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# Characterization, Symbiotic Effectiveness and Host range of Alfalfa Nodulating Rhizobia Isolated from Soils of Selected Kebele's in Hawella District, Sidama Regional State, Ethiopia.

M.Sc. Thesis by Bunamo Mitiku

JUNE, 2024

HAWASSA, ETHIOPIA

# Characterization, Symbiotic Effectiveness and Host Range of alfalfa nodulating Rhizobia Isolated from Soils of Selected Kebele's in Hawella District, Sidama Regional State, Ethiopia.

By: Bunamo Mitiku

M.Sc. Thesis Submitted To

The Department of Biology, College of Natural and Computational Sciences, School of Graduate Studies, Hawassa University in Partial Fulfilment of the Requirements for the Degree of Master of Science in Applied Microbiology

ADVISOR: KEDIR WOLIY (PhD)

JUNE, 2024

HAWASSA, ETHIOPIA

# DECLARATION

I declare that the work of this master of science thesis entitled “Characterization, symbiotic effectiveness and host range of alfalfa nodulating rhizobia isolated from soils of selected kebele, s Hawela district, sidama regional state, Ethiopia” is my original work and that it has not been presented for degree in any other university, and all sources of materials used for this thesis have been acknowledged.

Name: Bunamo Mitiku

Signature: _____

Date: _____

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# ADVISORS' THESIS APPROVAL SHEET-I

This is to certify that the thesis entitled "Characterization, Symbiotic effectiveness and Host range of alfalfa nodulating rhizobia isolated from soils of selected Kebele's in Hawella District, Sidama Regional State, Ethiopia. "Submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Microbiology, to Department of Biology has been carried out by BUNAMO MITIKU, ID: GPAPMicR/0001/13, under my supervision. Therefore, *I confirm the student has* fulfilled the requirements and hence can submit the thesis to the department for defense.

| Name of advisor | Signature | Date |
|---|---|---|
| Kedir Woliy (PhD) | _____ | _____ |

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# EXAMINERS THESIS APPROVAL SHEET-II

We, the undersigned, members of examiners of the final thesis defense by BUNAMO MITIKU have read and evaluated his thesis entitled on "Characterization, Symbiotic effectiveness and Host range of alfalfa nodulating rhizobia isolated from soils of selected Kebele's in Hawella District, Sidama Regional State, Ethiopia" and examined the student oral presentation. Therefore, this is to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Microbiology

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# ACKNOWLEDGMENTS

I would like to express my heartfelt thanks and gratitude to my advisor Kedir Woliy (PhD) for his constant expert guidance, advice and support through all the stages of the research work. I would like to acknowledge the laboratory technicians, Demere Takelegn, for his encouragement and assistance in materials and technical support.

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## LIST OF SYMBOLS AND ABBREVIATION

| | |
|---|---|
| ANOVA | Analysis of variance |
| BCP | Bromocresol purple |
| BNF | Biological nitrogen fixation |
| BTB | Bromothymol blue |
| CR | Congo red |
| DH2o | Distilled Water |
| DM | Dry Matter |
| Hr | Hour |
| HSD | Highest significance difference |
| Km | Kilo Meter |
| $Kno_3$ | Potassium Nitrate |
| LSD | Least significance difference |
| N | Normality |
| N2 | Dinitrogen |
| NDW | Nodule dry weight |
| NN | Nodule number |
| OD | Optical density |
| pH | Power of hydrogen |
| CRD | Completely randomized design |
| RDW | Root dry weight |
| Rev/min | Revolution per minute |
| SDW | Shoot dry weight |
| SE | Symbiotic effectiveness |
| SL | Shoot length |
| W/V | Weight by Volume |
| YEMA | Yeast extract mannitol agar |
| YEMA- CR | Yeast extract mannitol agar containing Congo red |
| YEMA-BTB | Yeast extract mannitol agar containing Bromothymol blue |
| YEMB | Yeast extract mannitol broth |

# ABSTRACT

*Feed shortage, low quality and seasonal fluctuations have constrained livestock production in Ethiopia. Using high-yielding, good quality and drought-tolerant forage legumes like alfalfa (*Medicago sativa *L.) has been suggested to overcome the feed constraints. Alfalfa is an important forage crop, which forms a symbiotic relationship with nodule-forming bacteria* Sinorhizobum meliloti. *The aim of this study was to characterize and evaluate the symbiotic effectiveness and host range of rhizobia isolated from smallholder farmers' fields in Hawella District, Sidama Regional State of Ethiopia using Alfalfa as a trap plant. To this end, a total of 12 bacterial isolates were trapped from different soil samples. The isolates showed differences in various presumptive, cultural, biochemical characteristics and physiological tolerances. Six (50 %) of the isolates( ANR 9, ANR 8, ANR 2, ANR 1, ANR 5 and ANR 11) could nodulate alfalfa with significant difference ($P<0.05$) in their capacity to infect and effectively fix nitrogen as evidenced from variations in nodulation parameters, shoot dry weights and shoot length. Accordingly, the isolates induced nodulation with nodule number ranging from 25.67 (ANR 9) to 9.00 (ANR 11) nodule per plant; nodule dry weight of 0.07 mg per plant (ANR 9) to 0.03 mg per plant (ANR 11); shoot dry weight ranging between 0.96 (ANR 9) and 0.17 (ANR 4) mg per plant and shoot length ranging from 30.50 (ANR 9) cm -11.47 (ANR 4) cm per plant. Using shoot dry weight as an indicator of the relative effectiveness, it was found that 33.3% of the isolates were highly effective (SE > 85 %) and 8.3 % were effective (SE ranging from 55 to 85 %) on alfalfa. Furthermore, the isolates showed broad host ranges on three legume species, namely, faba bean (*Vicia faba*), common bean (*Phaseolus vulgaris*), and lentil (Lens culinaris). All the six isolates (100 %) formed nodules with effective nitrogen fixation in faba bean, common bean and lentils exhibiting varying levels of effectiveness. Five of the six rhizobial isolates, ANR 9, ANR 8, ANR 2, ANR 1 and ANR 5 exhibited high $N_2$-fixation efficiency and are recommended for further evaluation to use them as inoculants for different legumes. We also recommend these isolates for molecular characterization to determine their phylogeny and taxonomic classes.*



***Keywords**: Alfalfa (Medicago sativa), Biological nitrogen fixation, Common bean (Phaseolus vulgaris), Faba bean (Vicia faba), Hawela District, Host range test, Lentil (Lens culinaris), Symbiotic effectiveness and Sinorhizobium.*

# 1. INTRODUCTION.

## 1.1. Background of Study.

Livestock production is a vital component of the Ethiopian agricultural system. It is a major source of income for agrarians and pastoralists (Feyissa et al., 2018), contributing to the overall economy of the country (Mengistu et al., 2017). However, production of livestock is challenged by inadequate and low-quality feed supply, disease, low genetic potential of the indigenous breeds, poor husbandry practices, and a weak marketing system. Among these, inadequate quality feed supply contributes to reduced livestock production in large extent (Tolera et al., 2012).

Livestock mainly rely on natural pasture and crop residues for feed, but these are often limited in both quantity and quality, which can hinder sustainable animal production. To address this issue, it is recommended to use high-yielding, drought-tolerant forage crops that are of good quality. Forage legumes are excellent choices as they provide better quality feed in sufficient quantities to support sustainable animal production. Legumes are highly nutritious and can thrive under poor soil conditions (Sheaffer *et al.*, 2018).

They are an important component of sustainable agriculture because they help maintain soil health through their ability to fix nitrogen biologically. Forage legumes provide various benefits, such as quality feed, nectar, green manure, and soil cover, making them an essential part of sustainable agricultural systems (Sheaffer et al., 2018). Globally, several forage legumes, including alfalfa (*Medicago sativa*) Trifolium repens (white clover), *Trifolium pratense* (red clover), *Trifolium subterraneum* (subterranean clover), *Lotus corniculatus* (birdsfoot trefoil), Vicia (vetch spp.), and *Lablab purpureus* (Lablab, Poor-man's bean), have been adopted and integrated into livestock production systems (Phelan et al., 2015).

In Ethiopia, cultivated forage legumes such as buffel grass, desho grass, rhodes grass, elephant grass, phalaris grasses from grass forage species. axillaris, green leaf, vetch, alfalfa, and white clover among legume forage species and leucaena, susbania, pigeon pea, and tree lucerne from tree and shrub legumes are growing and being integrated into livestock sector in highlands, midlands and lowlands (Mengistu et al., 2017).

Alfalfa (Medicago sativa) or Lucerne is a herbaceous perennial forage legume species that belongs to the leguminosae family. Alfalfa is called the queen of the forage plants because it has many desirable characteristics and outstanding economic importance (Hanson et al., 1988). Alfalfa is a type of plant that has a deep root system, allowing it to access water in the soil that other plants cannot reach. This makes it drought-tolerant and a popular choice as hay or pasture for farm animals because of its high nutritional value (Frame, 2005).

Alfalfa has a high nutritional value, serving as a good source of protein, calcium, vitamin A, and energy while being low in fibers (Frame, 2005). Additionally, including alfalfa in crop rotation provides several benefits, such as increasing soil organic matter, improving soil nutrition by adding fixed nitrogen to the soil, and reducing the need for nitrogen fertilizers for subsequent crops (Hanson et al., 1988; Giller et al., 2013). Alfalfa, as a member of the legume family, has the ability to form a symbiotic association with a member of the genus sinorhizobium which supplies the plant with fixed nitrogen.

Alfalfa not only contributes to fixed nitrogen supply through biological nitrogen fixation, but it can also help to protect and improve the environment by reducing nitrate contamination in groundwater. Biological nitrogen fixation by alfalfa provides substantial amounts of nitrogen (N) to livestock operations, subsequent crops, and soil organic matter. Alfalfa fixes most (70-90%) of its N needs from air through Rhizobia residing in alfalfa root nodules, much of which is stored in the crown and roots. An alfalfa stand can fix as much as 300 pounds of N per acre per year (Frame, 2005).

The use of efficient rhizobia to inoculate legumes and forage legumes is believed to increase their yield and yield components while also maintaining soil health. This process is eco-friendly and improves the availability of fixed nitrogen to other crops. However, research on forage legume production and the availability of compatible rhizobia in the soil of Sidama regional state's Hawella district is scarce. To address this issue, a study was conducted to isolate rhizobia using alfalfa, morphologically, biochemically and physiologically characterize the isolates, and evaluate their symbiotic effectiveness and host ranges.

## 1.2 Statement of Problem

Livestock production is one of the major components of agriculture in Hawella district (Atumo et al., 2021). Despite the large livestock population, the sector performed below its potential because of various constraints. Among these are the lack of sufficient supplies of fixed nitrogen and low-quality forages from the existing natural pastures (Kebede et al., 2022). Such problems can be circumvented by producing good quality forage legumes with minimal or no chemically fixed nitrogen inputs into the system. This can be achieved by using elite rhizobia inoculants that can effectively fix atmospheric nitrogen with different legume hosts.

Biological nitrogen fixation provides an economically feasible and environmentally sound source of nitrogen for forage legume production. However, many soils may not have adequate populations of such elite rhizobia in terms of number, quality, and effectiveness. This necessitates a search for compatible indigenous rhizobia that can effectively fix atmospheric nitrogen with a wide variety of legume hosts. Such differences in symbiotic performances between different legume-rhizobia combinations therefore require careful strain selection to achieve high N2 fixation rates and optimum plant development (Terpolilli *et al*., 2012). However, research on forage legume production and the availability of compatible rhizobia in the soil of the study area is scarce.

The aim of the current study was, therefore, to trap isolate rhizobia from smallholder farmers' fields in Hawella district of Sidama region using a forage legume alfalfa; and characterize the isolates morphologically and biochemically, and evaluate their host ranges and symbiotic effectiveness.

## 1.3 Objectives of the Study

### 1.3.1 General Objectives.

To isolate, characterize, and identify symbiotic effectiveness and host range tests of rhizobia nodulating alfalfa from the smallholder farmer fields in Hawella district of Sidama region, Ethiopia.

### 1.3.2 Specific objectives

- To isolate and characterize rhizobia nodulating alfalfa based on different cultural, biochemical and physiological characteristics.
- To assess the symbiotic effectiveness of the isolates under greenhouse conditions
- To determine the host ranges of the isolates against different legume hosts

## 1.4 Research Question

The following are the research questions that will be assessed and determined:

Are there indigenous alfalfa nodulating rhizobia in the soils of selected smallholder farmers' fields of different Kebele's in Hawella District of Sidama regional state?

What are the morphological, physiological, and biochemical attributes of these native alfalfa nodulating rhizobia?

What are the symbiotic efficiencies of the isolates?

What other legume hosts do the isolates are capable of nodulating?

## 1.5 Significance of the study

The study has attempted to isolate indigenous rhizobia capable of nodulating alfalfa and other legume crops. To our knowledge, this is the first report about the alfalfa nodulating rhizobia from the study area and the report may serve as a baseline data for further study. The rhizobia isolates were found to be symbiotically effective, and overall this research provides valuable insights that can be used by farmers, agricultural researchers, and rhizobial strain producers to enhance alfalfa (Medicago sativa) production, promote sustainable agriculture practices, and improve overall crop productivity and can be recommended for further field test in order to determine their suitability for inoculant production.

