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HAWASSA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTITIONAL SCIENCE

DEPARTMENT OF BIOLOGY

ISOLATION, CHARACTERIZATION, SYMBIOTIC EFFECTIVENESS AND HOST RANGE OF RHIZOBIA TRAPPED USING COMMON BEAN (*Phaseolus vulgaris*) FROM FARMERS FIELD IN HAWELLA DISTRICT; SIDAMA REGION

MSC THESIS

BY

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MAY, 2024

HAWASSA, ETHIOPIA

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A MASTER'S THESIS SUBMITTED TO THE DEPARTMENT OF BIOLOGY, COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES, SCHOOL OF GRADUATE STUDIES, HAWASSA UNIVERSITY,

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MAJOR ADVISOR: KEDIR WOLIY (PhD)

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HAWASSA, ETHIOPIA

CANDIDATE DECLARATION

I, the undersigned, declare that this is my original work and has never been presented in this or any other University and that all the source materials used for this thesis have been duly acknowledged;

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The thesis has been submitted for examination with my approval as a University advisor.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BCP	Bromcresol purple
BNF	Biological nitrogen fixation
BTB	Bromothymol Blue
CBR	Common Bean Rhizobia
CR	Congo red
CRD	Completely randomized design
GPA	Glucose Peptone Agar
LAI	Leaf Area Index
LSD	Least significant difference
MLSA	Multi Locus Sequence Analysis
MPN	Most Probable Number
RE	Relative efficiency
SNF	Symbiotic Nitrogen Fixation
SPSS	Statistical Package for Social Sciences
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth

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ABSTRACT

Rhizobia differ greatly in their symbiotic properties and efficiency in nitrogen fixation and therefore, the selection of highly effective strains is of great importance when attempting to achieve a successful preparation of rhizobial inoculants. The aim of this study was to isolate, characterize, and evaluate symbiotic effectiveness and host range of rhizobia trapped from small holder farmers' fields in Hawella district of Sidama regional state using common bean. A total of 8 bacterial isolates were trapped from different soil samples. Of these, only five isolates were authenticated as rhizobia. These five isolates showed differences in various presumptive, cultural, biochemical and physiological characteristics. The results revealed that the indigenous common bean rhizobia isolates considerably differ in their symbiotic efficiency. Two rhizobial isolates (CBR7 and CBR8) significantly produced high nodule number, nodule dry weight and shoot dry weight ($p<0.05$). The remaining three isolates were not effective in fixing atmospheric nitrogen with the host plants. In addition, all the five isolates formed nodules faba bean, alfalfa and lentils exhibiting potential host promiscuity. Isolates CBR7 and CBR8 exhibited high N_2-fixation efficiency and can be recommended for further evaluation to use them as inoculants for different legumes. Furthermore, we recommend detailed molecular characterization to determine their phylogenetic and taxonomic positions.

Keywords: Biological inoculation, nitrogenfixation, Rhizobia, symbiotic effectiveness.

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1. INTRODUCTION

1.1 Background

Rhizobium is a gram negative soil bacterium capable of infecting legume roots, inducing the formation of nodules and fixing nitrogen through the process known as Biological Nitrogen Fixation (BNF) in the soil (Mohammadi and Sohrabi, 2012; Namkeleja *et al.,* 2016; Poonia, 2011). The BNF takes place when rhizobia form a symbiotic relationship with legume plants as a result; they trap and fix atmospheric nitrogen into a form such as ammonia (Laguerre *et al.,* 1996; Namkeleja *et al.,* 2016; Pawar *et al*., 2014; Poonia, 2011). This form of mutualistic symbiotic relationship has been described as the most important biological mechanism for providing nitrogen to the plants as alternative to expensive synthetic nitrogen fertilizers in agriculture (Freiberg *et al*., 1997). The Biological Nitrogen Fixation has grown attention in recent years in Africa because it is an environmental friendly farm input as it prevents groundwater pollution by nitrates and its less expensive (Berrada and Fikri-Benbrahim, 2014; Bhaganagare *et al*., 2013). It is a useful technology in agricultural systems in order to overcome the problems associated with depletion of soil fertility as it manages soil acidity and salinity, which is accumulated due to frequent application of synthetic nitrogen fertilizers (Loganathan *et al*., 2010; Nyoki and Ndakidemi, 2014; Tairo and Ndakidemi, 2013). Moreover, the use of inorganic nitrogen fertilizers usually get lost by volatilization, de-nitrification and leaching of nitrate into ground water and report shows that only 30% to 50% is used by the crop (Graham and Vance, 2003).

Common bean (*Phaseolus vulgaris* L.) is an essential source of proteins, carbohydrates, vitamin B complex (riboflavin, thiamine, niacin, and folic acid), and vital minerals in human nutrition.

Flavonoid and isoflavonoid mainly produced as defense compounds against pathogens, and antioxidants as protectors against UV radiations act as anti-cancer agents that inhibit the tyrosine kinase, cyclooxygenase, protein kinase and lipoxygenase enzyme activities). Common bean is therefore not only a source of essential nutrients but also has medicinal features. In many parts of the world, the production of common bean is restricted by poor soil fertility, particularly nitrogen limitations). The farmers cannot compensate for this shortage by applying fertilizers, mainly because of economic reasons (Khonje, 1989; Onim, 1993; Savci, 2012**).** Rhizobia not only supply the plants with nitrogen through nitrogen fixation but also protect them against pathogens by activating their immune system (Beardon *et al*., 2014). They also confer tolerance to extreme temperatures, drought, salinity, or non-appropriate soil pH, often restricting common bean yield (Mabrouk *et al.*, 2018). Another feature of the rhizobia is phosphorus provision to the host plants through phosphate solubilization (Hao *et al*., 2014); however, solubilizing phosphate's ability differs substantially among Rhizobial strains.

Poor soil fertility is the major challenge that deters crop production in the tropics and sub-tropics (Giller *et al*., 1998; Mwandemele and Nchimbi, 1990). Legumes including Phaseolus vulgaris are among the crops whose production is lowered by the soil nutrient deficiencies. In Tanzania soil fertility loss and increased demand for food production because of high population pressure has led to increased use of inorganic fertilizers in most cropping systems. But, excessive use of inorganic fertilizers has an adverse impact on the environment. Suprapta (2012) reported that, excessive use of inorganic fertilizers has undesirable effects on agriculture, food, and biodiversity and health status of communities. Use of organic resources such as animal manure and compost is a traditional practice of farmers in Northern Tanzania to restore soil fertility, but its continued use is limited by the availability of these resources. Incorporation of grain legumes,

including *P. vulgaris* in combinations with inorganic fertilizers is a practical way to achieve sustainable food production, taking into consideration of annual population increase rate of 2.7% *(National Bureau of Statistics*, 2012).

Rhizobia-legume symbiosis is the most studied plant-bacteria mutualism (Subramanian *et al*., 2009). Rhizobia are soil bacteria that are well known for their symbiotic relationship with legumes even though they are also found in soil devoid of legumes (West *et al*., 2002; Denison and Kiers, 2004a, b; Lindström and Mousavi, 2010). Rhizobia are grouped into two major groups which are fast grower and slow growers (Somasegaran and Hoben, 2012). Fast grower takes 3-5 days to grow on the media and when grown in media containing bromothymol blue (BTB) indicator, they undergo alkaline reaction. While slow growing rhizobia takes about 7 to 10 days to grow on the media and show acidic reaction on BTB (Bala *et al*., 2010). Most of rhizobia which nodulate Phaseouls beans are from genus Rhizobium and species belong to genus Rhizobium are fast growers (Somasegaran and Hoben, 2012). Rhizobia range from symbiotic which nodulate legumes, to non-symbiotic which are unable to nodulate legumes at all. Symbiotic rhizobia are divided into two groups which are mutualistic and parasites. Mutualistic rhizobia supply their hosts with nitrogen at a reasonable carbon cost while parasite rhizobia infect legume plants but then fix little or no nitrogen inside their nodules (*Denison and Kiers,* 2004a). This review is based on Mutualistic rhizobia.

