EXPLORING AND SCREENING RHIZOBIA NODULATING FORAGE LEGUMES OF ALFALFA (*Medicago sativa*) AND VETCH (*Vicia villosa*) GROWING IN ETHIOPIA

M.Sc. THESIS BY AMISALECH MARTSA

JULY, 2023

ARBA MINCH, ETHIOPIA

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M.Sc. THESIS SUBMITTED TO
THE DEPARTMENT OF BIOLOGY, COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES, SCHOOL OF GRADUATE STUDIES, ARBA MINCH UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

I declare that the work of this Master of science thesis entitled ‟ Exploring and screening rhizobia nodulating forage legumes of alfalfa (*Medicago sativa)* and vetch *(Vicia villosa)* growing in 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 duly acknowledged.

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ADVISERS' THESIS APPROVAL SHEET

This is to certify that the thesis entitled **"Exploring and screening rhizobia nodulating forage legumes of alfalfa (*Medicago sativa)* and vetch *(Vicia villosa)* growing in Ethiopia"** submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology, to Department of Biology has been carried out by **AMISALECH MARTSA, ID: PRNS/004/14,** under our supervision. Therefore, we confirm that the student has fulfilled the requirements and hence hereby can submit the thesis to the department for defense.

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We, the undersigned, members of examiners of the final thesis defense by **AMISALECH MARTSA** have read and evaluated her thesis entitled on **"Exploring and screening rhizobia nodulating forage legumes of alfalfa (*Medicago sativa)* and vetch *(Vicia villosa)* growing in 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 Master's of Science in Biotechnology

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ACKNOWLEDGEMENT

I take this opportunity to express my gratitude to God for facilitating this laborious step towards my accomplishment. I am thankful to the Dita Woreda Administration for granting me this special occasion and to Arba Minch University, Department of Biology, for accepting and educating me with the provision of all necessary materials and facilities throughout the study.

I would like to express my heartfelt thanks and gratitude to my principal adviser Ashenafi Hailu (PhD) for his constant expert guidance, advice and support through all the stages of the research work. My gratitude also goes to my co-advisors Endalkachew Woldemeskel (PhD), Melkamu Derseh (PhD) and Kindu Mekonnen (PhD) from ILRI campus for arranging supports from Africa RISING, ILSSI projects, and Mixed Farming Initiative (MFS), guiding forage legume seed access, organizing soil sample and root nodule collections from various locations across Ethiopia and overseeing this collaborative research work.

I would like to acknowledge the laboratory technicians, Beyene Kushe, Dawit Albene, Fitsum Dejene and Yonas Siraj, for their encouragement and assistance in materials and technical support.

Appreciation also goes to my mother, Addis Coma, for her support during the long period of my study. Special thanks also go to my dear children, Bamlak Merid and Naod Merid; they made my work easier and tolerated my absence from home at a time that they needed me most. My heartfelt thanks and love go to my family for their support, encouragement, and understanding during the period of this study. Without your prayers and support, this study would surely not have been possible.

I extend my heartfelt appreciations to Africa RISING, ILSSI projects, and Mixed Farming Initiative (MFS) for their financial support through ILRI and Arba Minch University.

Finally, last but not least, I thank all others who contributed in one way or another to my M.Sc. study success.

ABBREVIATIONS

ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
RCBD	Completely randomized block design
CR	Congo red
IAR	Intrinsic Antibiotic Resistance
MLJ	Modified Leonard Jars
MPN	Most probable number
NN	Nodule number
RSE	Relative symbiotic efficiency
rpm	Revolutions per minute
SDW	Shoot dry weight
SNNPRS	Southern nation, nationalities and people's regional state
SE	Symbiotic effectiveness
UPGMA	Unweighted Pair Group Mean with the Average
YEMA	Yeast extract mannitol agar
YMB	Yeast extract mannitol broth

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ABSTRACT

Livestock production in Ethiopia has been constrained by feed shortage, low quality and seasonal fluctuations. The use of high-yielding, good quality and drought-tolerant forage legumes like alfalfa and vetch has been suggested to overcome the feed constraints. These forage legumes fix atmospheric nitrogen when being in symbiosis with rhizobia, but show variations in terms of N_2-fixation, calling for exploring the best symbionts. Thus, this study was intended to isolate and screen potential rhizobia nodulating forage legumes alfalfa (*Medicago sativa*) and vetch (*Vicia villosa*) growing in Ethiopia. A total of 90 bacterial isolates were obtained from field standing root nodules or from soil by plant trap method. The isolates were evaluated for preliminary symbiotic effectiveness and characterized phenotypically. Simultaneously, soil rhizobia population in the sampling sites were estimated using most probable number (MPN). Among the total isolates, 75 were authenticated as rhizobia. 27% of the isolates were preliminarily highly effective in terms of symbiotic effectiveness and 15% of which performed equivocally or better than artificial N-fertilization. There was significant difference ($p < 0.05$) between isolates regarding nodulation, shoot biomass and symbiotic effectiveness. Culturally, the isolates were rod shaped, gram-negative and fast-growing with colony morphology varying from 2mm to 5mm and adapted to different eco-physiological conditions. The isolates also metabolized a broader range of carbon and nitrogen sources and tolerated varying concentrations of heavy metals and antibiotics; these would enable them to thrive on marginalized soils. Numerical analysis of 55 phenotypic traits clustered vetch isolates into 3 and alfalfa isolates to 3 phenotypic groups at 70% of similarity coefficient. In general, the isolates were phenotypically diverse and symbiotically efficient rhizobia nodulating forage legumes vetch and alfalfa. According to symbiotic effectiveness tests, the rhizobial isolates VB23, VL40, VB76, VK33, VL11, VL34, VB63, AD50, AK88, AB20, and AB60 had high N_2-fixation efficiency and are recommended for field evaluation under different environmental conditions. We also recommend those strains for molecular characterization and use as inoculants on sympatric forage legumes in soils where they perform best.

Key words: Elite strains, forage legumes, physicochemical characterization, rhizobia screening, Symbiosis

1. INTRODUCTION

1.1. Background

Livestock production is a vital component of 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, the production of the livestock is constrained by inadequate and low quality feed supply, disease, low genetic potential of the indigenous breeds, poor husbandry practices, and a weak marketing system (Tolera et al., 2012). Among these, inadequate and poor quality feed supply contributes to reduced livestock production in large extent (Tolera et al., 2012; Feyissa et al., 2019). The main feed resources for livestock are natural pasture and crop residues, which are low in quantity and quality for sustainable animal production. To overcome with these limitations, it is advised to use high-yielding, good quality and drought-tolerant forages (Mengistu et al., 2017). Hence, forage legumes fulfill the requirements of better quality feed in adequate quantities for sustainable animal production.

Forage legumes are thus believed to contain high nutritional quality, survive under marginalized soil conditions and maintain soil health via their biological nitrogen fixation processes. Thus, they became an integral part of sustainable agricultural systems, providing quality feed, nectar, green manure, soil cover (Sheaffer et al., 2018) and adaptation to the prevailing climatic and soil conditions, ease of establishment, herbage productivity, seed yield potential, reduce soil erosion, resistance to pests and diseases (Tolera et al., 2012; Feyissa et al., 2019). These attributes make forage legumes ideal for livestock production.

A number of forage legumes such as *Medicago sativa* (lucerne, alfalfa), *Trifolium repens* (white clover), *Trifolium pratense* (red clover), *Trifolium subterraneum* (subterranean clover), *Lotus corniculatus* (birdsfoot trefoil), *Vicia* (vetch spp.), *Lablab purpureus* (Lablab, Poor-man's bean), and etc. have been globally adopted, 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 low lands (Mengistu et al., 2017).

Among the different forage crops, alfalfa is one of the most useful forage species recommended for diverse agro-ecological zones of Ethiopia due to its high yield, nutritional value, and better adaptability under diverse environmental conditions (Moreira & Fageria, 2010; Kebede et al., 2022). Due to its appropriateness for low-input, rain fed environments, benefits to soil fertility and nitrogen balance, high protein content and quality of its fodder, and compatibility with organic crop livestock systems, alfalfa can play a significant role (Annicchiarico & Pecetti, 2010). It has been reported that animal productivity increased and milking cows gave 50–100% higher milk when fed on alfalfa (Atumo et al., 2021). Furthermore, Symbiotic nitrogen fixation in alfalfa reduces chemical N usage and improves the environment by enhancing soil nitrogen fixation and providing N_2 accessibility to post-alfalfa plants (Latif et al., 2023). As a result, alfalfa is considered one of the most popular forage legumes worldwide (Atumo et al., 2021).

Another forage legume named Vetch (*Vicia villosa*) is the most significant annual forage legume for livestock production globally (Georgieva et al., 2016). It is typically planted for forage, consumed under direct or indirect grazing, or used as green manure due to biological fixation potential. One attraction of vetch is its versatility, which permits diverse utilization as either ruminant feed or green manure It is one of the popular cover crop that is advised for use in organic farming, soil and water conservation, and its ability to produce biomass in a short period of time (Renzi et al., 2020). Forage legumes including vetches are rich sources of N for livestock with cheaper prices compared to concentrates especially in developing countries due to its high protein content (Kebede et al., 2013). Due to its high capacity for nitrogen fixation, hairy vetch has received special consideration for cultivation as an annual feed crop for rotation with other crops to make up for nitrogen deficiency (Dastikaitė et al., 2009; Seyedeh et al., 2010).

Integration of these forage legumes into local production systems for improved livestock productivity by increase quantity and quality of feed since they fix nitrogen with association of root nodule bacteria known as rhizobia (Shimekite, 2006; Mengistu et al., 2017). Thus, the association between Alfalfa and Sinorhizobium meliloti is thought to be the most effective association for nitrogen fixation in the soil and fix nitrogen to the tune of Alfalfa 500 kg N ha^{-1}

with maximum production of 640 kg N ha^{-1} (Lynge et al., 2023). Similarly, hairy vetch can provide considerable amounts of nitrogen (N) to farming systems through symbiosis with Rhizobium leguminosarum biovar viciae (Rlv), and inoculating hairy vetch with this efficient Rlv strain raises N fixation (Mothapoa et al., 2013). Vetch have been reported to fix between 25 and 190 kg N ha−1 and effective at increasing soil N (Ashworth et al., 2017)..

Inoculation of legumes and forage legumes with efficient rhizobia is believed to increases the forage yield and yield components of legumes while maintaining soil health. It is also supposed to be eco-friendly practices used for improvement of N fixation, resulted in increased shoot growth and number of pods (Shimekite, 2006; Gedamu et al., 2021). But in Ethiopia, the work on the inoculant production and use for forage legumes is understudied area of research (Shimekite, 2006; Minalku et al., 2020; Gedamu et al., 2021). Therefore, this research project was initiated to exploring and screening rhizobia nodulating the forage legumes alfalfa (*Medicago sativa*) and vetch (*Vicia villosa*) growing in Ethiopia. Exploring rhizobia inoculants required nodule collection, rhizobial isolation, and identification, as well as the selection of effective nitrogen-fixing isolates under controlled environmental conditions.

1.2. Statement of problem

Livestock production is one of the major components of agriculture in Ethiopia (Atumo et al., 2021). Despite the large livestock population in Ethiopia, the sector performed below its potential due to various constraints. The lack of sufficient supplies and low-quality forages from the existing natural pastures is a leading problem affecting livestock productivity in Ethiopia (Kebede et al., 2022).

In the country majority of soils are unproductive due to their poor nutrient status, particularly with regard to nitrogen (N), and mineral fertilizers are hardly accessed or farmers do not frequently fertilize legumes, especially forage legumes (Hailu et al., 2015). The use of forage legumes in agricultural systems and utilization of associated BNF systems provides economically feasible and environmentally sound ways of decreasing external inputs and improving the soil nutrient content and, hence, can be suggested for the nutrition of sustainable agriculture. But, many soils do not have adequate populations of native rhizobia probably in terms of number, quality, or effectiveness to enhance BNF. Rhizobia are not universally present in all soils, and often those that are present gather little nitrogen. Therefore inoculation of legumes and forage legumes with efficient rhizobial inoculants is a cheap and clean N sources alternative for soil fertility improvement and good production for forage legumes. However, the production and use of rhizobia inoculants against forage legumes in Ethiopia is very scarce. Therefore, exploring and screening potential rhizobia isolates that nodulate forage legumes of alfalfa and vetch is a base for developing inoculants of forage legumes.

1.3. Objectives of study

1.3.1. General objective

To explore rhizobial nodulating forage legumes alfalfa (*Medicago sativa*) and vetch (*Vicia villosa*) growing in Ethiopia.

1.3.2. Specific objectives

- To isolate and authenticate rhizobia nodulating alfalfa (*Medicago sativa)* and vetch (*Vicia villosa*).
- To phenotypically characterize authentic rhizobia
- To screen the isolates in terms of preliminary relative symbiotic effectiveness

1.4. Research questions

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

- Is there potential diversity of rhizobia nodulating alfalfa and vetch growing in Ethiopia?
- Do applied N fertilizers and fix N have the same importance for legumes that are grown forage under controlled conditions?
- Is there variation among alfalfa and vetch rhizobia in terms of symbiotic effectiveness or phenotypic diversity?

1.5. Significance of study

The study has importance to overcome the problems of poor forage legume productivity and livestock production. The use of rhizobial inoculants as cheap and clean N source to cropping systems would mean a lot to our smallholder farmers. Besides, there was no commercial inoculant for forage legumes in Ethiopia. This work will be a breakthrough in providing elite strains as a basis for developing rhizobium inoculants for the forage legumes. Briefly, it may have the following contributions.

- It will reduce the use of expensive inorganic fertilizers and problems associated with it.

- Use of inoculants for the forage legumes will improve soil fertility and support sustainable forage production with the minimum cost.
- It will improve the environmental sustainability and crop yield

1.6. Scope of study

Rhizobia nodulating forage legumes alfalfa *(Medicago sativa*) and vetch (*Vicia villosa*) growing in Ethiopia was the target of this investigation. It constituted isolation of rhizobia from the legume root nodules, authentication of the isolates, phenotypic characterization and evaluation of the strains for symbiotic effectiveness to screen elite for designing inoculants to the forage legumes.

2. LITERATURE REVIEW

2.1. Forage legumes

Forage legumes belong to Fabaceae family and used to feed livestock, hence named forages (Phelan et al., 2015). Members of Fabaceae are distinguished by having seeds produced in pods, compound leaves with several leaflets, and bacterial root connections that provide symbiotic N_2 fixation (Sheaffer et al., 2018). Food and Agriculture Organization of the United Nations' lists 153 different species of legumes as forages. Alfalfa (*Medicago sativa*) and hairy vetch (*Vicia villosa*) are included in top 20 of the list with major global commercial importance (Phelan et al., 2015). They contribute to N-economy of agricultural land due to their association with N_2-fixing rhizobia. They increase herbage production, feed value, and ultimately ruminant production of meat and milk (Phelan et al., 2015). They also increase the quality of the herbage, speed up sward establishment and consolidation (Mengistu et al., 2018). Currently, forage legumes have been widely used in different countries and minimized the use of synthetic fertilizers (Seyedeh et al., 2010).