# 2. LITERATURE REVIEW

## 2.1. Legumes

Leguminosae or Fabaceae is the third most populous family of flowering plants (behind Asteraceae and Orchidaceae) with 670 to 750 genera and 18,000 to 19,000 species. Legumes include important grain, pasture and agro-forestry species. They are harvested as crops for human and animal consumption as well as used as pulp for paper production, fuel-woods, timber, oil production, sources of chemicals and medicines, and are also cultivated as ornamental, used as living fences and firebreaks among others (Lewis *et al.,* 2005).

The legumes provide many benefits to the soil so they are usually utilized as cover crop, intercropped with cereals and other staple foods. They do produce substantial amounts of organic nitrogen (Improving legume yield by inoculation with rhizobia), increase soil organic matter, improve soil porosity and structure, recycle nutrients, decrease soil pH reduce soil compaction, diversify microorganisms and mitigate disease problems (U.S Department of Agriculture [USDA], 1998).

## 2.2 Characteristics of Forage Legumes in the Study

### 2.2.1 Alfalfa (Medicago sativa)

Alfalfa is a perennial flowering plant in the pea family Fabaceae cultivated as main forage crop in many countries around the world. Alfalfa is native to warmer temperate climates and it has been cultivated as livestock fodder. It normally lives four to eight years, but can live more than 20 years, depending on variety and climate. The plant grows to a height of up to 1 m and produces deep, fibrous roots, with up to five feet of root growth per year, extending 10 to 15 feet, or more, below the ground.

Alfalfa has very special features: first, it is drought tolerant. The deep root system allows it to draw on soil moisture reserves in water-limited settings while improving soil structure (Confalonieri and Bechini, 2004). Alfalfa has ability to recover fast after continued dry conditions that helps in maintaining better yield than other forage crops. Secondly, alfalfa is moderately salt tolerant. Soil salinity restricts crop growth and subsequently reduces crop yield (Tanji and Kielen, 2002). It is more adapted to dry conditions with high soil salinity than other salt sensitive crops.

Third, alfalfa fixes nitrogen in its roots, which improves soil fertility. As nitrogen fixer that is both salt and drought resistant, alfalfa has become well adapted to the arid western regions of the U.S. that are under irrigation. Alfalfa has ability to fix nitrogen which can reduce farmers' fertilizer costs. Alfalfa fields can be rotated with corn, wheat, barley, tomatoes and lettuce. Because alfalfa can produce nitrogen for itself, it does not require extra nitrogen fertilizer for optimal growth (Putnam et al. 2001). Lastly, alfalfa is a key rotation crop capable of suppressing plant disease.

### 2.2.2 History of Alfalfa (Medicago sativa)

Alfalfa yield and the nutritive value of dry matter make it a leading perennial leguminous forage crop (Dinić and Đorđević, 2005). It originated from the Mediterranean basin and southwest Asia (Iran, Afghanistan) and was one of the first forage crops to be domesticated (Cook et al., 2005). However, the evolution of cultivated alfalfa has been greatly influenced by its winter hardy progenitor (Michaud et al., 1988). Alfalfa is one of the few cultivated plants that can produce high level of biomass with minimum inputs. Sustainability of farming system under organic management may be increase by the introduction of alfalfa in the crop rotation (Annicchiarico *et al.*, 2006).

There are numerous cultivars of alfalfa, selected for specific abilities, such as winter hardiness, drought resistance, tolerance to heavy grazing or tolerance to pests and diseases (Frame, 2005). Current breeding targets also include feeding value parameters such as digestibility and fiber content (Julier et al., 2000). Due to its high nutritional quality, high yields and high adaptability, it is one of the most important legume forages of the world as major source of protein for livestock and it is a basic component in rations for dairy cattle, beef cattle, horses, sheep, goats and other classes of domestic animals. It is cultivated in more than 80 countries in an area exceeding 35 million hectare (Radovic *et al*., 2009). World production of alfalfa was around 436 million tons (FAO, 2006).

The best conditions for its growth and development are in moderately warm or subtropical zones and in the uplands with the temperature of about 15-30 °C with a balanced amount of rainfall and plentiful sunshine (Mauriès 1991, 1994). This species tolerates short term shortage of moisture in the soil. Most often, it is associated with animal feed but also it is rarely used in human nutrition. Folk medicine uses this species because of its rich chemical composition and it is used

it as an adjunct to pharmacological agents in the treatment of gastrointestinal, cardiovascular and immune system diseases (Zanin, 2009).

Alfalfa contains features essential for organic farming because of its nutritional quality, nitrogen fixation and adaptive capacity (Torricelli, 2006). Specific varieties may be recommended depending on region-specific adaptation and management (Annicchiarico et al., 2010).

## 2.3 Importance of Alfalfa (Medicago sativa)

### 2.3.1 Alfalfa as a Feed for Animals

Alfalfa is used for livestock nutrition in different forms, most frequently as hay, but also dried/dehydrated in form of briquettes, as silage, haylage or for grazing. Alfalfa is harvested and stored primarily as hay or silage for use on the farm. The feeding value of harvested alfalfa may be changed by post-harvested factors as much as by pre-cutting environment and history of plant (genetics). According to Radović *et al.* (2009) report, Conservation and storage system are designed to minimize the loss and deterioration of nutrients. Alfalfa is widely used in ruminant livestock diets, impacting the performance of beef and dairy animals as well as the cost of production.

The decline in forage nutritive value with increasing harvest interval is a consequence of progressing maturity, along with the associated effects of increasing stem growth and decreasing leaf proportion, and decreasing stem nutritive value. The implications of greater maturity for animal performance are generally negative (Brink et al., 2011). It is a highly valued animal feed and a rich source of proteins, fibers, minerals and vitamins used in the diet of livestock. Alfalfa does not tolerate close grazing well, and some form of rotational grazing is necessary to maintain the persistence and production of plants, with rest intervals that replenish the crown and roots of plants in carbohydrates and nitrogen (Frame, 2005).

The duration of rest intervals depends on growth conditions, but 5 to 6 weeks are likely to be necessary. In a continuous grazing system, intensive defoliation can damage the plant crowns. In mixed pastures, stocking rates and grazing intensity should be controlled to prevent the selective overgrazing of alfalfa (Leach, 1983). Some cultivars are better adapted to grazing than others, including continuous grazing (Brummer et al., 1991). Its forage quality is determined by two

main components: protein digestibility and protein content (Hill et al., 1988) and Produce good nutrients with 15-22 % protein content and high vitamin and mineral contents (Wu, 2004).

It is one of the most popular high-yielding plants harvested in 3–5 cuts per year and has high content of nutrients and digestibility in cattle. The content of nutrients in feedstuffs varies, however, depending on the vegetation stage (maturity), season of harvesting and plant origin (cultivars) (Elizalde *et al.,* 1999). Ruminants benefit from two major characteristics of alfalfa. Firstly, its high protein content is readily digestible (protein digestibility varies from 81 % to 73 % in green alfalfa during the first cycle) and this digestibility surpasses that of competing forages.

Secondly, alfalfa fiber is very valuable as it is rapidly digested in the rumen, which is beneficial to rumen activity due to its buffering effect (INRA, 2007). Ruminants fed on alfalfa have higher nutrient intake and digestibility than when fed on other forage legumes and grasses (Frame, 2005). It may supply more than 30 % of the total digestible nutrients supplied by the same quantity of maize grain (Bruce *et al.,* 2008). The value of high-quality alfalfa for dairy cows is that it reduces grain and protein needs by providing variable protein content and solubility, as well as relatively high energy.

But the unique value is that it promotes greater intake and milk production by containing low NDF, faster rates of digestion, particle size reduction (its coarse structural fiber that stimulates ruminative chewing and salivation which results in rumen buffering), and rate of passage (Robinson, 2003). The beta-carotene content of alfalfa is much higher than in other forages and it has beneficial effects on the reproductive performance of dairy cows, as it increases calf weight at birth and reduces the interval between mating and calving.

It also has a stimulatory effect on milk yield (Mauries, 2003). Recent studies showed that cattle, sheep, and goats preferred alfalfa harvested on a clear day at sunset compared with the same forage harvested at the following sunrise because of potential to accumulate carbohydrates during the daytime (Fisher *et al.,* 2002). The ability of alfalfa to provide approximately 25 % more high quality feed than pasture, results in higher production potential. This is significant in economic terms (Moot, 2009). In addition to the nutritional components (proteins and

carbohydrates) that are important in the use of alfalfa and other plants as animal feed or food supplements (Sylwia *et al.*, 2010).

### 2.3.2 Nutritive Value of Alfalfa (Medicago sativa)

There are a number of chemical components of alfalfa hay that can be determined by currently available laboratory techniques. These allow the alfalfa hay to be divided into its ash (i.e., mineral) component (9 to 13 % of hay dry matter (DM)), fat (2 to 3 % of DM), protein (15 to 25 % of DM), non-structural carbohydrate such as sugars, pectin and starches (20 to 35 % of DM), and structural carbohydrates (30 to 50 % of DM). Ash, protein, fat and structural carbohydrate (usually defined as fiber insoluble in a solution of boiling detergent at a neutral pH, or neutral detergent fiber (NDF)), are generally assayed directly, while the level of non-structural carbohydrate (NSC) is calculated by difference.

Energetically, ash has no value while fat; NSC and proteins are generally almost fully digestible somewhere in the digestive tract. Thus the energy value of the hay, exclusive of the NDF, can be calculated with some accuracy. However it is the NDF portion of the hay, due to its relatively high contribution to the overall weight of the hay and its variable digestibility that makes it a key variable in estimating the energy value of alfalfa hay (Robinson, 1999). Protein content in alfalfa dry matter varies from 18 to 25% depending on the growth stage (cutting cycle), cultivar difference and other factors.

Alfalfa nutritive value is identified with protein content which depends on the share of leaves in dry matter yield which in its turn is positively correlated with protein content. The proportion of leaves and stems in alfalfa hay can vary greatly, depending on maturity at harvest, cultivars, handling, and rain damage (Katic et al., 2006). The nutritive value of alfalfa may also be improved by increasing its DM digestibility. Digestibility of alfalfa decreases with maturity as a result of increased concentration of cell wall material in stems, decreased stem digestibility, and decreased leaf weight ratio (LWR) (Albrecht *et al.*, 1987).

Decreasing protein content is a dilution effect related with the decreasing leaf to stem ratio; the leaves have stable protein content and their protein level is much higher than the protein content in stems. The decline of digestibility is the consequence of two processes: (a) the reduction of the highly digestible component (leaves) because of an increase of the less digestible component

(stems) and (b) the decreasing average digestibility of the stem component, with more cell walls (NDF) and lignin (Veronesi *et al.,* 2010).

When determining the nutritive value of alfalfa, ligneous cellulose content should be taken in account in addition to crude protein content. Neutral detergent fiber (NDF) content indicates the intake rate of alfalfa dry matter. The higher the NDF, the lower the alfalfa quality, the content of nutrients is reduced and livestock consumes such alfalfa less readily. In consequence, the livestock grows at a slower rate and the production of livestock products is proportionally reduced. ADF content indicates the potential production energy. Increase in ADF indicates a reduced energy, i.e., reduced quality (Katić *et al.,* 2008).

The term alfalfa forage quality is a broad term, referring to a number of factors that affect nutritive value of the forage. Among these factors, dry matter digestibility is considered to be the most important one (Posselt, 1994). Digestibility of alfalfa organic matter depends on the contents of cellulose and lignin. As lignin is virtually indigestible, intensive lignifications of cell wall in late stages of alfalfa development tends to reduce the coefficient of digestibility. Since alfalfa leaf is preferably eaten by animals and has better nutritive value than stems, appropriate stage of maturity and cultivars having high leaf yield is important for livestock feed (Anacleto, 2004).

Alfalfa has the potential to produce quality forage that is high in protein and carotene but low in fiber. Growing alfalfa is easy, but to produce a high yield of good quality forage and still maintain the stand demands attention to sound management practices namely: variety selection, insect and weed control, soil fertility and cutting management (James *et al.,* 1984). There is an optimum quality for alfalfa that should be fed to dairy cows. For forage that serves as the primary fiber source in the diet, NDF is the principal forage quality variable of concern.

NDF is defined as the remnants of a feedstuff that is retained offer dissolving in a neutral detergent; consisting of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are wall carbohydrates and are available for degradation by rumen microbes, which in turn produce volatile fatty acids. Lignin is anti-nutritive phenolic compound that is indigestible by rumen microbes. The ideal NDF level in alfalfa hay for dairy cows is 40 % (of dry matter). NDF levels

below 40 % are to low and the hay have high rates of passage through the rumen; resulting inefficient dry matter conversion.

NDF levels greater than 40 % begin to slow rate of passage down, creating a gut-fill effect. Higher gut-fill results in lower dry matter intake; and dry matter intake drives milk production (Găvan *et al.,* 2013). For forage trading (i.e., buying or selling), one number to describe different hays is more convenient rather than comparing their full nutrient analyses profiles. Such a Relative Feed Value (RFV) has been in place and proven very useful for livestock producers and hay farmers for long time to price hay and predict animal performance.