Common bean (*Phaseolus vulgaris*) was domesticated 8000 years ago in America and today is a staple food consumed worldwide for its edible seeds and pods (Maiti and Singh 2007). It is widely appreciated in developing countries for its affordability and its long storage life.

It is widely distributed throughout the world with total global production of about 25 million MT with a productivity of 792 kg/ha (FAOSTAT, 2013) out of which about 6 million MT is

produced in Africa (FAOSTAT, 2015). Latin America and sub-Saharan Africa are the leading producers of common beans. It is also cultivated in many parts of the tropics and sub-tropics as well as throughout the temperate regions with approximate contributions of three-quarters of the global production (Katungi *et al*., 2009) due to its high in starch, protein, and dietary fiber and is an excellent source of potassium, selenium, molybdenum, thiamine, vitamin B6, and folic acid (Maiti and Singh 2007). The ripe seeds are cooked for soups and broth in the world (Brucher *et al.*, 1977). Common bean also has the economic and environmental benefit of associating with N_2-fixing bacteria that gives the advantage to fix atmospheric nitrogen and leaving phosphorous (P) for plant growth.

Though common bean is grown in most of Ethiopia, production is mainly concentrated in the east (Hararghe highlands), the south and south-west (Sidama), in the Rift valley, and other arid and semiarid zones. Productivity of the crop is often constrained by problems of soil acidity and low soil fertility. Particularly, soil acidity is major constraint, since it causes low P availability and Al and Mn toxicity is of localized importance (CIAT, 1989).

Girma (2009) reported that low soil fertility status especially low levels of N and P was the major constraint for common bean production and responsible for the loss of grain yield up to **1.2 million tons in Africa**.

In general, an increase in grain yield and other agronomic parameters of common bean was observed as the rate of nitrogen and phosphorus increased till 27 kg N ha-1 and 69 kg P_2O_5 ha^{-1} (**150 kg DAP** $\mathbf{ha^{-1}}$) (Girma, 2009).

This fertilizer rate also gave yield advantages of 39% over the control. Among the nutrients, nitrogen is the critical limiting element for the growth of most plants including common beans due to its unavailability and poor fixation (Vance, 2001).

1.2. Statement of the Problems

Smallholder farmers productivity is threatened by poor soil fertility exacerbated by the depletion of essential nutrients including nitrogen and phosphorous, there by jeopardizing the efforts to sustain food security (Ndakidemi *et al*., 2006; Okalebo *et al.,* 2007; Pereira *et al*., 2006). Feasible and adaptable options to address the problem of depleted soil nitrogen include, among others, the use of BBNF technology (Bull *et al.,* 2002; Chisholm *et al.,* 2006). BNF through rhizobia inoculants is one of the best alternatives to increase N in the system because apart from increasing crop yield it also enhances environmental conservation (Bloem *etal.,* 2009). Unfortunately, in most African countries including Ethiopia awareness and the accessibility of the inoculants is not well established. In many African countries, most of Rhizobial inoculants are imported from abroad. Moreover, where produced (e.g. in Kenya and Zimbabwe), inoculants are manufactured using imported commercials which are necessarily not as effective as some of the land race strains. Therefore, it is necessary to isolate and investigate local rhizobial strains for use as effective inoculants. To develop effective inoculants and maximize legume productivity, it is essential to assess the symbiotic effectiveness of indigenous rhizobia isolates adapted to local environmental conditions. In this regards, the availability of compatible rhizobia nodulating common bean in the soil of the study area is scarce. Therefore the objective of this study was to isolate, characterize and evaluate symbiotic effectiveness of indigenous rhizobia isolates using common bean as trap host.

1.3. Objectives of the Study

1.3.1. General objective

The overall objective of this study was to isolation, characterization, symbiotic effectiveness and host range tastes of rhizobia trapped from smallholder farmer's in Hawela district of Sidama regional state, Ethiopia using common bean

1.3.2. Specific objectives

The specific objectives were;

i. To trap isolate, Characterize and authenticate rhizobia from small holder farmers in Hawella district of Sidama regional state

ii. To evaluate symbiotic effectiveness of the rhizobial isolates

iii. To evaluate host range of the rhozobial isolates using different legume hosts.

1.3.3. Research questions

The purpose of this resea]rch is to answer the following questions:

- What are the isolates morphological and biochemical characteristics?
- Which of the isolates are symbiotically effective?
- What other hosts the isolates can nodulate?

1.3.4. Significance of the study

Because there is insufficient information on the characterization and utilization of native rhizobia nodulating common bean in the study area this study would provide information on whether indigenous common bean nodulating rhizobia was present in the study area, characterize their physiological and biochemical properties, and screen their symbiotic effectivity. The study can be used as a baseline data for further study of such indigenous rhizobia in the study area.

2. LITERATURE REVIEW

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).

2.2. Isolation of elite rhizobia strains

Rhizobia strain isolation is done by taking nodules from the host legume, sterilizing them by using ethanol and sodium hypochlorite, followed by crushing it in a sterile Petri dish by using blunt tipped sterilized forceps and lastly streaking drop of the nodule suspension on the media. Yeast-Mannitol agar (YMA) and peptone glucose agar are used as growth media while bromothymol blue (BTB) or Congo Red (CR) is used as indicators (Bala *et al.*, 2010; Somasegaran and Hoben, 2012). In most cases, YMA containing CR or BTB indicators are used as evidenced in many studies (Zahran *et al*., 2013; Deshwal and Chaubey, 2014; Hassen *et al*., 2014). The use of indicator media reported to camouflage real morphologies and distort the growth rate of the rhizobia (Bala *et al*., 2010). Because of this drawback, some isolation is done

on YMA plates without indicator media. Rhizobia isolation which does not involve the use of indicator media, aims at investigating uniformity of colonies growth across the plates, uniformity indicate a pure culture while non-uniformity indicate contamination.

Isolation of rhizobia is a valuable process to maximize agricultural production (Berrada *et al.*, 2012; Berrada and Fikri-Benbrahim, 2014; Simon *et al*., 2014). It helps to get strong strain for nitrogen fixation, because effectiveness in nitrogen fixation by soil rhizobia population does vary widely between species (Singleton and Tavares, 1986) and the number of rhizobia that are not yet known is big and exceed the known one (Giller, 2001; Wolde-meskel *et al*., 2005). Therefore, isolation of indigenous rhizobia is a stepping stone towards discovering effective strain that will be more efficient in fixing nitrogen for various legumes. Specifically isolation of rhizobia strain from nonspecific (promiscuous) legumes gives a wider chance of identifying new effective strains for such legumes.

Common bean (Phaseolus vulgaris L.) is amongst the promiscuous legume hosts (Valverde *et al*., 2006; Aserse *et al*., 2012) and several rhizobia species have been reported to nodulate this legume, although not always effective in terms of fixing N_2 (Dall'Agnol *et al*., 2014). Since Phaseolus bean is a most important legume crop in most African countries as earlier mentioned, there is a need of isolating elite rhizobia strains nodulating it in areas where its production is practiced but yield potential has never been realized.

2.3. Biological Nitrogen Fixation

Nitrogen (N_2) is one of the most abundant elements on earth, which constitutes about 80% of the earth's atmosphere, but its availability often limits plant growth and crop production. This situation arises because the N_2 molecule is very stable chemically and so is unusable by most

biological organisms. It must be "fixed" before it can be assimilated. In nature, there are two main ways of" "fixing" nitrogen: lightning and biological nitrogen fixation (BNF). Atmospheric nitrogen fixation probably contributes at most about 10% of the total annual yield of fixed nitrogen. By far the most important source of fixed nitrogen derives from the activity of certain soil bacteria (hence is called biological N fixation) that absorb atmospheric N_2 gas and convert it into ammonium(Fisher & Newton, 2002).

