentrations were resisted by 14, 14, 11, 10 and 6 isolates, respectively in sub cluster CIIIa. 3 isolates of Sub-cluster CIIIb resisted salt concentrations 0.5%, 1.0%, 1.5% and 2.0%, respectively; but, only 1 isolate of them resisted 2.5% salt concentration.The salt concentration ranging 0.5% to 2.0% were tolerated by 3 isolates in sub cluster CIIIb and only strain KB2b was found to grow at 2.5% concentration. In temperature changes in the study, 100% isolates in cluster III were grown at 15^0C, 20^0C and 30^0C. None of the isolates from sub cluster CIIIa was found to grow at the temperature of 4^0C, but only FBH1 was grown from CIIIb sub cluster. Of isolates from CIIIa, 10isolates and 5 isolates were grown in higher temperatures 40^0C and 45^0C, respectively. But, from that of cluster CIIIb, FBH1 and KB2b were able to grow in the temperatures 40^0C and 45^0C. The reference “35” was not found to grow at extreme temperatures 4^0C, 40^0C and 45^0C (Table 11).

Interms of antibiotic resistance, 5 and 6 isolates resisted streyptomycin 80µgml^{-1} and 40µgml^{-1}, respectively. Most of the isolates resisted the antibiotics studied, whereas none of the isolates in the cluster CIIIb was found to resist streyptomycin 40 µgml^{-1}, erythromycin 20 µgml^{-1}, chloromaphenicol 10µgml^{-1} and 20µgml^{-1} and spectinomycin 250µgml^{-1} and 500µgml^{-1}. Isolates such as FBGE5, FBGE8 and FBH1 were found to resist streyptomycin 80µgml^{-1}, erythromycin

10μgml^{-1} and neomycin10μgml^{-1}, respectively. All isolates in sub cluster CIIIb were resistant to novobiocin (Table 11).

From the heavy metals included in the study (Table 11), $CoSO_4.7H_2O$ was resisted by 100% isolates including reference strain "35" in cluster III. Of the isolates in sub cluster CIIIa 14 were resisted $AlCl_3$ and $MnSO_4.H_2O$. 86.6% and 40% isolates resisted $Pb_2(CH_3COOH)_2$ and $ZnCl_2$. None of the isolates in sub cluster CIIIb was found to tolerate $ZnCl_2$, whereas 4 isolates resisted $MnSO_4.H_2O$ and 3 and 1isolates were able to resist $AlCl_3$ and $Pb_2(CH_3COOH)_2$ (Table 11).

Most of the isolates in cluster III utilized all of the nitrogen and carmbon sources. All solates from CIIIb did not utilize Frucose and 1 isolate from CIIIb utilized starch and mannose.

Unique features of sub-cluster CIIIa (Table 11) include: they were characteristically associated with reference strain "35". Compared to other clusters 8 isolates in this cluster were able to grow at pH 4.0, whereas 10 of the isolates were adapted to resist higher basic pH 10.0. They also had shown better performance in resisting antibiotics such as erythromycin, chloromapfenicol, neomycin and novobiocin. All of them resisted heavy metal $CoSO_4.7H_2O$; 14 isolates resisted $MnSO_4.H_2O$, $AlCl_3$ and 86% of them were resistant to $Pb(CH_3COOH)_2$. In terms of N-source and C-source utilization isolates had shown potent progress in reference to others. Accordingly L-glycin was utilized by 11 isolates whereas L-asparagine and L-histadine were utilized by 100% isolates of the sub-cluster. With regard to C-source, 15 isolates utilized D-xylose, L-arabinose, D-galactose and sucrose and comparably isolates also utilized the rest C-sources.

Unique features associated with sub cluster CIIIb include, isolate FBH1 that was grown at salt concentration of 2.5%; compared to sub-cluster CIIIa and other clusters they were 4 resistant to neomycin 5μgml^{-1}; all isolates were resistant to heavy metals stress of $MnSO_4$ and $CoSO_4.7H_2O$,

resistance to former heavy metal made them different from CIIIa. L-arganine and L-proline as N-source were utilized by 100% isolates and all of them were unable to utilize fructose as C-source (Table 11).

Unclustered isolates were FBAy and KK1c which belonged to East Badawacho and Kindokoysha. Both were grown in pH range of 4.5 to 8.0; none of them was able to grow in the pH value of 4.0 and FBAy was failed to grow on pH value of 8.5 and 10.0. The isolates were grown in salt concentration of 0.5% and 1.0%, but FBAy tolerated concentration of 1.5%. Both failed to grow at the temperature of 4^0C and 10^0C and grown in the temperature range of 15^0C and 40^0C. But isolate KK1c was grown at 45^0C. The isolates found to resist erythromycin 10µgml^{-1}, neomycin 5µgml^{-1} and 10µgml^{-1}, $CoSO_4.7H_2O$ and $Pb(CH_3COOH)_2$. They utilized glucose, sucrose, D-rhminose and starch as carbon source and L-isolucine as nitrogen source. Isolate FBAy was found to resist chloromaphenicol 10µgml^{-1}and 20µgml^{-1}; $AlCl_3$; utilized L-histadine, L-valine, L-arganine and giycine as nitrogen source. KK1c resisted novobiocin 5µgml^{-1} and $MnSO_4.H_2O$. They also utilized L-isolucine as nitrogen source. None of the isolates were found to resist streyptomycin 40 µgml^{-1} and 80 µgml^{-1} erythromycin 20µgml^{-1}and spectinomycin 250µgml^{-1} and 500µgml^{-1}. D-trehalose, D-mannose, D-rhaminose, D-arabinose and D-xylose were utilized as sole carbon sources(Table 11)..

Unique features of unclustred were non-growth on pH 4.0 and 10.0; their resistance to salt stress were limited to 1.5%; they were resistant to erythromycin 0.5µgml^{-1}concentration and $Pb(CH_3COOH)_2$; all of them were utilized D-rhaminose as C-ource and L-isolucine as N-source(Table 11).