### 2.3.3 Nutritional benefits of alfalfa for humans

The alfalfa plant is naturally high in many essential vitamins, including A, D, E, K, and even the full family of B vitamins. Each individual vitamin has an abundance of health benefits in itself, making them crucial to overall human health. It is also loaded with extremely important minerals such as biotin, calcium, folic acid, iron, magnesium, potassium, and many others. The alfalfa plant has an unusual, extensive root system that can reach as far as 60 feet into the soil. The alfalfa herb is believed to have a direct connection to lowering cholesterol, which is once again in direct connection with all of the positive vitamins and minerals it contains. The alfalfa herb is very good at detoxifying and better purifying the blood. Consuming alfalfa herb on a routine basis has an abundance of positive health results (Yong-Han, 2009).

### 2.3.4 Role of Alfalfa in nitrogen fixation

One of the key values of alfalfa is its ability to 'fix' nitrogen gas ($N_2$) from the air so that N is available for plant growth. Available N is very limited in the Earth's crust and is frequently deficient in plants. Nitrogen is a basic building block for plant proteins, and for human protein nutrition (Miriam, 2019). While cereal crops require millions of tons of N fertilizers per year. The alfalfa requires essentially no N fertilizers for optimum growth. Estimates for $N_2$ fixation in alfalfa range from 120 to 540 lbs of N per acre per year. $N_2$ fixation is accomplished by symbiotic association with the bacteria, which lives in nodules in alfalfa roots. Dinitrogen fixation by alfalfa has several important environmental benefits, which are not broadly recognized (Jennifer *et al.,* 2007).

### 2.3.5 Role of Alfalfa in preventing soil erosion

The soil erosion has always been a significant environmental hazard of agriculture. Soil erosion is permanent loss of productive potential, since the most fertile soil layers erode, only to pollute streams and lakes with sediment. Alfalfa protects the soil from erosion by reducing the amount of cultivation, by holding the soil in place through extensive rooting, by providing a vigorous above-ground canopy, and by improving 'tilth' and water penetration (Kristen et al., 2009).

Alfalfa's roots go much deeper than other crops. This deep rooting pattern is highly beneficial to soils. Alfalfa roots are commonly 9 - 16 feet (3 - 5 meters) and may extend much deeper. The deep, vigorous alfalfa root system holds the soil in place and creates many channels in the soil that encourage water infiltration, biological activity in the root zone, and improved nutrient cycling. Water use efficiency may be improved subsequent crops (Junying *et al.,* 2009).

## 2.4 Examples of other legume crops

### 2.4.1 Lentils (Lens culinaris)

Lentil belongs to the family Fabaceae and it is a nutritious food legume. It is one of the oldest annual grains legumes more consumed and cultivated in the world and mostly eaten as dhal. Lentil was originated from South Western Asia as early as 6000 B.C. Lentil is rich in protein and also contains high concentration of essential amino acid as isoleucine and lysine, as well as other nutrients like minerals and fiber, folate, vitamin B1 (Rozan *et al.,* 2001). Lentil is also known as a "poor man's meat" because of its rich protein content. In South East Asia lentil is also equally liked by all socioeconomic groups (Bhatty, 1988).

Lentil crop requires nitrogen for their growth and development approximately 85 % of nitrogen necessity of lentil is fulfilled with the help of atmospheric nitrogen fixation during symbiotic relationship of lentil roots with Rhizobia in the field and due to which yield could be increased up to 2 ton $ha^{-1}$ (Bisen et al., 1980). Small doses of N fertilizers applied to an annual pulse are beneficial if nodule initiation is delayed (Mahon and Child, 1979). Nutrition is essential for proper growth and high grain yield of lentil. Genotype of lentils may show a differential response to nutrients. Like mort annual legumes, lentil can provide a part of its own N requirement though synoptic $N_2$ fixation when the plants are inoculated.

Sonulski and Buchan (1978) reported that Rhizobia inoculation alone is not enough for obtaining high yield of legumes because of poor nodulation & nitrogenase activity. They concluded that annual legumes may require a high level of plant N fertility to achieve maximum yield. Small doses of N fertilizers applied to an annual pulse are beneficial if nodulation initiation is delayed (Mohon and Child, 1979), In dry land cultivation pea N application at 20 to 60 kg $ha^{-1}$increased seed yield by an average of 9 % in one quarter of 58 trials conducted in similarly application of fertilizer N increased dry bean seed yield proportionally in southern Manitoba (Mc Andrew and Mills, 2000).

### 2.4.2 Faba Bean (Vicia faba)

Faba bean is a major grain belonging to the legume family and widely cultivated in many countries for source of dietary and feed purposes (Sillero, J.C, 2010). It accounts major food and feed legumes because of the high nutritional value of its seeds, which are rich in protein and starch (Duc *et al.,* 2010). Faba bean plays a major role by fixing atmospheric nitrogen to plant-available form (Siczek and Lilies, 2016). Biological fixation of atmospheric nitrogen in legume-Rhizobia is well known eco- friendly practice used for the improvement of N fixation resulted in increased shoot growth, number of pods, and grain yield of faba bean.

However, the fixation of nitrogen depends on the genotype of legume, Rhizobia strain, and the interactions of these with the bio-physical of nitrogen vary with legume species and/or variety (Abdul-Aziz, 2013) and effectiveness of partner micro symbiont (Argaw, S. 2012). The report of Ouma *et al.,* 2016 also confirmed the host-specific rhizobia strains of common bean and soybean adapted better to the local soil and environmental condition. To have a successful establishment, inoculants strain must be able to survive in soil environment because the better survival rate and soil persistence of Rhizobia enhanced the possibility of effective nodulation and nitrogen fixation.

### 2.4.3 Common Bean (Phaseoulis vulgaris)

*Common bean* is the important sources of dietary protein, food for human, fodder for livestock and source of soil nitrogen (Iberia et al., 2013). Common bean and other grain obtain nitrogen through a process known as biological nitrogen fixation, which is done by the soil bacteria found in their root nodules known as rhizobia (Giller, 2001). Although P. vulgaris yield in Tanzania is below its production potential (< 3 t ha-1), the crop is widely grown in most regions of the

country (Semoka, 2006). Of recent, there is renewed interest in the use of biological nitrogen fixation technology in agricultural systems mainly to overcome problems associated with depletion of soil nitrogen and as an alternative to excessive use of inorganic nitrogen fertilizers.

Apart from the soil fertility aspect, BNF helps to improve food safety and enhances conservation of biodiversity since it poses no adverse impact to the environment (Jonah et al., 2012).Inoculation of legumes with rhizobia is the oldest and mostly used BNF technology in agriculture (Lindström *et al.,* 2010). Rhizobium is a genus of gram negative bacteria that lives symbiotically in root nodules of legumes which converts atmospheric nitrogen to ammonia and provide organic nitrogenous compounds to the plants (Pawar *et al.,* 2014).

## 2.5 The Rhizobia and their biology, physiology and taxonomy

Rhizobia or root nodule bacteria are medium-sized, rod-shaped cells, 0.5-0.9 µm in width and 1.2-3.0 µm in length. They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two to six peritrichous flagella. Rhizobia are predominantly aerobic chemo-organotrophs and are relatively easy to culture. They grow well in the presence of $O_2$ and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been found to fix N in the free-living form except under special conditions (Somasegaran and Hoben, 1994).

Some strains of rhizobia require vitamins for growth. Optimal growth of most strains occurs at a temperature range of 25-30$^{0C}$ and a pH of 6.0-7.0. Despite their usual aerobic metabolism, many strains are able to grow well under micro-aerophilic conditions at $O_2$ tensions of less than 0.01 atm. Generally, most rhizobia Produce white colonies, only weakly absorb Congo red (diphenyldiazo-bis-a-naphthylaminesulfonate) dye, which is included in culture media for isolating rhizobia. However, if the culture medium is not buffered, acid-producing rhizobia cause the dye to turn purple.

Other interesting and useful characteristics of rhizobia are their growth reactions in the standard YEMA medium containing bromthymol blue as the pH indicator. Fast-growing rhizobia produce an acid reaction in the YEMA medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction (Somasegaran and Hoben, 1994). Rhizobia are soil bacteria that fix nitrogen (diazotrophs) after becoming established inside root nodules of legumes (Fabaceae). In

order to express genes for nitrogen fixation, rhizobia require a plant host; they cannot independently fix nitrogen.

In general, they are Gram-negative, motile, non-sporulating rods (Oelke et al., 1991). Rhizobium is a genus of soil bacteria whose members are best known for their ability to establish symbiotic relationships with legumes of agricultural and environmental importance, in a process of biological nitrogen fixation. In the legume production, the application of high quality rhizobia inoculants substantially contributes to the N cost efficiency of farming systems through inputs from biological N fixation. (Mihaela *et al.,* 2007).

Rhizobia are bacteria that induce the root hairs of the plant to form nodules in which nitrogen is stored. Rhizobia are found in most soils, but they do not always form nodules. Sometimes there are not enough bacteria in the soil to form nodules, or they might not be the right type of rhizobium for the plants. Just as there are different sorts of legumes there are also different sorts of rhizobia. For nitrogen fixation to take place, the correct combination of rhizobium and legume is needed (Rienke and Joke, 2005). Rhizobia are a paraphyletic group that fall into two classes of the proteobacteria—the alpha- and beta-proteobacteria. Most belong to the order Rhizobiales, but several rhizobia occur in distinct bacterial orders of the proteobacteria (Oelke et al., 1991).

The development of molecular techniques accelerated the systematic evolution and led to the identification of many new rhizobia genera. Based on the sequence of the 16Sribosomal RNA (rRNA) gene rhizobia could be grouped into , β and gamma subdivision of the Proteobacteria (Young and Haukka, 1996) recently rhizobia systematic consisting of 13 genera.10-belonging to α- Proteobacteria; *Allrhizobium, Bradyrhizobium Azorhizobium, Devosia, Mesorhizobium Methylobacterium,Ocrobacterium,Phyllobacterium, Rhizobium,* and *Ensifer* (Ochman *et al.,* 2005).

## 2.6. Biological Nitrogen fixation

Biological nitrogen fixation is the process whereby atmospheric nitrogen (N=N) is reduced to ammonia in the presence of nitrogenase (Oelke et al., 1991). It is a natural process through which several species of bacteria convert atmospheric nitrogen into plant available nitrogen, usually ammonium ($NH_4$) (Nape Victoria Mothapo, 2011). Nitrogen is an essential and often limiting plant nutrient in crop production. Nitrogen fixation resulting from mutual symbiosis of rhizobia

and cultivated legume plants is therefore critical to food security as it directly affects agricultural production. Biological nitrogen fixation is brought about both by free-living soil microorganisms and by symbiotic associations of microorganisms with higher plants (Oelke et al., 1991).

## 2.7 Symbiotic Nitrogen fixation and Nitrogenase enzyme activity

### 2.7.1 Symbiotic Nitrogen fixation

Symbiotic nitrogen fixation is performed by rhizobia inside the nodules, which are the symbiotic organs formed on the roots of the host legume. The bacteria supply ammonium to the plant, while the sources of carbon and energy necessary for SNF are obtained from the plant photosynthates (Gisele *et al.,* 2007). The symbiotic nitrogen fixation is used to maximum advantage in case of leguminous crops. There is no doubt that specificity exists between rhizobia strain and the legume, and compatibility between the two is essential for successful nodulation.

This necessitates using specific cultures for different legumes (Tamiru *et al.,* 2012). Leguminous plants fix atmospheric nitrogen by working symbiotically with special bacteria, rhizobia, which live in the root nodules. Rhizobia infect root hairs of the leguminous plants and produce the nodules. The nodules become the home for bacteria where they obtain energy from the host plant and take free nitrogen from the soil air and process it into combined nitrogen. In return, the plant receives the fixed N from nodules and produces food and forage protein (Oelke *et al.,* 1991).

### 2.7.2 Nitrogenase enzyme and its activity

Nitrogenase is a biological catalyst found naturally only in certain microorganisms such as the symbiotic Rhizobia and Frankia, or the free-living Azospirillum and Azotobacter (Oelke *et al.,* 1991). Nitrogenase is an oxygen sensitive enzyme. The low oxygen tension condition is realized through compartmentation in cyanobacteria (heterocysts in Anabaena azollae), active respiration (in Azotobacter), and synthesis of leghemoglobin (in Rhizobia legume). Leg hemoglobin is a macromolecule synthesized by symbiotic partners, the rhizobia and the host plant.

Rhizobia synthesize the heme portion, and the plant the globine. Like human hemoglobin, leg hemoglobin fixes $O_2$. It is responsible for the red or brown colour of active ($N_2$-fixing) nodules. Non-$N_2$-fixing nodules have white nodule content or a green content when the globine has degenerated (Oelke *et al.,* 1991). Nitrogen fixation, which involves the chemical reduction of $N_2$ to $NH_3$ or $NH_4$, requires a source of electrons. Sources of electrons for the nitrogenase activity

vary with the organism. They are all small proteins and highly reductive molecules such as flavodoxin, ferredoxin, nicotinamide, or adenine dinucleotide (phosphate) (Oelke *et al.,* 1991).

