ISOLATION, CHARACTERIZATION AND EVALUATION OF SYMBIOTIC EFFICIENCY OF INDIGENOUS RHIZOBIA FROM TRAP CULTURES IN BORICHA AND LOKKA ABAYA DISTRICT, SIDAMA REGION, ETHIOPIA

MSc. THESIS

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BY

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LIST OF ABBREVIATIONS

A.s.l	Above Sea Level
BNF	Biological Nitrogen Fixation
DNA	Deoxyribonucleic Acid
cm	Centimeter
G	Gram
hr.	Hour
M	Meter
Ml	Milliliter
Mt.	Metric Ton
N	Nitrogen
rRNA	Ribosomal Ribonucleic Acid
Sec.	Second
V/V	Volume by Volume
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth

ABSTRACT

The excessive use of chemical fertilizers to boost crop yields has significant environmental drawbacks, including soil degradation, water contamination, and greenhouse gas emissions. Additionally, these fertilizers are often costly, making them inaccessible to many smallholder farmers. Leguminous crops play a crucial role in sustainable agriculture, contributing to soil fertility and crop productivity through their symbiotic relationship with rhizobia, which fix atmospheric nitrogen. Identifying and utilizing effective indigenous rhizobia is vital for improving legume yields, as these microorganisms are well-adapted to local environmental conditions, offering a cost-effective and eco-friendly alternative to chemical fertilizers. This study was aimed at isolation and characterization of indigenous rhizobia from soils in Boricha and Lokka Abaya districts in Sidama, Ethiopia, using groundnut and chickpea plants as a trapping host. Fourteen rhizobial isolates were successfully obtained after presumptive and authentication tests. All isolates tolerated alkaline conditions at pH 8, with 10 showing acid tolerance at pH 4. Most isolates grew at pH 5, except NiG2, NiG8, and NiC12, while nine tolerated pH 10. None of the isolates grew at pH 11. Notably, NiG8 was the only isolate capable of solubilizing tri-calcium phosphate. In metal resistance assessments, most isolates demonstrated growth on YEMA medium with aluminum, copper, and zinc, with 10 showing tolerance. Eight isolates exhibited resistance to manganese, and six to cobalt, while only NiG5, NiG8, and NiC12 tolerated mercury. Host range tests revealed significant host specificity and variability; Eight isolates nodulated soya bean effectively, six nodulated the common bean variety "Tafach," seven the "Nasir" variety, and five were effective on fava bean. No isolates nodulated lentil. Further analysis of symbiotic effectiveness showed significant differences ($p<0.05$) in nodule number, nodule dry weight, and shoot dry weight in fava bean, soya bean, groundnut, Tafach and Nasir. Most isolates exhibited high symbiotic effectiveness, often surpassing the nitrogen control and commercial inoculants. Isolates NiC4 and NiC7 demonstrated high effectiveness on chickpea, with values of 129% and 99.1%, respectively. On groundnut, NiG3 and NiG8 reached 103.4% effectiveness, with NiG6 also achieving 120% on the "Tafach" variety. For variety Nasir NiG6 showed 118.9% effectiveness, and NiG10 recorded 95.9%. These findings highlight the diversity and potentials of indigenous rhizobia to enhance legume productivity and soil fertility.

Key words: Indigenous rhizobia, Legume nodulation, Nitrogen fixation, Symbiotic effectiveness.

1. INTRODUCTION

1.1. Background of the Study

Ensuring food security, mitigating global climate change, and meeting the growing demand for food without affecting the environment are the challenges the world is facing today and in the years to come (FAO, 2024). Nitrogen (N) plays a vital role in agriculture since it is the most essential nutrient for plants; it is required in large quantities and serves as a fundamental building block for proteins and other biomolecules (Einarsson, 2024). Its presence is crucial for promoting healthy plant growth and ensuring overall agricultural productivity (Al-Shammary *et al.*, 2024). Estimates show that over 50 percent of the food produced worldwide depends on industrially fixed N (nitrogen) fertilizer (Ritchie and Roser, 2024). Agricultural inputs, such as nitrogen fertilizers, play a crucial role in boosting crop yields, but their excessive use has severe environmental consequences (Reay *et al.*, 2012).