Table 11: Physiological and Biochemical Characteristics of the Isolates within the Clusters

Characteristics		Cluster I, N=2	Cluster II, N=11	Cluster III, N=19		Unclustered, N=2
				CIII a N=15	C III b, N=4	(U)
pH	4	1	3	8	2	-
	4.5	+	10	+	3	+
	5	+	+	+	+	+
	5.5	+	+	+	+	+
	6	+	+	+	+	+
	8	+	+	+	+	+
	8.5	1	+	+	+	1
	9	1	+	+	+	+
	9.5	-	10	+	+	+
	10	-	2	11	1	1
Salt tolerance	0.5%	+	+	14	3	+
	1%	+	+	14	3	+
	1.5%C	+	+	11	3	1
	2%	1	10	10	3	-
	2.5%	-	-	6	1	-
	3%-5%	-	-	-	-	-
Temperature	4^0C	-	-	-	1	-
	10^0C	1	6	7	3	-
	15^0C	+	+	+	+	+
	20^0C	+	+	+	+	+
	30^0C	+	+	+	+	+
	35^0C	+	6	13	-	+
	40^0C	+	5	10	1	+
	45^0C	+	4	5	1	+
Phosphate sol.	$Ca_3(PO_4)_2$	-	-	-	-	-
	Streptomycin 40 µgml^{-1}	-	1	6	-	-
	Streptomycin 80 µgml^{-1}	-	2	5	1	-
	Erythromycin 10 µgml^{-1}	+	3	9	1	+
Antibiotic resistance	Erythromycin 20 µgml^{-1}	+	-	11	-	-
	Chloromaphinico 5µgml^{-1}	1	-	10	-	1
	Chloromaphinicol20µgml^{-}	1	-	10	-	1
	Neomycin 5 µgml^{-1}	1	2	13	+	+
	Neomycin 10 µgml^{-1}	1	1	6	1	+
	Novobicin 5 µgml^{-1}	-	-	13	+	1
	Spectinomycin250 µgml^{-1}	-	-	6	-	-
	Spectinomycin 500 µgml^{-}	-	-	-	-	-
Heavy metals	$CoSO_4$.7H2O	1	8	+	+	+
	$AlCl_3$	-	3	14	3	1
	$ZnCl_2$µgml^{-}	-	1	6	-	-
	$MnSO_4$ .H_2O	-	4	14	+	1
	$Pb(CH3COOH)_2$	-	3	13	1	+
N –source	Proline	-	2	12	+	-
	Isolucine	-	5	13	3	+
	Histadine	-	3	15	+	1

	Asparagine	1	6	16	+	-
	Asparatic acid	-	4	14	3	1
	Valine	-	3	14	+	1
	Arganine	1	6	13	1	-
	Glycine	-	3	11	+	+
C – source	Glucose	1	8	12	2	+
	Sucrose	+	+	+	+	+
	Fructose	-	3	5	-	1
	Trehalose	1	8	+	-	1
	Mannose	-	2	10	1	1
	Rhaminose	-	4	14	2	+
	Galactose	+	9	+	3	1
	Arabinose	+	9	+	3	1
	Starch	+	7	14	1	+
	Xylose	1	7	+	3	1

”+” the growth of the isolates on the provided test parameter; “-” the inability of the isolates to grow on the provided test parameter

5. Disscusion

5.1. Chickpea strains symbiosis effectiveness test

The study result in axenic sand culture showed that plants inoculated with M4b scored highest NN (51 plant^{-1}), but accumulated SDW was 0.95g. The plants inoculated with strain CA10 and ICRE18 had nodulated moderately (Table 3), but they accumulated 1.17g and 0.82g , respectively. Similar results were seen in the work of Assefa Funga, (2016), on the study of symbiosis effectiveness of elite chickpea strains on selective chickpea seed variety. On the other hand the infectiveness of the isolates were manifested by presence of leg hemoglobin in nodules when spitted. Moreover, the symbiosis effectiveness comparison showed that 94.35%, 77% and 66.11% were symbiosis values for plants inoculated with CA10, M4b and ICRE, respectively. But for that of reference strains EAL029 and CPM20b SE values were 46 and 45.2%.

In Pearson’s coefficient correlation comparison NN was positively correlated with NDW and SDW was negatively correlated with NDW (Table 4). These result implied that as number of nodule increases success of SDW accumulation decreases. The reason is that nutrient supply

from the plant for micro symbionts decreases due to higher population of root nodule bacteria (Somasegaran and Hoben, 1994).

Ready-made nitrate solution was the means of growth for the plant to have highest shoot dry matter accumulation. That is why; N-fertilized plants had higher shoot dry matter than negative controls (Table.3). A similar finding was reported by Muhammed Abdulbasit, (2013).

According to Lupwayi and Haque (1994), Peoples *et al.* (2002) and Mulongoy (2008), shoot dry matter is a good indicator of the relative effectiveness of an isolate and there exists a sound correlation between the nitrogen fixing capacity of legumes and their shoot dry matter accumulation. Based on these evidence and symbiosis effectiveness values CA10, M4b and ICRE18 were selected together with reference strains EAL029 and CPM20b for further symbiosis effectiveness study over pot filled field soil samples.

Studied results for soil samples pH, MPN, and soil total nitrogen (Table5) showed that soil pH content was higher for Damotgale 7.22; medium for Bulgita soil sample 6.57 and lowest 6.41 was for soil sample from Taba. Elizabeth et al., (2012), showed that the optimum range of pH for chickpea production was between 6.0 and 8.5. The work of Geletu (1996), indicated that chickpea plants can grow at pH range between 6.4 and 7.9. Acordingly the soil samples were suitable for chickpea plants growth.

MPN result has showed that Bulgita contains 1.09x106 cells g^{-1} of soil which was the highest, Taba 2.92×10^5 cells g^{-1} of soil medium and Galebuge 2.76×10^3 cells g^{-1} of soil the least. According to Elizabeth et al., (2012) the number of rhizobia needed for prompt nodulation lies between 10^2 and 10^3cells $g^{-1}$of soil. Due to this reason inoculation is not necessary for all soil samples. But simply based on standard inoculants amount 10^6 cells $g^{-1}$of inoculants, Galebuge and Taba require inoculation after studying the efficiency of resident population. Chickpea plants

shoot growth over Galenuge soil sample was greener compared to same isolates inoculated plants at Bulgita and Taba. Keeping other soil factors in mind, for the case of this study the isolates nitrogen fixation was higher for Galrbuge site compared to Bulgita and Taba. Because, most of the test and reference strains inoculated chickpea accumulated more shoot total nitrogen compared to Bulgita and Galebuge (Table 6). Shoot color varies from green to deep green due to the nitrogen fixing capacity of rhizobia Somasegaran and Hoben (1994)..

The result of mean distribution at $P<0.05$ indicated in Table 6 above about strains effectiveness test requirements shown that, chickpea plants inoculated with effective strains had nodulated a lot and accumulated relatively higher SDW. The chickpea plant inoculated with highly effective strain ICRE18 on studied soil samples from Bulgita and Galebuge scored high SDW 3.95g and 4.36g; high NN 71.33 $plant^{-1}$ and 38.0 $plant^{-1}$, and high NDW 0.17g and 0.27g, respectively compared to batch strains inoculated ones. On site Taba chickpea plants inoculated with CA10 scored higest NN, NDW and SDW as 57.67$plant^{-1}$, 0.5g and 3.18, respectively. This implied that nodulation enhaced shoot dry weight accumulation. Abere *et al*., (2009) indicated in his work that nodulation status correlated with plant biomass. Ali *et al*., (2009) and Muhammed (2013) reported that inoculated chickpea plants had higher nodules and accumulated higher SDW. Shoot total nitrogen was relatively higher for plants treated with effective strains that accumulated higher SDW, which is the best estimator of total nitrogen fixed in plants (Somasegaran and Hoben, 1994). Similarly the symbiosis effectiveness values were highest for effective strains having highest SDW at each study site (Table 6).