2.2. Characteristics of Forage Legumes in the Study

2.2.1. Alfalfa (*Medicago sativa*)

The genus *Medicago* is the most important of the temperate pasture legume genera containing as it does the widely cultivated perennial plant Medicago sativa, (alfalfa/ Lucerne). Alfalfa is an important perennial forage legume globally and well adopted due to high productivity, herbage quality and providing cheap forages of high nutritive value and digestibility (Radovic et al., 2009). The domestication history of alfalfa is poorly known and first cultivated in its center of origin (Near-East to Middle East) 9,000 years ago, is the oldest cultivated forage crop and one of the oldest crops in the world. It spread to North Africa and Europe through invaders and was introduced to Asia (India and China) more than 2,000 years ago. From the 15th Century, alfalfa became a popular crop in France and other European countries from where it accompanied explorers and migrants to America from the 16th Century onwards and to austral countries by the 19th Century. It is now grown in temperate regions worldwide and has recently become a cornerstone crop in organic agriculture (Prosperi et al., 2014; Ghaleb et al., 2021).

The crop's worldwide cultivation area is about 30 million hectares, with a total production of about 450 million tons. Major alfalfa producers are North and South America, Europe and Asia, with a total production of around 41, 23, 25, and 8%, respectively (Latif et al., 2023). Alfalfa is one of the most useful feed legume growing in various agro-ecological zones of Ethiopia (Kebede et al., 2022). The land suitability analysis of Alemayehu et al. (2020) indicated that rainfall is the most influencing factor for alfalfa production in Ethiopia. Areas located in western, central, and eastern parts of Ethiopia that receive annual rainfall above 350 mm are identified as highly suitable areas for alfalfa production, while 1500 mm of annual rainfall is required for optimal growth. It has been reported that a significant part of the Ethiopian land is 472,153 km^2 (43% of the total land in Ethiopia) is highly suitable, whereas 397,133 km^2 (36%) is moderately suitable and 16,165 km^2 is marginally suitable land for alfalfa production under the current climate conditions.

The crop adapted a wide range of environments in Ethiopia (Kebede et al., 2022) and it can grow in a variety of soil types but grows well on deep, well-drained sandy to fertile loamy soils with 6.5 to 7.5 soils PH. Generally, alfalfa grows in higher altitudes of up to 4000 m above sea level and tolerates drought but it cannot stand prolonged flooding and heat stress (Worqlul et al., 2022). But it has properties of quick recovery following cutting, longevity, and environmental stress tolerance (Radovic et al., 2009). The stage of maturity, soil fertility, climatic conditions, and cultivar differences are the main factors affecting alfalfa yield and quality (Kebede et al., 2022). Depending on soil and environment conditions, alfalfa achieves dry matter yields which ranged from 50 to 100 t/ha and 12 to 19 t/ha, respectively (Radovic et al., 2009). And also the mean dry matter yield of alfalfa ranging from 6.49 to 7.87 t/ha in which a higher dry matter value was recorded at Arba Minch in 2017 compared to other testing sites with the lowest yield at Dilla (Atumo et al., 2021).

Alfalfa is a major source of protein for livestock, it is a basic component in rations for dairy cattle, beef cattle, horses, sheep, goats and other classes of domestic animals (Radovic et al., 2009;Tolera et al., 2012). The alfalfa can be used as hay, silage, green chop, pelleted or cubed products, and grazing (Higginbotham et al., 2008; Sheaffer et al., 2018). The major value of feeding alfalfa to livestock is its high protein contents compared to other common forage crops. For development, maintenance, lactation, and reproduction, animals need to consume high

protein in their diet. Since, alfalfa is an important source of protein (estimated 15–22% crude protein), carbohydrates and crude fiber as well as several other important vitamins (A, B, C, E, and K), essential amino acids and minerals such as calcium, phosphorus, copper, and potassium, which are directly or indirectly vital for most of the livestock and dairy industries (Markovic et al., 2007; Higginbotham et al., 2008; Latif et al., 2023).

Another excellent contribution of alfalfa to agriculture is its symbiotic association with soil bacteria, rhizobia and N_2 fixation potential adds fixed nitrogen to the soil and improves soil nitrogen content, which in turn reduces artificial N fertilizer requirement. Hence, it provides a positive carryover impact to crop rotation, as well as protecting and enhancing the environment (Radovic et al., 2009). In saline environments, alfalfa has the ability to develop nodules that can fix atmospheric nitrogen. Therefore, it might be useful as a fodder or green manure crop in saline agriculture, especially for systems using practically nitrogen-free seawater for irrigation (Elgharably & Benes, 2021).

2.2.2. Vetch (*Vicia villosa*)

The *Vicia* genus, of the Fabaceae family, includes several winter annual legumes, generically grouped as "vetches." Within this complex, *Vicia villosa* ssp. *villosa* Roth, commonly known as hairy vetch (HV), is a relevant member. It is native in Europe and West Asia, being introduced as a crop or a weed to temperate climate regions and being the second most important vetch in agricultural systems worldwide. Vetches are annual forage legumes used as short-term fodder crops, green manure, or ruminant feed. They grow on black and reddish-brown clay soils in highlands with elevations ranging from 1500 to 3000m altitude in temperate regions and are suited to a wide range of rainfall. It has been grown successfully in areas of acid soil with pH of 5.5 to 6 (Kebede et al., 2013; Mengistu et al., 2017), and it grow under rainfed or irrigated conditions in different climates. Specifically, vetch grow in temperate and cold-temperate climates (Mothapo et al., 2013; Bamford & Entz, 2017). The dry matter yields achieved by *vicia villosa* ranging from 4.70 to 6.98 and 7.16 to 8.60 t ha^{-1} at Holetta and Ginchi, respectively, at different agroecological conditions in Ethiopia (Kebede et al., 2013).

Like alfalfa, many vetch species are grown for direct grazing, hay, green forage, and/or seeds. It has received a lot of attention as a crop with a high protein content (Seyedeh et al., 2010),

drought resistance, high productivity (Dastikaitė et al., 2009), fast mineralization of residues and substantial groundcover formation (Roper et al., 2020) and high N_2-fixation capacity (Seyedeh et al., 2010). In addition, as a legume cover crop and green manure, hairy vetch is frequently employed in agroecosystems for its advantages in erosion management, weed suppression by surface forming mulch and insect control, and increased soil N fertility (Mothapo et al., 2013; Bamford & Entz, 2017) and it is among the best of the legumes in its ability to be productive in low fertility or acid soils (Dastikaitė et al., 2009). Vetch has been cultivated in a number of agroecological regions, where it is frequently utilized as N source in organic agriculture (Mothapo et al., 2013).

2.3. Biological N_2 fixation (BNF)

Biological nitrogen fixation (BNF) is the process by which atmospheric nitrogen is biologically converted into nitrogenous compounds (ammonia), a suitable form of nitrogen for plant use. The conversion occurs in legume roots symbiotically by soil bacteria called rhizobia and supplied to the legumes that in turn provide the bacteria with carbon produced by photosynthesis (Drew et al., 2012). The conversion of the N_2 into ammonia is performed by nitrogenase enzyme of the rhizobia (Howieson & Dilworth, 2016).

Symbiosis is a multi-stage process that involves the exchange of molecular signals between symbiotic partners. Plant roots secrete flavonoids that trigger the rhizobial regulatory NodD protein, which causes the stimulation of nodulation genes that produce lipo-chito-oligosaccharide (Nod factors) (Wielbo et al., 2012). Due to nod factors and symbiotic exopolysaccharides, the plant forms infection threads, which are small tubules filled with bacteria that enter the cortical tissue of the plant and deliver the bacteria to their target cells. Invading bacteria are taken up by plant cells in the inner cortex and internalized in host-membrane-bound compartments that develop into symbiosome-like structures. Once absorbed, the bacteria transform into bacteroids, a specialized form that can fix nitrogen with in nodules (Jones et al., 2007).

Rhizobia produce two types of nodules in general: determinate and indeterminate. The indeterminate nodules are elongated and have a persistent meristem that continuously produces new nodule cells, which are then infected by rhizobia present in the nodule. The determinate nodules are usually round, lack a persistent meristem, and do not show an obvious

developmental gradient. Historically, the production of indeterminate nodules has been studied using models such as *Medicago sativa* (alfalfa), *Medicago truncatula*, *Pisum sativum* (pea), *Vicia* species (vetches), and *Trifolium* species (clovers) (Jones et al., 2007; Gage, 2017). The rhizobia inside the nodules reproduce; receive nourishment and protection from the plant. They, absorb N_2 from the air, and transform it into amino acids that the plant needs for growth (Russelle, 2004).

Rhizobia

Rhizobia are gram-negative, rod-shaped, aerobic bacteria that often live in soil. Based on their growth properties, they are divided into two groups as "fast" and “slow” growers. The fast growers include *Rhizobium* spp., which grows within 3-5 days when cultured at 28°C on a solid medium like yeast extract mannitol agar (YEMA). The slow growers, that include *Bradyrhizobium* spp., on the other hand, take 5-12 days to achieve maximum colony size when cultured under the same conditions as fast growers (Somasegaran & Hoben, 1994). The rhizobia do not produce spore-like structures for survival, making them extremely sensitive to environmental challenges. Stresses like temperature, salt, extreme pH, and toxic chemicals can easily destroy them (Drew et al., 2012).

Sinorhizobium meliloti are among the rhizobia from alpha-protobacteria that forms symbiotic associations with the alfalfa (*Medicago sativa L.), Melilotus* (sweet clover *Melilotus alba*), M. truncatula and Fenugreek (*Trigonella spp.*). On the roots of these plants *S. meliloti* forms nodules within which the differentiated bacteroids reduce nitrogen gas to ammonia (Kajić et al., 2019). As *S. meliloti* nodulates alfalfa, *Rhizobium leguminosarum* bacteria of the *symbiovar (sv) viciae* (Rlv) establishes a nitrogen-fixing symbiotic partnership with vetch *(Vicia villosa*), field pea (*Pisum sativum*), faba bean(*Vicia faba*), and lentil (*Lens culinaris* ssp. *Culinaris)* plants (Bartoli et al., 2009; Mothapoa et al., 2013). The rhizobia are often specific in terms of nodulating legumes. The specificity of the legume-rhizobia interaction is accomplished at a number of checkpoints, beginning with exchange of distinct plant flavonoids and bacterial nod factors; hence, not every rhizobial species can infect every legume species (Wippel & Long, 2019).

2.4. Factors affecting symbiotic biological N_2 fixation

Rhizobia dwell and interact with legumes growing in various soil environments. The varying conditions of the soil and its environments frequently exposes the rhizobia to numerous biotic and abiotic stress conditions such as varying pH levels, salt concentrations, humidity, temperature, and other physical and chemical characteristics of the soil, all of which have an impact on biological N_2 fixation (Zahran, 1999). Besides the soil and environmental variables, the inherent gene of the rhizobia and the host plant determines the symbiotic association between the partners and subsequently affects their biological nitrogen fixation. So, the success of inoculation depends not only on high-quality inoculants and good inoculation practices but also on the establishment of effective and efficient BNF through optimization of the factors that affect its performance, such as legume genotype, climatic, soil, and management factors (Dabessa et al., 2018) .

2.4.1. Temperature stress

Every bacterium has its own optimum condition, under which it grows at its best. *Rhizobium* that is very active within a certain temperature range is less active within another temperature range. For these reasons, greater nitrogen gains can probably be achieved by improvements in the heat resistance of the symbiosis. For most rhizobia, the optimum temperature range for growth is 28 to 31°C, and many are unable to grow at 38°C. High soil temperature (35 to 40°C) usually result in the formation of ineffective nodules; however, there is exception for some strains of rhizobia (Mabrouk & Belhadj, 2012). Exposure of both symbiotic partners to the extreme temperatures impairs infection, nodulation and nodule functioning as well as plant growth and productivity. Increased temperature directly affects the synthesis or release of nod-gene inducers, modifies nodule functioning due to nitrogenase enzyme denaturation and reduced leghemoglobin biosynthesis, and additionally speeds up nodule senescence (Gebremedhin, 2019).

2.4.2. Water stress

Rhizobia and the majority of soil microorganisms are influenced by soil water content through the processes of diffusion and mass flow, and nutrient concentration is correlated with soil pore space. By lowering water activity below critical tolerance limits, soil moisture content directly

affects the growth of rhizosphere microorganisms such as rhizobia (Gebremedhin, 2019) and is a significant factor in low N_2 fixation and nodulation failure (Hungria & Vargas, 2000). It also affects plant growth, root architecture, and exudates. Water stress affects not only nodule function but also nodule formation and longevity, leghemoglobin production, rhizobial survival, growth, and population organization in soil. Significant stress may also result in the permanent cessation of N_2 fixation (Gebremedhin, 2019).

2.4.3. Soil acidity

Acidity of the soil hinders symbiotic N_2 fixation, reduces *Rhizobium* survival and persistence in soils, inhibits nodulation, and results in nutritional imbalance. The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 and relatively few rhizobia grow well at pH less than 5.0. Acidity affects early steps in the infection process including the exchange of molecular signals between symbiotic partners and attachment to the roots (Hungria & Vargas, 2000). Furthermore for crop production, soil acidity is a complex of numerous factors involving nutrient/element deficiencies and toxicities, low activity of beneficial microorganisms and reduced plant root growth that limit nutrient and water uptake (Moreira & Fageria, 2010).

Soil acidity has become a serious threat to crop production in most highlands of Ethiopia. Most of the potential arable land in Ethiopia is acidic. Low soil pH is often associated with increased Al and Mn ion toxicity and lack vital nutrients like calcium (Ca), phosphorus (P), magnesium (Mg) and molybdenum (Mo), acidic soils result in poor plant growth (Hungria & Vargas, 2000; Bekere, 2013). The extreme pH of the soil can inhibit rhizobial activity in the rhizosphere surrounding the roots of legume plants. Low levels of phosphorus, calcium, and molybdenum, as well as aluminum and manganese toxicity, are features of extremely acidic soils ($pH < 4$), which have an impact on plants and rhizobia and the symbiosis (Somasegaran & Hoben, 1994; Dabessa et al., 2018).

2.4.4. Salt stress

Globally, the issue of soil salinization is a curse on agricultural productivity. Due to insufficient rainfall to eliminate salts and an excess of sodium ions in the rhizosphere, salinity and sodicity issues are common in arid and semiarid regions. These issues arise in both irrigated and non-

irrigated croplands, as well as rangelands, in almost every irrigated region of the world. As a result, salinization affects almost all land (Gebremedhin, 2019). Crops grown on saline soils suffer on an account of high osmotic stress, nutritional disorders and toxicities, poor soil physical conditions and reduced crop productivity. Salts that are water-soluble exist in all soils. Essential nutrients are taken up by plants in the form of soluble salts, but an excessive accumulation severely inhibits plant growth.

Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants, and thus limits water uptake from soil (Shrivastava & Kumar, 2015). Disruption of ionic equilibrium and high Na+ levels also lead to reduction in photosynthesis (Ahmad & Umar, 2010). Soil salinity significantly reduces plant phosphorus (P) uptake because phosphate ions precipitate with Ca ions (Bano & Fatima, 2009). Some elements, like sodium, chlorine, and boron, have definite harmful effects on plants. Rapid osmotic stress and cell death can result from an excessive buildup of salt in cell walls (Shrivastava & Kumar, 2015). Legumes are extremely salt-sensitive crops, and in water-scarce regions where evapotranspiration exceeds precipitation, a high concentration of Na^+ and Cl^- ions near the root zone, limits the geographical range of legumes.

2.5. Rhizobia inoculation

Nitrogen is likely the most significant nutrient needed by plants, being an essential component of all proteins, nucleic acids, and other nitrogen compounds. Despite the fact that nitrogen gas makes up 78.1% of the Earth's atmosphere, nitrogen availability is restricted in many soils. Given that they use biological processes to fix atmospheric N_2, legumes have a large impact on the nitrogen cycle. A beneficial substitute for N-fertilizer is BNF, which provides high sustainability for ecosystems (Bomfeti et al., 2011). To BNF and meet N requirements while restoring soil fertility, rhizobia are introduced into legume crops, the process called inoculation. The success of symbiotic connections can be seen in the effective N-fixation symbiosis between rhizobia and legumes. However, a number of agricultural soils lack a sufficient number of effective rhizobia. The utilization of effective rhizobial strains is a key strategy for combining their importance in increasing crop output with biological control of plant diseases and pests

(Athul et al., 2022). Rhizobia strains as inoculants have been considered as a common approach to improve the effectiveness of symbiotic nitrogen fixation and legume productivity.

The total nitrogen fixation in the world is estimated to be about 1.75×10^{11} Kg, of which symbiotic nitrogen fixation in legumes accounts for about 8.0×10^{10} Kg by fixing, on average, 20–200 kg N fixed ha^{-1} $year^{-1}$ more than 70% of legumes, and the other near half is industrially fixed while producing N fertilizers (about 8.8×10^{10} Kg) (Shah et al., 2021). Thus, the association between Alfalfa and *Sinorhizobium meliloti* is thought to be the most effective association for nitrogen fixation in the soil. It has been reported that BNF of alfalfa ranged from 4–650 kg of nitrogen per hectare each year, 70 to 250 kg N in North America, 93–258 kg N for eastern Canada, 174–466 kg N for western Canada, and 200–650 kg N in Argentina (Latif et al., 2023).

Similarly, hairy vetch can provide considerable amounts of nitrogen (N) to farming systems through symbiosis with *Rhizobium leguminosarum biovar viciae* (Rlv), and inoculating hairy vetch with this efficient Rlv strain raises N fixation (Mothapoa et al., 2013). The crop provides greater total N (100 to 135 kg N ha-1) than Austrian pea (*Pisum sativum subsp. arvense*) and white clover (*Trifolium repens*), two other common winter-hardy legume cover crops (Roper et al., 2020). It has been reported that the estimated amounts of N fixed by Lablab species (215 and 140 kg N ha-1), Vicia species 163.0 and 103.6 kg N ha-1, Medicago 121.9 and 108.5 kg N ha-1), Trifolium species (45 and 40 kg N ha-1) and increased grain yields of the succeeding wheat crop by 16-125% in the Ethiopian Highlands where no N fertilizer was applied (Haque & Lupwayi, 2000).

Another reports indicated that inoculation of vetch and alfalfa by *R. leguminosarum* var *viceae* and *Sinorhizobium meliloti* showed significant differences in percent of symbiotic effectiveness on vetch, and alfalfa respectively and there were found to be the most efficient strains with percentage effectiveness in terms of dry matter accumulation (Shimekite, 2006). Gedamu et al. (2021) indicated that inoculation of faba bean strain, EAL- 1018 brought biologically higher significant grain and biomass yield and also economically maximum net benefit on vertisol of Wereillu District, South Wollo, in Ethiopia. Furthermore inoculation of native rhizobia isolates from soils of major cowpea producing areas in Ethiopia had significantly increased nodule

number per plant, nodule dry weight per plant and shoot dry weight per plant compared with the uninoculated treatment (Kebede et al., 2020). It has been indicated that inoculation increased chickpea grain yield by 21% in Ethiopia (Wolde-meskel et al., 2018).

3. MATERIALS AND METHODS

3.1. Sampling sites

In this study, root nodules and soil samples were collected from different locations in Ethiopia based on the history of growing the forage legumes alfalfa *(Medicago sativa*) and vetch (*Vicia villosa*). The sampling sites included different agro-climatic regions of Ethiopia (Appendix 10), in SNNPRS (Lemo and Kedida Gamela), Amhara (Debrebirhan), and Oromia (Debreziet) that are known to grow forage legumes with no history of previous rhizobium inoculation.

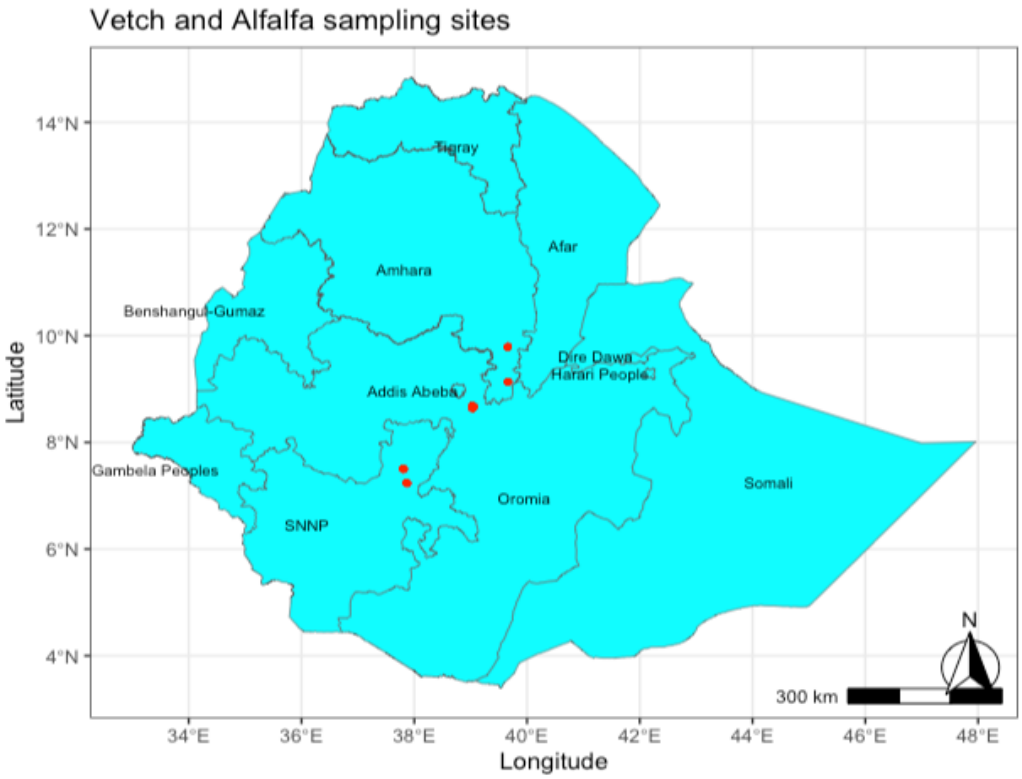

Figure 1: Map of the study area

3.2. Research Design

Soil and nodule samples were collected from different locations in Ethiopia, and the rhizobia were isolated and trapped. The isolated rhizobia strains were subjected to morphological, physiological and biochemical tests. The rhizobia were authenticated based on their ability to re-infect the alfalfa and vetch host legumes. The experiment was arranged in a completely

randomized block design (RCBD) with three replications. Treatment groups included were uninoculated controls; N fertilized controls and isolated strains. The symbiotic effectiveness of the rhizobia isolates was evaluated by inoculating them on their host plants grown under greenhouse conditions by supplying with N-free nutrient solution till 50% flowering. Data on plant growth parameters such as nodule number, shoot dry weight, root, and nodule dry weight per plant were examined after 45 days of planting. A multivariate cluster analysis of phenotypic properties was employed to group the isolates using similarity coefficients by the Unweighted Pair Group Mean with the Average (UPGMA) clustering method with the NTSYSpc version 2.1. Furthermore, analysis of variance was employed to determine the mean variations between the strains using the R package *agricolae* and screen the symbiotic nitrogen fixation in terms of plant biomass.

3.3. Soil and Nodule sample collections

3 kg of soil samples were excavated from different spots of a field with M pattern at a depth of 0–30cm where alfalfa and vetch plants have been growing. The soil samples were composted and separately resampled into dry, clean plastic bags (Woldemeskel et al., 2004) and transported to Arba Minch University, Abaya Campus for trapping rhizobia using plant trap method. In addition to soil samples, nitrogen-fixing nodules from field standing legumes were collected from the sites mentioned above. From each site, 15 or more nodules were carefully collected and maintained in vials containing silica gel and cotton. The nodules were collected during early flowering stages and brought to the Advanced Molecular and Microbial Biotechnology Laboratory, Arba Minch University, for isolation of nitrogen-fixing bacteria. The passport information for the sites was being assessed during the sample collection (Appendix 10).

3.4. Trapping Rhizobia from Soil samples

Rhizobia were isolated from soil samples by the plant infection method (Somasegaran and Hoben, 1994). Three kg of the collected soil samples were potted into surface-sterilized pots by soaking in 3-5% sodium hypochlorite ($NaHClO_3$) overnight and then rinsing with sterilized distilled water. Each soil sample was filled into surface-sterilized (95% ethanol) plastic pots. The soil was moistened to water-field capacity before transplanting seedlings. For the transplanting, seeds of alfalfa and vetch were surface sterilized by 95% ethanol for 10 seconds, followed by 3-

5% sodium hypochlorite for 4 minutes. The sterilizing chemicals were neutralized by rinsing the seeds in six changes of sterilized water. The surface-sterilized seeds were germinated in pre-sterilized petri dishes containing 1% water agar. The germinated seeds were aseptically transplanted into the pots containing moistened soil. The seedlings were watered and grown for 45 days in the greenhouse at the Abaya campus of Arba Minch University. The seedlings were carefully uprooted for nodule assessment and collection. Healthy and big nodules were carefully collected and maintained in vials containing silica gel and cotton, as described above. The vials were preserved in a refrigerator at 4°C until used to isolate rhizobia.

3.5. Isolation of Rhizobia from Root Nodules

Rhizobia were isolated from both fresh and desiccated nodules (Howieson and Dilworth, 2016; Somasegaran & Hoben, 1994). Dehydrated or desiccated root nodules were immersed in sterile distilled water overnight in petri dishes. The fresh and imbibed nodules were surface-sterilized with 95% ethanol for 10 seconds and transferred to a 3-5% solution of sodium hypochlorite for 4 minutes. The surface sterilized nodules were rinsed with sterile distilled water six times to completely remove the sterilizing agents and crushed aseptically in a drop of sterile distilled water in a sterile test tube or beaker. Finally, the nodule suspension was streaked on Yeast Extract Mannitol Agar containing Congo Red Dye (YEMA-CR) and the pH of the medium was adjusted to 6.8 with 10% (0.1N) solution of NaOH or HCl. After adjusting the pH, the medium was autoclaved at 121°C and 15 psi for 15 minutes, cooled to 55°C, dispended into sterile Petri dishes and solidified in a laminar hood flow. The plates were incubated at 28°C for 3–7 days and daily assessed for colony appearance (Somasegaran & Hoben, 1994). To obtain pure isolates, a single colony was picked from the plates and subsequently re-streaked on a new YEMA-CR. The re-streaking continued several times until a pure and clean colony was obtained.

3.6. Gram staining of the rhizobia isolates

The test was conducted by using loopful of pure culture grown on YEMA and stained as standard Gram's test procedure according to Somasegaran & Hoben (1994). The isolates which retained primary stain (Crystal Violet), blue in color, were recorded as Gram positive discarded realizing rhizobia are not gram positive; whereas the isolates which retained counter stain

(Safranin), red or pink in color, were recorded as Gram negative. Finally, it was observed using oil emersion objectives of a light microscope for their rod shape.

3.7. Designation of the isolates

Pure isolates were designated by A and V for alfalfa and vetch respectively. Nomenclature of the isolates was carried out representing their host plant (first letter), region of origin (second letters) and isolate number that corresponded to the isolate information.

3.8. Preservation of the pure rhizobia isolates

A loopful of the pure colony was transferred to a test tube containing 10ml of sterile yeast extract mannitol broth (YMB). The test tubes were incubated at 28±2°C for 3-7 days in a shaker incubator at 120 revolutions per minute (rpm). The isolates were grown in YEM broth verified by formation of a cloudy appearance and 1 OD (optical density), and 700µl of the broth culture was transferred into a 2-ml Eppendorf tube containing 300µl of 50% sterile glycerol solution. The mixture was vortexed and maintained in a refrigerator at -20°C for future use.

3.9. Authentication of the isolates

All the rhizobial isolates were subjected to host plant re-infection test according to Somasegaran & Hoben (1994). Seeds of alfalfa and vetch were surface sterilized using 95% ethanol, bleached with 3-5% sodium hypochlorite for 4 minutes, rinsed with six changes of sterile water, and allowed to germinate as described above. Germinated seeds were transplanted into modified Leonard jars (MLJ) containing pre-treated river sand. MLJ were designed from two plastic cups. The upper cups were filled with pretreated river sand to support the seedlings that had been cleaned by 98% sulfuric acid solution for overnight and washed with tap water till the pH of the wash water becomes nearly 7.0. Then the second cups were connected to the upper cups by cotton wick, the two cups covered with aluminum foil and autoclaved at 121°C and 15 psi for 15 minutes. After the seedlings are established in the jars, 1 ml of each broth culture at its logarithmic growth phase is inoculated at the base of the seedlings growing in the jars. The seedlings were supplemented once a week with 300–500ml of Jensen's N-free nutrient solution and sterilized distilled water as needed. Negative controls (uninoculated seedlings) and positive controls (0.5% KNO_3 supplemented seedlings) were included for contamination counter checks

and comparative purposes. The experiment was statistically arranged in a completely randomized block design (RCBD) with three replications.

3.10. Cultural, Eco-Physiological and Substrate Utilization Characteristics Tests

3.10.1. Cultural Characterization

The cultural characteristics of rhizobia are very important for identification of the rhizobial colonies from other contaminating bacteria on YEMA medium during the initial isolation process. The major colony characteristics include shape, size, texture and color. The characteristics of the isolates was determined on yeast extract mannitol medium after incubation for 3-7 days at 28°C (Somasegaran & Hoben, 1994).

Rhizobia colonies are usually discrete; having round shapes varying from flat to domed and even conical on YEMA medium. A colony size is a diameter of a single colony measured by using a caliper. Rhizobial colony size varies from 2-5mm. Texturally, the colonies often vary from buttery or elastic and the colony color was identified as watery or white-translucent, white opaque and milky.