BNF is a process by which N_2 in the atmosphere is reduced into a biologically useful, combined form of N-ammonia by living organisms (Giller, 2001). This process is carried out by a small group of bacteria, in either free-living condition, associated with different plants such as epiphytes or endophytes, or establishing endocellular symbiosis with legumes. The rhizobium-legume symbiosis involves the exchange of carbon sources produced by the plant and ammonium fixed by the bacteria in specialized organs known as nodules. This symbiotic relationship aids legumes in naturally colonizing nitrogen-deficient soils and benefits agriculture not only by saving nitrogen (N) fertilizers, but also by reducing the negative impact on the environment, which is essential for achieving sustainable agriculture. The use of legume crops substantially reduces the N requirement from external sources (Bhattacharyya &Jha, 2012).

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 about 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. Symbiotic Nitrogen Fixation in Legumes

The *Fabaceae*, also known as *Leguminosae* or legumes, are the third largest family of flowering plants, with a diverse range of economically and scientifically important genera and species. The family contains nearly 770 genera and over 19,500 species, resulting in a staggering amount of diversity. The *Faboideae*, formerly known as the Papilionoids, are the largest subfamily of legumes and possibly the most important economically. This clade contains almost all of the legumes that are widely known or economically important, such as soybean (*Glycine max*), peanut (*Arachis hypogaea*), common bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), pea (*Pisums ativum*), licorice (*Glycyrrhiza glabra*), cowpea (*Vignaungui culata*), chickpea (Cicer*arie tinum*) (Nadon & Jackson, 2020). These hold immense agricultural significance worldwide contributing an area ~14% of total land under cultivation (Sulieman& Tran, 2016). They significantly contribute to global food and nutritional security, as well as soil health. They also generate income for millions of smallholder farmers on a regional and global scale, and their role in environmental safety measures is well documented (Guardia *et al.,* 2016; Peoples *et al.,* 2009; Yadav *et al.,* 2015).

2.6. 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).

2.6.1. 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 PH than fast-growers, strain to strain difference exists. *Rhizobium meliloti* is particularly sensitive to acid conditions.

2.7. Recognition and determinants of nodulation

Successful Symbiotic interaction requires compatibility at various stages starting from initial recognition, through successful differentiation to nitrogen fixation. The initial interaction between the host plant and free-living rhizobia is by the Plant roots secrete many different organic compounds into the soil, some of which allow microorganisms to grow in the Rhizosphere and include carbohydrates, amino acids, organic acids, vitamins and phenolic derivatives (People and Crawswell,1992). Reactions between certain compounds in the bacterial cell wall and the root surface are responsible for the rhizobia recognizing their correct host plant and attaching to the root hairs.

Flavonoids secreted by the root cells activate the nod genes in the bacteria which then induce nodule formation because Nod genes direct the various stages of nodulation. The whole nodulation process is regulated by highly complex chemical communications between the plant and the bacteria. Even if a strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. Such rhizobia are termed ineffective. Effective strains induce nitrogen-fixing nodules. Effectiveness is governed by a different set of genes in the bacteria from the specificity genes (Ott, 2005).

2.8. Root infection and nodule formation

The legume-rhizobia symbiosis is highly specific and depends on complex signaling processes between the host plant and rhizobia partner. Symbiotic N fixation between legumes and rhizobia takes place in plant-derived root organ called nodules, and competent nodulation is critical for efficient BNF (Nape Victoria Mothapo, 2011). The symbiotic relationship implies a signal exchange between both partners that leads to mutual recognition and development of symbiotic structures. Rhizobia live in the soil where they are able to sense flavonoids secreted by the roots of their host legume plant. Flavonoids trigger the secretion of nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses, such as ion fluxes. The best-known infection mechanism is called intracellular infection; in this case the rhizobia enter through a deformed root hair in a similar way to endocytosis, forming an intracellular tube called the infection thread. A second mechanism is called "crack entry"; in this case, no root hair deformation is observed and the bacteria penetrate between cells, through cracks produced by lateral root emergence. Later on, the bacteria become intracellular and an infection thread is formed like in intracellular infections (Oelke *et al*., 1991).

The infection triggers cell division in the cortex of the root where a new organ, the nodule, appears as a result of successive processes (Oelke *et al*., 1991). Infection threads grow to the nodule, infect its central tissue and release the rhizobia in these cells, where they differentiate morphologically into bacteroids and fix nitrogen from the atmospheric, elemental N2 into a plant-usable form, ammonium (NH3 + H+ → NH4 +), using the enzyme nitrogenase. The reaction for all nitrogen-fixing bacteria is: N2 + 8 H+ + 8 e−→ 2 NH3 + H2 12 In return, the plant supplies the bacteria with carbohydrates, proteins, and sufficient oxygen so as not to interfere with the fixation process. Hemoglobin's, plant proteins similar to human hemoglobin's, help to provide oxygen for respiration while keeping the free oxygen concentration low enough so as not to inhibit nitrogenase activity (Oelke *et al*., 1991).

2.9. Indigenous rhizobia population

Inoculation of legumes with introduced rhizobia strain is a common agricultural practice intended to promote nitrogen fixation and increasing crop yield (Thies *et al*., 1991a). But for 11 Rhzobium inoculants to be efficient in fixing nitrogen as well as increasing crop yield, population size of indigenous rhizobia strains play an important role (Meade *et al*., 1985). The size of the indigenous rhizobia population is the most powerful environmental factors that determine the competitive success of inoculated rhizobia versus indigenous rhizobia strain found in the area (Thies *et al*., 1992). It has been found that the likelihood of a response to inoculation with Rhizobium strains decreased as the numbers of indigenous rhizobia increased (Thies *et al*., 1991a, b). Singleton and Tavares (1986) found the same inverse correlation between rhizobia inoculants with native rhizobia population.

According to Singleton and Tavares (1986), introduced rhizobia strains (inoculants) are always outcompeted with the native rhizobia strains, thus, it is not possible to enhance N2-fixation when indigenous soil rhizobia populations were above threshold number (102 rhizobia cells per gram of soil) and had some effective strains. Amijee and Giller (1998) reported that, the response of Phaseolus vulgaris to Rhizobium inoculants is not common in areas with a large number of indigenous rhizobia. Furthermore, Meade *et al*. (1985) explained that the number of indigenous rhizobia present in the soil before inoculation affect the concentration of inoculants required in that area. A Study done by Weaver and Frederick (1974) indicated that for rhizobia inoculums to be able to form 50% or more of the nodules must be applied in a rate of 1,000 times higher than the size of the indigenous population in the soil. Also increase in economic yield due to inoculation is a function of indigenous rhizobia present in the soil (Thies *et al*., 1991b). That means, economic yield due to inoculums application increase as a number of indigenous rhizobia per gram of soil decrease and vice versa.

It is important to note that the indigenous rhizobia population is not the only factor that determines inoculums responses as there are other factors such as soil N availability, physiochemical constraints (like soil pH and salinity) and climatic conditions such as temperature, moisture content and oxygen stresses (Singleton and Tavares, 1986; Thies *et al*., 1991a; Giller *et al*., 1998; Hungria and Vargas, 2000). However, the population of indigenous rhizobia is among of the most important factor. For that reason, many findings recommended that for successful use of rhizobia inoculants, there should be prior knowledge of the size of the indigenous rhizobia population (Meade *et al*., 1985; Peterson and Loynachan, 1981; Singleton and Tavares, 1986). Therefore, in order to know which areas need inoculation and at which quantity, knowing the population of indigenous rhizobia found in that area is of importance.