Moreover, from the Pearson’s correlation comparison result (Table 7) SDW was positively correlated with NDW and STN at site Bulgita at $P<0.05$; SDW was positively correlated with STN at $P<0.01$ at Galebuge soil; and SDW was positively correlated with NDW at $P<0.05$ and

strongly and positively correlated with STN at $P<0.0001$ at Taba soil. According to Somasegaran and Hoben (1994), positive correlation between shoot dry weight and nodule dry weight and (or) shoot total nitrogen, SDW is used as standard to select symbiotically effective strain from those included in the experiment. Based on this fact higest SDW scored for strain ICRE18 at Bulgita and Galebuge sites, was symbiotically effective. But for the case of Taba highest SDW scored for CA10, and effective over soil sample from Taba.

Finally ICRE18 outcompeted the indigenious populations in Bulgita and Galebuge soils, and CA10 at Taba in most studied parametres. Therefore, strain ICRE18 is proposed as inoculant for Bulgita and Galebuge kebeles, and Strain CA10 is a promising inoculant for Taba kebele. Accordingly, those promissing strains must further be checked under field conditions before formulating them as inoculants.

5.2. Phenotypic and symbiotic characterization of faba bean rhizobia

The study result of presumptive test showed that all of the 33 isolates and reference strain 35 were not absorbed CR dye from YMA-CR medium (Fig, 5b); turned green color of YMA-BTB medium yellowish at colony growth area, which was indicator for fast growth and acid production (Fig, 5c); none of them was found to grow in PGA-BCP medium (Fig. 5a); and all of them were gram negatve rod shaped under Gram stain test. The results were concomitant with standard indicated in Vincent (19730) and Somasegaran and Hoben (1994). The study result was similar to Rao (1983); Ayneabeba Adamu *et al.,* (2001); Zerihun Belay(2006), Abere Minalku., *et al.,* (2009); Solomom Legesse and Fassil Assefa (2014) and Getahun Negash (2015), who wrked on faba bean root nodule bacterial isolates. But, the study result was not matched with finding of Anteneh Argaw (2012), who reported that about 11 isolates with rhizobial growth characteristics were found to grow over PGA-BCP medium. The finding confirmed that all of the

33 isolates were responded in a similar manner with that of reference strain 35 over the growth mediums and gram stain test. Therefore, they were presumptive bacteria with similar characteristics test result as reference strain 35.

The result from the authentication test all of the 33 faba bean root nodule bacteria including reference strain35 were showed nodulation on host faba bean plants by re-infection. Their nodules were dichotomous, lobed in shape, aggregated to the base of the root, for some plants the nodules were pink colored. The dichotomous nodules were indeterminate, as showed by Prel and Pool (2006). The interior pinkish colour of nodules was the indication of the synthesis of leg hemoglobin by root nodule bacteria, and efficient infection and BNF Somasegaran and Hoben, (1994). The results were similar with Getahun Negash (2015), who worked on faba bean nodulatig rhizbia characterization.

Based on symbiosis equation proposed by Date *et al*., (1993) cited by Purcino *et al.,*(2000), study isolates were categorized in to four groups. Isolate FBM11 was groped under highly effective with SE value 130.5% and showed better performance from N-fertilized positive control. Solomon Legesse and Fassil assefa (2014), also reported that one of their isolates was shown better performance from N-fertilized one. In work of Getahun Negash (2015), isolate FBW140 showed highest symbiosis value 103.4% compared to positive control. Nitrogen fixation could be the possible reason for its performance Nimnoi (2009). 11 isolates with that of reference strain 35 were scored SE values in between (50%-80%), which were effective; 10 isolates were scored SE values between (35%-50%), they were moderately effective and 12 isolates scored SE values <35%. The work of Ashenafi Haylu and Endalkachew Woldemeskel (2011), on field pea isolates characterization showed grouping them in SE categories.

Current study result mean distributions of NN, NDW and SDW at P<0.05 showed variation (Table 9). From the isolates included in the study faba bean plant inoculated with isolate FBM11 scored highest shoot dry weight 2.28g and have highest SE value 130.5%, which was highly effective. Other plants inoculated with isolates FBD1, KB2d, FBGE1 and FBD1 which had SDW 1.58g, 1.43g, 1.29g and 1.27g, and SE values (Table 9), 73.1%, 66.2%, 59.7% and 58.8%, respectively were symbiotically effective.

Positive correlation was seen between SDW and NN (r=0.31) at P<0.001 and SDW with NDW (r=0.34) at P<0.001 (Table 10), which were positive indicator to take SDW as measure for symbiosis effectiveness screening Somasegaran and Hoben, (1994). Accordingly FBM11, FBD1, KB2d, FBGE1 and FBD1 were symbiotically effective. Therefore it could be better to test them in field or field soil sample.

The morphological characteristics study result (Appendix 3) showed that isolates showed different colony growth features. 29 isolates were texturally showed buttery colonies and the rest showed milky and watery translucent colonies. Their colony elevation for most of them was domed, about 3 of the 33 isolates (KK1c, KK1d and FBA12) produced extracellular polysaccharide and their colony appearance was circular with copious mucus production. Colony diameter ranged between 1.7mm to 5.4mm and mean generation time was in the range of b1.59hr and 3.65hr. Short time in mean generation indicates fast growth and the shortest colony diameter of rhizobia begins with 1mm.The characteristics growth features of the isolates were similar with rhizobia based on standard given by Somasegaran and Hobben, (1994).

Numerical analysis based on different phenotypic characteristics (Table 11) puts the isolates in to three different clusters and 2 un-clustered categories. Pongsilp *et al.,*(2010), reported that isolates

belonging to the same genera varied in phenotypic features of utilizing carbon and nitrogen sources, resistance to antibiotics and other substrate requirements. Accordingly number of isolates in cluster I (Phenon-I) were 2, cluster II (Phenon-II) consisted 11 isolates and 19 isolates were grouped under cluster III (Phenon-III) which was subdivided in to two sub clusters, sub clusterIIIa and IIIb. The remaining 2 isolates were unclustered. Of the isolates under cluster III the 14 isolates were grouped with reference strain 35 which nodulated faba bean. Rhizobial isolates which were nodulated faba bean taken as reference strain (Jordan, 1986; Zaharan *et al.,* 1999 and Wier, 2006).