## 2.8 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. (Society for General Microbiology, 2002). Specificity genes determine which Rhizobia strains infect which legume. 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).

### 2.8.1 Root infection and Nodule formation

The legume-rhizobia symbiosis is highly specific and depends on complex signalling 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 $N_2$ into a plant-usable form, ammonium ($NH_3 + H+ \rightarrow NH_4 +$ ), using the enzyme nitrogenase.

Reaction for all nitrogen-fixing bacteria is: $N_2 + 8\ H^+ + 8\ e^- \longrightarrow 2\ NH_3 + H_2$. In return, the plant supplies the bacteria with carbohydrates, proteins, and sufficient oxygen so as not to interfere with the fixation process. Leg haemoglobins, plant proteins similar to human hemoglobins, 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 Ecological factors that affecting biological nitrogen fixation

### 2.9.1 Soil temperature

Extreme temperatures affect $N_2$ fixation adversely. This is easy to understand because $N_2$ fixation is an enzymatic process. However, there are differences between symbiotic systems in their ability to tolerate high ($35^0_C$) and low ($<25^0_C$) temperatures (Oelke *et al.,* 1991). High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops (Michiels *et al.,* 1994). High root temperatures strongly affect bacterial infection and $N_2$ fixation in several legume species, including soybean, guar, peanut, cowpea, and beans (Hungria and Franco, 1993).

Critical temperatures for $N_2$ fixation are $30^0_C$ for clover and pea and range between 35 and $40^0_C$ for soybean, guar, peanut, and cowpea (Michiels et al., 1994). Nodule functioning in common beans is optimal between 25 and $30^0_C$ and is hampered by root temperatures between 30 and $33^0$C (Piha and Munnus, 1987). Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to the plant cultivar (Arayankoon *et al.*, 1990). Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (Roughly, 1970).

### 2.9.2 Soil pH

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic $N_2$ fixation. (Zahran, 1999) reported that pasture and grain legumes acidify soil to a greater extent and that the legume species differ in their capacity to produce acids. Legumes and their rhizobia exhibit varied responses to acidity. Some species, like lucerne, are extremely sensitive to acidity, while others, such as Lotus tenuis, tolerate relatively low soil pH.

Soil acidity constrains symbiotic $N_2$ fixation in both tropical and temperate soils, limiting Rhizobium survival and persistence in soils and reducing nodulation. Soil acidity and related problems of Calcium deficiency and aluminum and manganese toxicity adversely affect nodulation, $N_2$ fixation and plant growth. Research work on the identification of symbioses adapted to acid soil should focus on the host plant, because effective rhizobia adapted to soil acidity can be found naturally and can be produced through genetic manipulations (Oelke *et al.*, 1991).

### 2.9.3 Soil moisture

Excessive moisture and waterlogging prevent the development of root hair and sites of nodulation, and interfere with a normal diffusion of $O_2$ in the root system of plants. Sesbaniarostrata and Aeschynomene sp. can actively fix $N_2$ under these conditions because they are located on the plant stems, rather than on the roots (Oelke *et al.*, 1991). The occurrence of rhizobia populations in desert soils and the effective nodulation of legumes growing there in emphasize the fact that rhizobia can exist in soils with limiting moisture levels; however, population densities tend to be lowest under the most desiccated conditions and to increase as the

moisture stress is relieved (Zahran, 1999). Drought reduces the number of rhizobia in soils, and inhibits nodulation and $N_2$ fixation. Prolonged drought will promote nodule decay. Deep-rooted legumes exploiting moisture in lower soil layers can continue fixing $N_2$ when the soil is drying (Oelke *et al.,* 1991).

### 2.9.4 Salt Stress

Salinity is a serious threat to agriculture in arid and semiarid regions. Nearly 40% of the world's land surface can be categorized as having potential salinity problems (Cordovilla *et al.,* 1994); most of these areas are confined to the tropics and Mediterranean regions. Increases in the salinity of soils or water supplies used for irrigation result in decreased productivity of most crop plants and lead to marked changes in the growth pattern of plants (Cordovilla *et al.,* 1994). Increasing 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).

Soil salinity and acidity are usually accompanied by mineral toxicity (specific ion toxicity), nutrient deficiency, and nutrient disorder. Salt damage to non-halophytic plants grown in nutrient solution is often due to the effect of ion imbalance (disorder) rather than the osmotic potential. This disorder might occur by specific toxicity of ions such as $Na^+$ and $Cl^-$ and might be balanced by increasing the concentration of counter ions, like $K^+$ and $Ca_2$+, against $Cl^-$ (Zahran, 1999). The salinity response of legumes varies greatly and depends on factors; climatic conditions, soil properties, and the stage of growth.

The legume-Rhizobium symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Rao *et al.,* 2002). Salt stress inhibits the initial steps of Rhizobium-legume symbioses. The reduction of $N_2$-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leg hemoglobin, by nodules. The depressive effect of salt stress on $N_2$ fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Rao *et al.,* 2002).

### 2.9.5 Soil Nutrients

Nodulation and $N_2$-fixation by many legumes are limited by deficiencies in soil nutrients such as P, Mo, Ca, Fe and S, (Giller and Wilson, 1991). A group of these essential nutrients are required

at specific stages in the development of legume symbiosis and the symbiotic N fixation to the extent that their deficiencies limit the productivity of host legumes in some agricultural systems (Aira, 2003).Phosphorus deficiency is commonplace in tropical Africa and reduces nodulation, $N_2$ fixation and plant growth. Identification of plant species adapted to low-P soils is a good strategy to overcome this soil constraint (Oelke *et al.,* 1991).

Phosphorus is one of several elements which affects $N_2$ fixation, and, along with N, it is a principal yield-limiting nutrient in many regions ofthe world (Zahran, 1999). Plants engaged in symbiotic $N_2$ fixation generally have a higher requirement for P than those grown with N fertilization (Panda *et al.,* 2002). P is required for signal transduction, membrane biosynthesis, nodule development and function (Grusak, 2000), and nitrogenase activity. The addition of P-solubilizing microorganisms, particularly of the genera *Pseudamaias,* Bacillus, *Penicillium,* and *Asprigillus* solubilize rock phosphate and organically bound soil P (which constitutes 95 - 99% of the total phosphate in soils).

However, the use of these microorganisms is not widespread (Oelke *et al.,* 1991).Mineral N inhibits the Rhizobium infection process and also inhibits $N_2$ fixation. The former problem probably results from impairment of the recognition mechanisms by nitrates, while the latter is probably due to diversion of photosynthates toward assimilation of nitrates. Application of large quantities of fertilizer N inhibits $N_2$ fixation, but low doses (<30 kg N ha-1) of fertilizer N can stimulate early growth of legumes and increase their overall N2 fixation. The amount of this starter N must be defined in relation to available soil N (Oelke*et al.,* 1991).

# 3. MATERIALS AND METHODS

## 3.1 Soil Sampling Sites

Soil samples were collected from some selected fields of smallholder farmers in Hawela district in Sidama region. Hawela is located at a distance of 17 KM from Hawassa and 294KM from Addis Ababa. Geographically, it is located between 6.45 degree and 38.7 degree longitude east, and between 6.33 degree and 6.62 degree latitude north. The district has a total population of 138,979. The average annual rainfall is 1124 mm. The average altitude is 1710 meters, with a maximum temperature of 32 degrees Celsius and a minimum temperature of 28 degrees Celsius. Maize is the dominant crop grown in the area.

Cattles are the most common livestock in the area and the local community heavily relies on cattles for various purposes, including milk production, meat, and as a source source of income through trade (Teklemariam and Cochrane, 2021). Regarding current forage resources, the Hawela District is rich in natural vegetation and grasslands, which serve as essential grazing areas for cattle. The areas has a diverse range of grass species that provide nutritious forage for livestock. Alfalfa, a highly nutritious legume forage crop, is cultivated in the district.

In terms of employment and livelihood, the majority of the local population in the Hawela District engages in agriculture, particularly in livestock farming. Cattle rearing and related activities provides employment opportunities and serve as the primary source of income for many households. The districts economy heavily relies on the livestock sector, making it vital component of the local livelihood. Overall Hawela district is a suitable study for this research due to its abundant cattle resources, husbandry practices, diverse forage resources, and reliance on livestock for livelihood. The selection of the Kebele’s from where the soil samples collected had no history alfalfa production and prior inoculation.

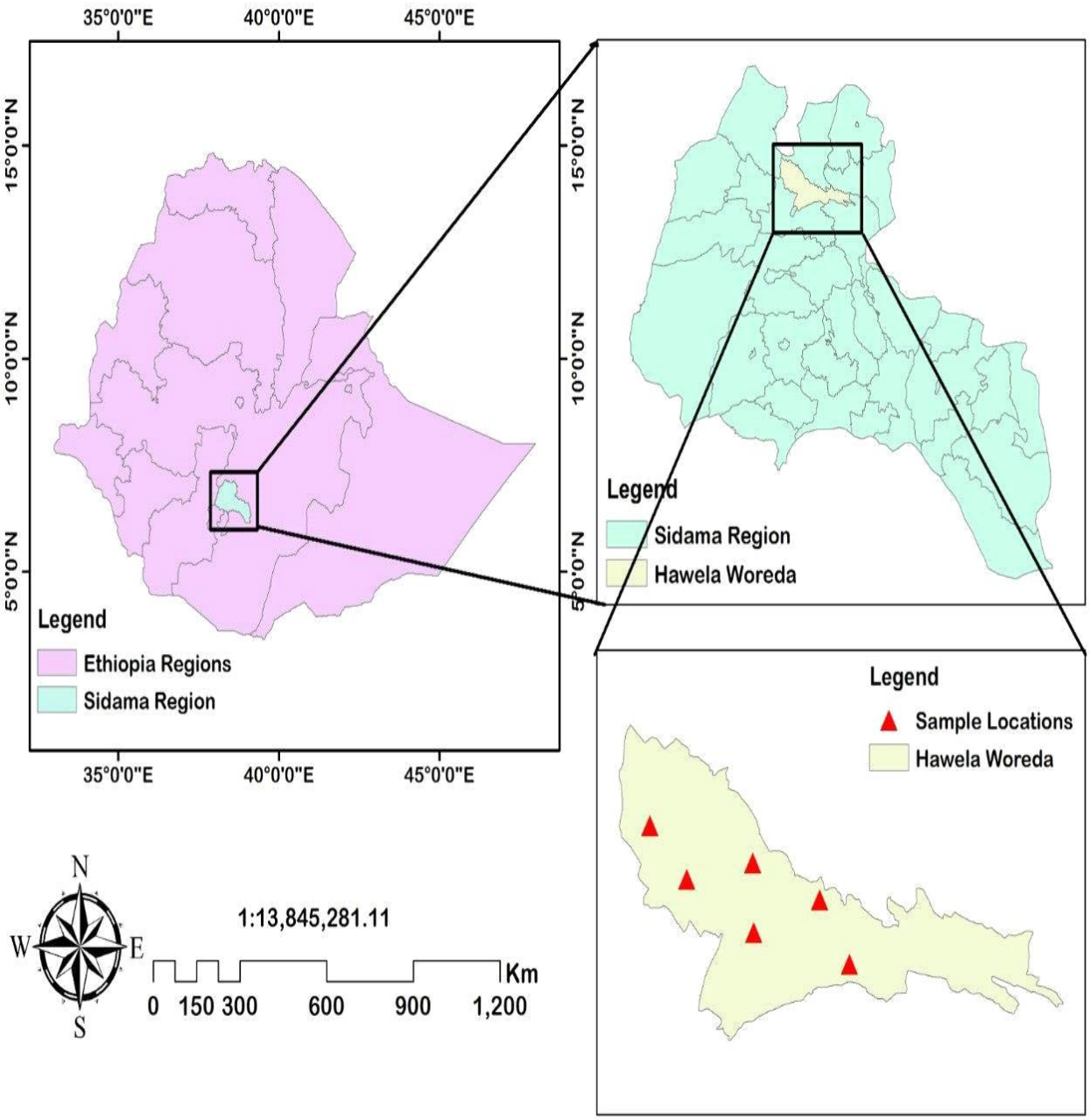


Figure 3. 1: Geographic distribution of sampling sites in Hawela, Sidama, Ethiopia.

The map illustrates key locations where soil samples were obtained providing a visual overview of the diverse sites selected for the study their significance in relation to alfalfa cultivation in the region. [Source (Researcher's Manipulation)].

### 3.2 Soil Sampling Procedure

About 3 Kg soil samples were randomly gathered from three farmers' fields from each of the selected kebeles' in a zigzag pattern from the soil surface to a depth of 20 cm in sterile plastic bags. Composite samples were made by thoroughly mixing the soil sample collected from different spots and transported to Hawassa University's College of Agriculture, soil Microbiology Laboratory for nodule trapping and rhizobial isolation.