2.10. Phosphorus acquisition

In addition to Rhizobium, phosphate-solubilizing microorganisms belonging to Pseudomonas, Bacillus, Aspergillus, and other groups increase the supply of phosphorus. These microorganisms produce organic acids and other bioactive molecules which in turn increase the availability of soil phosphorus, and consequently P uptake, growth, and yield of crops. Soil microorganisms possessing phytase activity can contribute to plant-phosphorus nutrition through phytate mineralization (Idriss *et al*., 2002). In addition, an obligatory symbiotic AM fungus can also stimulate P absorption in crops by spreading their extensive hyphal growth beyond the nutrient depletion zone of plant roots (Smith and Smith, 1990) and supply plants with major nutrients such as phosphorus, nitrogen, and basic cations such as Mg, Ca, K and even micronutrients (Fe, Cu, Zn). Arbuscular mycorrhizal fungi, besides supplying nutrients to the plants, support plants in the alleviation of stressed environmental conditions such as salinity, acidity, and moisture stress.

3. MATERIALS AND METHODOLOGY

3 .1 Description of study area

The soil sample for the study was collected from smallholder farmers fields of selected Kebele's in Hawela district of Sidama regional state. Hawela district is located about 20 km south of Hawassa, the capital city of the Sidama National Regional state. The Kebele's were selected based on their accessibility and production status of Common beans in the area,

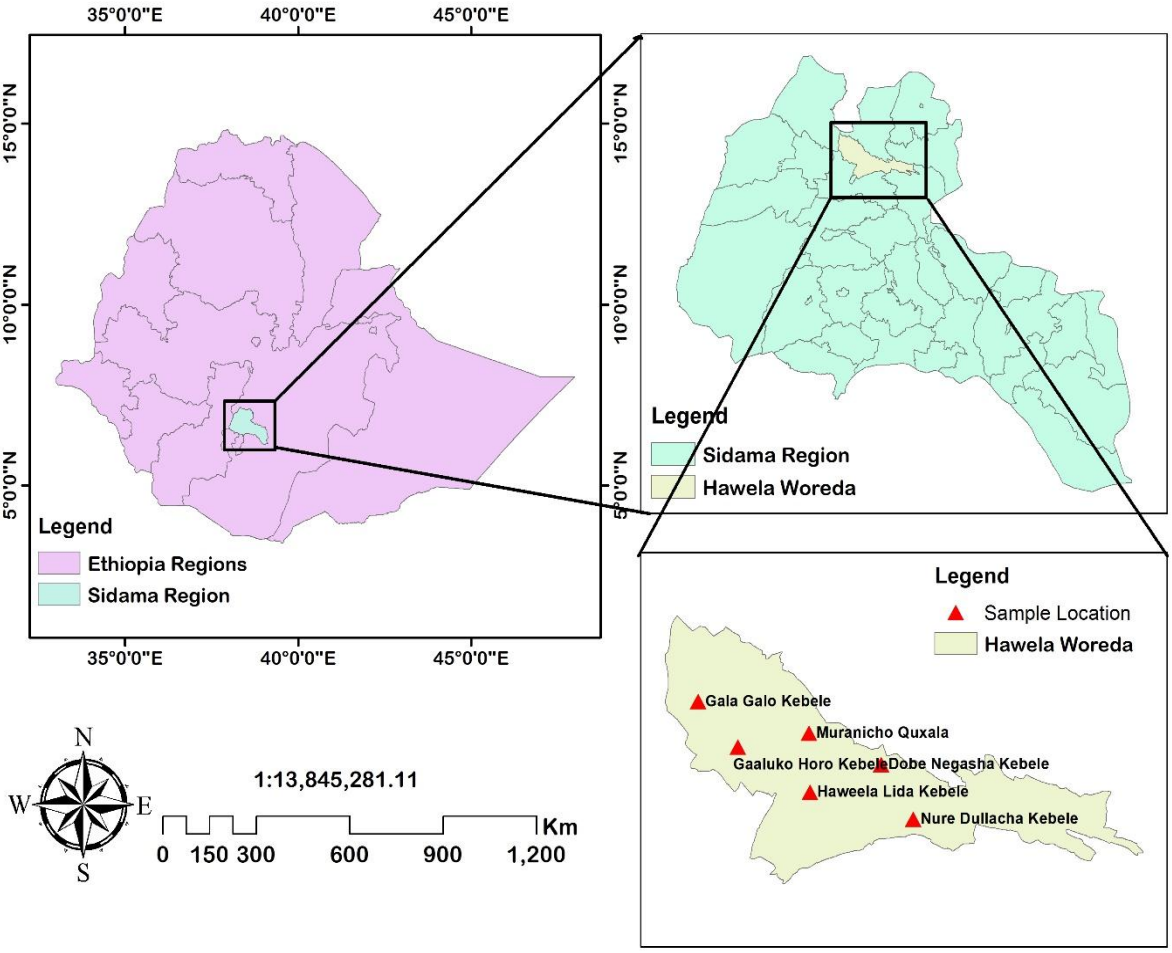

Figure 3. 1: Geographic distribution of sampling sites in Hawela, Sidama, Ethiopia.

The map, created using ArcGIS Desktop: Release 10 (ESRI 2011), illustrates key locations where soil samples were obtained, providing a visual overview of the diverse sites selected for soil sampling.

3.2. Soil Sampling Technique and Trapping of Rhizobia

Soil samples were collected randomly from different farmer's fields of the selected kebele's in sterile plastic bag. The sampling was conducted in a zigzag pattern from the soil surface to a depth of 20 cm in fields. The samples collected from different spots within the field were thoroughly mixed to create a homogeneous soil sample for each field, each weighing approximately 3 kg as described by Erena Kebede et al. (2020). The samples were transported to soil microbiology laboratory of Hawassa University's College of Agriculture for further experimentation involving nodule trapping and rhizobia isolation

3.3 Greenhouse Nodulation Induction and Assessment

The 'plant trap' method, as per Vincent (1970), was used to induce rhizobia nodulation on a common bean variety obtained from Sidama Agricultural research center. The seeds were surface sterilized using a process involving (1) 70% ethanol and (2) 3% sodium hypochlorite solution, then sown five seeds into sterilized 3-kg capacity plastic pots filled with soil samples. Post-planting care included thinning of seedlings to two per pot and regular watering with approximately 100 ml of water daily. After 45 days, plants were uprooted, and the presence of nodules was assessed to measure the soil's nodulation potential.

3.4. Isolation of Rhizobia from Root Nodules

Rhizobia isolation was conducted using protocols established by Vincent (1970) and Somasegaran & Hoben (1994). Initially, nodules were placed in a Petri dish and soaked in sterilized distilled water for four hours to rehydrate them. Then, the nodules were surface sterilized by immersing them in 70% ethanol for one minute, followed by a three-minute immersion in a 3% sodium hypochlorite solution. After sterilizing the nodules, they were washed six times with sterile distilled water.

Then, the rinsed nodules were crushed with a drop of sterile distilled water in aseptic conditions. A loop full of the crushed nodule material was streaked across Petri dishes containing Yeast Extract Mannitol Agar media (YEMA) supplemented with 0.0025% (w/v) Congo red (CR). The composition of YEMA included 0.5 g di-potassium hydrogen phosphate (K_2HPO_4), 0.2 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.1 g sodium chloride (NaCl), 0.5 g yeast extract, 10 g mannitol, and 15 g agar per liter. The Petri dishes were incubated at 28˚C for 7 to 10 days, and their growth was checked every 24 hours to monitor the growth of rhizobia and any contaminant strains.

3.5 Purification, Designation and Preservation of Isolated Rhizobia

The purification process of rhizobial isolates involved selecting a single well-grown colony. This colony was then transferred to a fresh YEMA plate with Congo Red (CR). The plate was incubated at a temperature of 28±2°C for a period of 2-3 days. Sub-culturing was done repeatedly until purity and uniformity were achieved. To preserve the isolates, isolated colonies were transferred to YEMA slants that contained 0.3% (W/V) Calcium carbonate (CaCO3). These slants were stored at a temperature of 4°C for subsequent characterization.