The Isolates phenotypic characteristics were studied using physiological and biochemical test parameters in a cluster wise (Table 11). The entire test isolates (cluster I-III) were grew in pH values range of 5.0, 5.5, 6.0, and 8.0. In higher acidic value one isolate from cluster I, 3 isolates from cluster II and 10 isolates from cluster III were grew at pH 4.0, and at higher basic pH 10.0 none grew from cluster I, 2 isolates grew from cluster II and 12 isolates grew from cluster III. Rhizobial growth response to different pH valuer approaching towards acidic or basic pH decreased compared to standard neutral medium for good growth. Rhizobia have been reported to grow best at neutral pH (Singh *et al.,* 2008). On the work of Droun *et al.,*(1996), isolates studied were grown at pH values between 5.0 anf 9.4.

With rgard to isolates response to different concentrations of salt and changes of temperatures, most isolates in clusters showed growth response to salt concentrations range between 0.5 and 2.0%. at concentration of 2.5% only 6 isolates showed growth response. But the rest isolates in all clusters were failed to grow at concentration greater or equals to 2.5%. Isolates growth response decreases as salt concentration increased. The reason is the effect of osmotic stress. Increased salt concentration have determenrtal effect to the rhizobia (Negales *et al.,*2002).

The effect of changes of temperatures over isolates in the cluster showed that, all of the isolates under clusters grew at 15ºC, 20ºC and 30ºC; but, the growth response continued to 45ºC for isolates in cluster I. The growth response of isolates decreased towards higher extreme changes of temperatures, only 1 isolate under sub cluster IIIb was showed growth at 4ºC. The mesophillic rhizobia species can grow at temperature range of 10ºC and 37ºC (Graham,1992; Zaharan 1994). The failure or growth of rhizobia in salt concentrations and changes of temperatures resulted from their environmental back ground or genetic variability. All isolates in cluster I resisted erythromycin (10&20)

µgml^{-1}, and chloromaphinico (5 &20) µgml^{-1}and neomycin (5&10) µgml^{-1} each resisted by 1 isolate. Most isolates under sub-clusters in cluster III were grew in most tested antibiotics, but in case of cluster II the isolates were grew weakly and intermitentlty over some antibiotics mediums. None of the isolates was found to grow in mediun of spectinomycin 500 µgml^{-1}. With regard to heavy metals resistance test isolates in Clusters II and III showed varied resistance. But only one isolate from first cluster was resisted cobalt. The intrinsic antbiotics and heavy metals resistance pattern among studied isolates were varied in the woks over field pea and faba bean isolates phenotypic characterization (Ashenafi Haylu and Endalkachew Woldemeskel, 2011; and Getahun Negash, 2015). Isolates resistance to heavy metals and/or antibiotics is important characteristics to select elite straions in order to inoculate at the sites contaminated with them.

Isolates capacity to utilize carbon and nitrogen source showed that, aii isolates in cluster I utilized galactose starch, sucrose and arabinose but, showed intrmitent utilization for the rest remaining carbon and niteogen source. Isoltes from ciuster III were showed substrates utilization superiority over cluatr II. The potency to utilize carbon and ntrogen source with in isolates in the

cluster and among clusters varied (Table 11). The reson for that matter is adaptational or varied enzyme secretion. Pogsilp *et al.,*(2010), reported that isoltes belonging to the same genera varied in phenotypic featurwes including carbon and ntrogen sources and antibiotic resistance. finally none of the isoltes were not found to solublize phospha in tricalcium phosphate medium. tha possible rwason may be lack of enzyme that solublize phosphate from the rock. soil phosphate level must be considered before the elite strains from isoolates prepared as inoculats.

6. Conclusions and recommendation

6.1. Conclusion

Modified Leonard Jars enabled us to screen strains varying in symbiotic effectivness. Strains CA10, M4b and ICRE18 were symbiotically screened for their effectiveness in modified Leornard jars. Symbiotically screened strains significantly improved nodulation and shoot dry matter, shoot total nitrogen content and symbiosis effectiveness of chickpea plant. The improvement is due to the interaction among the symbionts and soil physical and biological properties. ICRE18 is compitent in Bulgita and Galebuge soils and CA10 is compitent in Taba soil.

Faba bean nodulating rhizobia are acid producers, fast growers, circular in colony shape, 1.7mm to 5.4mm in colony diameter and produces copious mucoidy colonies. They induce nodules on host plant up on re-infection. These morphological phenotypic properties are similar to reference strains suggeresting that the isolates are rhizobia.

They survive at wide range of pH, temperature and salt concentrations. They utilize diverse carbon and nitrogen sources and resist different antibiotics and heavy metals. This implies that the strains have various physiological and bichemical charactersitics.

Numercal analysis indicated that Ethiopian soils harbour diverse rhizobia nodulating faba bean.

6.2. Recommendations

It is recommended that symbiotically effective chickpea strains ICRE18 and CA10 which were checked over soil samples from different sites in lath house should be tested in the field

Molecular characterization should be carried out on rhizobia nodulating faba bean to support the phenotypic characteristics thus to unravel the true diversity in our collection.

Symbiotically effective faba bean isolates FBM11, FBD1, KB2d and FBD9 in sterile sand pot experiment under controlled condition should be checked in field soil samples or on the field to check their competency.