Approximately half of the nitrogen fertilizer applied to the soils can be lost through ammonia (NH_3) volatilization, nitrate (NO_3^-) leaching, or emitted as nitrous oxide (N_2O) (Woliy *et al.*, 2019; Wu *et al.*, 2021). Likewise the heavy reliance on chemical fertilizers has contributed to a range of environmental problems, such as soil degradation, water pollution, and loss of biodiversity (Pahalvi *et al.*, 2021).

Furthermore, excessive fertilizer use can result in soil acidification, which gradually reduces land fertility and necessitates even more fertilizers to sustain crop yields (Abebe *et al.*, 2022). This cycle not only undermines the sustainability of agriculture but also threatens food security especially in sub-Saharan Africa countries as the land becomes increasingly unproductive and dependent on chemical inputs (Hailemariam and Asfaw, 2015). In this context, minimizing the amount of N fertilizer added to agricultural systems appears an obvious option to reduce the effect of synthetic N fertilizer on the environment (Zhang *et al.*, 2022). One such solution is the

enhancement of grain and forage Legume production by improving soil fertility through biological nitrogen fixation (BNF) using rhizobia. The term 'rhizobia' is commonly used to represent all the bacteria that can make nodules in symbiotic association with taxonomically and phylogenetically diverse groups of leguminous plants and convert atmospheric nitrogen into ammonia (Wang *et al.*, 2019).

BNF provides a cheaper and usually more effective agronomic practice for ensuring adequate N nutrition for the legumes, compared with the application of N fertilizer (Howieson and Dilworth, 2016). Adoption of this technology will have paramount importance, particularly for smallholder farmers in Ethiopia who are challenged by the high cost of chemical fertilizers (Zerssa *et al.*, 2021). By utilizing indigenous rhizobia for nitrogen fixation, farmers can reduce their reliance on expensive synthetic fertilizers, leading to more sustainable and cost-effective agricultural practices (Megersa, 2021).

Ethiopia, with its diverse agroecological zones, presents a unique opportunity for advancing research on rhizobia (Megersa, 2021). Various studies have shown that Ethiopian soils host a wide array of indigenous rhizobial species (Wolde-Meskel *et al*., 2005; Degefu *et al*., 2013; Amsalu *et al*., 2012; Degefu *et al*., 2018a), which are effective in fixing atmospheric nitrogen in association with different leguminous plants. These rhizobial species hold significant potential for inoculant development (Kebede *et al*., 2020; Temesgen and Assefa, 2020; Gunnabo *et al*., 2021). Developing inoculants from indigenous rhizobia is particularly beneficial because these bacteria are naturally adapted to local environmental conditions, making them more efficient at nitrogen fixation (Hailemariam and Asfaw, 2015; Megersa, 2021). Tapping into the potentials of indigenous rhizobia can potentially improve soil fertility, increase crop yield and reduce reliance on chemical fertilizers thereby benefiting the farmers and the environment (Wekesa *et al.*, 2022). Moreover, this technology has the potential to enhance agricultural sustainability, promote biodiversity, and contribute to long-term food security. It underscores the importance

of exploring and identifying Ethiopia's rich rhizobial diversity, which possesses significant nitrogen-fixing potential and could play a crucial role in sustainable farming practices (Hailemariam and Asfaw, 2015; Zerssa *et al*., 2021).

In this study, indigenous rhizobia isolates were trapped from smallholder farmlands in Boricha and Lokka abaya districts of Sidama Regional state. The trapped isolates were authenticated, and characterized to select elite indigenous rhizobia strains with outstanding symbiotic performance that hold the potential to be included in inoculant formulations for grain legume production.

1.2. Statement of the Problem

In Ethiopia, the application of chemical fertilizers on cropland forms the backbone of the farming system. According to the Ethiopian Central Statistical Agency (2021), during the 2020-21 production years a total of 680,033 quintals of urea, 2,339,559 quintals of NPS, 479,705 quintals of mixed fertilizers, 10,832,220 quintals of urea and NPS, and 4,877,244 quintals of both urea and mixed fertilizer were used at the national level. However, extensive use of chemical fertilizers can have significant environmental impacts including greenhouse gas emission and contamination of water bodies (Woliy *et al.,* 2019; Wu *et al.,* 2021).

Rhizobial inoculants represent a cost-effective and highly efficient agronomic solution for supplying nitrogen to legumes (Howieson and Dilworth, 2016). This technology is particularly vital for smallholder farmers in Ethiopia, who are often burdened by the prohibitive costs of chemical fertilizers (Abebe *et al*., 2022).