### 3.3 Trapping of Nodules

Alfalfa seeds (Variety 1086) used for trapping of the nodules were provided by Hawassa Agricultural Research Centre. Nodulation were induced by 'plant trap' method under greenhouse condition as described by Vincent (1970).The soil samples were filled in 3 kg capacity plastic pots that had been surface sterilized (using 70% alcohol for 5sec.). Similar sized seeds of the Alfalfa were also surface sterilized with 70% ethanol for 5 seconds and with 3 % (v/v) solution of sodium hypo-chlorate for 3-4 minutes, and washed thoroughly with five changes of sterile distilled water.

Five seeds were sown in one pot and the seedlings were thinned down to three per pot after germination. The plants were watered every two day for 45 days. After 45 days the pink and undamaged nodules were collected at flowering stage of the plants. The presence of nodules on each plant was assessed to measure the soil's nodulation potential, and plants with nodules were recorded. As stated by Somasegaran and Hoben (1994), the presence of nodules on plants indicated that the soil contained rhizobia compatible with alfalfa. The nodules were then transferred in to sterile empty petri-dishes using forceps sterilized by dipping in alcohol and flaming.

### 3.4 Isolation of rhizobia from Nodules

Collected nodules were surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo-chlorate for 3-4 minutes. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals (Lupwayi and Haque, 1994). Then nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood (Somasegaran and Hoben, 1994). Then after 0.1ml (loopful) of the

suspensions were streaked on plate containing Yeast Extract Mannitol Agar (YEMA) and incubated at 28 ± 2°C from 2-3 days.

### 3.5 Purification and preservation of isolates

Single colonies were picked up and repeatedly streaked on sterile YEMA plates, and incubated at 28°Cto ensure the purity and uniformity of colony types. Purified colonies were transferred in YEMA slant containing 0.3% (W/V) Calcium carbonate (CaCO3) and preserved at 4°C for further use (Vincent, 1970). Isolates were labeled as '*ANR*' (Alfalfa Nodulating Rhizobia), followed by unique numbers.

### 3.6. Characterization of isolates

#### 3.6.1. Cultural characterization of isolates

##### 3.6.1.1. Colony morphology of isolates

The isolates were cultured on Yeast Extract Mannitol Agar (YEMA) plates to assess a variety of characteristics. Morphological characteristics such as colour change, opacity, colony elevation, consistency, texture, shape, size, exo-polysaccharide gum, border, transparency and mucosity were used for identification of the rhizobia isolate.

##### 3.6.1.2. Gram staining test of isolates

Pure cultures grown on YEMB for 3 days were used for the staining. A loopful of each isolate was spread on a slide, air-dried, and heat fixed. The slide was then flooded with crystal violet for 1 minute, followed by Gram's iodine for 1 minute. After rinsing with water, ethanol was applied for 10 seconds, followed by safranin for 1 minute. The color development was recorded after washing off the excess safranin and air-drying the smear. Gram staining was followed by a microscopic examination to differentiate rhizobia from other bacteria. All isolates were observed microscopically for shape and Gram reaction to confirm their identities. Gram-negative organisms observed as pink to red whereas gram positive bacteria showed purple color /retain the primary dye.

##### 3.6.1.3 Congo Red absorption test

Stock solution of Congo red was prepared by dissolving 0.25 g of Congo red in 100 ml of sterile distilled water. From stock solution, 10 ml 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 aluminium foil and incubated at 28°C for 2 to 3 days to detect Congo red absorption by the colonies (Vincet, 1970).

### 3.6.1.4 Keto-Lactose Test

The rhizobia isolates were streaked on Keto-Lactose agar medium(Composition: lactose 10 g; $K_2HPO_4$ 0.52 g; $MgSO_4 \cdot 7\ H_2O$ 0.2 g; NaCl 0.12 g; $CaCO_3$ 3 g; yeast extract 1 g; agar 15 g; per litre). The isolates were streaked at the center of yeast extract lactose agar plates to tentatively proof whether they belong to rhizobia. The plates were incubated at 28°C for 48 hours. After 48 hrs the plates were flooded with Benedict's reagent incubated at room temperature and the presence of ring around the colonies were checked (Holt, 1994).

### 3.6.1.5 Glucose Peptone Agar (GPA) Test

The glucose-peptone medium was prepared according to Lupwayi and Haque (1994) by dissolving 5 g of glucose, 10 g of peptone, 15 g of agar and 10 ml of Bromcresol purple (BCP, prepared by dissolving 1 g 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 glucose- peptone agar medium to observe the presence of growth by incubating at 28°C.

### 3.6.1.6 Acid-Base Production on Bromothymol Blue

To determine the ability of the rhizobia isolates to produce acid or alkaline in the medium, YEMA containing Bromothymol blue (BTB) 0.025% (w/v) was used. A loopfull of the isolates from a 48 hrs old broth culture was streaked on to the YEMA-BTB medium and incubated for 2-3 days so as to record the color changes of the medium. Formation of yellow color was recorded as positive results (Jordan, 1984).

### 3.6.1.7 Motility Test

Motility Test Medium is recommended for detection of bacterial motility. Composition of culture media that was used to detect the motility of rhizobia, g/L Tryptone 10.000 g, Sodium chloride 5.000 g and Agar 5.000 g. 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- 48 hrs at 28°C-30°C.

#### 3.6.1.8 Growth in Presence of 8% $KNO_3$

Strains were tested for the ability to grow in the presence of 8% $KNO_3$ in YEM broth for a 7 days incubation period at 28°C (El Idrissi *et al.*, 1996).

## 3.7. Biochemical characterization of isolates

### 3.7.1 Catalase test

Catalase test was performed to study the presence of enzyme catalase in rhizobia isolates. Firstly, smear of strain was made on a clean and dry glass slide, and then a few drops of $H_2O_2$ were added to the slide. Production of gas bubbles and effervescence showed a positive test (McFadden, 1980).

### 3.7.2 Citrate Utilization Test

The Simmon's citrate agar slant was prepared by replacing mannitol from YEMA with equal amount of sodium and Bromothymole blue (25 mg/l) and the culture was streaked heavily on the surface of the agar slant and incubated for 24-48 hrs at 28-30°C. After incubation for 24-48 hrs, color change of the slants for isolates were observed and recorded. The blue color indicates the positive, while green color indicates negative tests (Koser, 1923).

### 3.7.3 Urease Test

To perform this test Christensen's Urea Agar was prepared. The composition of urea agar slant in gram per liter of deionized water: Urea 20.00, Sodium Chloride 5.00, Mono potassium Phosphate 2.00, Peptone 1.00, Dextrose 1.00, Phenol Red 0.12 and agar 15.00. The medium was heated to dissolve completely. The urea is heat-labile and should be filter sterilized. Test culture was streaked on the surface of a urea agar slant. The cap was left loosely and the tube was incubated at 28-30°C in ambient air for 2-3 days. After the incubation period, the appearance of deep pink color indicates positive results.

### 3.7.4 Starch hydrolysis

The test was performed so as to determine capability of microorganism to use starch as carbon source (de Oliveira, 2007). Starch agar media (5 g/L peptone, 2 g/L potato starch, 3 g/L beef extract, 15 g/L agar, pH 7.0) were inoculated with bacterial isolates, incubated and analyzed. In

the presence of starch, the production of extra cellular enzymes occurs indicating the potential of the organism to use starch as carbon source. Iodine test was used to determine capability of microorganisms to use starch. Drops of iodine solution (5 ml) were spread on 24 hr old cultures grown on Petri-plates. Formation of clear zone around the colonies indicated utilization of starch and vice versa.

### 3.7.5 Methyl Red Test

To perform methyl red test, Glucose phosphate broth (GPB) and methyl red indicator were required. The composition of Glucose phosphate broth Buffered peptone 7.000 g, Dextrose 5.00 g and Dipotassium phosphate 5.00 g. Final pH (at 25°C) 6.9±0.2).Then, these components were dissolved well in 1000 ml of distilled water. The medium were heated to dissolve completely.

The medium was distributed in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then, the test culture were inoculated into GPB and incubated at 28°C-30°C for 48-72 hr. After incubation 5 drops of methyl red indicator were added to the medium. The Isolate that forms red color at the top of medium is positive, while yellow color indicates negative tests.

### 3.7.6 Indole Production test

Tryptone broth medium was prepared. The medium was poured into the test tubes. The rhizobia isolates were inoculated separately to the broth and incubated at 28°C for 2 days. The inoculated 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 at top is indicating the positive results; whereas yellow color ring indicates negative result.

### 3.7.7 Methylene Blue Test

Methylene Blue Test was performed to check the growth of the isolates. In this test, methylene blue dye (1 mL) was added to the YMB (10 mL) and inoculated with Rhizobia. Incubation was given at 30°C for 24 – 48 hours and observations were made as blue/ purple bacterial cells indicates positive growth (Gao *et al.,* 1994).

## 3.8 Physiological characteristics of isolates

### 3.8.1 NaCl (2%) Tolerance of isolates

To the basal medium of YEMA, 2% NaCl was added to check the purity of the isolates. As 2% NaCl is inhibitory for most rhizobia isolates it can serve as an identification tool (Sadowsky *et al.,* 1983).

### 3.8.2 pH (4 and 9) tolerance of isolates

Tolerance of isolates to pH was tested on YEMA medium adjusted to pH of 4 and 9 (Amarger *et al.,* 1997).

## 3.9 Authentication and Symbiotic effectiveness of isolates on sand pot culture under greenhouse condition

### 3.9.1 Preparation of Sand and Pots

Collected sand were socked with Hydrochloric acid for 24 hours and thoroughly washed with tap water several times until its pH becomes neutral. Then the sand was drained and autoclaved at 121°C for 1 hr. About 0.3 kg of the autoclaved sand was added to surface sterilized normal plastic pots and saturated with sterile distilled water prior to sowing seeds.

### 3.9.2 Seed Germination, Planting and Inoculation

The seeds were rinsed in 95% ethanol for 10 seconds and surface sterilized by soaking them in 3 % (v/v) sodium hypo chlorate solution for 3-4 minutes as described by Lupwayi and Haque (1994). After draining of the solution, the seeds were washed seven times with sterile distilled water. Then, seeds of alfalfa were plated into sterilized 0.75 % (w/v) water agar and incubated at 28°C for pre/germination. Five germinated seeds of alfalfa were then transferred to the pots by using sterile forceps to the depth of 1cm after two days. Each seedling was inoculated with 1 ml of a broth culture containing about $10^9$ cells of the isolate eight days after transplanting. The pots were then placed in the greenhouse. The seedlings were thinned down into three after a week.

### 3.9.3 Treatments and Experimental design

The potted experiment was laid out on a Completely Randomized Design (CRD) in the greenhouse. The treatments were consisted a bacterial inoculated, plus-nitrogen (N+ control) and minus-nitrogen (N- control). All treatments were replicated three times. For positive control treatment, 120 ml of 0.05% $KNO_3$ solution was added to each pot once every week (Matallah *et*

*al*., 2002) and for negative control were supplied 100 ml N-free nutrient solution per week as described in Somasegaren and Hoben (1994).

The plants were uprooted after 6 weeks of planting to check for the presence or absence of nodules. Shoots were dried at 70°C for 48 hr, and shoot dry weights (SDW) were recorded. The roots and adhering sand were dislodged into a coarse sieve and were washed with a gentle tap water to collect the nodules. The numbers of nodules (NN) were counted and nodules dry weight (NDW) was determined following similar procedure used for determining SDW.

### 3.10. Determination of symbiotic effectiveness indices of rhizobia isolates

The symbiotic effectiveness was calculated according to the equation proposed by Date *et al*., (1993) cited in purcino *et al.*, (2000).

$$SE = \frac{\text{Inoculated plant shoot DM}}{\text{N-Fertilized plant shoots DM}} \times 100$$

Where, D.M. = dry matter, S.E. = symbiotic effectiveness

The rate of nitrogen fixing effectiveness is ranked as: Highly effective > 85 %, Effective= 55-85 %, Lowly effective 35-54 % and Ineffective <35 %.

### 3.11. Host range of the isolates

Host ranges of the six symbiotically effective isolates were assessed by inoculating on three legume hosts; namely: lentil (Lens culinaris), Faba bean (Vicia faba) and Common bean (Phaseuolis vulgaris). Seeds of each crops were surface sterilized, pre-germinated and seedlings were transplanted aseptically into surface sterilized pots as described earlier. Similarly, all greenhouse activities including experimental design, number of replications, thinning, inoculation, watering and N-free nutrient application were as described above for authentication and preliminary screening of alfalfa rhizobia.

Treatments without inoculation and a $N^+$ treatment at a rate of 120 ml of 0.05 % $KNO_3$ solution applied once a week were included as controls for each legume species. Plants were grown for 45 days in the greenhouse and harvested for determination of nodule number, nodule dry weight, shoot dry weight and shoot length. The relative efficiency (RE) of each isolate was calculated as described before using the methods of Purcino *et al.,* (2000).

## 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 symbiotic effectiveness percentage was calculated by the method described in Purcino *et al*., (2000) as: Effectiveness (%) = (Shoot Dry Weight of inoculated plant/Shoot Dry Weight of N-fertilized treatment) × 100. The analysis of variance (ANOVA) and list significant difference (LSD) at $P< 0.05$ were determined using SAS statistical package version 10.