3.6. Cultural characterization of isolates

3.6.1 Colony morphology of isolates.

Morphological characteristics such as colour change, opacity, colony elevation, consistency, texture, shape, size, exo-polysaccharide gum, border, transparency and mucosity was used for identification of the rhizobia isolate

3.6.2 Gram staining test of isolates

Bacterial smear of different strains was prepared separately and fixed in flame. Smears were fixed by passing over a Bunsen burner flame and then stained with ammonium oxalate crystal violet for one minute. Then, it was washed with tap water and immersed in Gram's iodine for one minute. Again washed with tap water and blot dried smear was flooded with 95% ethyl alcohol (decolorize) for 30 sec. It was again washed with water and blot dried carefully. Then it was counter stained with safranin, again washed with tap water and finally dried and examined under oil immersion objective on the microscope. Gram-negative bacteria retain the pink/red colour while Gram-positive bacteria retain the crystal-violet.

3.6.3 Congo red absorption test

Stock solution of Congo red was prepared by dissolving 0.25g of Congo red in 100ml of sterile distilled water. From stock solution, 10ml were added to a liter of YEMA and autoclaved. Loop full of test isolates grown on YEMA were streaked on the medium. The plates were covered with aluminum foil and incubated at 28^0C for 3 to 7 days to detect Congo red absorption by the colonies (Vincet, 1970).

3.6.4 Glucose Peptone Agar GPA Test

The peptone-glucose medium was prepared according to Lupwayi and Haque (1994) by dissolving 5g of glucose, 10g of peptone, 15g of agar and 10ml of Bromcresol purple (BCP, prepared by dissolving 1g of BCP in 100 ml of ethanol) in a liter of distilled water and the pH was adjusted to 6.7 with 1N NaOH and HCl. Three days old Yeast extract Mannitol broth culture was streaked on to the peptone- glucose medium to observe the presence of growth by incubating at 28 ^{o} C.

3.7. Biochemical Characterizations

3.7.1 Catalase test

Catalase test was performed to study the presence of enzyme catalase in rhizobial isolate which hydrolyzes H_2O_2 into H_2O and O_2 in bacterial strains. Firstly, smear of the isolate was made on a clean and dry glass slide, and then a few drops of H2O2 were added to the slide. Production of gas bubbles and effervescence showed a positive test (McFadden, 1980).

3.7.2. Citrate Utilization Test

In this medium citrate is the only carbon source available to the bacteria; however, the rhizobia isolate cannot grow on the citrate and results in no colour change. To inoculate the slant, a loop full of culture of the isolate was used; the slant was inoculated following stab and streak method and finally observed after incubation period of 24 h at 28ºC.

3.7.3. Urease Test

With an aim to detect the production of urease enzyme capable of hydrolyzing urea to release ammonia by the test bacterial isolate, all the five isolates were grown in five separate test tubes with Urease Test Broth (purchased from HI Media®, containing 20g/L urea and Phenol Red as the pH indicator dye). The tubes were then incubated at 28 °C temperature for 24-48 hrs and were observed for the color change of the broth media from yellow to red that indicates a positive reaction.

3.7.4. Motility Test

Motility test medium is recommended for detection of bacterial motility. Composition of culture media used to detect the motility of the isolates was, Tryptose(10gms, Sodium chloride (5gms) and Agar (5gms). Final pH (at 25°C) 7.2±0.2 was adjusted and the ingredient were dissolved in 1000 ml distilled water. The medium was heated to dissolve completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The tube medium was allowed to cool in an upright position; the medium was inoculated with culture and incubated for 24-48hrs at 28^0C-30^0C.

3.7.5. Growth in Presence of 8% kNO3

Isolates were tested for the ability to grow in the presence of 8% KNO3 in YEM broth for a 7 days incubation period at 28°C (El Idrissi et al., 1996).

3.7.6. Indole test

Tryptone broth medium was prepared. The medium was poured into the test tubes. The bacterial isolates were inoculated separately to the broth and incubated at 28°C for 2 days. The

uninoculated broth was maintained as control. After the period of incubation, 1 ml of Kovac's reagent was added to each tube, including control. The tubes were gently shaken at an interval of 10–15 min and allowed to stand until the reagent reaches the top. The formation of red color ring is indicating the positive results; whereas yellow color ring indicates negative result.

3.7.7. Lysine decarboxylase test

In this test bacterial isolates were streaked on Bromocresol Purple Falkow medium (peptone 5 g, yeast extract 3 g, glucose 1g, Bromocresol purple 0.02 g, distilled water 1 liter). Then the isolates were streaked on the media and kept for incubation at 34 ^{0}C for 24 hours.

3.7. 8. Hydrogen sulphide production test

The hydrogen agar medium was prepared and the loopful of culture was inoculated and incubated for about 2 to 3 days at 30^{0}c and then results are recorded. The formation of black precipitate on the medium indicates the positive result. Absence of blackening of the medium indicates the negative result.

3.8 Physiological characteristics of isolates

3.8.1 NaCl (4%) Tolerance of isolates

To the basal medium of YMA, 4% NaCl was added to check the purity of the isolates. As 6% NaCl .is inhibitory for most Rhizobial isolates it can serve as an identification tool.

3.8.2 pH tolerance of isolates

The ability of the bacterial isolates to tolerate extremes of pH was evaluated using YEMA agar media with pH adjusted to 4.0, and 8.5 using 1 M HCl and 1 M NaOH as indicated by

Kapembwa (2016). All isolates were tested for the development of distinct colonies at each pH level by incubating at 28±2°C for 2-3 days (Somasegaran & Hoben, 1994).

3.9. Authentication and Screening of the Isolates for Symbiotic Effectiveness on Sand Culture in the Greenhouse

To determine the definitive purity of all the isolates, a nodulation test was performed on each purified isolate. By re-inoculating the isolate on the host plant, common bean using acid-treated and sterilized river sand as the growth medium. Each pure isolate was authenticated as root nodulating bacteria for infectivity and effectiveness (Somasegaran & Hoben, 1994).

3.9.1. Growth on the sterile sand medium

The seeds were grown in sterilized plastic cups which had two sections: a base for nutrient solution and a top filled with sterile river sand. The sections were connected by a cotton wick (Yates et al., 2016). The sand was treated with sulfuric acid, washed, dried, and autoclaved at 12°C for 30 minutes, and then cooled for 24 hours before use.

3.9.2 Culturing of Isolates and Seed Preparation

Isolates were cultured in YEMB for 2-3 days before planting. Seeds were surface sterilized by immersing in 95% alcohol for 10 seconds, then immersed in a 3% sodium hypochlorite solution for 3-5 minutes. They were washed six times in distilled sterile water and left in the final change of water for one hour until fully imbibed. Afterwards, they were washed twice more and transferred to a 1% water agar petri dish to incubate at 25°C (Bala et al., 2019).

3.9.3. Planting and Inoculating

Pre-germinated seeds were planted one centimeter below the surface of the rooting medium in the plastic cups. Each seedling was inoculated with 1 ml of a broth culture containing about 10^8 cells of the isolate. The cups were then placed in the greenhouse. Five days after emergence, the plants were thinned to one per cup.

3.10 Experimental design, growth conditions

The greenhouse experiment was conducted in a completely randomized block design (CRD) with three (3) replicates and different treatments. These treatments included pots inoculated with rhizobia isolates, the positive control treatments that were not inoculation but with nitrogen fertilizer applied as a 0.05% KNO3 (w/v) solution weekly and the negative control treatments with no inoculation and no chemical nitrogen fertilizer added (Somasegaran & Hoben, 1994).