Beyond their economic benefits, rhizobial inoculants offer an environmentally sustainable alternative by enabling biological nitrogen fixation; This process converts atmospheric nitrogen (N_2) into plant-available ammonia (NH_3) within root nodules, thereby meeting the nitrogen

demands of legume crops (Wu *et al.*, 2021). Additionally, after harvest, the nitrogen residues left by legumes enrich the soil, reducing dependence on synthetic fertilizers and promoting long-term soil fertility (Peoples et al., 2009).

Various studies have shown that Ethiopian soils across various agro-ecological zones host a wide diversity of rhizobial species. (Wolde-Meskel *et al*., 2005; Degefu *et al*., 2013; Amsalu *et al*., 2012; Degefu *et al*., 2018a). Despite this diversity, the nitrogen fixation potentials of these rhizobia have not been fully explored (Abebe *et al*., 2022). In addition, isolating and using indigenous rhizobia from different agro-ecologies is highly recommended (Gunnabo *et al*., 2021). These strains are naturally adapted to the local environment, and can easily establish and perform optimally under specific soil and climatic conditions (Gunnabo *et al*., 2021; Wekesa *et al*., 2022). In view of this, indigenous rhizobial isolates were trapped from smallholder farmlands in Boricha and Lokka abaya districts of Sidama regional state. The trapped isolates were authenticated, and characterized to select elite indigenous rhizobia strains that have outstanding symbiotic performance and hold the potential for inoculant formulations.

1.3. Objective of the Study

1.3.1. General Objective

- The general objective of this study was to, isolate, and characterize indigenous rhizobia by trap culture method from agricultural soil samples collected from smallholder farmers' fields in the Boricha and Lokka Abaya districts in Sidama region and assess their host ranges and symbiotic effectiveness.

1.3.2. Specific Objectives

- To trap and isolate indigenous rhizobial isolates using potted soil using different host plants.
- To authenticate and characterize the isolates using morphological, biochemical, and physiological studies.
- To determine the symbiotic effectiveness and host range of the isolates under greenhouse condition.

1.4. Significance of the study

This research provides essential insights into the specific types of rhizobia that naturally associate with local legume crops, which are crucial for enhancing nitrogen fixation and improving soil fertility. This work also enriches the existing knowledge of rhizobial diversity, revealing phenotypic and symbiotic variations among indigenous strains, adapted to local environmental conditions. The study identifies superior rhizobia strains with high symbiotic effectiveness, which can be developed into inoculants as an environmentally sustainable alternative to chemical fertilizers. Ultimately, this research supports efforts to increase crop productivity, promote sustainable agricultural practices, and strengthen food security in Ethiopia.

2. REVIEW OF RELATED LITERATURE

2.1. Rhizobia

Rhizobia are characterized as gram-negative, motile, rod-shaped bacteria (Somasegaran and Hoben, 1994). They possess the ability to move in damp soil by utilizing the water films surrounding soil particles, although their self-driven movement is notably slow (Garg and Sharma, 2012).

In 1889, the Dutch microbiologist and botanist Martinus Willem Beijerinck delved into the mechanism of plant root nodules and made a groundbreaking discovery by identifying microbes in them. He became the first to isolate a bacterium from plant root nodules, naming it *Bacillus radicicola*. Initially, the term "rhizobia" was used to designate bacteria within the genus Rhizobium (Jarvis *et al.,* 1997). However, over time, it became apparent that only a fraction of rhizobia belonged to this genus, and additional genera were later identified, including *Bradyrhizobium, Sinorhizobium*, and *Mesorhizobium* (Jarvis *et al.*, 1997).

2.2. Taxonomy of rhizobia

The first classifications of rhizobia were based on cross-inoculation tests between rhizobia and their host plants (Zakhia and De Lajudie, 2001). The host plant was not the only criteria taken into account for the classification of rhizobia for which species were classified into two groups: Strains are classified into fast-growing and slow-growing categories based on their generation time. and their growth rate on culture medium (Berrada and Fikri-Benbrahim, 2014).