# 4. RESULT

## 4.1 CULTURAL CHARACTERIZATION OF THE ISOLATES

### 4.1.1 Gram staining, Congo red, Ketolactose agar and GPA tests of isolates

All 12 isolates were gram-negative and did not grow on either glucose peptone agar (GPA) or ketolactose agar media, except for three isolates (ANR 3, ANR 6 and ANR 12) that showed limited growth on GPA (Table 1 and annex 1). Furthermore, none of the colonies of the current bacterial isolates absorbed Congo red, indicating that the isolates were rhizobia members.

**Table 1: Colony Characteristics of isolates**

| Rhizobia isolates | Cong red test | Growth on GPA | Growth on ketolactose agar | Gram reaction |
|---|---|---|---|---|
| ANR 1 | White translucent | No growth | No growth | _ |
| ANR 2 | White translucent | No growth | No growth | _ |
| ANR 3 | Watery translucent | Poor growth | No growth | _ |
| ANR4 | Watery translucent | No growth | No growth | _ |
| ANR 5 | White translucent | No growth | No growth | _ |
| ANR 6 | milky | Poor growth | No growth | _ |
| ANR 7 | milky | No growth | No growth | _ |
| ANR 8 | White translucent | No growth | No growth | _ |
| ANR 9 | White translucent | No growth | No growth | _ |
| ANR 10 | White opaque | No growth | No growth | _ |
| ANR 11 | milky | No growth | No growth | _ |
| ANR 12 | White opaque | Poor growth | No growth | _ |

### 4.1.2 Growth of Isolates on YEMA-BTB and its Colony Characteristics, and motility test and growth of isolates on 8% $KNO_3$

The isolates displayed distinct morphological characteristics, indicating diversity among the rhizobia nodulating alfalfa (Table 2 and annex 1). The colonies of the isolates had a buttery or elastic texture. The color of the colonies ranged from white-translucent to opaque and milky, with round shapes varying from flat to dome and even conical on the YEMA medium.

The bacterial isolates demonstrated a rod-shaped morphology, with colony diameters ranging from 1-5mm after 2-3 days of incubation at 28 °C, suggesting their classification as rhizobia (Somasegaran & Hoben, 1994) (annex 1). Additionally, all isolates caused a noticeable color change from green to yellow in the yeast extract mannitol agar medium containing bromothymol

blue (YEMA-BTB) (Table 2 and annex 1). To test motility of isolated rhizobia, we inoculated them on medium and incubated them at 28℃ for 24 and 48 hrs. All twelve tested isolates came to be motile. Six of Rhizobia strains showed growth in the presence of 8% $KNO_3$ in the broth.

**Table 2 : cultural characterization of isolates**

| Strain | colony characteristics | | | | | Growth on BTB | Motility test | |
|---|---|---|---|---|---|---|---|---|
| | Color | size | Shape | Texture | Cell Shape | | | 8% $KNO_3$ |
| ANR 1 | White translucent | 3 | flat | buttery | rod | Yellow | + | + |
| ANR 2 | White translucent | 3 | flat | buttery | rod | Yellow | + | + |
| ANR 3 | Watery translucent | 1 | conical | elastic | rod | Yellow | + | _ |
| ANR 4 | Watery translucent | 2 | flat | elastic | rod | Yellow | + | _ |
| ANR 5 | White translucent | 3 | dome | buttery | rod | Yellow | + | + |
| ANR 6 | Milky | 2 | dome | elastic | rod | Yellow | + | _ |
| ANR 7 | Milky | 3 | flat | Elastic | rod | Yellow | + | _ |
| ANR 8 | White translucent | 4 | dome | buttery | rod | Yellow | + | + |
| ANR 9 | White translucent | 5 | dome | buttery | rod | Yellow | + | + |
| ANR 10 | White opaque | 2 | flat | elastic | rod | Yellow | + | _ |
| ANR 11 | Milky | 2.5 | Flat | buttery | rod | Yellow | + | + |
| ANR 12 | White opaque | 2 | conical | elastic | rod | Yellow | + | _ |

## 4.2 Biochemical characterization of isolates

Table 3 and annex 1 summarizes the results of the biochemical characteristics of the isolates. All isolates tested positive for the catalase, citrate, urease and methyl red tests. Six of the isolates were positive for the indole production test. Six of the isolates did not hydrolyze starch and

tested negative for the starch utilization test. All twelve of the isolates did not grow on methyl blue dye.

**Table 3 : Biochemical tests of isolates**

| isolates | Catalase test | Citrate test | Urease test | Starch utilization test | Indole test | Methyl red test | Methyl blue test |
|---|---|---|---|---|---|---|---|
| ANR 1 | + | + | + | _ | + | + | _ |
| ANR 2 | + | + | + | _ | + | + | _ |
| ANR 3 | + | + | + | + | _ | + | _ |
| ANR 4 | + | + | + | + | _ | + | _ |
| ANR 5 | + | + | + | _ | + | + | _ |
| ANR6 | + | + | + | + | _ | + | _ |
| ANR 7 | + | + | + | + | _ | + | _ |
| ANR 8 | + | + | + | _ | + | + | _ |
| ANR 9 | + | + | + | _ | + | + | _ |
| ANR10 | + | + | + | + | _ | + | _ |
| ANR11 | + | + | + | _ | + | + | _ |
| ANR12 | + | + | + | + | _ | + | _ |

## 4.3 Physiological characterization of isolates

### 4.3.1. Growth on 2% NaCl and pH 4 and 9

The isolates showed different levels of tolerance to pH levels of 4 and 9. They grew better at pH 9.0 and four even tolerated pH 4, showing their ability to adapt to different pH ranges (Table 4). However, only six of the rhizobial isolates grew in the presence of 2% NaCl (Table 4), while the others did not.

**Table 4 : Physiological Tolerance Tests of Isolates**

| Isolates | Physiological Tolerance of Isolates | | |
|---|---|---|---|
| | pH 4 | pH 9 | 2% NaCl |
| ANR 1 | - | + | + |
| ANR 2 | - | + | + |
| ANR 3 | - | + | - |
| ANR 4 | - | + | - |
| ANR 5 | + | + | + |
| ANR 6 | - | + | - |
| ANR 7 | - | + | - |
| ANR 8 | + | + | + |
| ANR 9 | + | + | + |
| ANR 10 | - | + | - |
| ANR 11 | + | + | + |
| ANR 12 | - | + | - |

## 4.4 Pot Experiment

### 4.4.1 Evaluation of Symbiotic Effectiveness on Sand Culture

The infectivity and efficacy of twelve rhizobial isolates were assessed in sand pots within a controlled greenhouse environment. Among these, six isolates were confirmed to be capable of forming nodules on the test host plant (annex 1). These rhizobial isolates (Table 5) displayed significantly varied shoot dry mass ($P<0.05$). The observed nodule numbers ranged from 9.00 to 25.67, highlighting notable diversity in performance across the isolates. The dry weight of the nodules ranged from 0.03 mg per plant to 0.07 mg per plant, while the shoot dry weight ranged from 0.96 g to 0.17 g per plant. The longest shoot length recorded was 30.50 cm for a plant inoculated with ANR 9, and the shortest was 11.47 cm for ANR 4. The mean shoot dry weight for positive control is (0.71 g per plant) and (0.14 g per plant) for the N negative control were observed. The relative effectiveness, expressed as a percentage of the shoot dry mass of the

inoculants over the N positive control, varied from highly effective (>85%) to ineffective (<35%). Four of the 12 isolates exhibited very high symbiotic effectiveness compared to the N positive control. The highest relative effectiveness was 135.21% in plants inoculated with the ANR 9 rhizobia isolate. The reason could be due to the higher presence of native population of rhizobia at Hawela district.

Table 5 : Shoot and nodule dry weight, nodule number, shoot length and relative Effectiveness of Nitrogen fixation of Alfalfa rhizobia isolates of Hawela District tested of alfalfa on the sand culture.

| **Rhizobia** | **Alfalfa** | | | | **SE%** | **SE rate** |
|---|---|---|---|---|---|---|
| | **NN** | **NDW** | **SDW** | **SL** | | |
| **ANR 9** | 25.67±0.88$^{a}$ | 0.07±0.00$^{a}$ | 0.96±5.77e-03$^{a}$ | 30.50±1.26$^{a}$ | 135.21 | HE |
| **ANR 8** | 22.67±1.45$^{b}$ | 0.06±5.77e-03$^{b}$ | 0.89±5.77e-03$^{ab}$ | 28.85±0.93$^{a}$ | 125.35 | HE |
| **ANR2** | 12.67±0.88$^{c}$ | 0.05±3.33e-03$^{c}$ | 0.83±5.77e-03$^{b}$ | 25.53±0.78$^{b}$ | 116.90 | HE |
| **ANR 1** | 19.33±0.88$^{c}$ | 0.05±0.00$^{c}$ | 0.71±0.00$^{c}$ | 22.95± 0.49$^{c}$ | 100 | HE |
| **ANR 5** | 16.67±1.20$^{d}$ | 0.04±0.00$^{d}$ | 0.52±5.77e-03$^{d}$ | 20.49±1.85$^{c}$ | 73.24 | E |
| **ANR 11** | 9.00±0.58$^{r}$ | 0.03±0.00$^{r}$ | 0.36±8.82e-03$^{r}$ | 20.10±0.53$^{c}$ | 50.70 | LE |
| **ANR 12** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.35±0.08$^{r}$ | 16.48±0.63$^{d}$ | 49.30 | LE |
| **ANR 6** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.31±0.05$^{rf}$ | 12.10±0.54$^{f}$ | 43.70 | LE |
| **ANR 10** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.31±0.01$^{rf}$ | 15.07±0.75$^{cr}$ | 43.70 | LE |
| **ANR 3** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.22±6.67e-03$^{fg}$ | 13.38±1.07$^{rf}$ | 31.00 | IE |
| **ANR 7** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.22±5.77e-03$^{fg}$ | 14.23±0.58$^{crf}$ | 30.98 | IE |
| **ANR 4** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.17±0.08$^{d}$ | 11.47±1.89fg | 23.94 | IE |
| **N+** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.71±3.33e-03$^{c}$ | 29.02±0.27$^{a}$ | 100 | HE |
| **N-** | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 0.14±0.02$^{g}$ | 9.08±1.12$^{g}$ | 19.72 | IE |

NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, SL=Shoot length, % SE= percent of symbiotic effectiveness, and treatments with the same letter are not significantly different from one another. HE= >85%, E= 55-85%, LE= 35-54%, IE= < 35% and HE= Highly effective, E= Effective, LE= Low effective and IE= Ineffective.

## 4.5 Host range of the rhizobia isolates

Six rhizobia isolates were chosen for their symbiotic performance and were subsequently tested for their capacity to form nodules on different legume hosts faba bean (Vicia faba), common bean (Phaseoulis vulgaris), and lentils (Lens culinaris). While all isolates were able to nodulate all three legume hosts, there were noticeable variations in the nodulation parameters of the inoculated plants (Tables 6, 7& 8). The number of nodules ranged from 15.67 to 28.38 per plant on faba bean, 17.67 to 29.67 per plant on common bean, and 14.00 to 21.67 per plant on lentils.

There were also differences in nodule dry weight, which ranged from 0.27 to 0.32 mg per plant on faba bean, 0.14 to 0.28 mg per plant on common bean, and 0.20 to 0.74 mg per plant on lentils. The response of the host legumes to inoculation in terms of shoot dry weight also varied greatly, ranging from 1.23 to 2.19 g per plant faba bean, 0.13 to 2.37 g per plant on common bean, and 0.44 to 0.80 g per plant on lentils. The inoculated plants also showed variations in their Shoot length. Accordingly, the shoot length ranged from 53.67 to 74.67cmper plant on faba bean, 43.67-75.00 cm per plant on common bean and 20.82-43.38 cm per plant on lentil.

The relative effectiveness, expressed as a percentage of the shoot dry mass of the inoculants over the N positive control, varied from highly effective (>85%) to effective (55- 85%) on faba bean. Four of the six isolates exhibited very high symbiotic effectiveness compared to the N positive control on faba bean. The highest relative effectiveness was 110.10% in faba bean plants inoculated with the ANR 8 rhizobial isolate. Using shoot dry weight as an indicator of the relative effectiveness, it was found that 66.67% of the isolates were highly effective (SE > 85%) and 33.33% were effective (SE ranging from 55 to 85%) on faba bean than trapped host plant (alfalfa), which was found that 33.33% of the isolates were highly effective (SE>85) and 8.3% were effective (SE 55 to 85).

The relative effectiveness, expressed as a percentage of the shoot dry mass of the inoculants over the N positive control, varied from highly effective (>85%) to ineffective (<35%) on common bean. Five of the six isolates exhibited very high symbiotic effectiveness compared to the N positive control on common bean. The highest relative effectiveness was 113.68 % in common bean plants inoculated with the ANR 8 rhizobial isolate. Using shoot dry weight as an indicator of the relative effectiveness, it was found that 66.67% of the isolates were highly effective (SE > 85%) and on common bean than trap host plant (alfalfa), which was found that 33.33% of the

isolates were highly effective (SE>85) and 33.33% isolates were showed equal ineffectiveness (SE < 35) on both plant (common bean and alfalfa).