3.10.2. Eco-physiological tolerance test of isolates

For each eco-physiological characteristics test, a loop full of three- to five-day-old broth culture was streaked onto the YEMA medium according to the range of tests specified below. The inoculated YEMA plates were incubated at 28 ± 2°C for 3-7 days for the pH and salt concentration tests, while different temperature ranges were used for the temperature tolerance tests. Intrinsic resistance (IAR) to antibiotics and heavy metals was tested on YEMA medium containing the following filter-sterilized antibiotics or heavy metals at the concentrations mentioned below. The growth of each rhizobial isolate under such conditions was determined as (+) for positive growth and (-) for no growth.

Temperature tolerance test

The growth of each isolate at different incubation temperatures was evaluated by inoculating each isolate on YEMA plates. According to Lupwayi and Haque (1994), the inoculated plates were incubated at a temperature of 5°C, 10°C, 15°C, 40°C and 45°C.

Salt tolerance test

The ability of the isolates to grow at different levels of salt concentrations was determined by inoculating each isolate on YEMA media containing 0.5%, 1%, 2%, 3%, 4%, 5%, and 6% NaCl solutions (Lupwayi & Haque, 1994).

pH tolerance test

The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating each isolate on YEMA adjusted at a pH of 4.0, 4.5, 5.0, 5.5, 8, 8.5, and 9 using NaOH or HCl adjustments as mentioned above (Amarger et al., 1997)

Intrinsic Antibiotic Resistance (IAR) test

The intrinsic antibiotic resistance of isolates to different antibiotics at different concentrations was evaluated by streaking each isolate on YEMA containing freshly prepared antibiotics at the following concentrations: Ampicillin 5mg/ml, Cephalexin (2.5 and 10mg/ml), Amoxicillin 5mg/ml, Bacteriocin 5mg/ml, Kanamycin (1 and 10mg/ml), Streptomycin (2.5 and 10 mg/ml), and Penicillin 1mg/ml. The stock solution of each antibiotic was prepared as described in Lupwayi and Haque (1994) and kept in a refrigerator until it was used. Stock solution of each antibiotic was filter sterilized using sterile 0.22µm disposable membrane filters and plastic hypodermic syringes (10ml) with the Luer-Lok system and aseptically used to autoclaved YEMA at 55^{o}C in a laminar hood flow (Somasegaran & Hoben, 1994). The plates were incubated at 28 ± 2°C for 3-7 days, and growth was recorded.

Intrinsic Heavy Metal Resistance test

The intrinsic heavy metal resistance of isolates to different heavy metals at different concentrations was evaluated by streaking each isolate on YEMA containing freshly prepared filter-sterilized heavy metals of $Al_2(SO_4)_3$ 2.5mg/ml, $ZnCl_2$ 5mg/ml, CsCl 10mg/ml, $MnSO_4.H_2O$ 50mg/ml, $CuSO_4$ 10mg/ml, $CoCl_2$ 2.5mg/ml, $ZnSO_4$ 10 mg/ml, and $(CH_3COO)_2Pb$ 2.5mg/ml at different concentrations. The plates were incubated at 28 ± 2°C for 3-7 days, and growth was recorded (Wubie & Adal, 2021).

3.10.3. Substrate utilization test of rhizobia isolates

Carbon source utilization

Isolates were checked for their ability to utilize different carbon sources to determine their capability to survive on limited soil nutrients. Different sources of carbons such as D-glucose, D-fructose, maltose, lactose, sucrose, sorbitol, citric acid, and dextrose were tested according to Somasegaran & Hoben (1994). Ten percent distilled water solution of each carbohydrate source (1g carbohydrate in 10ml distilled or deionized water) was prepared, and heat-stable carbohydrates (D-glucose, D-fructose, lactose and sucrose) were autoclaved together with the medium, but heat-labile carbohydrates were filter sterilized using disposable membrane filters of 0.22 μm sizes and added to the basal medium (essentially similar to YEMA except for the reduction of yeast extract from 0.5 g/l to 0.05 g/l) after sterilization when the medium temperature was reduced to 55°C and allowed to solidify. A loop full of each rhizobium isolate was streaked on the plates of incorporated carbohydrates under test and incubated at 28±2°C for 3-7 days. The growth of the rhizobial isolate was examined and scored qualitatively either as (+) for growth or (-) for no growth.

Nitrogen source utilization

Different types of amino acids including L-leucine, L-alanine, glycine, L- asparagine, L-arginine L-proline, L-tryptophan, and vitamins; Thiamine, folic acid and Nicotinic acid were used in this experiment in order to determine the ability of the rhizobial isolates to utilize the amino acids and vitamins as a nitrogen source (Amarger et al., 1997). These amino acids and vitamins were added at a final concentration of 0.5g/l to a basal medium (g/l: 0.2 $MgSO_4.7H2O$, 0.1 NaCl, 0.5 K_2HPO_4, 0.05 Yeast extract, 1 D-manitol, 15 Agar and 1000 ml distilled water). The membrane filter sterilized amino acids and vitamins were added to the autoclaved and cooled (approximately 55°C) basal media. Finally, each rhizobium isolate was inoculated into these basal media containing nitrogen source and incubated at 28±2°C for 3-7 days. The growth of rhizobial isolate was examined and scored qualitatively either as (+) for growth or (-) for no growth.

3.11. Relative effectiveness of the isolates

At the 50% flowering, the plants were uprooted to measure nodule number, nodule dry weight and shoot dry weight. The effectiveness of the isolates in accumulating plant shoot dry matter was calculated as described in Gunnabo et al. (2019)

$$\%SE= \frac{\text{Inoculated plant SDW} - \text{Negative control SDW}}{\text{N – Fertilized plant SDW} - \text{Negative control SDW}} X\ 100$$

Where SDW = shoot dry weight, SE = symbiotic effectiveness, Negative control SDW = shoot dry weight of the plants that were not inoculated or not received any N-fertilizer. The rate of nitrogen-fixing effectiveness was evaluated as highly effective ≥80%, effective 50–80%, slowly effective 35–50%, and ineffective <35% (Mulongoy, 2004).

3.12. Most probable number (MPN) for soil samples

The number of viable soil rhizobial background population was estimated by most probable number (MPN) according to Somasegaran & Hoben (1994). The number of vetch and alfalfa rhizobia in soils was estimated through the most probable number (MPN) method with a plant infection technique. Modified Leonard Jars were used to estimate the population of rhizobia by most probable number (MPN). 1g of each soil sample was serially diluted in sterile distilled water up to 10^{-6}. 1ml from each dilution was inoculated to the base of each one of the four replicates in the set vetch and alfalfa seedlings growing in the jars. Seed surface sterilization and germination steps were similar within the authentication. The seedlings were transferred to green house and supplemented once a week with 300–500 ml of Jensen's N-free nutrient solution and sterilized distilled water as needed. After 45 days of growth the MPN estimations of plants based on nodulation were determined and scored as '+' for nodulation and '-' for the absence of nodulation. Finally MPN of cell per gram of soil was calculated following procedures described in Somasegaran & Hoben (1994) and the equation:

$$MPN = \frac{m*d}{v}$$

m = Likely number from the MPN table for the lowest dilution of the series

d = Lowest dilution (first unit used in the tabulation)

v = Volume of aliquot applied to plant

3.13. Data analysis

A computer cluster analysis of 55 phenotypic variables was carried out using similarity coefficient by the Unweighted Pair Group Mean with the Average (UPGMA) clustering method with NTSYSpc2.1 program. The data generated from experiments were subjected to statistical analysis to determine the mean variations between the treatments. The analysis of variance (ANOVA) and list of significant difference (LSD) at $p < 0.05$ was determined using R package *agricolae.*

4. RESULT AND DISCUSSION

4.1. Rhizobial isolates, authentication and soil population estimates

A total of 40 vetch and 50 alfalfa nodulating bacteria were isolated from the root nodules of vetch and alfalfa. Most of the isolates were originated from Bishoftu using both of the legumes, and least number of isolates trapped from Debrebirhan using vetch and Kedida Gamela using alfalfa (Table1). The variation of isolates at sampling locations is due to the sites with higher number of rhizobia could have grown the legumes for longer years and their rhizobia have well proliferated in the soil, while the sites with poor rhizobia recovery could have recently started growing the legumes and their rhizobia are yet to establish well in the soil.

Among the total isolates, 31 (77.5%) from vetch and 44(88%) from alfalfa were induced nodules upon re-infecting their host plant were authenticated as true rhizobia (Somasegaran and Hoben, 1994). This is in agreement with the findings of Miressa (2018), who reported 61.3% field pea isolates indicated root nodulating rhizobia. Others reported that 80%-89% alfalfa bacterial isolates successfully elicited nodulation on the host plant (Mohamed et al., 2014; Latrach et al., 2017) .

The isolates that failed to elicit nodules on the host legume could be considered nodule endophytes or *Agrobacterium* related strains, capable of co-inhabiting in root nodules with the rhizobia (Aserse et al., 2013; Latrach et al., 2017). Several studies demonstrated the presence of non-rhizobial bacteria in the root nodules of legumes. For example, micromonospora strains were recovered from alfalfa nodules, strongly suggesting as endophytes that commonly associate with the symbiotic organ of legumes (Martínez-Hidalgo et al., 2014).

In order to use rhizobial inoculants in different soil, it has been a trend to investigate the background population of the rhizobia in the soil. Accordingly, number of viable rhizobial soil population's nodulating vetch and alfalfa were determined as MPN (cells g^{-1} soil) (Table 1). The result indicated that the highest number of rhizobia per gram of soil ($1.8x10^5$) was recorded in soils collected from Bishoftu and Lemo, while the lowest number ($3.1x10^4$) was estimated from Debrebirhan using alfalfa. In opposite to this, most of the soils where vetches have been grown contained rhizobia below detection level of MPN. Soils collected from Bishoftu were estimated

to contain 1.0 x10^3 rhizobia cells g^{-1} of soil. It indicated that soils are heterogeneous in physicochemical properties varying from farm to farm or from location to location. Such variability and history of legume crop production determines soil rhizobial populations, which in turn defines the soil effectiveness. Soils with null or very low levels of rhizobia population require inoculation. The current result is in line with numbers of rhizobia nodulating alfalfa ranged from undetectable to 3.5 x 10^4 rhizobia g^{-1} soil (Langer et al., 2008) and grass pea (*Lathyrus sativus*) nodulating MPN of rhizobia from Ethiopia contain 2.3 x 10^1 g^{-1} soil (Adal, 2018). According to current study, Ethiopian soil harbor indigenous rhizobia cells per gram of soil nodulating alfalfa and vetch. But based on standard high inoculants amount (>10^8 cells g^{-1}) of inoculants (Somasegaran & Hoben, 1994), almost all of the vetch and alfalfa growing sites require inoculation after studying the efficiency of potential population for better nodulation and plant yield.

Table 1: Number of vetch and alfalfa rhizobia isolates and soil population estimates

Legumes	Soil sampling sites	No. of isolates	No. of authenticated isolates	Soil population (MPN cells g^{-1})
Vetch	Debrebirhan	5	4	NA
	Bishoftu	19	11	1.0 x 10^3
	Lemo	7	7	NA
	Kedida Gamela	9	9	NA
	Total	40	31	-
Alfalfa	Debrebirhan	14	10	3.1 x 10^4
	Bishoftu	18	18	1.8 x 10^5
	Lemo	14	12	1.8 x 10^5
	Kedida Gamela	4	4	5.9 x 10^4
	Total	50	44	-
Sum total		90	75	-

4.2. Cultural, Eco-Physiological and Substrate Utilization Characteristics tests

4.2.1. Cultural Characterization

The authenticated isolates showed different morphological characteristics, indicating the existence of diversity among rhizobia nodulating vetch and alfalfa (Table 2). The colonies of the isolates appeared to have a buttery or elastic texture. The colonies color varied from watery or white-translucent to opaque and milky, with round shapes varying from flat to domed and even conical on the YEMA medium. Furthermore, all of the vetch and alfalfa isolates displayed gram-

negative reaction, rod-shaped, fast growing with colony diameters ranging between 2-5mm within 3–7 days of incubation, indicating the isolates were rhizobia (Somasegaran & Hoben, 1994).

These characteristics were concurrent with previous reports of the finding that *R. leguminosarum* bv. *viciae* strains from Northern Gondar, Ethiopia (Belay & Assefa, 2011). Furthermore, the characteristics developed by our strains were consistent with the phenotypic appearance previously described in rhizobial isolates nodulating alfalfa (Mohamed et al., 2014; Azib et al., 2022).

Table 2 Colony characteristics of vetch and alfalfa isolates

Colony Characteristics		**No. of vetch isolates**	**%**	**No. of alfalfa isolates**	**%**
Colour	Watery Translucent	14	45.2	21	47.7
	White Translucent	9	29.0	16	36.3
	White Opaque	5	16.1	5	11.3
	Milky	3	9.7	2	4.5
Size	2-3mm(medium)	12	38.7	15	34.1
	>3mm(large)	19	61.3	29	65.9
Shape	Dome	19	61.3	32	72.7
	Flat	9	29.0	8	18.2
	Conical	3	9.7	4	9.1
Texture	Buttery	21	67.7	36	81.8
	Elastic	10	32.3	8	18.2
Congo red Absorption	Not absorbed	31	100.0	42	95.5
	Slightly absorbed	-	-	2	4.5
1st Colony Appearance Date	3-5days	31	100.0	44	100.0
Cell shape	Rod	31	100.0	44	100.0

4.2.2. Eco-physiological tolerance test of isolates

All of the isolates showed variability in their eco-physiological tolerance to pH, salt, temperature, intrinsic antibiotics, and heavy metals. The isolates were able to grow within 3–7 days of inoculation on the test media. The growth of the isolates on the test media indicated that

they were capable of tolerating the specified values of the tests. However, the absence of growth indicated that the isolates were sensitive to the given tests (Tables 3; 4).

Temperature tolerance test

It has been reported that the optimum temperature for growth ranges from 26 and 32°C. Some strains of rhizobia were reported to tolerate temperature as high as 42°C (Dabessa et al., 2018) while others were able to grow at lower temperature of 10°C. Accordingly, the current test isolates exhibited a wide range of variations in their temperature tolerance. A significant number of vetch (58%) and alfalfa (52.2%) rhizobia grew at 10 °C, while 32.2% vetch and 50% alfalfa rhizobia grew at 40°C, but none grew at 5°C and 45°C temperature extremes. Similarly the isolates of alfalfa and vetch were reported to sensitive growth temperature of 5°C and 45°C (Shimekite, 2006), but only four isolates resist lowest temperature of 5°C, indicating their ability to further survive at even very lower temperatures. The ability of the strains to grow at lower and higher temperature extremes could be a survival advantage for the strains under varying temperature conditions. Additionally, Belay & Assefa (2011) reported sensitivity *Rhizobium leguminosarum* bv. *Viciae* isolates for highest extremes of 45°C from Northern Gondar, Ethiopia, which agrees with the previous report by Mitiku et al. (2016).