However, there was considerable question about the validity of this classification due to data that were inconsistent between the host range and the idea of bacterial growth speed (Garg and Sharma, 2012).This creates room for comparison techniques like plasmid analysis, serology, the coefficient of Chargaff, RNA/DNA or DNA/DNA hybridization, etc. (Shamseldin, 2017)

Based on the 16S ribosomal DNA sequence, the three primary different phylogenetic subclasses of legume symbionts that are currently known are α, β, and γ-Proteobacteria (Fig. 1).

In the subclass γ-Proteobacteria, there are eleven genera with over 98 species, two genera in the order Burkholderiales in the subclass β-Proteobacteria, and one genus in the order. (Berrada and Fikri-Benbrahim, 2014; Shamseldin, 2017).

The alpha-proteobacterial genera *Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, Devosia, Methylobacterium, Ochrobactrum,* and *Phyllobacterum* all harbor nodule-forming bacteria, and so do the beta-proteobacterial *Burkholderia* and *Cupriavidus* (Berrada and Fikri-Benbrahim, 2014; Shamseldin, 2017)

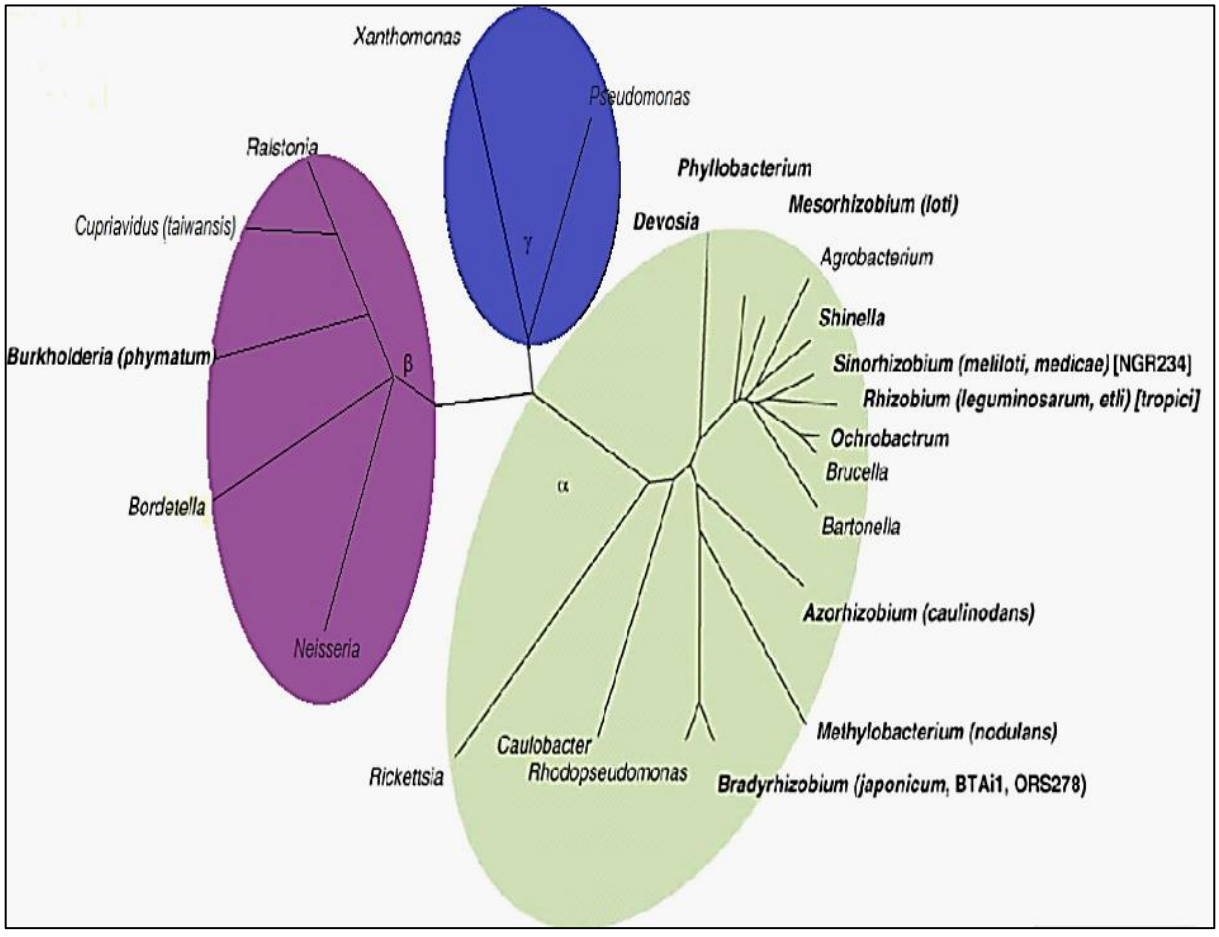

Figure 1. Simplified phylogenetic tree of Proteobacteria based on the 16S rRNA gene sequences. The rhizobial genera are shown in bold-face. Adopted from Berrada and Fikri-Benbrahim (2014).