Seedlings were irrigated with nitrogen free nutrient solution prepared according to Broughton and Dilworth (1971) as described by Somasegaran and Hoben (1994). After 45 days, plants were harvested, and nodules in the roots were assessed. Shoots were dried at 70°C for 48 hours, and shoot dry weights (SDW) were recorded as described by Somasegaran and Hoben (1994). After collecting and counting the number of nodules (NN) per plant, the nodules were dried their dry weight (NDW) was determined. .

3.11. Determination of Symbiotic effectiveness of the isolates

Relative symbiotic effectiveness (RSE) of the isolates was calculated according to the equation proposed by Date et al. (1993). g

$$\text{RSE} = \frac{Shoot\ dry\ weight\ (SDW) of\ plants\ inoculated\ with\ rhizobia\ isolates}{Shoot\ dry\ weight\ of\ N\ fertilized\ plants}\ X\ 100$$

The nitrogen fixing effectiveness was evaluated as highly effective RSE > 85%, effective 55-85%, less effective 35-54% and ineffective <35%

3.12 Host range of rhizobia isolates

Five selected isolates, authenticated on Common bean, were tested for their ability to nodulate three legumes, alfalfa, lentils and faba bean. Seeds of each legume crops were surface sterilized, pre-germinated and seedlings were transplanted aseptically into surface sterilized pots as before. Similarly, all greenhouse activities including experimental design, number of replications treatments, thinning, inoculation, watering and N-free nutrient application were as described above for authentication and preliminary screening of the rhizobial isolates. Plants were grown for 45 days in the greenhouse and harvested for determination of nodule number, nodule dry weight and shoot dry weight. The relative symbiotic efficiency (RSE) of each isolate was calculated as described before using the methods of Purcino et al. (2000)

3.13 Data 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.

4. RESULTS AND DISCUSSION

4.1Results

4.1.1 Isolation and authentication of the isolates

Eight Rhizobia isolates (CBR1, CBR2, CBR3, CBR4, CBR5, CBR6, CBR7 and CBR8) were isolated from the root nodules of Common bean grown in pots. These eight isolate were re-inoculated to the trap host plant for authentication (Table 1). Five of these isolates (62.5%) formed nodule on host plant while the three isolates did not. The five isolates that were able to form nodules on the original host were considered as the true rhizobial isolates while the remaining three isolates were rejected.

4.1.2 Morphological and Cultural Characteristics and Gram staining

The colonies appeared either dull or shiny with entire margin and convex elevation on YMA media. The colony transparency was either opaque or translucent with firm, dry, or smooth viscous texture. The colony diameter ranged between 1.0 mm and 4 mm (Table1). Upon streaking on the YEMA-BTB media, the isolates acidified and turned the green media to moderately yellow and deep yellow color after 3 days of incubation in the dark. The Gram staining and microscopic examination revealed that all isolates were Gram-negative (Table1).

Table 1: Cultural characterization of isolates

Rhizobial isolates	Colony morphology					
Isolates	Colony color	Colony shape	Colony size (mm)	Gram staining	opacity	Elevation
CBR1	Red	Oval	1	_	T	R
CBR 2	Red	Oval	2	_	T	R
CBR 3	Red	Oval	2	_	T	R
CBR 4	White	Round	3	_	T	R
CBR 5	White	Round	3.5	_	T	R
CBR 6	White	Round	4	_	T	R
CBR 7	White	Round	2	_	T	R
CBR 8	White	Round	2.5	_	T	R

T=Transparency, R= Raised.

4.1.3 Biochemical characteristics of the isolates

Several qualitative biochemical tests were conducted as described by Lowe (1962) and summarized in table 4.2 below. Initial screening of the nodule bacteria on YEMA media with Congo red showed little or no absorption of Congo red indicating that the isolates were rhizobia. All the isolates were positive for catalase, indolent, urease and citrate tests. However, response

to other biochemical tests were variable among the different isolates as described in table 4.2 below.

Table 2: Biochemical characterization of isolates

Isolates	Biochemical reaction of isolates										
	Urease test	Catalase test	Indolent test	Amylase test	Citrate test	GPA Test	Motility test	8% kNO_3	Turbidity test	Congo red test	H_2S test
CBR1	+	+	+	_	+	_	+	_	_	_	+
CBR 2	+	+	+	_	+	_	+	-	-	+	+
CBR 3	+	+	+	_	+	-	+	-	-	+	+
CBR 4	+	+	+	-	+	-	+	+	+	-	-
CBR 5	+	+	+	-	+	-	+	+	+	-	-
CBR 6	+	+	+	-	+	-	+	+	+	-	-
CBR 7	+	+	+	-	+	-	+	+	+	-	-
CBR 8	+	+	+	-	+	-	+	+	+	-	-

Note: In the table, "+" indicates positive growth or a positive test result, while "-" indicates no growth was observed

4.1.4 Physiological characterization of isolates

All the isolates showed growth on pH 6 and most on pH 8.5. But none of the isolates showed growth on pH 2, thus indicated the isolated were rhizobial isolated. Similarly all of the isolates shown growth on 4% NaCland none of isolates grew on 6% NaCl (Table3).

Table 3: Physiological characterization of isolates

Isolates	Physiological tolerance of isolates				
	pH2	pH 6	pH 8.5	4% NaCl	6% NaCl
CBR 1	_	+	+	+	_
CBR 2	_	+	+	+	_
CBR 3	_	+	+	+	_
CBR 4	-	+	+	+	-
CBR 5	-	+	+	+	-
CBR 6	-	+	+	+	-
CBR 7	-	+	+	+	-
CBR 8	-	+	+	+	-

Note: In the table, "+" indicates positive growth or a positive test result, while "-" indicates no growth was observed

4.1.5 Symbiotic effectiveness of isolated rhizobia

4.1.5.1 Nodule number

Nodule numbers differed significantly ($p<0.05$) between the rhizobial isolates. The nodule number ranged from 13.00±1 per plant to 27.00±1 per plant (Table 4). The common bean with N+ and N- treatments and inoculated with CBR1, CBR2 and CBR3 produced no nodule

4.1.5.2 Nodules dry Weight

Nodules dry weight differed significantly ($p<0.05$) between the rhizobial isolates, and ranged from 0.06±0.005 mg per plant to 0.22±0.01mg per plant (Table 4).The common bean with N+ and N- treatments produced no nodule.

4.1.5.3 Shoot dry Weight

Shoot dry weight was also significantly different ($p<0.05$) between the different treatments Common bean plants inoculated with CBR8 produced the highest shoot biomass (0.21±0.009 g per plant) followed by CBR8 (0.19±0.01 g per plant). The smallest shoot dry weight was produced by CBR4 (0.05±0.02 g per plant). The result of this study indicated that CBR7 and CBR8 isolates were symbiotically highly effective with more than 100% RSE compared to the positive control treatment. Furthermore, CBR5 and CBR6 were also effective with RSE of 72% compared to the positive control.

Table 4: Symbiotic effectiveness of rhizobial isolates on common bean. Average values are given (n=3) ± standard error of the mean.

Rhizobial strain	NN	NDW(mg)	SDW(g)	RSE%	Effectiveness
CBR1	-	-	0.04 ± 0.01^{c}	22.2%	IE
CBR2	-	-	0.06 ± 0.015^{c}	33.3%	IE
CBR3	-	-	0.07 ± 0.01^{c}	38.9%	LE
CBR4	27.00 ± 1.00^{b}	0.22 ± 0.01^{a}	0.05 ± 0.02^{c}	27.8%	IE
CBR5	33.00 ± 1.00^{a}	0.22 ± 0.017^{a}	0.13 ± 0.01^{b}	72%	E
CBR6	13.00 ± 1.00^{d}	0.10 ± 0.01^{b}	0.13 ± 0.026^{b}	72%	E
CBR7	22.66 ± 1.52^{c}	0.07 ± 0.002^{c}	0.19 ± 0.01^{a}	105.5%	HE
CBR8	21.00 ± 1.00^{c}	0.06 ± 0.005^{c}	0.21 ± 0.009^{a}	116.7%	HE
N^{+}	-	-	0.18 ± 0.01^{a}	100%	
N^{-}	-	-	$0.03\pm.001^{c}$	16.7%	

Key: NN-Nodule number, NDW-nodule dry weight, SDW, shoot dry weight, N+ positive control; N- negative control. In columns, superscripts with similar letter show no significant differences.