Salt tolerance test

The current isolates also revealed variations in their tolerance to different concentrations of salt (Table 3). The number of the surviving vetch and alfalfa rhizobial isolates instantly decreased when the salt concentration gradient increased from 0.5 to 5%. None of the isolates from both host legumes grew at the salt concentration of 6%, reflecting the serious effect of the salt concentration on the rhizobial survival.

The results indicated that almost all of the isolates were resistant to low salt concentrations of 0.5%, while a few isolates were tolerated up to salt concentrations of 5% (Table 3). The rhizobial isolates that resist high salt concentrations (>3%) 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 test

Most of vetch isolates better tolerated pH ranges of 5-8, but their growth decreased when the pH lowered below 5 and increased above pH8 (Table 3). Relatively, most of the isolates loved higher pH range than the lower ones. Thus, the growth of the isolates decreased in acidic extremes of pH4. The sensitivity of the isolates to lower pH coincides with the sensitivity of the lentil isolates reported by Mitiku et al. (2016). The ability of the current isolates to survive at wide pH range agrees with the faba bean nodulating *R. leguminosarum* bv. *viciae* isolates' growth at pH 5.5 to 9 in Northern Gondar, Ethiopia (Belay & Assefa, 2011). Like vetch isolates, alfalfa isolates showed better growth on pH ranging from optimum (6.8 ± 2) to pH 9.0 and even some of isolates (15.9%) tolerated acidic settings (pH 4), indicating that they were adapted to varying pH ranges. Hameed et al. (2014) and Shimekite (2006) reported that *Sinorhizobium meliloti* strains grew at pH 4 and 9, reflecting the ability of the strain to grow at wide pH range. Similarly, microsymbionts nodulating *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.

Table 3 Response of vetch and alfalfa rhizobial strains to eco-physiological changes

Physiological test values		Vetch		Alfalfa	
		No. of isolates	Percentage (%)	No. of isolates	Percentage (%)
Temperature (°C)	5	0	0.0	0	0.0
	10	18	58.0	23	52.2
	15	26	83.8	42	95.4
	40	10	32.2	22	50.0
	45	0	0.0	0	0.0
NaCl (%)	0.5	27	87.0	44	100.0
	1	11	35.4	44	100.0
	2	7	22.5	42	95.4
	3	6	19.3	5	11.3
	4	2	6.45	3	6.8
	5	1	3.2	2	4.5
	6	0	0.0	0	0.0
pH tolerance	4	5	16.1	7	15.9
	4.5	28	90.3	9	20.4
	5	30	96.7	10	22.7
	5.5	30	96.7	13	29.5
	8	31	100.0	44	100.0
	8.5	31	100.0	44	100.0
	9	26	83.8	44	100.0

Intrinsic antibiotic resistance (IAR) test

The results showed that the isolates were diverse in their tolerance to different types and concentrations of antibiotics. Some of the vetch isolates revealed intermediate resistance to Penicillin 1mg/ml, Amoxicillin 5mg/ml, and Bacteriocin 5mg/ml, while few resisted Streptomycin 10mg/ml, Streptomycin 2.5mg/ml, Kanamycin 1mg/ml, Cephalexin 10mg/ml, Cephalexin 2.5 mg/ml, and Ampicillin 5mg/ml (Table 4). Similarly, alfalfa isolates survived different type and concentrations of antibiotics mentioned above. On the other hand, they were sensitive to different concentrations of Streptomycin and Kanamycin. One of the strategies to develop inoculants for a given legume crop is evaluating the competitive ability of the isolates in the soil, which could be defined by resistance to antibiotics released by the antagonistic microbes in the soil. Hence, the evaluated vetch and alfalfa rhizobial isolates showed abilities to resist antibiotics supposed to be released by soil microbes. The ability of the isolates in responding to

different concentrations of antibiotics would render them competitive to strains adapted to soil. Our current result is in agreement with the result of Kajić et al. (2019), who reported that alfalfa nodulating rhizobial isolates were sensitive to different concentrations of kanamycin. Mitiku et al. (2016) also observed that lentil isolates were sensitive to streptomycin and kanamycin at doses of 5 and 10µg/ml,

Intrinsic Heavy metal resistance test

It has been reported that rhizobial strains are affected by heavy metal concentrations in the soil. Some of the Ethiopian soils have been exposed to soil erosion that make the soil either calcareous in the highlands or salty marshes in the lowlands. In addition, most of the soils in the highlands of Ethiopia become acidic due to high levels of Al, Mn and their compounds. Hence, we evaluated the effects of heavy metals and Al toxicity on vetch and alfalfa rhizobial isolates to suggest resistant strains for such locations. We found that most of the vetch and alfalfa isolates grew on a medium containing $Al_2(SO_4)_3$, CsCl, and $ZnSO_4$, whereas they depicted intermediate resistance to $MnSO_4.H_2O$ and $ZnCl_2$ (Table 4). The result agreed with the report that fast-growing strains were found to be more sensitive to antibiotics than slow-growing rhizobia (Maatallah et al., 2002).

The toxic compounds $Al_2(SO_4)_3$, CsCl, $ZnSO_4$, $MnSO_4.H_2O$ and $ZnCl_2$ were the major constraints limiting crop growth and productivity in acidic soils (Hungria & Vargas, 2000), which are common in arable lands of Ethiopia (Bekere, 2013), containing those heavy metal compounds. The isolates, resistant to such compounds are ideal for acidic soils and can be recommended acidic soils of Ethiopia.

Table 4: Responses of vetch and alfalfa rhizobial isolates to antibiotics (IA) and heavy metals

Physiological test values		Vetch		Alfalfa	
		No. of isolates	**Percentage (%)**	**No. of isolates**	**Percentage (%)**
Intrinsic antibiotics resistance test (mg/ml)	Bacteriocin 5	11	35.4	3	6.8
	Penicillin 1	12	38.7	6	13.6
	Streptomycin 10	7	22.5	0	0.0
	Streptomycin 2.5	7	22.5	0	0.0
	Kanamycin 10	0	0.0	0	0.0
	Kanamycin 1	7	22.5	0	0.0
	Cephalexin 10	9	29.0	2	4.5
	Cephalexin 2.5	9	29.0	2	4.5
	Amoxicillin 5	10	32.2	2	4.5
	Ampicillin 5	9	29.0	3	6.8
Heavy metal resistant test (mg/ml)	$Al_2(So_4)_3$ 2.5	31	100	44	100
	$ZnCl_2$ 5	14	45.1	19	43.1
	CsCl 10	27	87.0	44	100
	$MnSo_4.H_2O$ 50	15	48.3	21	47.7
	$CuSo_4$ 10	0	0.0	0	0.0
	$CoCl_2$ 2.5	0	0.0	0	0.0
	$ZnSo_4$ 10	24	77.4	22	50.0
	$(CH3COO)_2Pb$ 2.5	0	0.0	0	0.0

4.2.3. Substrate utilization test of rhizobia isolates

Carbon source utilization

It has been a trend to evaluate rhizobial isolates for their ability of using different carbohydrates as a sole source of carbon. Accordingly, our current investigation revealed that all isolates of vetch and alfalfa utilized D-Glucose, D-Sorbitol, D-Lactose, dextrose, and sucrose as their sole sources of carbon (Table 5). This finding agrees with Mitiku et al. (2016), who reported that most lentil nodulating isolates from Shewa, Ethiopia, utilized almost all of the evaluated carbohydrates. In the current study, among the evaluated carbon sources, citric acid (18.2%) and fructose (15.9%) have been utilized by very few isolates (Table 5). Soils reach in such carbon sources might have specialized rhizobial strains that can be a target for future investigations. However, Shimekite (2006) reported that fructose and maltose were utilized as sole sources of carbon by vetch and alfalfa nodulating strains, indicating differences from location to location

among rhizobia in terms of carbon source utilizations. This validates the current activity of screening strains in terms of their carbon source utilizations for future field applications.

Rhizobia are soil dwelling bacteria that are considered to survive on diverse sources of carbons. In marginalized soils, the rhizobia are expected to use any available carbon source by shifting their metabolic machineries but some others fail to do so and their survival in the soil is limited.

Generally, it has been suggested that rhizobia strains can oxidize a wide range of carbon as their sole carbon source gives them their survival and nutritional advantage. The isolates are known to use a greater variety of carbon sources for their survival; giving them to compete with other microbes, so they are more likely to be selected for inoculants after being evaluated under diverse field conditions.

Nitrogen source utilization

Amino acids and vitamins are important nitrogen sources for all living organisms, which use it for nucleic acid synthesis, co-enzyme synthesis, ATP (energy storage molecule) synthesis, etc. The living organisms obtain nitrogen from different sources: some fix their own nitrogen (e.g., rhizobia), or some others obtain it from organic matter or from soil that is either provided as N-fertilizer or from N-ores. The microbes show variations in terms of utilizing available nitrogen sources in the soil. Hence, this investigation targeted at evaluating the nitrogen source utilization potential of vetch and alfalfa rhizobial isolates. We observed that all of the vetch isolates (100%) utilized the amino acids L-Asparagine and L-Alanine as sources of nitrogen, and the rest of the amino acids under study were utilized by more than 80% of the isolates. Similarly, almost all of the alfalfa isolates utilized the amino acid L-Asparagine and L-Arginine as a source of nitrogen. Vitamins such as Folic acid and Thiamine have been also utilized by 90% of the isolates (Table 5). The nitrogen source utilization pattern of the current isolates concomitantly agrees with the previous report by Mitiku et al., (2016), who revealed that lentil nodulating rhizobial isolates utilized a wide range of amino acids as nitrogen sources, except glycine and alanine. The capacity of the isolates to use various nitrogen sources would provide them with an ecological, nutritional and competition advantage under nutrient-deficient soil conditions.

Table 5: Response of vetch and alfalfa isolates to C- and N- sources in a growth medium

Biochemical test values		Vetch		Alfalfa	
		No. of isolates	**Percentage (%)**	**No. of isolates**	**Percentage (%)**
carbon source utilization test	D-Glucose	31	100.0	44	100.0
	D-Sorbitol	31	100.0	44	100.0
	D-Lactose	31	100.0	44	100.0
	Dextrose	31	100.0	43	97.7
	D- Fructose	16	51.6	7	15.9
	citric acid	17	54.8	8	18.2
	Sucrose	31	100.0	44	100.0
	D-Maltose	18	58.0	22	50.0
Nitrogen source utilization test	L-Leucine	25	80.6	38	86.3
	L-Asparagine	31	100.0	43	97.7
	Folic Acid	30	96.7	40	90.9
	L-Tryptophan	30	96.7	38	86.3
	L-Proline	29	93.5	37	84.0
	L-Arginine	28	90.3	42	95.4
	Thiamine	30	96.7	40	90.9
	L-Alanine	31	100.0	38	86.3
	Glycine	26	83.8	39	88.6
	Nicotinic Acid	26	83.8	28	63.6

4.3. Relative effectiveness of the isolates

Nitrogen fixation potential of newly explored rhizobia isolates can be evaluated by relative symbiotic effectiveness in terms of shoot biomass. The shoot dry weight of plants harvested at floral initiation or after significant plant biomass accumulation is the generally accepted criterion for N_2-fixing effectiveness, but nodule dry weight may also be employed. Nodule numbers are a less reliable indicator of evaluating strain effectiveness, since nodulation rarely correlates with the amount of fixed nitrogen and accumulated plant biomass. Besides measuring the amounts fixed nitrogen and plant biomass, a protein called leghemoglobin in the nodule tissue is often used to characterize legume-rhizobia symbiosis as effective symbiosis (Somasegaran & Hoben, 1994). Based on the principles of shoot biomass and observations of leghemoglobin, the newly isolated vetch and alfalfa rhizobia were discriminated in terms of symbiotic effectiveness. The result indicated that most of the inoculated plants showed deep green leaves, long and branched stems, and pink nodules compared to negative controls.

Figure 2: Leaf and nodule color of plants inoculated with test isolates of vetch and alfalfa, negative & positive control plants

The analysis of symbiotic effectiveness on sand culture in MLJ revealed a significant ($p < 0.05$) variation among strains in terms of inducing nodule number, nodule dry weight, shoot dry weight, and relative symbiotic effectiveness. The vetch isolates induced nodules on the host plant; with the mean nodule number (NN) ranging 22.33 per plant (VD6) to 101.00 per plant (VL40) and nodule dry weight ranging from minimum 0.02g per plant to maximum 0.18g per plant (Table 6). The result agreed with previous reports that vetch isolates showed differences in nodule numbers and nodule dry weight (Shimekite, 2006; Roper et al., 2020). There was also a significant variation among the strains in terms of inducing shoot dry weight. Up to 0.56g shoot biomass was recorded from a single plant that inoculated with VB23 was grown on sand culture for 45 days in the greenhouse. Comparatively, some of the current isolates accumulated higher shoot biomass than those reported by Shimekite (2006), with maximum shoots biomass of 0.347g per plant. Some of the inoculated plants accumulated more shoot biomass (e.g., 0.56g shoot dry weight) than the N-fertilized (positive control) plants (0.43g), indicating that some strains fix and contribute higher amount of nitrogen to the plant than the recommended amount of N-fertilizer for sand cultures. The recovery of such strains, from natural habitat advocates the alternative use of inoculants instead of N-fertilizers. In contrast, few strains accumulated dry matter below the dry matter of the negative control plants, suggesting the need for screening high nitrogen fixers. Others also showed that some strains fixed lower Nitrogen that could be due to low nitrogen-fixing performance or by competition effect (Checcucci et al., 2017). Strains that

accumulated more dry matter than positive controls, indicates that their superiority in symbiotic effectiveness.

The analysis of variance also revealed a significant difference ($p < 0.05$) between strains regarding symbiotic effectiveness (SE) that ranged from -16.51% (VK2) to 176.07 (VB23). The negative value indicates that some strains accumulated lower shoot biomass than the negative controls for vetch plants. Generally, 29.0% of isolates were highly effective, 12.9% were effective, 3.2% were low effective, and 54.8% were ineffective (Table 6; Appendix 1)). Isolates with SE ≥80% were used for further evaluation to select the best elite and recommend for evaluation under field conditions.