2.3. Rhizobia Diversity in Ethiopian Soils

Ethiopia, with its diverse agroecological zones and legume species, harbors a rich diversity of rhizobia (Asfaw *et al.,* 2020). The country's agro-ecological zones, which range from highlands to lowlands, create varied ecological niches that support the growth of different legume species. This, in turn, contributes to the diversity of rhizobia in Ethiopian soils (Wolde-Meskel, 2007).

According to the study conducted by Wolde-Meskel *et al.,* (2004), cluster analysis of metabolic and genomic fingerprints showcased 18 and 25 groups, respectively. This finding underscored significant diversity within the indigenous rhizobial population in Ethiopian soils. Notably, 29% of the strains, corresponding to 25 strains, were associated with members of *Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium.*

Once again, the phylogenetic analysis conducted by Wolde-Meskel *et al.,* (2005) revealed that strains native to Ethiopia were classified within the genera *Mesorhizobium, Agrobacterium, Bradyrhizobium, Methylobacterium, Rhizobium,* and *Sinorhizobium.*

Aserse *et al.,* (2012b) investigated the diversity and phylogeny of 32 rhizobial strains isolated from common bean nodules collected from 30 different locations in Ethiopia. The examination of diversity, based on cluster analysis of AFLP fingerprints, revealed that the strains formed six genomic clusters and six single positions. In a tree constructed from concatenated sequences of glnII, rpoB, recA and partial 16S rRNA genes, these strains were distributed into seven monophyletic groups. Specifically, strains in groups B, D, E, G1, and G2 were identified as *Rhizobium phaseoli, Rhizobium etli, Rhizobium giardinii, Agrobacterium tumefaciens* complex, and *Agrobacterium. radiobacter*, respectively. On the other hand, strains in group C appeared to represent a novel species. Notably, *Rhizobium. phaseoli, Rhizobium. etli*, and the novel group emerged as the major bean-nodulating rhizobia in Ethiopia.

In Ethiopia, a diverse range of *Bradyrhizobium* species has been identified as symbionts of woody legumes such as *Crotalaria spp*., *Indigofera spp*., and *Erythrina brucei*. Besides *Bradyrhizobium spp*., sporadic symbiotic bacteria, including those belonging to *Rhizobium spp*., have been found in nodules of *Indigofera spp*. and *E. brucei.* (Aserse *et al.,* 2012a).

Through phylogenetic analysis of the housekeeping gene *recA* and symbiotic genes *nodC* and *nifH*, various Rhizobium species were identified in the root nodules of lentil (*Lens culinaris*) and faba bean (*Vicia faba*) collected from southern Ethiopia. These *included R. pisi, R. binae, R. bangladeshense, R. lentis, R. leguminosarum, R. anhuiense, R. laguerreae, R. aethiopicum,* and *R. aegypticum.* (Asfaw *et al.,* 2020).

Jaiswal *et al.,* (2016) conducted a study on the diversity of soya bean-nodulating *bradyrhizobia* in various agro-climatic regions of Ethiopia and they utilized restriction fragment length polymorphism (RFLP) analysis of the 16S–23S rRNA region, revealing the presence of 30 distinct restriction pattern types within the population. The research also involved estimating the differences in *bradyrhizobial* communities among pairs of soil samples through alpha, beta, and gamma diversity measures. The findings indicated that Southern Ethiopia exhibited the most diverse *bradyrhizobial* populations, as evidenced by the numerous RFLP clusters present. Moreover, the ratio of beta to gamma diversity in the *bradyrhizobial* community compositions was higher in the northwestern region compared to the southern region of Ethiopia.