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APPENDIXES

Appendix 1: Chickpea strains sand culture screening experiment

Strain	Rep	NN	NFW	NDW	RFW	RDW	SFW	SDW	NC	NS	SC
CP1	1	28	0.3	0.08	2.79	0.38	2.07	0.39	P	L	G
CP1	2	53	1.03	0.21	3.95	0.44	2.03	0.46	P	L	G
CP1	3	29	0.32	0.06	3.34	0.32	2.01	0.43	P	L	G
CP26	1	19	0.25	0.05	2.16	0.28	2.09	0.42	P	M	G
CP26	2	32	0.8	0.13	2.42	0.35	2.14	0.49	P	L	G
CP26	3	10	0.18	0.03	2.54	0.31	1.69	0.34	P	L	G
CP67	1	37	0.48	0.11	3.6	0.43	2.13	0.38	P	L	G
CP67	2	53	0.81	0.12	3.67	0.4	2.12	0.42	P	L	G
CP67	3	58	0.94	0.25	3.53	0.37	2.68	0.45	P	L	G
CP68	1	42	1.16	0.12	2.48	0.34	1.92	0.44	P	L	G
CP68	2	16	0.17	0.02	1.71	0.27	1.86	0.32	P	M	G
CP68	3	26	0.32	0.04	2.12	0.43	1.72	0.37	P	L	G
CP98	1	44	0.47	0.09	3.88	0.45	1.77	0.41	P	L	G
CP98	2	62	0.57	0.11	1.74	0.38	2.25	0.51	P	L	G
CP98	3	37	0.42	0.07	2.72	0.29	1.69	0.38	P	L	G
CP99	1	40	0.52	0.08	2.53	0.32	2.54	0.54	P	M	G
CP99	2	44	0.45	0.09	2.47	0.4	2.58	0.63	P	L	G
CP99	3	38	0.25	0.05	2.51	0.3	2.38	0.39	P	L	G
CP129	1	40	0.22	0.09	2.86	0.26	2.84	0.54	P	M	G
CP129	2	20	0.12	0.05	3.26	0.36	2.67	0.37	W	M	G
CP129	3	33	0.19	0.07	2.26	0.46	2.78	0.45	P	L	G
CP130	1	48	0.92	0.23	3.09	0.3	2.58	0.41	P	L	G
CP130	2	45	0.52	0.15	1.95	0.26	1.78	0.39	P	L	G
CP130	3	50	0.6	0.11	2.83	0.42	2.63	0.41	P	M	G
CP151	1	45	0.63	0.12	3.36	0.46	2.16	0.39	P	L	G
CP151	2	50	0.78	0.24	2	0.2	2.25	0.51	P	L	G
CP151	3	47	0.67	0.12	1.85	0.28	2.13	0.41	P	L	G
ICRE18	1	43	0.95	0.27	2.44	0.31	2.65	0.96	P	L	G
ICRE18	2	33	0.95	0.15	3.25	0.43	2.34	0.64	P	M	G
ICRE18	3	39	0.4	0.11	2.62	0.24	2.51	0.87	P	L	G
ICRE25b	1	40	0.61	0.18	3.13	0.35	2.33	0.52	P	M	G
ICRE25b	2	65	0.61	0.2	2.37	0.3	2.45	0.42	P	L	G
ICRE25b	3	16	0.13	0.05	2.16	0.25	2.06	0.4	P	M	G
M4b	1	48	0.43	0.19	2.07	0.32	2.34	0.86	P	L	G
M4b	2	49	0.61	0.23	2.4	0.31	2.56	0.91	P	L	G
M4b	3	57	0.98	0.37	4.09	0.55	2.88	1.07	P	L	G
ICNRE03	1	38	0.47	0.04	3.34	0.39	1.92	0.36	P	M	G
ICNRE03	2	42	1.03	0.49	5.8	0.54	2.33	0.47	P	L	G

ICNRE03	3	52	0.64	0.28	5.07	0.48	2.28	0.48	P	L	G
M7	1	23	0.21	0.06	2.43	0.38	1.7	0.38	P	L	G
M7	2	32	0.45	0.21	3	0.31	2.1	0.42	P	L	G
M7	3	56	0.95	0.27	3.63	0.45	2.77	0.58	P	L	G
CA10	1	32	1.42	0.09	5.01	0.56	2.82	1.34	p	L	G
CA10	2	43	1.46	0.21	3.24	0.4	2.97	1.42	p	L	G
CA10	2	24	0.61	0.09	3.1	0.42	2.26	0.75	p	L	G
EAL-029	1	35	0.39	0.07	2.87	0.24	2.25	0.62	P	M	G
EAL-029	2	47	0.91	0.31	3.01	0.36	2.07	0.67	P	M	G
EAL-029	3	33	0.28	0.04	1.96	0.18	1.89	0.38	WP	L	G
CPM-20b	1	58	0.57	0.09	2.14	0.26	2.31	0.57	P	L	G
CPM-20b	2	34	0.25	0.06	2.25	0.33	2.14	0.38	WP	M	LG
CPM-20b	3	45	0.91	0.19	3.07	0.35	2.01	0.75	P	M	LG
N+	1	0	0	0	4.06	0.56	6.41	1.27			G
N+	2	0	0	0	4.5	0.92	7.32	1.36			G
N+	3	0	0	0	6.3	1.19	4.68	1.08			G
N-	1	0	0	0	5.06	0.48	1.42	0.26			YG
N-	2	0	0	0	1.53	0.18	1.32	0.27			YG
N-	3	0	0	0	3.44	0.34	1.31	0.26			YG

P=pink, W=white, L=large, S=small, M=medium, LG=light green, Y=yellow, DG=deep green, NN=nodule number, NFW, NDW, RFW, RDW, SFW, SDW=nodule, root, and shoot fresh and dry weight, NC & NS= nodule color and size, LC=leaf color

Appendix 2: Chickpea strains potted field sample soil experiment data

Kebele	Strain	Rep.	NN	NFM	NDM	RFM	RDM	SFM	SDM	TN%
Bulgita	CPM20b	1	25	0.4	0.11	5.48	0.64	9.89	2.56	3.10
	CPM20b	2	32	0.48	0.21	4.91	0.51	10.92	3.56	3.40
	CPM20b	3	55	0.71	0.28	4.63	0.51	11.25	3.78	3.53
	CA10	1	31	0.31	0.12	5.8	0.54	8.42	1.27	2.80
	CA10	2	56	0.67	0.24	4.7	0.42	9.31	1.25	2.80
	CA10	3	28	0.23	0.06	4.5	0.41	6.71	1.09	2.29
	M4b	1	49	0.63	0.19	3.66	0.46	9.96	1.57	3.20
	M4b	2	55	0.76	0.2	6.5	0.69	10.78	3.08	2.95
	M4b	3	50	0.68	0.12	3.67	0.54	9.56	2.46	3.50
	ICRE18	1	80	1.11	0.39	6.07	0.56	11.03	3.64	3.30
	ICRE18	2	68	0.99	0.13	5.38	0.63	10.67	4.13	4.85
	ICRE18	3	66	0.87	0.14	7.65	0.53	11.6	4.09	3.12
	CPEAL029	1	69	0.88	0.31	5.09	0.49	11.5	3.94	3.32