The relative effectiveness, expressed as a percentage of the shoot dry mass of the inoculants over the N positive control, varied from highly effective (>85%) to ineffective (< 35 %) on lentil. Four of the six isolates exhibited very high symbiotic effectiveness compared to the N positive control on lentil. The highest relative effectiveness was 131.51 % on lentil plants inoculated with the ANR 9 rhizobial isolate. Using shoot dry weight as an indicator of the relative effectiveness, it was found that 66.67% of the isolates were highly effective (SE > 85%), 16.67% were effective (SE=55-85%) and 16.67 %( SE<35%) on lentil than trap host plant (alfalfa), which was found that 33.33% of the isolates were highly effective (SE>85) and 8.3% were effective (SE =55 to 85).

Table 6 : Nodulation and Symbiotic Characteristics of 6 selected Alfalfa rhizobia isolates on faba bean (Vicia faba) legume species.

| Rhizobia | Faba Bean | | | | **SE%** | **SE rate** |
|---|---|---|---|---|---|---|
| | **NN** | **NDW** | **SDW** | **SL** | | |
| **ANR 1** | 9.33±6.17$^{ab}$ | 0.27±0.04$^{a}$ | 2.19±0.29$^{a}$ | 70.67±4.33$^{ab}$ | 110.61 | HE |
| **ANR 8** | 27.67±8.84$^{a}$ | 0.31±0.05$^{a}$ | 2.18±0.31$^{a}$ | 76.44±8.22$^{ab}$ | 110.10 | HE |
| **ANR 2** | 25.00±3.60$^{a}$ | 0.300±5.774e-03$^{a}$ | 2.16±0.10$^{a}$ | 74.67±1.85$^{ab}$ | 109.10 | HE |
| **ANR 9** | 28.33±6.69$^{a}$ | 0.32±0.03$^{a}$ | 1.99±0.31$^{a}$ | 72.43±7.78$^{ab}$ | 100.50 | HE |
| **ANR 5** | 20.33±4.91$^{ab}$ | 0.29±0.02$^{a}$ | 1.65±0.13$^{a}$ | 63.34±6.49ab | 83.33 | E |
| **ANR11** | 15.67±0.88$^{ab}$ | 0.27±8.89e-03$^{a}$ | 1.23±3.333e-03$^{a}$ | 53.67±2.33$^{ab}$ | 62.12 | E |
| **N+** | 0.00±0.00$^{b}$ | 0.00±0.00$^{b}$ | 1.98±0.33$^{a}$ | 80.0±0.58$^{a}$ | 100 | HE |
| **N-** | 0.00±0.00$^{b}$ | 0.00±0.00$^{b}$ | 1.37±0.05$^{a}$ | 66.82±0.88$^{ab}$ | 69.20 | E |

NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, SL=Shoot length, % SE= percent symbiotic effectiveness, and treatments with the same letter are not significantly different from one another and HE= >85%, E= 55-85%, LE= 35-54%, IE= < 35%.

**Table 7: Nodulation and Symbiotic Characteristics of 6 selected Alfalfa rhizobia isolates on Common bean (Phaseolus vulgaris) legume species.**

| Rhizobia | Common bean | | | | SE% | SE rate |
|---|---|---|---|---|---|---|
| | **NN** | **NDW** | **SDW** | **SL** | | |
| **ANR 9** | 28.67±0.88[a] | 0.28±0.01[a] | 2.37±0.07[a] | 75.00±2.89[a] | 124.74 | HE |
| **ANR 8** | 29.67±1.45[a] | 0.31±0.05[a] | 2.16±0.19[a] | 69.33±0.67[a] | 113.68 | HE |
| **ANR 2** | 27.67±1.45[a] | 0.24±0.07[a] | 1.98±5.774e-03[a] | 53.00±0.58[b] | 104.21 | HE |
| **ANR 1** | 27.61±0.20[a] | 0.16±0.08[a] | 1.92±0.01[a] | 49.67±0.33[bc] | 101.10 | HE |
| **ANR11** | 17.67±6.23[a] | 0.14±0.12[a] | 0.45±0.34[b] | 43.67±0.33[c] | 23.68 | IE |
| **ANR 5** | 24.67±2.90[a] | 0.15±0.10[a] | 0.13±3.333e-03[b] | 52.67±3.18[b] | 6.84 | IE |
| **N+** | 0.00±0.00[b] | 0.00±0.00[b] | 1.90±5.774e-03[a] | 69.07±1.24[a] | 100 | HE |
| **N-** | 0.00±0.00[b] | 0.00±0.00[b] | 0.16±0.00[b] | 45.50±2.30[bc] | 8.42 | IE |

NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, SL=Shoot length, % SE= percent symbiotic effectiveness, and treatments with the same letter are not significantly different from one another and HE= >85%, E= 55-85%, LE= 35-54%, IE= < 35%.

**Table 8 Nodulation and Symbiotic Characteristics of 6 selected Alfalfa rhizobia isolates on lentil (Lens culinaris) legume species.**

| **Rhizobia** | **Lentil** | | | | **SE%** | **SE rate** |
|---|---|---|---|---|---|---|
| | **NN** | **NDW** | **SDW** | **SL** | | |
| **ANR 9** | 21.67±5.92[a] | 0.28±0.02[b] | 0.80±3.333e-03[a] | 43.38±1.68[a] | 131.15 | HE |
| **ANR 8** | 20.00±5.80[a] | 0.25±0.03[bc] | 0.73±0.02[a] | 33.36±2.21[bc] | 119.67 | HE |
| **ANR 5** | 19.33±2.85[a] | 0.21±6.667e-03[bc] | 0.70±0.02[a] | 27.31±1.14[cde] | 114.75 | HE |
| **ANR 1** | 16.67±4.05[ab] | 0.25±0.00[bc] | 0.70±0.07[a] | 29.20±0.88[cd] | 114.75 | HE |
| **ANR 11** | 14.00±1.73[ab] | 0.20±5.774e-03[c] | 0.44±0.06[bc] | 20.82±0.39[e] | 72.13 | E |
| **ANR 2** | 20.33±3.84[a] | 0.74±0.03[a] | 0.17±3.333e-03[a] | 25.69±1.34[de] | 27.87 | IE |
| **N+** | 0.00±0.00[b] | 0.00±0.00[b] | 0.61±0.00[ab] | 38.49±1.01[ab] | 100 | HE |
| **N-** | 0.00±0.00[b] | 0.00±0.00[b] | 0.29±0.04[c] | 23.42±2.18[de] | 47.54 | LE |

NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, SL=Shoot length, % SE= percent symbiotic effectiveness, and treatments with the same letter are not significantly different from one another and HE= >85%, E= 55-85%, LE= 35-54%, IE= <35.

# 5. DISCUSSION

## 5.1 Cultural Characterizations of Isolates

In this study on the basis of morphological observations on Yeast extract mannitol agar (YEMA) medium, colonies of all the strains were found to be round, white, raised, opaque, translucent and produced mucous when grown on YEMA plates). All of the alfalfa isolates displayed gram- negative reaction, rod-shaped, fast growing with colony diameters ranging between 1-5 mm within 2-3 days of incubation, indicating the isolates were rhizobia (Somasegaran & Hoben, 1994).

Furthermore, the characteristics developed by our strains were consistent with the phenotypic appearance previously described in rhizobia isolates nodulating alfalfa (Mohamed et al., 2014). Thus, the diversity observed in the colony morphology suggests the existence of genetic diversity among the different isolates. Bacterial strains showed very fast growth on YEMA-BTB and change the medium into yellow color (Table 2) which indicated the feature of Rhizobia along with other endosymbionts.

Initial growth was started on second day of culturing and usually took 2-3 days for the complete growth on the YEMA media plate. These results were in agreement with Begom et al., (2021) who reported fast grower nature of Rhizobial isolates which can nodulate lentil for biological $N_2$ fixation. Similar findings were also reported by Keyser et al., (2022) who reported fast grower nature of rhizobia which can nodulate soybean for biological N2 fixation. This could be considered as an indicator of production of acidic and verifying the common characteristics of fast growing rhizobium sp. (Somasegaren and Hoben, 1994).

According to Jordan, 1984 classification those isolates were classified as fast growing root nodule bacteria. However, these results should be confirmed by further experiments on generation time to determine their growth rate. Similar results were found by Fano (2010), Aregu *et al.,* (2012) and Kassa et al., (2015) from previous observations on field pea nodulating rhizobia. Presumptive Tests of rhizobia for confirmation of all the isolates as rhizobia spp., all the 12 rhizobia spp. were screened for different confirmatory tests using different media viz. Congo red test, ketolactose test, and glucose peptone agar test and gram staining reaction (Table 1).

The colonies did not absorb the Congo red color and such nature, differentiate Rhizobia from *Agrobacterium*. Somasegaran and Hoben (1994) also indicated that species of rhizobia nodulating legumes do not absorb CR dye or may absorb a little amount to give a pale pink appearance. In Keto-lactose test, no yellow zone was observed around the colonies after the addition of Benedict's reagent which is the characteristic of rhizobia and the same results were observed (Deshwal and Chaubey, 2014).

The result showed all the bacteria to be Gram negative (Table 1). Similar results were found by Hameed et al., (2004) who reported that most of the rhizobia and *Bradyrhizobium* (class of the Alpha proteobacteria, order of the *Rhizobiales*) are Gram-negative nitrogen-fixing bacteria that occur either as free-living soil bacteria or in interaction with the roots of leguminous plants. Similar findings were also obtained by (Datta et al., 2015); they found that all isolated strains shown to be gram negative and rod shaped and motile. Ismail et al., (2022) also reported that isolated strains were found to be gram negative as the cells appeared pink in color after gram staining.

In Glucose peptone agar test, all the isolates showed no growth on Glucose peptone agar medium that indicated the features of rhizobia (Table 1). Regarding the growth in Glucose peptone agar, Vincent et al., and 1970) reported that rhizobia showed either no growth or grew very poorly. From the above observations we can conclude that all the bacterial isolates were rhizobia sp.

All tested isolates came to be motile. A positive motility test is indicated by a red turbid area (hazy appearance) extending away from the line of inoculation. Our data came to be in coherence with many authors such as Fathy et al., (2021) on testing their isolates, this considers one of the specific characters of rhizobia isolates as motile. Rhizobia strains showed growth in the presence of 8% $KNO_3$ in the broth (Idrissi et al., 1996).

## 5.2 Biochemical characterization of isolates

The results of biochemical characteristics of bacterial isolates were represented (Table 3). All the isolates were catalase positive. Some researchers also observed bubble formation around bacterial colonies (Mahana et al., 2000). Panek and Brian (2004) reported that aerobically grown *Bradyrhizobium japonicum* cells express a similar catalase activity. Six of the isolates showed negative results for amylase and six of the gave positive result for it. When the inoculated plates were flooded with iodine solution, clear zones around the colonies were not observed while blue colour appears on no growth areas. The same results match with those who observed that rhizobium isolates have capability to use starch (De Oliveira *et al.,* 2007).

Isolates also showed positive result for urease test. Similar test results were reported by Sadowsky *et al.,* (1983). The urease test was done to determine the ability of the isolates to break down urea, to simple forms of nitrogen which can be readily absorbed by the plants to promote growth. The positive test is an important aspect in growth and development of rice in the case where fertilizers are applied. All isolates are capable of utilizing citrate as carbon source. All twelve isolates gave positive result of methyl red test.

On addition of methyl red (pH indicator) the methyl red medium remained red indicating that the test organism is acid producer. Therefore the test organism is methyl red positive. Selected isolates were unable to grow on medium containing 1 ml methylene blue earlier studies also indicated that Rhizobia cells were unable to grow in the presence of these two dyes (Wei *et al.,* 2003).

## 5.3 Physiological tolerance tests of isolates

All strains in this study were able to tolerate 2% NaCl, which is in accordance with the characteristics of rhizobia isolates (Holt *et al.,* 1994). When the isolated strain of rhizobia was inoculated on YEMA plates having 2% NaCl and then incubated at 30°C for 24 hr., growth was observed on the medium concentrations on 2% NaCl. Thus we could conclude that the isolated strains of bacteria are tolerant to medium salt (NaCl) concentrations. But according to Hashem *et al.,* (1998) salt stress may decrease the efficiency of the rhizobia-legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemo taxis and root hair colonization, thus directly interfering with root nodule function.