Agricultural practices, including crop rotation, intercropping, and fallow systems, further influence rhizobial diversity in Ethiopian soils, these practices create diverse ecological conditions and introduce different legume species into the soil, resulting in the establishment of varied rhizobial populations; traditional farming systems, such as mixed cropping and agroforestry, also play a role in maintaining rhizobial diversity by providing habitats for diverse legumes and their associated rhizobia (Wolde-Meskel, 2007; Kumar *et al.,* 2013)

2.4. Legume

A legume is a plant that belongs to the *Fabaceae* (*Leguminosae*) family. The seed is also known as a pulse when it is utilized as a dry grain (Edwards *et al.*, 2019). In agriculture, legumes are mostly produced for human food, animal feed and silage, and green manure that improve soil. Legumes that are commonly known include mesquite, carob, tamarind, alfalfa, clover, beans, soya beans, chickpeas, peanuts, lentils, lupins, grass peas, etc. (Herridge *et al.*, 2008).

One of the most significant features of legumes is their ability to form symbiotic associations with nitrogen-fixing bacteria, primarily rhizobia; these bacteria reside in specialized structures called root nodules, where they convert atmospheric nitrogen into a biologically available form, facilitating nitrogen fixation (Kumar *et al.,* 2013). This process enhances soil fertility by supplying nitrogen to the plant and surrounding vegetation which gives them a vital role in agriculture due to their ability to fix atmospheric nitrogen and enrich soil fertility (Shamseldin, 2017). They are often used as cover crops, green manures, and rotation crops in agricultural systems; Legume crops, such as soya beans, peas, lentils, and alfalfa, are an essential source of protein for both human and animal consumption. Additionally, legumes contribute to sustainable farming practices by reducing the dependence on synthetic nitrogen fertilizers and promoting soil health (Herridge *et al.*, 2008).

According to Edwards *et al.,* (2019) Nineteen (19) leguminous species are cultivated as crops in various parts of Ethiopia, these are Groundnut/Peanut (*Arachis hypogea)* Soya bean (*Glycine max*), Jack bean (*Canavalia ensiformis*), Common bean (*Phaseolus vulgaris*), Lima bean (*Phaseolus lunatus*), The scarlet runner bean (*Phaseolus coccineus*), Bambara groundnut (*Vigna subterranean*), cowpea (*Vigna unguiculata*), mung bean (*Vigna radiata*), Hyacinth bean/Lablab (*Lablab purpureus)* pigeon pea (*Cajanus cajan*), white lupin (*Lupinus albus*), pearl lupin (*Lupinus mutabilis*), fenugreek (*Trigonallafoenum-graecum*), chickpea (*Cicer arietinum*), pea

(*Pisum sativum*), fava bean (*Vicia faba),* lentil (*Lens culinaris*), grass pea (*Lathyrus sativus*) the seeds of these crops are often called pulses that are valued for their high protein content.

In Ethiopia, pulse crops cover 10.7% of the total cultivated land and contribute to 10.1% of the total production of major crops (CSA, 2021). Ethiopia is the world's second-largest producer of faba beans. (0.92 Mt.) in the world after China (Dobocha and Bekele, 2022). Common beans and chickpea are also major legumes, both with a production of more than 200,000 Mt., Ethiopia ranks 6^{th} in chickpea production, and 14^{th} in the production of common beans among African countries (Megersa, 2021).

2.5. Biological nitrogen fixation (BNF)

Biological nitrogen fixation (BNF) is the process whereby atmospheric nitrogen (N_2) is reduced to ammonia in the presence of nitrogenase (Liu *et al.,* 2011). It is a natural process through which several species of bacteria convert atmospheric nitrogen into plant available nitrogen, usually ammonia (NH_3) (Mothapo, 2011).

Nitrogen is an essential and often limiting plant nutrient in crop production, Nitrogen fixation resulting from mutual symbiosis of rhizobia and cultivated legume plants is therefore critical to food security as it directly affects agricultural production (Mothapo, 2011). Biological nitrogen fixation is brought about both by free-living soil microorganisms and by symbiotic associations of microorganisms with higher plants (Liu *et al.,* 2011).

2.6. Symbiotic Interaction between Rhizobia and Leguminous Plants

The symbiotic interaction between rhizobia and leguminous plants is a remarkable example of mutualistic symbiosis, Rhizobia are soil bacteria that can fix atmospheric nitrogen and convert it into a form that can be utilized by plants and leguminous plants provide a specialized environment for rhizobia to live within their root nodules (Liu *et al.,* 2011).