Table 6 Shoot and nodule dry weight, nodule number and relative symbiotic effectiveness of vetch inoculated with test rhizobial isolates

Strains	SDW (g plant^{-1})	NN plant^{-1}	NDW(g plant^{-1})	%SE	SE level
VB23	0.56^{a}	73.33^{ab}	0.06^{ab}	176.07^{a}	HE
VL40	0.53^{ab}	101.00^{a}	0.07^{ab}	158.22^{ab}	HE
VB76	0.50^{a-c}	63.67^{a-d}	0.18^{a}	142.27^{a-c}	HE
VK33	0.45^{a-d}	63.67^{a-d}	0.06^{ab}	114.91^{a-d}	HE
N+	0.43^{a-e}	0.00^{e}	0.00^{b}	100.00^{a-d}	HE
VL11	0.43^{a-f}	63.33^{a-d}	0.04^{b}	122.74^{a-d}	HE
VL34	0.42^{a-g}	65.67^{a-d}	0.05^{ab}	104.00^{a-d}	HE
VB63	0.40^{a-h}	82.67^{ab}	0.07^{ab}	100.62^{a-d}	HE
VB79	0.35^{a-i}	35.00^{c-e}	0.03^{b}	84.37^{a-d}	HE
VD27	0.32^{b-j}	68.67^{a-d}	0.06^{ab}	80.33^{a-d}	HE
VB43	0.30 $^{b-j}$	71.67^{a-c}	0.05^{b}	54.96^{a-d}	E
VL35	0.29^{c-j}	41.67^{b-e}	0.03^{b}	54.07^{a-d}	E
VL7	0.28 $^{c-j}$	71.67^{a-c}	0.05^{b}	72.44^{a-d}	E
VB32	0.27^{d-j}	38.33^{b-e}	0.04^{b}	57.90^{a-d}	E
VB25	0.23 $^{d-j}$	46.67^{b-e}	0.03^{b}	38.92^{a-d}	SE
VL10	0.23 $^{d-j}$	38.33^{b-e}	0.02^{b}	31.85^{a-d}	IE
VK5	0.21^{e-j}	43.00^{b-e}	0.03^{b}	32.90^{a-d}	IE
VK3	0.20^{f-j}	49.33^{b-d}	0.04^{b}	19.73^{a-d}	IE
VL1	0.20^{g-j}	33.33^{c-e}	0.02^{b}	27.90^{a-d}	IE
VK16	0.19^{g-j}	53.00^{b-d}	0.03^{b}	20.63^{a-d}	IE
VB28	0.18^{h-j}	65.67^{a-d}	0.04^{b}	16.97^{b-d}	IE
VK22	0.18^{h-j}	32.00^{c-e}	0.03^{b}	27.05^{a-d}	IE
VK9	0.17^{h-j}	35.00^{c-e}	0.02^{b}	18.49^{b-d}	IE

VD24	0.17^{h-j}	61.33^{a-d}	0.03^{b}	15.20^{b-d}	IE
VK26	0.16^{ij}	23.00^{de}	0.02^{b}	8.79^{b-d}	IE
VD6	0.15^{ij}	22.33^{de}	0.02^{b}	12.12^{b-d}	IE
VD4	0.15^{ij}	36.33^{b-e}	0.02^{b}	2.20^{b-d}	IE
VK30	0.15^{ij}	44.00^{b-e}	0.03^{b}	10.28^{b-d}	IE
N-	0.14^{ij}	0.00^{e}	0.00^{b}	0.00^{cd}	IE
VB15	0.13^{ij}	60.67^{a-d}	0.03^{b}	-3.16^{cd}	IE
VB14	0.12^{j}	40.67^{b-e}	0.02^{b}	-6.81^{cd}	IE
VB17	0.11^{j}	41.67^{b-e}	0.02^{b}	-8.57^{cd}	IE
VK2	0.09^{j}	32.67^{c-e}	0.02^{b}	-16.51^{d}	IE
CV%	1.99773	1.996564	1.996564	1.996564	-
$LSD_{0.05}$	0.113981	23.211	0.06600096	75.93507	-

Treatments with the same letter are not significantly different. HE= >80%, E= 50-80%, LE= 35-50%, IE= < 35%, NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, %SE= percent symbiotic effectiveness.

There was also significant ($p < 0.05$) variation in nodulation, shoot dry matter, and symbiotic effectiveness among alfalfa isolates (Table 7; Appendix 2). This finding is in line with those of Jia et al. (2008) and Langer et al. (2008), who reported that alfalfa strains showed differences in their nodulation, shoot dry weights, and effectiveness. The current isolates induced nodule number (NN) ranging from 8.33 per plant (AL83) to 114.00 (AD36). Regarding nodule dry weight, the plant inoculated with AD50 showed a maximum NDW ($0.139g/p^{-1}$); while a minimum NDW ($0.012g/p^{-1}$) was recorded from isolate AD64.The largest shoot dry weight was 0.85g (recorded on plant inoculated by strain AD50) while the lowest was 0.14g per plant from AD64. This indicated the existence of variations in rhizobial effectiveness during the sand culture study on nodule induction and symbiotic effectiveness, which corresponded to variations in physical appearance, plant color that ranged from yellow to deep green leaves, and related characters mentioned (Figure 2). Among the inoculated plants, all of them accumulated a higher shoot dry weight than the negative control (0.13g per plant), and some of them accumulated a higher shoot dry weight than the positive control plants (0.77g per plant). There was a significant difference ($p < 0.05$) among alfalfa strains in terms of symbiotic effectiveness (SE) (Table 7). The SE ranged from 1.05% (ineffective isolate AD64) to 113.22% (the most effective isolate AD50). Like, vetch isolates, four alfalfa nodulating isolates AD50, AK88, AB20 and AB60 had significantly higher ($p < 0.05$) SE than the N-fertilized positive controls. Generally, 25.0% of the alfalfa isolates highly effective (Table 7) and such isolates can be further taken for field evaluations to screen the best isolates.

Table 7 Shoot and nodule dry weight, nodule number and relative symbiotic effectiveness of alfalfa inoculated with test rhizobial isolates

Strains	SDW (g plant^{-1})	NN plant^{-1}	NDW(g plant^{-1})	%SE	SE level
AD50	0.85^{a}	110.33^{a}	0.139^{a}	113.22^{a}	HE
AK88	0.84^{a}	94.67^{a-d}	0.079^{b-d}	111.20^{a}	HE
AB20	0.80^{ab}	83.33^{a-g}	0.057^{b-g}	104.67^{ab}	HE
AB60	0.79^{a-c}	106.33^{ab}	0.099^{a-c}	103.28^{a-c}	HE
N+	0.77^{a-d}	0.00^{l}	0.00^{g}	100.00^{a-d}	HE
AK91	0.76^{a-e}	69.67^{a-i}	0.057^{b-g}	99.78^{a-d}	HE
AD36	0.76^{a-e}	114.00^{a}	0.101^{ab}	98.49^{a-d}	HE
AK89	0.75^{a-f}	75.00^{a-i}	0.065^{b-f}	95.22^{a-e}	HE
AL84	0.73^{a-g}	84.67^{a-f}	0.069^{b-f}	93.04^{a-e}	HE
AL71	0.72^{a-g}	79.33^{a-h}	0.065^{b-f}	90.66^{a-f}	HE
AD80	0.68^{a-h}	79.67^{a-h}	0.068^{b-f}	85.39^{a-g}	HE
AL69	0.65^{a-i}	86.67^{a-e}	0.072^{b-e}	80.80^{a-h}	HE
AK92	0.60^{a-j}	44.00^{e-l}	0.030^{d-g}	72.24^{a-i}	E
AB77	0.56^{a-j}	71.00^{a-i}	0.043^{b-g}	68.65^{a-i}	E
AB52	0.54^{a-k}	95.33^{a-c}	0.080^{b-d}	66.12^{a-j}	E
AB54	0.53^{a-l}	93.33^{a-d}	0.072^{b-e}	61.31^{a-k}	E
AB78	0.53^{a-l}	79.33^{a-h}	0.056^{b-g}	61.94^{a-k}	E
AD82	0.52^{a-l}	55.00^{c-j}	0.042^{c-g}	61.78^{a-k}	E
AL87	0.48^{a-l}	46.00^{e-k}	0.045^{b-g}	55.20^{a-k}	E
AD31	0.47^{a-l}	45.33^{e-l}	0.031^{d-g}	52.69^{a-k}	E
AB72	0.42^{b-l}	43.67^{e-l}	0.030^{d-g}	45.05^{b-k}	SE
AB70	0.42^{b-l}	70.67^{a-i}	0.045^{b-g}	43.47^{b-k}	SE
AD13	0.39^{b-l}	64.33^{b-i}	0.042^{c-g}	39.14^{b-k}	SE
AB74	0.38^{c-l}	47.33^{e-k}	0.040^{c-g}	38.51^{c-k}	SE
AB38	0.37^{d-l}	52.67^{c-k}	0.035^{d-g}	35.24^{d-k}	SE
AL81	0.37^{d-l}	54.33^{c-j}	0.041^{c-g}	36.70^{d-k}	SE
AL59	0.37^{d-l}	74.00^{a-i}	0.065^{b-f}	38.23^{c-k}	SE
AL73	0.36^{e-l}	72.00^{a-i}	0.062^{b-f}	36.12^{d-k}	SE
AD66	0.36^{e-l}	53.00^{c-k}	0.046^{b-g}	35.44^{d-k}	SE
AB68	0.34^{f-l}	52.33^{c-k}	0.043^{b-g}	32.24^{e-k}	IE
AB51	0.33^{g-l}	60.00^{c-j}	0.038^{d-g}	32.09^{e-k}	IE
AB45	0.30^{h-l}	49.67^{d-k}	0.029^{d-g}	26.73^{f-k}	IE
AL61	0.29^{h-l}	55.00^{c-j}	0.042^{b-g}	25.38^{f-k}	IE
AB58	0.29^{h-l}	60.33^{c-j}	0.049^{b-g}	25.29^{f-k}	IE
AL41	0.29^{h-l}	36.67^{h-l}	0.041^{c-g}	24.86^{f-k}	IE
AB57	0.29^{h-l}	50.67^{c-k}	0.044^{b-g}	24.27^{g-k}	IE
AD42	0.27^{h-l}	38.67^{g-l}	0.027^{d-g}	21.37^{g-k}	IE

AB53	0.27^{i-l}	44.00^{e-l}	0.016^{e-g}	20.92^{g-k}	IE
AB49	0.26^{i-l}	39.33^{f-l}	0.030^{d-g}	20.86^{g-k}	IE
AD48	0.24^{j-l}	8.33^{kl}	0.012^{fg}	16.75^{h-k}	IE
AB46	0.23^{j-l}	18.33^{j-l}	0.017^{e-g}	16.92^{h-k}	IE
AL75	0.23^{j-l}	47.00^{e-k}	0.040^{c-g}	15.96^{h-k}	IE
AL56	0.21^{j-l}	53.00^{c-k}	0.043^{b-g}	12.78^{i-k}	IE
AL83	$0.\ 21^{j-l}$	8.33^{kl}	0.012^{fg}	11.60^{i-k}	IE
AD64	0.14^{kl}	32.67^{i-l}	0.012^{fg}	1.05^{jk}	IE
N-	0.13^{l}	0.00^{l}	0.00^{g}	0^{k}	IE
CV%	1.986675	1.986086	1.986086	1.986086	
$LSD_{0.05}$	0.1942898	21.76174	0.02785075	31.34062	

Treatments with the same letter are not significantly different.
HE= >80%, E= 50-80%, LE= 35-50%, IE= < 35%, NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, %SE= percent symbiotic effectiveness

4.4. Numerical taxonomy

Microorganisms adapt to environments varying in agroclimatic and edaphic properties. They develop eco-physiological traits that enable them to withstand this versatile environment. Such eco-physiological traits have been used to categorize microorganisms by a computer multivariate cluster analysis into distinct groups. The use of eco-physiological traits for classifying the microbes is named numerical taxonomy (Quesne, 1969). The current numerical analysis of the vetch and alfalfa rhizobial isolates indicated wide phenotypic diversity among the strains. It categorized vetch isolates into three distinct clusters and alfalfa isolates into three distinct clusters with two ungrouped of strains at 70% similarity cut point (Figure 5). Similarly, Azib et al. (2022) reported that 48 isolates of alfalfa were clustered in to 3 groups based on twenty seven phenotypic characters at 60% dissimilarity. Mohammed et al. (2020) also conducted grouping of 22 rhizobial isolates of grass pea based on 59 phenotypic features and found three distinct clusters at 25% dissimilarity level. Furthermore, (Ereso, 2017) reported three different clusters and 2 un-clustered isolates of *Rhizobium leguminosarum* at 65% of similarity cutoff point. This suggests that it is possible to categorize rhizobial isolates on the basis of similarities or dissimilarities of their phenotypic properties.

Zooming into the details of the phenotypic groups, Cluster I of vetch isolates consisted of four strains originating from all sampling sites, indicating even distribution of the isolates. Surprisingly, majority of the isolates with higher symbiotic effectiveness were grouped in this

cluster and all of them were sensitivity to low (5°C) and high (45°C) temperatures, grow in pH ranges of 4.5 – 9.0 and unable to grow at 3% salt concentration and beyond. Clustering of most of the symbiotically highly effective strains to one phenotypic group suggests that analysis of phenotypic traits would be ideal to screen best performing elite strains. This conventional analysis of phenotypes could be preferred than the fancy molecular analysis to screen best isolates, since phylogenetic analysis of chickpea strains were not congruent with the symbiotic performance of the strains (Gunnabo et al., 2021). Regarding antibiotics and heavy metal resistance tests, almost all of the isolates in this cluster were sensitive to most of the antibiotics tested. In addition, they were resistant to $Al_2(SO_4)_3$ CsCl and $ZnSO_4$ but sensitive to $CuSO_4$, $CoCl_2$, and $(CH_3COO)_2Pb$. As suggested above, resistance to antibiotics, toxic Al and Mn compounds and heavy metals would make the strains competitive in the soil as well as increase their survival even under toxic soils. Low soil pH is associated with increased Al and Mn toxicity. Around 41% of Ethiopia's arable land is acidic, characterized by Al toxicity that restricts crop growth and productivity (Hungria & Vargas, 2000; Bekere, 2013). Al and Mn tolerant isolates could be desirable to increase environmental adaptation and minimize the adverse impacts of acid soils on symbiosis and BNF. In this cluster almost all of the isolates utilized most of carbohydrate and nitrogen sources. The use of these diverse C- and N-sources would enable the strains to survive in soils with rare C- and N-sources and this can increase their adaptation to different environments. Like vetch isolates, alfalfa isolates in cluster I survived at temperatures of 10°C–40°C, pH 4.0 – 9.0 and salt concentrations of 0.5%–3%. They showed differences in responses to different antibiotics but none of them survived on heavy $CuSO_4$, $CoCl_2$, or $(CH_3COO)_2Pb$. All of the isolates in this cluster used all of the tested C- and N-sources, except fructose.

Most of the vetch isolates in cluster II were characterized by their potential to resist temperature, pH, salt, antibiotics, except Kanamycin and heavy metals $CuSO_4$,$CoCl_2$, $(CH_3COO)_2Pb$. Isolates in this cluster used all of the tested C- and N- sources, except fructose. In case of alfalfa, the second cluster consisted of 75% of the isolates. 82% of highly effective isolates were included in this cluster. This supports the above reflection that phenotypic properties are important indicators of best nitrogen fixer that gather them to one cluster. They differ from the cluster I by having quite different response to temperature variation and loving higher pH (8.0 – 9.0). This cluster was sensitive to all antibiotics except penicillin and ampicillin. They were also resistant to

$Al_2(SO_4)_3$, and CsCl (Table 7). With regard to C- and N-sources utilization, almost all of the isolates catabolized carbon and nitrogen sources, with a few exceptions in the case of carbon source maltose, which was catabolized by about 50% of the isolates, and fructose and citric acid, were utilized by a single isolate.