4.1.6. Host range and symbiotic effectiveness of isolated rhizobia

4.1.6.1. Plant nodulation and symbiotic effectiveness of rhizobial isolates with alfalfa

All the five rhizobial isolates nodulated alfalfa (table 5). Two of the symbiotic parameters, nodule number and nodule dry weight were not significantly different between the alfalfa plants inoculated with the different rhizobia isolates ($p>0.05$). However, shoot dry weight was significantly different between the different treatments ($p<0.05$). In this experiment, the number of nodules ranged from 14.00±1.00 to 17.33±3.21 per plant, while the nodule dry weight ranged 0.03±0.01 to 0.10±0.050 mg per plant. Similarly, shoot dry weight ranged from 0.16±0.02 g per plant in negative control to 0.81±0.076 g per plant in CBR7. The result of this study show that most of the all rhizobial isolates effectively fixed nitrogen with alfalfa with RSE ranging from 83.3% to more than 100%.

Table 5: Symbiotic effectiveness of rhizobial isolates on alfalfa. Average values are given (n=3) ± standard error of the mean.

Rhizobial isolates	NN	NDW(mg)	SDW(g)	RSE%	Effectiveness
CBR4	14.00±1.00[a]	0.022±0.002[a]	0.73±0.15[a]	100%	HE
CBR5	12.00±3.60[a]	0.10±0.050[a]	0.63±0.208[a]	83.3%	HE
CBR6	15.66±3.78[a]	0.028±0.010[a]	0.53±0.185[a]	72.6%	E
CBR7	14.00±2.64[a]	0.100±0.020[a]	0.81±0.076[a]	110.9%	HgE
CBR8	17.33±3.21a	0.03±0.01[a]	0.79±0.04a	108.2%	HE
N^+	-	_	0.73±0.05a	100%	HE
N^-	-	-	0.16±0.02b	21.9%	IE

***Key**: NN-Nodule number, NDW-nodule dry weight, SDW, shoot dry weight* -, N-control with nitrogen fertilizer and without nitrogen fertilizer. In columns, superscripts with Zero results show no significant effect in isolates.

4.1.6.2 Plant nodulation and symbiotic effectiveness of rhizobial isolates with Lentil

All the five rhizobial isolates nodulated lentil (table 6). Number of nodule was not significantly different between the lentil plants inoculated with the different rhizobia isolates similar to alfalfa ($p>0.05$). However, nodule dry weight and shoot dry weights were significantly different between the different treatments ($p<0.05$). In this experiment, the number of nodules ranged from 10.00±1 ±1.00 to 18.66±1.52 per plant, while the nodule dry weight ranged 0.03±0.01 to 1.19±0.79 mg per plant. Similarly, shoot dry weight ranged from 0.11±0.001 g per plant in negative control to 0.36±0.01 g per plant in CBR7. The result of this study show that most of the all rhizobial isolates effectively fixed nitrogen with alfalfa with RSE ranging from 70.6% to more than 100%.

Table 6: Symbiotic effectiveness of rhizobial isolates on lentil. Average values are given (n=3) ± standard error of the mean

Rhizobial isolate	NN	NDW(mg)	SDW(g)	RSE	Effectiveness
CBR4	18.66±1.52a	1.19±0.79a	0.34±0.04a	100%	HE
CBR5	15.66±0.57a	0.41±0.02b	0.24±0.01a	70.6%	E
CBR6	14.00±1.00a	0.38±0.01b	0.27±0.03a	79.4%	E
CBR7	13.33±4.93a	0.28±0.01b	0.36±0.01a	105.9%	HE
CBR8	10.00±1.00a	0.03±0.01c	0.35±0.22a	102.9%	HE
N^+	-	-	0.34±0.005a	100%	HE
N^-	-	-	0.11±0.0001b	32.3%	IE

***Key**: NN-Nodule number, NDW-nodule dry weight, SDW, shoot dry weight,* N-control with nitrogen fertilizer and without nitrogen fertilizer. In columns, superscripts with Zero results show no significant effect in isolates.

4.1.6.3 Plant nodulation and symbiotic effectiveness of rhizobial isolates with Faba bean

All the five rhizobial isolates nodulated faba bean (table 7). Number of nodule and nodule dry weight were not significantly different between the faba bean plants inoculated with the different rhizobia isolates ($p>0.05$). However, shoot dry weights were significantly different between the different treatments ($p<0.05$). In this experiment, the number of nodules ranged from 14 ±1 ±1.00 to 18.33±1.15 per plant, while the nodule dry weight ranged from 0.19±0.005 to 0.40±0.01 mg per plant. Similarly, shoot dry weight ranged from 0.13±0.001 g per plant in negative control to 0.25±0.02 g per plant in CBR7. The result of this study show that most of the all rhizobial isolates effectively fixed nitrogen with alfalfa with RSE ranging from 77.3% to more than 100%.

Table 7: Symbiotic effectiveness of rhizobial isolates on faba bean. Average values are given (n=3) ± standard error of the mean

Rhizobial isolate	NN	NDW(mg)	SDW(g)	RSE%	Effectiveness
CBR4	18.00±1.00a	0.40±0.01[a]	0.17±0.02a	77.3%	E
CBR5	16.66±2.51a	0.33±0.04a	0.17±0.06a	77.3%	E
CBR6	18.33±1.15a	0.28±0.11a	0.22±0.015a	100%	HE
CBR7	15.00±1.00a	0.27±0.10a	0.25±0.02a	113.6%	HE
CBR8	14.00±1.00a	0.19±0.005a	0.21±0.03a	95.4%	HE
N+	-	-	0.22±0.04a	100%	HE
N-	-	-	0.13±0.001b	59.1%	E

***Key**: NN-Nodule number, NDW-nodule dry weight, SDW, shoot dry weight, CBR- Common bean Rhizobium,* N-control with nitrogen fertilizer and without nitrogen fertilizer. In columns, superscripts with Zero results show no significant effect in isolates.

4.2 DISCUSSION

The formation of nodules on the trap host can be regarded as indicator of the presence of compatible of rhizobia present in the soil (Fentahun *et al.*, 2013). Hence, in the current study five of the eight bacterial isolates were able to form nodules on a common bean, a legume used for trapping potential rhizobia from the soils of the study area.

The five isolates were also authenticated as rhizobia and showed different morphological, biochemical and physiological characteristics, indicating the existence of diverse groups of rhizobia nodulating common bean (Tables 4). Colony characterization uncovered diverse growth and morphological traits among the indigenous common bean nodulating rhizobia from the study site. Most isolates formed mucoid and translucent colonies on YEMA, produced abundant mucus, and had circular colonies with entire margins. The colony diameter ranged between 1.0 mm and 4 mm after 2-3 days of incubation. (Table 4).] These findings demonstrate the phenotypic heterogeneity existing among indigenous rhizobia populations colonizing common beans in this region. All the isolated bacteria were gram negative, the colonies color varied from watery or white-translucent to opaque and milky, with round shapes varying from flat to domed and even conical on the YEMA medium. Furthermore, colony diameters ranging between 1-4mm within 2-3 days of incubation, indicating the isolates were rhizobia according to (Somasegaran & Hoben, 1994).