The host plants produce flavonoids (and related secondary metabolites) in the rhizosphere that attract the rhizobia for attachment to the root hair Attachment is mediated by plant lectin that binds polysaccharide of the bacteria, flavonoids produced by the host plant and is perceived by Nod D protein, Upon flavonoid binding, Nod D become a transcriptional activator of other nodulation genes that trigger a developmental program leading to construction of the root nodule and entry of rhizobia into the nodule (Terpolilli *et al.,* 2012).

Nodulation genes, such as nodABC, encode enzymes that synthesize and secrete Nod factors, which are lipochitooligosaccharides (LCOs) crucial for determining host specificity by interacting with plant flavonoids (Terpolilli *et al.,* 2012). The structure of Nod factors (LCOs) varies in the length of the oligosaccharide backbone, the type of fatty acid at the non-reducing terminus, and the number and types of substituent groups attached to the molecule (Laranjo *et al.*, 2014).

Nitrogen fixation genes, such as *nif* and *fix*, encode the structural components of nitrogenase (nif HDK), the enzyme responsible for atmospheric nitrogen fixation (Terpolilli *et al.,* 2012). The rhizobia-legume symbiosis exhibits a high degree of specificity, with each rhizobial strain having a distinct host range that can vary from very narrow to remarkably broad (Laranjo *et al.*, 2014).

Numerous studies have been conducted in Ethiopia to investigate the symbiotic relationships between various legume species and rhizobia, particularly in the context of nitrogen fixation. These studies have focused on identifying compatible rhizobial strains, assessing their symbiotic efficiency. For instance, For cowpeas (*Vigna unguiculata*), *Bradyrhizobium spp.* strains such as AC7C9, AC10B, AC7C9-2, AC10C, AC10E, and AC9C4 have been identified as highly effective in establishing symbiotic relationships, significantly contributing to nitrogen fixation (Woliy *et al.*, 2019). Similarly, fenugreek (*Trigonella foenum-graecum L.*) has shown a highly effective association with *Sinorhizobium meliloti*, further promoting nitrogen enrichment in soils

(Tsegaye *et al.*, 2015). Groundnut (*Arachis hypogaea*) also forms a highly efficient symbiosis with *Bradyrhizobium spp.* strain AC7C9, enhancing soil fertility and crop yield (Argaw, 2017; Woliy *et al.*, 2019).

Soya bean (*Glycine max*) demonstrates a strong symbiotic relationship with *Sinorhizobium spp.* strains GMR120C and GMR125E, contributing to highly effective nitrogen fixation (Temesgen and Assefa, 2020). Common beans (*Phaseolus vulgaris*) are compatible with *Rhizobium* and *Phaseoli spp.*, both of which exhibit high efficiency in nitrogen fixation, as supported by various studies (Wolde-Meskel *et al.*, 2018; Aserse *et al.*, 2012b).

Mung beans (*Vigna radiata*) similarly show a highly effective relationship with *Bradyrhizobium spp.*, optimizing nitrogen uptake and improving crop productivity; Chickpeas (*Cicer arietinum*), through their symbiosis with *Mesorhizobium ciceri* strains AC16 and AC10B, as well as *Rhizobium leguminosarum* bv. *Ciceri*, also demonstrate effective nitrogen-fixing abilities (Gunabo *et al.*, 2021).

Peas (*Pisum sativum*) form effective symbiotic associations with *Rhizobium leguminosarum* bv. *Viciae* strains AC7C9 and AC17, supporting nitrogen fixation and contributing to improved soil health (Argaw and Mnalku, 2017). Finally, lentils (*Lens culinaris*) show significant nitrogen-fixing potential through their relationship with *Rhizobium leguminosarum* bv. *Viciae* (Argaw *et al.*, 2017).

2.7. Host Range and Factors Influencing Host Range Specificity

The initial signaling events during legume-rhizobia associations provide the first opportunity for partner choice by both parties; the first step of this exchange is the exudation of flavonoids from the root of the prospective host plant (Shamseldin, 2017). Legumes possess an enormous diversity of flavonoids (Weston and Mathesius, 2013). However, evidence so far suggests that only subsets of these are involved in symbiosis, Flavonoids play key roles in various

developmental and allelopathic processes in both legume and non-legume plants. Their presence can influence the host range of rhizobia through two mechanisms: by acting as infection signals that stimulate the expression of rhizobial nod genes, or by functioning as phytoalexins that trigger antimicrobial activity (Liu and Murray, 2016).