Cluster III contained two (VB25 and VB43) vetch isolates originating from Bishoftu. All of the isolates in this cluster were grown at temperatures ranging from 10°C to 40°C, pH ranges from 4 to 9, and salt concentrations from 0.5% to 5%. None of the isolates in this cluster grown at temperature extremes (5 and 45°C) or high salt concentrations (6%), making the different from other clusters. They had no significant difference in terms of toxicity to Al, Mn and heavy metal sensitivity. The cluster was sensitive to all of the antibiotics in the test except for the growth of one isolate on bacteriocin and penicillin. While cluster III alfalfa isolates had mixed symbiotic effectiveness levels, distorting the suggestion we made above, in which we said phenotypic out comes would cluster effective strains to a single cluster and it could be ideal for screening the best. On the other hand, this suggests case specific nature of the phenotypic profiling. The isolates were sensitive to acidic settings and high salt concentrations. All of the isolates were sensitive to antibiotics and heavy metals under study, except they grew on $Al_2(SO_4)_3$, and CsCl. However, they did not use maltose, L-Leucine, L-Tryptophan, Thiamine, Glycine, and nicotinic acid C- and N-sources. Like the vetch strains, alfalfa strains in cluster III had different responses to different temperatures. But, they loved wide pH (4.0 – 9.0) and salt concentration (0.5–5%) ranges.

Unclustered isolates were distinct in that both of them were sensitive to all of the heavy metals and antibiotics being studied. In terms of the utilization of carbon and nitrogen sources, AD31 used the carbon sources D-glucose, D-sorbitol, D-lactose, and sucrose, as well as the nitrogen sources L-Arginine and thiamine; the isolate did not use the other carbon and nitrogen sources. AL73 used all carbon and nitrogen sources, but the isolate did not use L-Alanine or Nicotinic Acid.

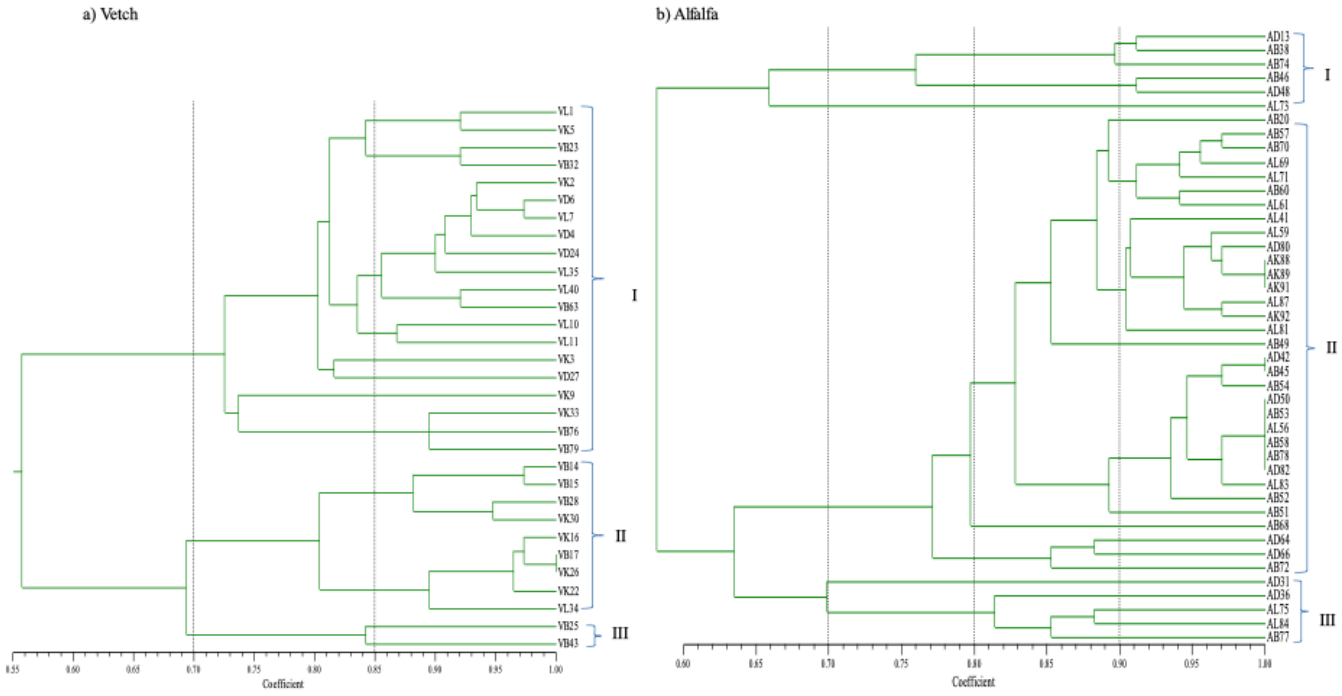

Figure 3 : Multivariate cluster analysis of 55 phenotypic properties of 31 vetch and 44 alfalfa isolates using NTSYSpc version 2.1

Table 8: Eco-physiological and Biochemical Characteristics of the Isolates within the Clusters

Characteristics		Vetch			Alfalfa				
		Cluster I N=20	Cluster II N=9	Cluster III N=2	Cluster I N=5	Cluster II N=33	Cluster III N=4	Unclustered N=2	
								AL73	AD31
Temperature (°C)	5	-	-	-	-	-	-	-	-
	10	8	8	+	+	17	-	+	-
	15	16	8	+	+	+	3	+	-
	40	1	8	1	4	15	2	+	-
	45	-	-	-	-	-	-	-	-
pH	4	-	4	1	4	2	-	-	+
	4.5	17	+	+	+	2	-	+	+
	5	19	+	+	+	3	-	+	+
	5.5	19	+	+	+	5	1	+	+
	8	+	+	+	+	+	+	+	+
	8.5	+	+	+	+	+	+	+	+
	9	17	7	+	+	+	+	+	+
NaCl	0.5	17	8	+	+	+	+	+	+

	1	5	4	+	+	+	+	+	+
	2	1	4	+	+	31	+	+	+
	3	-	4	+	4	-	-	+	-
	4	-	1	1	2	-	-	+	-
	5	-	-	1	1	-	-	+	-
	6	-	-	-	-	-	-	-	-
Antibiotic resistance(mg/ml)	Bacteriocin 5	2	8	1	3	-	-	-	-
	Penicillin 1	2	+	1	3	3	-	-	-
	Streptomycin 10	-	7	-	-	-	-	-	-
	Streptomycin 2.5	-	7	-	-	-	-	-	-
	Kanamycin 10	-	-	-	-	-	-	-	-
	Kanamycin 1	-	7	-	-	-	-	-	-
	Cephalexin 10	-	+	-	2	-	-	-	-
	Cephalexin 2.5	-	+	-	2	-	-	-	-
	Amoxicillin 5	1	+	-	2	-	-	-	-
	Ampicillin 5	-	+	-	2	1	-	-	-
Intrinsic Heavy Metal Resistance(mg/ml)	$Al_2(So_4)_3$ 2.5	+	+	+	+	+	+	+	+
	$ZnCl_2$ 5	3	+	+	+	15	-	-	-
	CsCl 10	16	+	+	+	+	+	+	+
	$MnSo_4.H_2O$ 50	5	8	+	+	16	-	-	-
	$CuSo_4$ 10	-	-	-	-	-	-	-	-
	$CoCl_2$ 2.5	-	-	-	-	-	-	-	-
	$ZnSo_4$ 10	13	+	+	+	16	1	-	-
	$(CH3COO)_2Pb$ 2.5	-	-	-	-	-	-	-	-
Carbohydrate	D-Glucose	+	+	+	+	+	+	+	+
	D-Sorbitol	+	+	+	+	+	+	+	+
	D-Lactose	+	+	+	+	+	+	+	+
	Dextrose	+	+	+	+	+	+	+	-
	D- Fructose	5	+	+	4	1	1	+	-
	Citric acid	6	+	+	5	1	1	+	-
	Sucrose	+	+	+	+	+	+	+	+
	D-Maltose	7	+	+	+	16	-	+	-
Nitrogen	L-Leucine	14	+	+	+	32	-	+	-
	L-Asparagine	+	+	+	+	+	+	+	-
	Folic Acid	19	+	+	+	32	2	+	-
	L-Tryptophan	19	+	+	+	32	-	+	-
	L-Proline	18	+	+	+	30	1	+	-
	L-Arginine	17	+	+	+	+	2	+	+
	Thiamine	19	+	+	+	+	-	+	+
	L-Alanine	+	+	+	+	32	1	-	-
	Glycine	15	+	+	+	+	-	+	-
	Nicotinic Acid	15	+	+	+	+	-	-	-

5. CONCLUSION

This study shows physiological characterization and evaluation of the symbiotic effectiveness of the rhizobia nodulating forage legumes alfalfa and vetch growing in Ethiopia. The isolates possessed different eco-physiological and biochemical characteristics indicating that there is great diversity among them, and their tolerance to temperature, alkaline, and acidic settings are important adaptations for the inoculant's selection. The tested rhizobial isolates also utilized a wide range of carbon and nitrogen sources, which is another point of view for their survival advantage. Based on these characteristics, the numerical cluster of 31 vetch and 44 alfalfa isolates evaluated formed three vetch clusters, and three alfalfa distinctive clusters with two unclustered isolates. The preliminary symbiotic effectiveness revealed that 29% of vetch and 25% of alfalfa isolates were found to be highly effective and indicated an increase in nodulation, shoot, and nodule dry weight of the plants with respect to the uninoculated control. Seven isolates of vetch (VB23, VL40, VB76, VK33, VL11, VL34, and VB63) and four of alfalfa (AD50, AK88, AB20, and AB60) performed better in the greenhouse trial than nitrogen fertilizer. This suggests that Ethiopian soils harbor phenotypically diverse and symbiotically highly effective rhizobia.

6. RECOMMENDATIONS

Rhizobia nodulating forage legumes have been understudied in Ethiopia. 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 phenotypic characterization. It is recommended to further characterize the strains genetically for correct identity.

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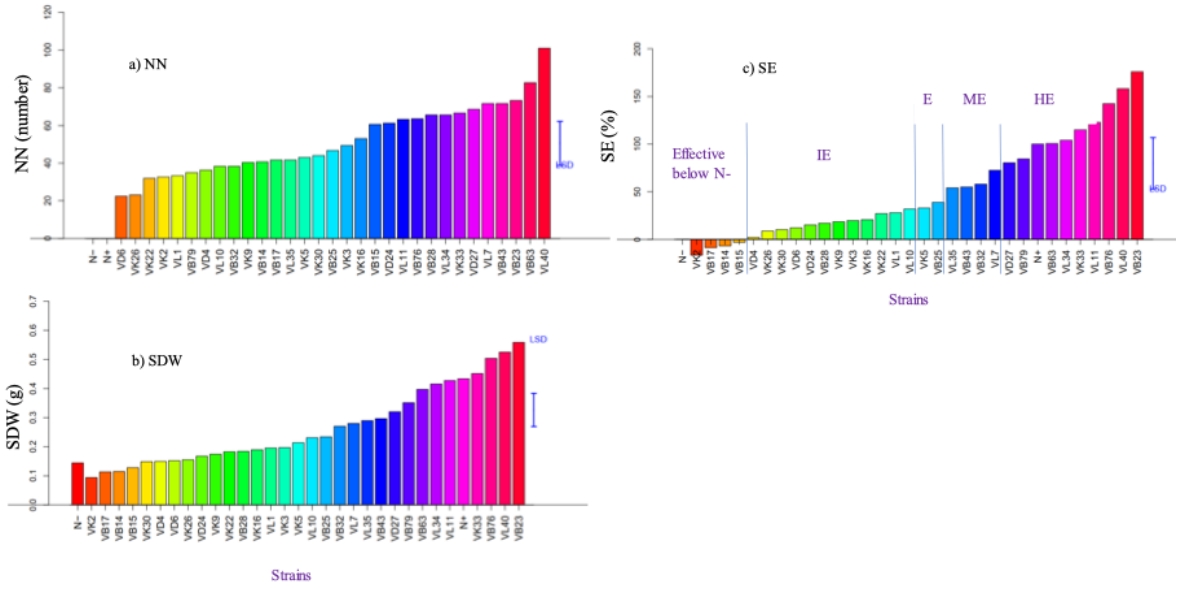
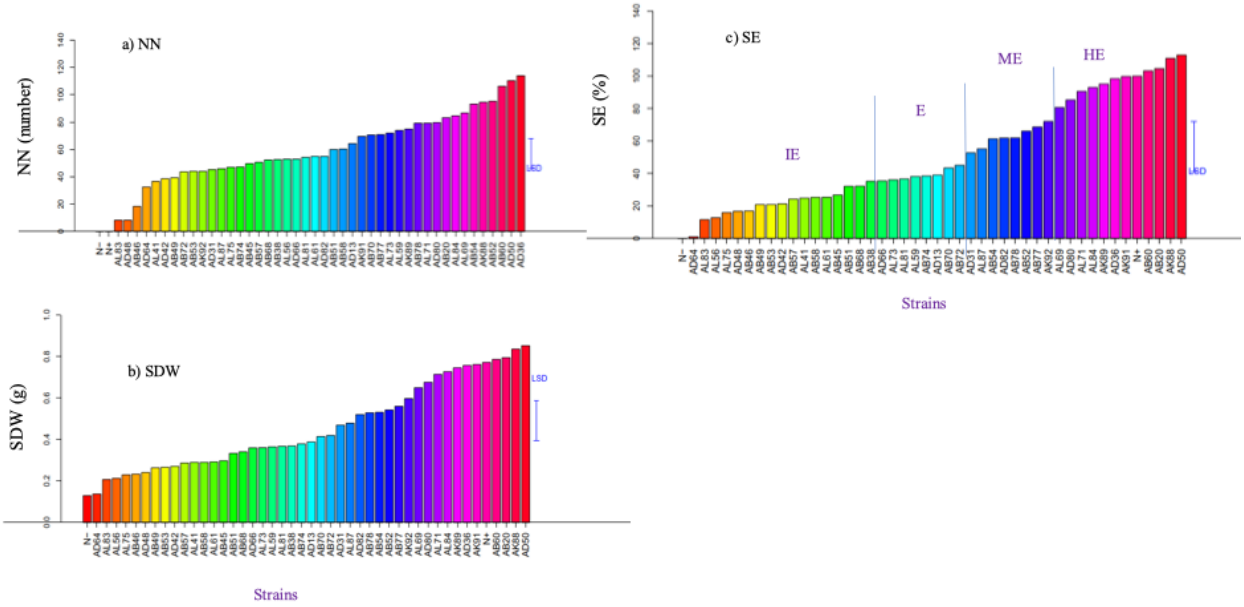
APPENDICES

Appendix 1 Nodulation, shoot dry weight and symbiotic effectiveness performance of vetch rhizobia isolates



HE=highly effective, E=effective, SE=slowly effective, IE=ineffective

Appendix 2 Nodulation shoot dry weight and symbiotic effectiveness performance of alfalfa rhizobia isolates



HE=highly effective, E=effective, SE=slowly effective, IE=ineffective

Appendix 3 ANOVA, rhizobia isolate effect on variability of shoot dry weight, nodule number, nodule dry weight, and symbiotic effectiveness of vetch and alfalfa plants under greenhouse test.