The results were observed that almost all of the alfalfa rhizobia isolates were resistant to 2% salt concentrations. So they have better adaptation abilities for stress conditions with salt. However, as the salt concentration increased, the tolerance of the isolates decreased; this may be due to the direct toxicity of $Na^+$ and the osmotic stress imposed by salinity. Similarly, previous results with strains of rhizobia nodulating alfalfa showed decreased growth with increasing salt concentration (Bhargava *et al.,* 2016). The result is also consistent with the result of Mohamed *et al.,* (2014), who said that beyond the concentration of 2 %, the growth of all strains decreased with an increase in NaCl in the medium, and a few isolates resisted up to 5 % concentration.

pH tolerance of alfalfa isolates showed better growth on pH 9.0 and even some of isolates (33.3%) tolerated acidic settings (pH 4), indicating that they were adapted to varying pH ranges. Hameed *et al.,* (2014) and Shimekite (2006) reported that *Sinorhizobium melitot* strains grew at pH 4 and 9, reflecting the ability of the strain to grow at wide pH range. Similarly, microsymbionts nodulating alfalfa (*Medicago sativa)* from the Algerian Sahara were found to survive at slightly acidic, neutral, and alkaline pH (Azib *et al.,* 2022), confirming the ability of alfalfa strains preference of diverse pH conditions. The findings reveal that rhizobial isolates thrive in acidic and alkaline pH environments, making them crucial for establishing and maintaining symbiosis on the majority of acidic soils in the Ethiopian highlands, as they can thrive in various pH ranges, particularly those that withstand acidic condition.

## 5.4 Evaluation of symbiotic effectiveness of isolates on sand pot culture experiment

Two features, infectivity (the ability to form nodules) and symbiotic effectiveness (capacity to fix nitrogen), are commonly used to assess the ecological and evolutionary relationship between rhizobia and their host (Brockwell, 1998). Six of the rhizobial isolate assessed in this study showed a capacity to induce nodule formation on the host plant (Table 5). The ineffective isolates showed different colony morphology (Table 2) and failed to nodulate the homologous host (Table 5); most of their physiological characteristics were found to be similar to the other isolates.

Therefore it is possible to conclude that the isolate may be either rhizobia that loss their nitrogen fixing capacity due to the loss of their plasmids (Segovia *et al.,* 1991) or some other rhizobia that penetrate the nodules (Johnston and Beringer, 1976). However, it must be experimentally supported before categorizing them as contaminant or genetically defective

rhizobia. The isolates that nodulated alfalfa (Medicago sativa) showed significant difference in shoot dry weight. Several reports showed that shoot dry mass is a good indicator of relative strain effectiveness, and there is a good correlation between shoot dry matter production and nitrogen fixation capacity of legumes (Peoples *et al.,* 2002).

The highest scores of 135.21% effectiveness of symbiotic nitrogen fixation were displayed by the isolates (Table 5). This indicated that Rhizobia isolates significantly increased plant growth compared with the control plant (without bacterial inoculation) in a measured parameters due to their role in symbiotic interaction with alfalfa for nitrogen fixation in root nodules (Mohammadi and Sohrabi 2012). Previously, Desta Beyene and Angaw Tsigie (1987) only managed to isolate 23 symbiotically effective strains (11%) from 108 isolates from Central Shewa, Ethiopia.

Such variability in symbiotic effectiveness of alfalfa rhizobia was found to be widespread in Ethiopia (Van Berkum et al., 1995). Ayneabeba Adamu Treatments with the same letter are not significantly different from one another. HE= >85%, E= 55-85%, LE= 35-54%, IE= < 35%, et al., (2001) also reported 66.87% effectiveness in nitrogen fixation on sand culture of Rhizobium leguminosarum var viceae isolates from Ankober, Molale, Keyt, and Mehalmeda sites from Northern Shewa, Ethiopia.

Generally the result of this study suggests that, the highly performed strains are worthy of further greenhouse and broad host range testing on faba bean, common bean and lentils in order to select effective and competent strains. Using shoot dry weight as an indicator of the relative effectiveness, it was found that 33.3% of the isolates were less highly effective (SE >85%) 0n alfalfa than faba bean , common bean and lentil which were show 66.67% , 66.67%, 66.67% highly effective (SE> 85) respectively. Similarly, the relative effectiveness isolates on faba bean, common bean and lentil which were showed equal highly effectiveness 66.67 % (SE>85%).

### 5.5 Host range test of alfalfa rhizobia isolates on other three different legume species

The ability of host range of six (6) selected alfalfa rhizobia isolates (ANR 9, ANR 8, ANR 2, ANR 1, ANR 5 and ANR 11) to form nodules and effectively fix nitrogen was determined on other cross-nodulating hosts. All of six isolates (100%) nodulated all faba bean (Vicia faba), common bean (Phaseoulus vulgaris) and lentil (lens culinaris) hosts (Table 7, 8 and 8). The data showed that 66.7%, 66.7% and 66.67% of these isolates displayed highly effective symbiosis

with their heterologous faba bean, common bean and lentil hosts, respectively and 33.3% and 16.7% of isolates were effective with their heterologous faba bean and lentil hosts respectively.

However, some (33.3%) of them formed ineffective on common bean. Musiyiwa *et al., (*2005) reported that all soybean isolates tested nodulated cowpea with 58% being moderately effective to very effective and about 82% nodulated pigeon pea 36% of which were effective.

Appunu *et al.,* (2009) also reported that all rhizobia isolated from soybean nodulated cowpea, 67 % nodulated mung bean and 22% formed nodules with pigeon pea and peanut. Yang and Zhou (2008) showed that two *Brady-rhizobium* strains isolated from soybean could also nodulate peanut growing in the same ecological niche that may have phylogenetic connection with one another. The study showed that with respect to nodulation ability; faba bean, common bean and lentil were equally promiscuous by forming nodules with 100% of the tested isolates (Table 6, 7 and 8).

Common bean in case was the most specific of the hosts examined, with about 33.3% of the isolates unable to nodulate or forming ineffective symbiosis. Several studies in the past also showed that common bean is one of the most promiscuous legume hosts in the so- called 'common bean' miscellany group of rhizobia (Musiyiwa et al., 2005). The promiscuity of their group was previously showed on the closest relative faba bean (Risal et al., 2012) and distantly associated lentil (Coutinho et al., 1999). Using shoot dry weight as an indicator of the relative effectiveness, it was found that 33.3% of the isolates were highly effective (SE > 85%) and 8.3% were effective (SE ranging from 55 to 85%) in alfalfa. The promiscuity of their group was previously showed on the closest relative mung bean and distantly associated pigeon pea (Pueppke and Broughton, 1999; Risal et al., 2012).

# 6 CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Conclusions

This study shows cultural, biochemical and physiological characterization and evaluation of the symbiotic effectiveness and host range of the rhizobia nodulating forage legumes alfalfa (Medicago sativa) in Hawela District, Sidama Region, Ethiopia. The isolates possessed different presumptive, cultural, physiological and biochemical characteristics indicating that there is great diversity among them, and their tolerance to alkaline and acidic settings are important adaptations for the inoculant's selection. The preliminary symbiotic effectiveness revealed that 33.3% of alfalfa isolates were found to be highly effective and indicated an increase in nodule number, nodule dry weight, shoot dry weight and shoot length of the plants with respect to the uninoculated control.

Four isolates of Alfalfa (ANR 9, ANR 8, ANR 2 and ANR 1) were performed better in the greenhouse trial than nitrogen fertilizer. This suggests that Hawella district soils harbor phenotypically diverse and symbiotically highly effective rhizobia. The study conducted using selected isolates, indicated that the isolates have wide host ranges and is highly effective on most of the legumes. Isolates (ANR 9, ANR 8, ANR 2 and ANR 1)were highly effective on the three legumes; faba bean (Vicia faba), common bean (Phaseulos vulgaris), and lentil (Lens culinaris) indicative of their potential to be used as inoculant strains for these legumes after testing their symbiotic effectiveness and competitiveness under field condition in the presence of other indigenous.

## 6.2 Recommendations

Rhizobia nodulating forage legumes have been understudied in Hawela district. Livestock production and productivity directly depend on the availability and quality of feed. The first choice is the use of adaptive, high-yielding, and improved drought-tolerant forage legumes that fix atmospheric nitrogen. Hence, we recommend highly effective strains (screened in this study) for field evaluation to check their competency and consistent nitrogen fixation under field conditions. Our screening was limited to preliminary cultural, biochemical and physiological characterization. It is recommended to further characterize the strains genetically for correct identification.

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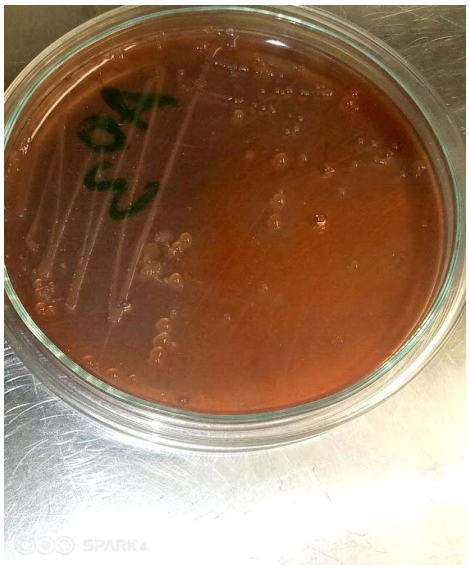
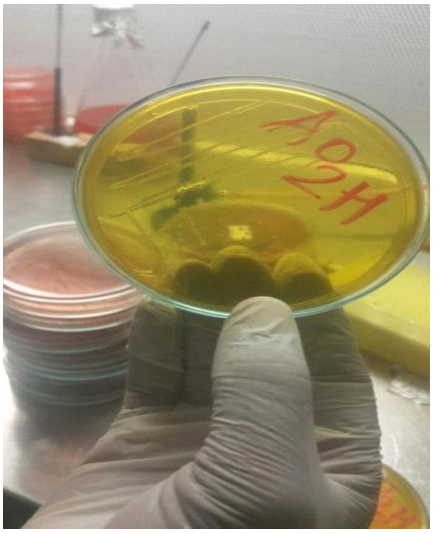
# 8 APPENDIXES

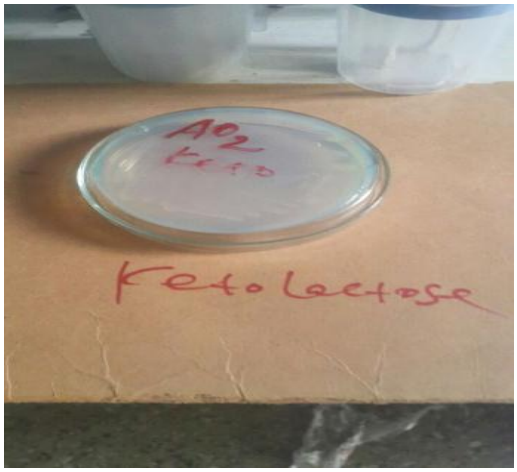
## 8.1 PICTURE DURING THE STUDY SESSIONS



(a) YEMA-Congo red



(B) YEMA-BTB Result




(c)Keto lactose positive result


(d) GPA positive result


(e) Indole positive result


(f) Citrate positive result


g) 8% KNO3

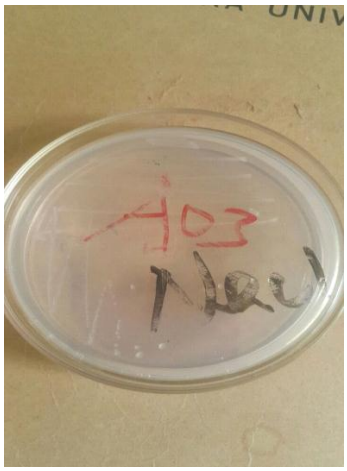
(h) Nodule sample measuring


(i)Starch

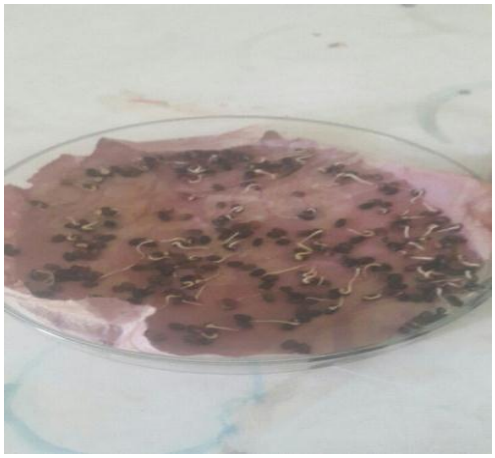
(j) YEMA- slant

(m) Trapping nodule sample

(n) pot preparation




(k) 2% NACL



(l) Alfalfa seed germination

(o) Common bean seed germination

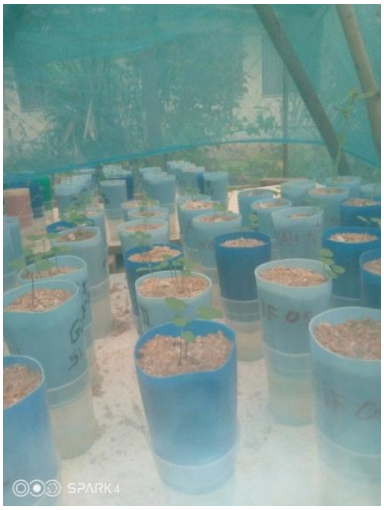
(p) YEMA-Broth

(q) Alfalfa greenhouse experiment

(r) lentil shoot negative


(s) Culturing rhizobia


(t) Alfalfa dry shoot



(u) Lentil shoots

(v) sterilizing the media

(w) faba bean young

(x) Alfalfa shoots

(z) common bean nodule appearance

(A) Common bean N-control

(B) Urease positive

(C) catalase Positive