On YEMA containing congo red, the isolates authenticatd as rhizobia formed whitish to pale pink colonies, with no dye absorption indicating that they were rhizobia (Somasegaran and Hoben, 1994). These isolates were also negative for H_2S test. In contrast, isolates that fails authenticity test had absorbed the congo red and were positive for H2S test. In this study, all the rhizobia isolates were subjected to growth on Peptone Glucose Agar (PGA) medium (Table 4). None of these

isolates show growth on the PGA medium. Similar results were reported by Sharma et al., (2010) most rhizobia isolated did no growth on the PGA medium. This confirms previous results that rhizobia have no ability to grow on PGA medium. However, recent studies have shown that some rhizobia isolates can indeed grow on PGA medium. This highlights the importance of continual resear]ch and testing to challenge and expand our current understanding of rhizobia. The ability of some rhizobia isolates to grow on PGA medium could have significant implications for future studies aiming to isolate and identify these bacteria.

All isolates authenticated as rhizobia were positive for urease, catalase, indole, motility and citrate tests (Table 2). These test results provide insights into the isolates' metabolic capabilities. The isolates were also positive for motility and grew on a medium containing 8% KNO3. These findings are in close agreement with Elsheikh and wood (1989); Javed and Asghari(2008) who also characterized the rhizobia from soil and root nodules with similar positive biochemical tests. Similarly Oblisami (2005) also studied the nodulation pattern in forage legume bacteria by screening through the same biochemical tests and obtained similar results.

All of isolates showed growth on pH 8.5 and 6. But none of the isolates showed growth on pH 2 thus indicating that the isolates poorly serves in the acidic pH. These pH tolerance findings align with previous in vitro research. Yifru Abera et al. (2019) reported nearly all isolates grew on YEMA at pH 5.0-9.5, regardless of taxonomic grouping. Diriba Temesgen and Fassil Assefa (2020) also found soybean rhizobia isolates tolerant to a wide pH range (5-10). Similarily all the isolates has shown growth on 4% Nacl and none of isolates were grow on 6%NaCl (table3). These observations demonstrate certain rhizobia isolates' adaptability under saline conditions, potentially enhancing competitiveness and nitrogen fixation efficiency.

When a rhizobial isolate forms a nodulation relationship with a legume, the nitrogen fixation outcome can range from none to maximum (Terpolilli *et al.*, 2008). Therefore, assessing the infectivity, nodulation, and symbiotic effectiveness of native rhizobial populations is crucial for selecting isolates for inoculant production. The study found that most of the indigenous isolates from the current were capable of forming nodules on the tested legume hosts. On the other hand, uninoculated control did not show any nodule, demonstrated that aseptic conditions were met in the experimental set up.

The variation in the number of nodules observed per plant, ranging from 13 to 27, further highlights the diversity of rhizobia populations within these soils. This suggests that different rhizobia strains may vary in their ability to induce nodule formation, which could be attributed to differences in their genetic makeup or environmental adaptation. Previous studies similarly confirmed the presence of large number of rhizobia in Ethiopian soils. Erena Kebede *et al.* (2020) reported that 93.3% of soil samples collected from different sampling sites in Ethiopia induced and supported nodulation on cowpea. These comparisons provide a broader context for understanding the distribution and activity of legume-nodulating rhizobia in Ethiopia and highlight areas for further research.

The analysis of symbiotic effectiveness on sand culture revealed a significant ($p < 0.05$) variation among isolates (Table 4.4). The estimated values for relative symbiotic effectiveness ranged from 27.8 % (isolate CBR4) to 116.7% (isolate CBR8) with a common bean. There was a significant variation among the isolates in terms of the shoot dry weight ($p<0.05$). Up to 0.21g shoot biomass was recorded from a single plant that was grown on sand culture for 45 days in the greenhouse. Comparatively, some of the current isolates accumulated higher shoot biomass than those reported by Shimekite (2006), Some of the inoculated plants accumulated more shoot

biomass (e.g., 0.21g shoot dry weight) than the N positive control plants (0.18g), indicating that some isolates fix and contribute higher amount of nitrogen to the plant than the recommended amount of N-fertilizers. These findings align with previous research demonstrating the variability in symbiotic efficiency among different rhizobia isolates in Ethiopia. Similarly, Diriba Temesgen (2017) reported that selected isolates from Ethiopian soils were either effective or highly effective, indicating that the indigenous rhizobia have differences in their nitrogen fixation capability.

The ability of 5 selected rhizobial isolates to form nodules with different legumes hosts (alfalfa, lentils and faba bean) and demonstrated that these isolates can be potentially promiscuous nodulating different legume pants (Tables 5,6 and 7). This results may indicates that the current isolates might better suits with these host than the trap host used in this study and call for further study to determine the true potential of the current isolates with regard to their hosts and nitrogen fixation effectiveness. Previous studies also showed that some rhizobia effectively fix nitrogen with several hosts. Appunu et al. (2009) reported that all rhizobia isolated from common bean nodulated cowpea and mung bean. Yang and Zhou (2008) also reported that two leguminosarum strains isolated from chick pea nodulated common bean growing in the same ecological niche that may have phylogenetic connection with one another. Contrary to the present finding, Ansari and Rao (2013) indicated that chickpea rhizobia did not nodulate common bean at all indicating selective nodulation of different strains of rhizobia.

The analysis of symbiotic effectiveness on sand culture revealed a significant ($p < 0.05$) variation among isolates in terms of shoot dry weight. The maximum shoot dry weights were recorded in plant inoculated with CBR7 in all the three legume hosts. Howe ever, there was no significant variation in terms of nodule number and nodule dry weight in all the three legume hosts

($p>0.05$). The isolates induced nodules on the host plant with the mean nodule number (NN) ranging 12 -17 mg per plant in alfalfa; 10 - 18.86 mg per plant in lentils and 14 to 18 mg per plant in faba bean (Tables 5,6 and 7). The result agreed with previous reports that vetch isolates showed differences in nodule numbers and nodule dry weight (Shimekite, 2006; Roper et al., 2020). The current isolates were relatively highly effective with alfalfa, lentils and faba bean compared to common bean which was used for trapping the isolates from the soil. The relative symbiotic effectiveness ranged from 72.6% -110.9% with alfalfa; 70.6% -105.9& with lentils and 77.3% -113.6% with faba bean.

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

- The result of this study show the presence of compatible rhizobial isolates in the small holder farmers' fields in Hawella district of Sidama regional states.
- In this study, most isolates inoculated on common bean plants induced different levels of nodulation.
- The isolated rhizobia have potentially wide host ranges nodulating four different legumes hosts.
- The isolates that elicited greater nodulation (nodule mean number per plant) and effective in nitrogen fixation ability with common bean were able to nodulate and effectively fix nitrogen in alfalfa, lentils and faba bean.
- Tests on symbiotic effectiveness showed that two of the current isolates, CBR7 and CBR8, showed extraordinary efficiency in fixing nitrogen with all the four legumes and can be further evaluated for their suitability as a potential inoculant.

5.2 Recommendation

This study recommends further study, especially to involve the field trial to test symbiotic effectiveness and molecular characterization of the isolates which showed outstanding performance in the greenhouse trial. Also there is a need to conduct bigger studies on indigenous rhizobia populations as well as isolation studies across different agro-ecological zones of Ethiopia and exploit their potential as a bio-fertilizer. Therefore**,** Additional research is needed under greenhouse and field conditions to elucidate the effectiveness and competitiveness of the of the rhizobial isolates.

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APPENDIXES

Appendixes- 1: Figures and Photographs in Greenhouse Work

Preparing of seed for sowing.

Nodule No

Pot arrangements, sowing and growing

Appendixes- 2: Figures And Photographs In Lab Work

Growing microorganisms under the laminar air flow hood

Pure culture of rhizobia