Source of Variation			Df	Sum of squares	Mean squares	F-value	Pr(>F)
Vetch	SDW	Replication	2	0.00965	0.004825	0.9703	0.3845
		Strains	32	1.74546	0.054546	10.9680	6.968e-16***
		Residuals	64	0.31828	0.004973		
	NN	Replication	2	120	59.83	0.2888	0.7502
		Strains	32	45980	1436.88	6.9350	3.043e-11***
		Residuals	64	13260	207.19		
		Replication	2	0.004112	0.0020558	1.2642	0.28942
	NDW	Strains	32	0.094626	0.0029571	1.8184	0.02124*
		Residuals	64	0.104074	0.0016262		
	SE	Replication	2	74027	37014	32.6784	1.662e-10***
		Strains	32	268465	8390	7.4069	7.205e-10***
		Residuals	64	72491	1133		
Alfalfa	SDW	Replication	2	0.0263	0.013141	0.9160	0.4038
		Strains	45	5.9430	0.132066	9.2056	<2e-16***
		Residuals	90	1.2912	0.014346		
	NN	Replication	2	90	45.22	0.247	0.7817
		Strains	45	100328	2229.51	12.178	<2e-16***
		Residuals	90	16478	183.08		
		Replication	2	0.000674	0.00033679	1.1454	0.3227
	NDW	Strains	45	0.096534	0.00214521	7.2958	9.194<2e-16***
		Residuals	90	0.026463	0.00029403		
	SE	Replication	2	943	471.3	1.2691	0.2861
		Strains	45	146128	3247.3	8.7447	<2e-16***
		Residuals	90	33421	371.3		

Where SDW = shoot dry weight; NN = nodule number; NDW = nodule dry weight; SE = symbiotic effectiveness

Appendix 4 Temperature, NaCl and pH tolerance test of vetch isolates

No	Strain	Tem 5	Tem 10	Tem 15	Tem 40	Tem 45	0.5%NaCl	1%NaCl	2%NaCl	3%NaCl	4%NaCl	5%NaCl	6%NaCl	pH 4	pH 4.5	pH 5	pH 5.5	pH 8	pH 8.5	pH 9
1	VL1	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+
2	VK2	-	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
3	VK3	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+
4	VD4	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+
5	VK5	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+
6	VD6	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
7	VL7	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
8	VK9	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+
9	VL10	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
10	VL11	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
11	VB14	-	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+
12	VB15	-	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+
13	VK16	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-
14	VB17	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
15	VK22	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
16	VB23	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
17	VD24	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
18	VB25	-	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
19	VK26	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
20	VD27	-	+	+	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+
21	VB28	-	+	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+
22	VK30	-	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+
23	VB32	-	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
24	VK33	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+

25	VL34	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-
26	VL35	-	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-
27	VL40	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+
28	VB43	-	+	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+
29	VB63	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-
30	VB76	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-
31	VB79	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+

Appendix 5 Antibiotics and heavy metal resistant test of vetch isolates

No	Strain	Bac 5mg/ml	Pen 1mg/ml	Strep 10mg/ml	Strep 2.5mg/ml	Kan 10mg/ml	kan 1mg/ml	Cep 10mg/ml	Cep 2.5mg/ml	Amo 5mg/ml	Amp 5mg/ml	Al2(so4)3 2.5mg/ml	ZnCl2 5mg/ml	CsCl 10mg/ml	Mnso4.H2o 50mg/ml	Cuso4 10mg/ml	Cocl2 2.5mg/ml	Znso4 10mg/ml	$(CH3COO)_2Pb$ 2.5mg/ml
1	VL1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
2	VK2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
3	VK3	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
4	VD4	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
5	VK5	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-
6	VD6	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
7	VL7	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
8	VK9	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
9	VL10	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
10	VL11	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
11	VB14	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
12	VB15	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-

13	VK16	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
14	VB17	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
15	VK22	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
16	VB23	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
17	VD24	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
18	VB25	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
19	VK26	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
20	VD27	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
21	VB28	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-
22	VK30	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-
23	VB32	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-
24	VK33	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
25	VL34	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	-
26	VL35	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
27	VL40	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-
28	VB43	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
29	VB63	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-
30	VB76	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
31	VB79	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-

Appendix 6 Carbon and nitrogen source test of vetch isolates

No	Strain	D-Glucose	D-Sorbitol	D-Lactose	Dextrose	D- Fructose	Citric acid	Sucrose	D-Maltose	L-Leucine	L-Asparagine	Folic Acid	L-Tryptophan	L-Proline	L-Arginine	Thiamine	L-Alanine	Glycine	Nicotinic Acid
1	VL1	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
2	VK2	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
3	VK3	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
4	VD4	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
5	VK5	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
6	VD6	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
7	VL7	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
8	VK9	+	+	+	+	-	-	+	-	-	+	-	+	+	+	-	+	-	-
9	VL10	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+
10	VL11	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
11	VB14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	VB15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	VK16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	VB17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	VK22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	VB23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	VD24	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
18	VB25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	VK26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	VD27	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
21	VB28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

22	VK30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	VB32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	VK33	+	+	+	+	-	-	+	-	+	+	+	+	+	-	+	+	-	-
25	VL34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	VL35	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+
27	VL40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
28	VB43	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	VB63	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
30	VB76	+	+	+	+	-	-	+	-	-	+	+	+	-	-	+	+	-	-
31	VB79	+	+	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-

Appendix 7 Temperature, NaCl and pH tolerance test of alfalfa isolates

No	strain	Temp 5	Temp 10	Temp 15	Temp 40	Temp 45	0.5%NaCl	1%NaCl	2%NaCl	3%NaCl	4%NaCl	5%NaCl	6%NaCl	pH 4	pH 4.5	pH 5	pH 5.5	pH 8	pH 8.5	pH 9
1	AD13	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+
2	AB20	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+
3	AD31	-	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+
4	AD36	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
5	AB38	-	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+
6	AL41	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
7	AD42	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
8	AB45	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
9	AB46	-	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+
10	AD48	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+
11	AB49	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
12	AD50	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+

13	AB51	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
14	AB52	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
15	AB53	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
16	AB54	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
17	AL56	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
18	AB57	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
19	AB58	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
20	AL59	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
21	AB60	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
22	AL61	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
23	AD64	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+
24	AD66	-	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+
25	AB68	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
26	AL69	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+
27	AB70	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
28	AL71	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
29	AB72	-	+	+	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+
30	AL73	-	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
31	AB74	-	+	+	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+
32	AL75	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
33	AB77	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+
34	AB78	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
35	AD80	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
36	AL81	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
37	AD82	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
38	AL83	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
39	AL84	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
40	AL87	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+

41	AK88	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
42	AK89	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
43	AK91	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
44	AK92	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+

Appendix 8 Antibiotics and heavy metal resistant test of alfalfa isolates

No	strain	Bac 5mg/ml	Pen 1mg/ml	Strep 10mg/ml	Strep 2.5mg/ml	Kan 10mg/ml	kan 1mg/ml	Cep 10mg/ml	Cep 2.5mg/ml	Amo 5mg/ml	Amp 5mg/ml	Al2(so4)3 2.5mg/ml	ZnCl2 5mg/ml	CsCl 10mg/ml	Mnso4.h2o 50mg/ml	Cuso4 10mg/ml	Cocl2 2.5mg/ml	Znso4 10mg/ml	(CH3COO)2Pb 2.5mg/ml
1	AD13	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
2	AB20	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
3	AD31	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
4	AD36	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
5	AB38	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
6	AL41	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
7	AD42	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
8	AB45	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
9	AB46	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-
10	AD48	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-
11	AB49	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
12	AD50	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
13	AB51	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-
14	AB52	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
15	AB53	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
16	AB54	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
17	AL56	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-

18	AB57	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
19	AB58	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
20	AL59	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
21	AB60	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
22	AL61	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
23	AD64	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
24	AD66	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
25	AB68	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-
26	AL69	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
27	AB70	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
28	AL71	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
29	AB72	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
30	AL73	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
31	AB74	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
32	AL75	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
33	AB77	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
34	AB78	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
35	AD80	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
36	AL81	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
37	AD82	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-
38	AL83	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-
39	AL84	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
40	AL87	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
41	AK88	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
42	AK89	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
43	AK91	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-

44	AK92	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-

Appendix 9 Carbon and nitrogen source test of alfalfa isolates

No	strain	D-Glucose	D-Sorbitol	D-Lactose	Dextrose	D- Fructose	Citric acid	Sucrose	D-Maltose	L-Leucine	L-Asparagine	Folic Acid	L-Tryptophan	L-Proline	L-Arginine	Thiamine	L-Alanine	Glycine	Nicotinic Acid
1	AD13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	AB20	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
3	AD31	+	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-
4	AD36	+	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-
5	AB38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	AL41	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
7	AD42	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
8	AB45	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
9	AB46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	AD48	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
11	AB49	+	+	+	+	-	-	+	-	-	+	-	+	-	+	+	+	+	-
12	AD50	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
13	AB51	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
14	AB52	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
15	AB53	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
16	AB54	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
17	AL56	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
18	AB57	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
19	AB58	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+

20	AL59	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
21	AB60	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
22	AL61	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
23	AD64	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
24	AD66	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
25	AB68	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-
26	AL69	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
27	AB70	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
28	AL71	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
29	AB72	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
30	AL73	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
31	AB74	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	AL75	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-
33	AB77	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-	+	-	-
34	AB78	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
35	AD80	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
36	AL81	+	+	+	+	-	-	+	-	+	+	+	-	+	+	+	-	+	-
37	AD82	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
38	AL83	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
39	AL84	+	+	+	+	-	-	+	-	-	+	+	-	-	+	-	-	-	-
40	AL87	+	+	+	+	-	-	+	-	+	+	+	+	-	+	+	+	+	-
41	AK88	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
42	AK89	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
43	AK91	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
44	AK92	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-

Appendix 10 Passport data for vetch and alfalfa sampling sites

Alfalfa(*Medicago sativa)* and Vetch(*Vicia villosa)* passport data									
Host plants	Study area	Sample site	Zone	Woreda	Kebele	Latitude (N)	Longitude (E)	Altitude	Sample code
Alfalfa(*Medicago sativa)*	Debrebrehan	S1	North shewa	Baso	Gudoberet	9º47′22″	39º 39′35″	2922	AD1
		S2	North shewa	Baso	Gudoberet	9º47′22.3″	39º 39′35.8″	2925	AD2
		S3	North shewa	Baso	Gudoberet	9º47′22.2″	39º 39′35.6″	2921	AD3
		S4	North shewa	Baso	Gudoberet	9º47′22.3″	39º 39′35.6″	2921	AD4
		S5	North shewa	Baso	Gudoberet	9º47′22″	39º 39′35″	2923	AD5
	Debrezeit	S1	East shewa	Adaa	Udee	8º40′46″	39º 2′22″	1866	AB1
		S2	East shewa	Adaa	Udee	8º40′46″	39º 2′22″	1872	AB2
		S3	East shewa	Adaa	Udee	8º40′46″	39º 2′22″	1866	AB3
		S4	East shewa	Adaa	Gebesay	8º38′34.8″	39º 2′10.3″	1899	AB4
	Lemo	S1	Hadiya	Lemo	Jawi	7º30′25.7″	37º 48′21.4″	2176	AL1
		S2	Hadiya	Lemo	Jawi	7º30′25.5″	37º 48′21.3″	2173	AL2
		S3	Hadiya	Lemo	Jawi	7º30′25.3″	37º 48′21.3″	2173	AL3
		S4	Hadiya	Lemo	Jawi	7º30′25″	37º 48′21.2″	2174	AL4
		S5	Hadiya	Lemo	Jawi	7º30′25.6″	37º 48′21.3″	2177	AL5
	Kedida gamela	S1	Kambata tambaro	Kedida gamela	Azedobo	7º13′52.6″	37º51′36.9″	1987	AK1
		S2	Kambata tambaro	Kedida gamela	Azedobo	7º13′52.6″	37º51′36.9″	1987	AK2
		S3	Kambata tambaro	Kedida gamela	Azedobo	7º13′52.6″	37º51′36.9″	1987	AK3
Vetch(*Vicia*	Debrebrehan	S1	North shewa	Baso	Gudoberet	9º41′12.7″	39º 39′54″	2956	VD1

villosa)		S2	North shewa	Baso	Gudoberet	9º8′6″	39º 39′55″	3183	VD2
		S3	North shewa	Baso	Gudoberet	9º8′6″	39º 39′55″	3183	VD3
		S4	North shewa	Baso	Gudoberet	9º8′6″	39º 39′55″	3183	VD4
		S5	North shewa	Baso	Gudoberet	9º8′6″	39º 39′55″	3183	VD5
	Debrezeit	S1	East shewa	Adaa	Udee	8º40′45″	39º 2′22″	1868	VB1
		S2	East shewa	Adaa	Udee	8º40′45″	39º 2′22″	1867	VB2
		S3	East shewa	Adaa	Udee	8º40′45″	39º 2′22″	1870	VB3
		S4	East shewa	Adaa	Gebesay	8º38′34″	39º 2′10.4″	1899	VB4
		S5	East shewa	Adaa	Gebesay	8º38′34″	39º 2′10″	1898	VB5
	Lemo	S1	Hadiya	Lemo	Jawi	7º30′2.6″	37º 48′15.9″	2200	VL1
		S2	Hadiya	Lemo	Jawi	7º30′2.6″	37º 48′15.9″	2200	VL2
		S3	Hadiya	Lemo	Jawi	7º30′2.6″	37º 48′16″	2201	VL3
		S4	Hadiya	Lemo	Jawi	7º30′2.4″	37º 48′16.4″	2203	VL4
		S5	Hadiya	Lemo	Jawi	7º30′2.5″	37º 48′16.5″	2202	VL5
	Kedida gamela	S1	Kambata tambaro	Kedida gamela	Azedobo	7º14′11.8″	37º52′4.7″	2002	VK1
		S2	Kambata tambaro	Kedida gamela	Azedobo	7º14′11.8″	37º52′4.7″	2004	VK2
		S3	Kambata tambaro	Kedida gamela	Azedobo	7º14′11.9″	37º52′4.7″	2004	VK3
		S4	Kambata tambaro	Kedida gamela	Azedobo	7º14′11.9″	37º52′4.6″	2002	VK4
		S5	Kambata tambaro	Kedida gamela	Azedobo	7º14′12″	37º52′4.6″	2001	VK5