	CPEAL029	2	38	0.42	0.09	3.89	0.51	9.83	3.09	3.59
	CPEAL029	3	92	1.49	0.47	4.63	0.81	12.16	3.67	3.43
	N+	1	41	0.39	0.18	5.84	0.6	14.59	4.22	3.59
	N+	2	49	0.64	0.25	6.78	0.64	12.56	3.56	3.41
	N+	3	36	0.38	0.08	4.63	0.48	11.82	3.35	2.85
	Control	1	53	0.92	0.21	6.55	0.49	10.41	4.03	3.37
	Control	2	49	0.96	0.32	6.46	0.54	10.34	3.94	3.89
	Control	3	45	0.64	0.25	5.17	0.47	10.27	3.43	3.29
Gale buge	CA10	1	29	0.47	0.24	4.55	1.85	11.49	3.64	4.08
	CA10	2	33	0.54	0.26	5.45	1.89	11.35	3.91	4.14
	CA10	3	25	0.37	0.16	6.26	1.63	9.47	2.98	3.88
	M4b	1	38	0.96	0.25	4.99	0.63	11.34	3.96	4.03
	M4b	2	23	0.67	0.2	4.09	0.56	11.26	4.08	3.79
	M4b	3	17	0.53	0.06	3.3	0.57	10.64	3.97	3.81
	CPEAL029	1	41	0.23	0.12	7.29	0.86	11.31	4.36	3.99
	CPEAL029	2	19	0.12	0.04	11.04	0.89	10.24	3.68	3.99
	CPEAL029	3	16	0.09	0.02	4.46	0.55	9.78	2.93	4.21
	CPM20b	1	43	0.55	0.26	5.97	0.83	11.19	4.03	3.86
	CPM20b	2	25	0.37	0.11	7.85	0.95	9.97	3.1	3.22
	CPM20b	3	48	0.61	0.29	5.13	0.75	11.88	3.61	3.59
	ICRE18	1	33	0.67	0.29	2.56	0.54	11.96	4.23	4.10
	ICRE18	2	31	0.51	0.18	2.02	0.51	12.31	4.44	4.10
	ICRE18	3	50	0.71	0.34	3.68	0.77	12.44	4.41	4.30
	Control	1	47	0.69	0.26	4.09	0.56	11.79	4.53	4.52
	Control	2	38	0.54	0.23	3.45	0.53	10.47	3.79	3.69
	Control	3	24	0.33	0.11	2.44	0.45	9.95	2.91	3.63
	N+	1	10	0.12	0.06	5.49	0.71	13.66	4.52	3.98
	N+	2	12	0.18	0.09	3.67	0.86	12.86	4.38	4.42
	N+	3	25	0.33	0.09	3.67	0.714	13.12	4.27	4.00
Taba	CA10	1	83	1.49	0.68	4.94	0.54	11.94	3.32	3.00
	CA10	2	52	1.23	0.49	1.05	0.07	10.78	3.27	2.70
	CA10	3	38	0.49	0.33	0.49	0.61	10.59	2.96	2.79
	CPM20b	1	39	0.67	0.17	5.23	0.67	9364	1.87	2.97
	CPM20b	2	52	1.14	0.27	5.72	0.39	10.24	2.63	2.96
	CPM20b	3	50	0.77	0.19	3.66	0.64	10.01	2.97	2.81
	M4b	1	59	1.09	0.29	8.61	0.86	10.57	2.94	2.93
	M4b	2	49	0.99	0.25	7.71	0.76	8.66	2.74	2.68
	M4b	3	47	0.87	0.24	6.24	0.64	10.53	2.63	3.04
	Control	1	59	1.06	0.39	5.68	0.81	10.53	2.91	3.20
	Control	2	37	0.79	0.19	6.99	0.76	9.23	2.56	3.03

Control	3	54	1.01	0.35	5.19	0.64	10.75	2.84	3.33
CPEAL029	1	35	0.64	0.23	4.23	0.49	11.33	3.86	2.94
CPEAL029	2	44	0.65	0.28	4.16	0.51	9.51	3.09	2.65
CPEAL029	3	48	0.78	0.43	8.35	0.57	9.28	2.38	2.78
N+	1	70	1.03	0.49	5.63	0.76	18.52	4.12	3.43
N+	2	20	0.29	0.16	5.45	0.75	11.53	3.17	3.15
N+	3	12	0.15	0.05	7.78	0.69	12.41	3.24	3.00
ICRE18	1	52	0.98	0.38	5.87	0.76	11.23	3.02	2.95
ICRE18	2	55	1.09	0.48	7.67	1.01	11.66	3.02	2.85
ICRE18	3	47	0.94	0.37	5.9	0.69	9.93	2.41	2.96

Control= chickpea plant grown over potted field soil, but neither strain inoculated nor N- fertilized, Bulgita= sample soil site of Eastbadawacho, Galebuge=sample soil site of Damotpullasa, Taba= sample soil site of Damotgale.

Appendix 3: Faba bean Authentication test

strain	Rep	NN	NFW	NDW	RFW	RDW	SFW	SDW	NC	NS	LC
KB2C	1	11	0.4	0.07	8.48	0.62	5.6	0.74	P	M	YG
KB2C	2	6	0.3	0.05	2.52	0.23	2.65	0.38	P	L	G
FBA4	1	3	0.1	0.001	1.54	0.29	3.84	0.63	W	S	YG
FBA4	2	12	0.13	0.08	3.1	0.29	4.82	0.38	W	S	YG
KB3e	1	4	0.08	0.03	2.53	0.72	2.48	0.39	P	S	YG
KB3e	2	11	0.17	0.06	2.18	0.36	6.89	1.01	P	S	YG
FBM11	1	20	0.17	0.09	7.42	0.81	10	2.34	P	M	G
FBM11	2	12	0.12	0.06	3.35	0.52	8.42	1.98	P	L	YG
KB1c	1	27	0.36	0.12	3.37	0.31	7.89	0.79	W	L	G
KB1c	2	15	0.29	0.06	5.91	0.41	6.09	0.65	W	M	YG
FBAy	1	9	0.06	0.02	4.79	0.31	4.8	0.54	W	L	YG
FBAy	2	12	0.09	0.03	4.05	0.66	6.5	0.98	W	L	YG
KK1c	1	6	0.05	0.002	2.16	0.48	4.24	0.65	WP	L	YG
KK1c	2	10	0.09	0.006	3.05	0.61	2.09	0.81	WP	M	YG
KK1d	1	16	0.18	0.06	4.85	0.39	7.5	0.56	W	M	G
KK1d	2	11	0.09	0.04	2.07	0.03	5.4	0.36	W	M	G
KB2e	1	6	0.04	0.02	3.34	0.18	4.5	0.35	W	M	G
KB2e	2	19	0.18	0.08	3.97	0.24	6.5	0.43	W	M	G
KB2e	3	15	0.14	0.06	2.9	0.21	5.92	0.39	P	M	G
KB2g	1	12	0.12	0.04	3.62	0.12	3.71	0.21	P	M	G
KB2g	2	19	0.15	0.06	2.89	0.11	4.54	0.25	P	M	G
KB2b	1	4	0.03	0.006	2.56	0.48	2.86	0.68	WP	S	G
KB2b	2	10	0.07	0.01	3.01	0.51	3.16	0.85	WP	M	G
KB2b	3	12	0.12	0.05	1.89	0.59	4.43	1.02	P	L	YG
FBA12	1	12	0.1	0.04	2.51	0.43	2.57	0.92	P	M	G
FBA12	2	25	0.25	0.13	2.04	0.52	5.25	0.99	P	M	YG
FBA12	3	8	0.09	0.03	1.84	0.09	1.97	0.21	W	L	G
FBM6	1	9	0.1	0.04	1.85	0.63	1.99	0.73	W	L	Y
FBM6	2	17	0.18	0.09	1.89	0.52	2.08	0.98	W	M	YG
FBM8	1	21	0.21	0.04	2.08	0.72	2.13	1.03	P	S	YG
FBM8	2	16	0.15	0.03	2.05	0.58	1.97	0.89	P	L	Y
FBA11	1	6	0.05	0.002	2.05	0.35	2.22	0.59	S	S	G
FBA11	2	12	0.09	0.004	1.04	0.29	1.84	0.63	S	M	G
KB2d	1	15	0.12	0.07	2.95	1.02	3.05	1.49	M	M	YG
KB2d	2	10	0.08	0.002	1.97	0.93	2.07	1.37	S	M	YG
FBGE1	1	39	0.39	0.11	1.92	0.5	8.09	1.81	W	S	G
FBGE1	2	35	0.38	0.1	3.84	0.32	7.36	1.15	P	L	G
FBGE1	3	29	0.25	0.09	5.68	0.66	5.87	0.91	P	L	G

FBGE5	1	46	0.15	0.09	3.34	0.4	7.65	1.38	P	S	LG
FBGE5	2	40	0.1	0.04	4.53	0.68	6.61	1.09	P	S	LG
FBGE5	3	18	0.03	0.01	1.32	0.51	4.36	0.86	W	S	LG
FBGE7	1	16	0.05	0.01	1.85	0.53	6.66	0.9	W	S	LG
FBGE7	2	53	0.45	0.11	4.09	0.5	8.01	1.02	P	M	LG
FBGE7	3	64	0.64	0.14	5.27	0.56	9.11	1.16	P	M	LG
FBGE8	1	13	0.08	0.01	6.58	0.58	3.73	0.67	W	S	LG
FBGE8	2	21	0.18	0.05	4.78	0.49	6.3	0.95	W	L	G
FBD1	1	12	0.25	0.08	3.71	0.78	6.84	1.34	P	L	G
FBD1	2	30	0.39	0.15	3.53	0.6	7.59	1.87	P	L	G
FBD1	3	18	0.09	0.04	2.68	0.63	6.97	1.54	W	S	G
FBD4	1	12	0.07	0.02	1.89	0.27	6.37	0.99	P	M	G
FBD4	2	28	0.23	0.1	2.14	0.45	7.02	1.09	P	M	G
FBD4	3	39	0.4	0.12	3.68	0.37	8.95	1.14	P	M	G
FBD5	1	22	0.08	0.03	3.87	0.54	5.5	0.74	W	S	G
FBD5	2	14	0.03	0.01	1.84	0.68	4.51	0.66	W	S	LG
FBD5	3	39	0.07	0.05	4.08	0.4	5.82	0.8	W	S	G
FBD7	1	35	0.08	0.03	1.07	0.71	6.99	1.24	P	M	LG
FBD7	2	12	0.05	0.01	1.54	0.24	3.55	0.34	P	M	LG
FBD8	1	51	0.16	0.09	2.97	0.61	5.75	1.38	P	L	G
FBD8	2	7	0.01	0.001	3.02	0.69	7.09	0.91	W	S	LG
FBD9	1	40	0.19	0.08	1.41	0.5	7.31	1.34	P	L	G
FBD9	2	92	0.69	0.12	2.76	0.53	6.97	1.21	P	L	DG
FBD11	1	23	0.12	0.05	1.75	0.2	5.35	1.05	W	S	LG
FBD11	2	18	0.09	0.03	2.89	0.63	5.56	0.67	W	S	LG
FBD14	1	3	0.07	0.01	4.11	0.68	8.96	1.01	P	M	G
FBD14	2	21	0.04	0.02	3.9	0.74	9.02	1.04	W	S	LG
FBD14	3	16	0.09	0.04	4.82	0.51	8.57	1.24	P	L	G
FBH1	1	7	0.01	0.001	0.46	0.36	2.12	0.64	W	S	G
FBH1	2	18	0.06	0.02	2.11	0.13	3.58	0.55	W	M	G
FBH1	3	3	0.02	0.001	2.83	0.24	4.9	0.35	W	S	LG
FBH2	1	39	0.13	0.06	1.79	0.47	5.16	0.86	P	M	G
FBH2	2	29	0.08	0.03	3.93	0.67	6.42	1.05	W	L	LG
FBH3	1	27	0.03	0.01	1.58	0.73	6.69	1.07	LP	M	LG
FBH3	2	39	0.16	0.09	4.14	0.59	7.09	1.24	P	L	LG
FBH3	3	23	0.12	0.05	4.15	0.52	5.82	0.96	P	M	G
FBSD2	1	25	0.13	0.08	2.6	0.53	7.95	1.08	P	S	LG
FBSD2	2	5	0.02	0.002	3.98	0.58	5.26	0.98	W	S	YG
FBSD2	3	62	0.36	0.12	4.62	0.64	8.97	1.38	P	M	G
FBSD7	1	19	0.08	0.04	2.47	0.71	7.37	1.04	P	S	LG
FBSD7	2	9	0.03	0.01	1.67	0.25	3.16	0.87	P	L	G

FBSD7	3	44	0.22	0.07	3.43	0.26	3.16	1.18	P	L	G
35	1	49	0.29	0.12	3.27	0.38	4.19	1.28	P	L	G
35	2	23	0.13	0.09	2.54	0.65	3.04	0.99	P	L	G
35	3	34	0.19	0.1	3.04	0.51	3.16	1.15	LP	M	G
N+	1	0	0	0	6.79	0.85	8.96	2.16			DG
N+	2	0	0	0	4.63	0.59	9.02	2.24			DG
N+	3	0	0	0	4.08	0.66	8.57	2.08			G
N-	1	0	0	0	1.23	0.26	3.48	0.38			YG
N-	2	0	0	0	0.54	0.15	2.02	0.38			YG
N-	3	0	0	0	1.84	0.28	1.67	0.52			PG

P=pink, W=white, L=large, S=small, M=medium, LG=light green, Y=yellow, DG=deep green, NN=nodule number, NFW, NDW, RFW, RDW, SFW, SDW=nodule, root, and shoot fresh and dry weight, NC & NS= nodule color and size, LC=leaf color

Appendix 4: Data for test results of isolates colony morphology characteristics

	CR-absorption	YMA-BTB	PGA-BCP	Gram test	Bacterial shape	Colony appearance	Size (mm)	colony Texture	EPS	Elevation	MGT
KB1c	-	+	-	-	Rod	circular	1.7	Buttery	-	Domed	1.59
KK1c	-	+	-	-	Rod	circular	3.5	Buttery	+	Flat	2.03
KK1d	-	+	-	-	Rod	Circular	3.3	Buttery	+	Domed	2.1
KB2b	-	+	-	-	Rod	Circular	4.8	Buttery	-	Domed	1.89
KB2c	-	+	-	-	Rod	circular	3.1	Buttery	-	Domed	1.98
KB2d	-	+	-	-	Rod	Circular	3.5	Buttery	-	Domed	2.31
KB2e	-	+	-	-	Rod	Circular	5.4	Buttery	-	Raised	2.23
KB2g	-	+	-	-	Rod	Circular	3.4	Buttery	-	Domed	1.25
KB3e	-	+	-	-	Rod	Circular	3.3	Watery	-	Flat	1.9
FBD1	-	+	-	-	Rod	Circular	2.5	Buttery	-	Domed	2.6
FBD4	-	+	-	-	Rod	Circular	3.3	Buttery	-	Domed	1.96
FBD5	-	+	-	-	Rod	Circular	3.1	Buttery	-	Raised	2.98
FBD7	-	+	-	-	Rod	Circular	2.9	Buttery	-	Domed	3.4
FBD8	-	+	-	-	Rod	Circular	3.0	Buttery	-	Domed	3.65
FBD9	-	+	-	-	Rod	circular	3.2	Buttery	-	Domed	2.5

FBD11	-	+	-	-	Rod	Circular	2.8	Buttery	-	Domed	2.5
FBD14	-	+	-	-	Rod	Circular	5.0	watery	-	Raised	2.4
FBGE1	-	+	-	-	Rod	Circular	2.7	Buttery	-	Raised	2.0
FBGE5	-	+	-	-	Rod	Circular	2.8	Milky	-	Domed	2.0
FBGE7	-	+	-	-	Rod	Circular	3.2	Buttery	-	Domed	1.8
FBGE8	-	+	-	-	Rod	Circular	4.0	Buttery	-	Domed	2.1
FBH1	-	+	-	-	Rod	Circular	2.4	Buttery	-	Domed	1.98
FBH2	-	+	-	-	Rod	Circular	5.2	Milky	-	Domed	1.8
FBH3	-	+	-	-	Rod	Circular	3.1	Buttery	-	Domed	2.0
FBA4	-	+	-	-	Rod	Circular	4.2	Buttery	-	Domed	2.4
FBA11	-	+	-	-	Rod	Circular	3.6	Buttery	-	Domed	1.95
FBA12	-	+	-	-	Rod	Circular	4.2	watery	+	Domed	2.0
FBAy	-	+	-	-	Rod	Circular	2.5	Buttery	-	Domed	1.8
FBSD2	-	+	-	-	Rod	Circular	3.1	Buttery	-	Domed	2.3
FBSD7	-	+	-	-	Rod	Circular	2.8	Buttery	-	Domed	2.1
FBM6	-	+	-	-	Rod	Circular	3.6	Buttery	-	Domed	2.2
FBM8	-	+	-	-	Rod	Circular	1.8	Buttery	-	Domed	2.1
FBM11	-	+	-	-	Rod	Circular	3.7	Buttery	-	Domed	2.0
35	-	+	-	-	Rod	Circular	4.5	Buttery	-	Raised	2.5

-: no absorption of congo red (CR) dye and no growth on Peptone Glucose agar with Bromocresol purple (PGA-BCP), += Conversion of Bromotymol Blue dye to yellow and acid production, marker for exo-polysaccharide production (EPS), -: code for gram negative, YMA-CE=Yeast mannitol agar with congored and MGT= mean generation time.#35= reference strain from Holota Agricultural Research Institute

Appendix 5: Response of the isolates and reference inoculant to extreme pH, Temperature and salt

Test character	Values	Growth response	Percentage (%)
	4	13	38.2
	4.5	32	94.1
	5	34	100
	5.5	34	100
PH concentration	6	34	100
	8	34	100
	8.5	32	94.1
	9	33	97.1
	9.5	30	88.2
	10	15	44.1
	0.5	33	97.1
	1	32	94.1
	1.5	26	76.5
	2	24	70.6
Salt concentration	2.5	6	17.65
	3	0	0
	3.5	0	0
	4	0	0
	4.5	0	0
	5	0	0
	4^0C	1	2.4
	10^0C	17	50
	15^0C	34	100
Temperature changes	20^0C	34	100
	30^0C	34	100
	35^0C	24	70.6
	40^0C	20	58.8
	45^0C	12	35.3

Appendix 6: C- and N-Source utilization of the Isolates and reference strains

C-source	Isolates	%	N-source	Isolates	%
Glucose	25	73.5	Proline	19	55.9
Sucrose	34	100	Isolucine	22	64.7
D-fructose	10	29.4	L-histadine	22	64.7
D-trehalose	28	82.4	L-asparagine	26	76.5
D-mannose	16	47.1	L-asparatic acid	21	61.8
Rhaminose	24	70.6	L-valine	22	64.7
Galactose	29	85.3	L-arganine	23	67.6
L-arabinose	30	88.2	Glycine	17	50.0
Starch	24	70.6			
L-Xylose	25	73.5			

Appedndix7: The test Isolates response to antibiotic concentrations (µgml-1)

Antibiotic	Concentration(µgml^{-})	Resistant isolate	Percentage (%)
Streptomycin	40	6	17.65
Streptomycin	80	8	23.53
Erythromycin	10	16	47.1

Erythromycin	20	13	38.2
Chloromaphinicol	5	13	38.2
Chloromaphinicol	20	12	35.3
Neomycin	5	23	67.65
Neomycin	10	12	35.3
Novobioycin	5	20	58.8
Spectinomycin	250	5	14.7
Spectinomycin	500	0	0

Appendix 8: Internisic heavy metal reisitance of the isolates

Metallic compound	Concen. (μg/ml)	Resistant isolates	Percentage (%)
$CoSO_4$.7H2O	100	29	85.3
$AlCl_3$	500	20	58.8
$ZnCl_2$	500	8	23.53
$MnSO_4 .H_2O$	500	23	67.65
Pb(CH3COOH)$_2$	500	17	50

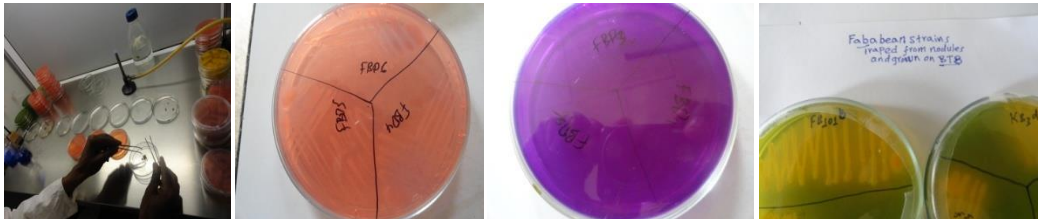
Appendix 9: Some sample photos taken during experiment

1. Sand prepared, seed germinated, transplanted and strain inoculated (photo by Tadele E.)

2. Grown and after 45 days harvested (photo by Tadele E.)

3. Chickpea rhizobia strains inoculated for symbiotic effectiveness test in potted sample soils photos from inoculation to harvesting

4. Faba bean nodulating rhizobia isolation and authentication photos



Germinating Fababean seeds
KB3e
FBM11
FBD9
FBD14