Although the ability of the host plant to recognize Nod factors during the initial cross-talk between the two parties is also a determinant of host range as subsequent events depend on the activity of nodulation-related genes such as *NIN*, which are themselves expressed in response to Nod factor signaling. Although Nod factors share the same basic structure of a chitooligosaccharide chain connected to a fatty acid, they can be extensively modified by the bacteria (Terpolilli *et al.*, 2012).

This has given rise to an exceptional diversity of Nod factors across rhizobial species specifically, variations in the extent of chitooligosaccharide polymerization, the nature of the attached fatty acid and chemical substitutions at either terminus of the molecule allow for different rhizobial species to produce chemically distinct combinations of Nod factors (Liu and Murray, 2016).

In much the same way that the ability to recognize flavonoids is the primary rhizobial determinant of host range, the ability to recognize Nod factors is the primary determinant of symbiosis specificity from the perspective of the host. Transfer of Nod factor genes between rhizobial strains may allow the recipient to colonize natural hosts of the donor strain (Liu and Murray, 2016).

Here several factors contribute to the specificity of the rhizobia-host plant interaction like Nodulation Gene Variation: Variation in nodulation genes among rhizobial strains determines their compatibility with specific legume hosts (Howieson and Dilworth, 2016).These genes encode proteins involved in Nod factor production, secretion, and perception, influencing the

molecular recognition process (Oldroyd and Downie, 2008). Also, Legume-Specific Receptor Proteins that leguminous plant possess specific receptor proteins that recognize and respond to the Nod factors produced by compatible rhizobial strains (Hungria *et al.,* 2006). Again Co-evolution between rhizobia and leguminous plants has shaped their mutualistic interactions and over time, rhizobia strains have developed adaptations to specific legume hosts, leading to host specificity (Oldroyd and Downie, 2008).

2.8. Mechanisms and Regulation of Rhizobia Nodulation

The nodulation process between rhizobia and leguminous plants is a complex and highly regulated interaction that enables nitrogen fixation and mutualistic symbiosis (Radutoiu *et al.,* 2007). Rhizobia, a diverse group of soil bacteria, establishes a symbiotic relationship with leguminous plants by colonizing specialized structures known as root nodules; the nodulation process begins with the recognition and signaling between rhizobia and leguminous plants; Rhizobia releases signaling molecules called Nod factors, which are perceived by receptor proteins on the root surface of the host plant and this recognition triggers a signaling cascade in the plant, leading to the activation of nodulation-related genes (Murray, 2011).

Upon Nod factor recognition, the plant responds by initiating a series of events leading to the formation of infection threads. These infection threads are tubular structures that grow through the root hair cell walls and into the root cortex. Root hair cells undergo a process called "curling" to accommodate the growing infection thread (Murray, 2011; Radutoiu *et al.*, 2007).

The infection threads serve as conduits for the rhizobia to enter the root cortex. Inside the root cortex, rhizobia are released from the infection threads and invade plant cells. Rhizobia differentiate into specialized forms called bacteroids within the plant cells, where nitrogen fixation occurs (Radutoiu *et al.,* 2007). Within the nodules, bacteroids undergo physiological changes to become specialized for nitrogen fixation. They possess the enzyme nitrogenase, which catalyzes the conversion of atmospheric nitrogen into ammonia, a form that can be utilized by the

plant. The plant provides bacteroids with energy-rich carbohydrates to fuel nitrogen fixation (Oldroyd and Downie, 2008).

The nodulation process is tightly regulated to ensure proper symbiotic development and prevent excessive nodulation that could be detrimental to the plant. One of the regulatory mechanisms involved in the autoregulation of nodulation is where long-distance signaling between roots and shoots suppresses nodulation in already nodulated plants (Murray *et al.*, 2011).

2.9. Nitrogen Fixation by Rhizobia

To carry out nitrogen fixation, rhizobia bacteroids require a low-oxygen environment and it is achieved through oxygen diffusion barriers and the production of oxygen-scavenging enzymes like leghaemoglobin, .then the bacteroid fix nitrogen by using nitrogenase enzyme (reduce N_2 to $NH4^+$) $NH4^+$ and it is actively transported out of the bacteroid to host plant where it is fixed into glutamine by glutamine synthase (Murray, 2011; Radutoiu *et al.*, 2007).

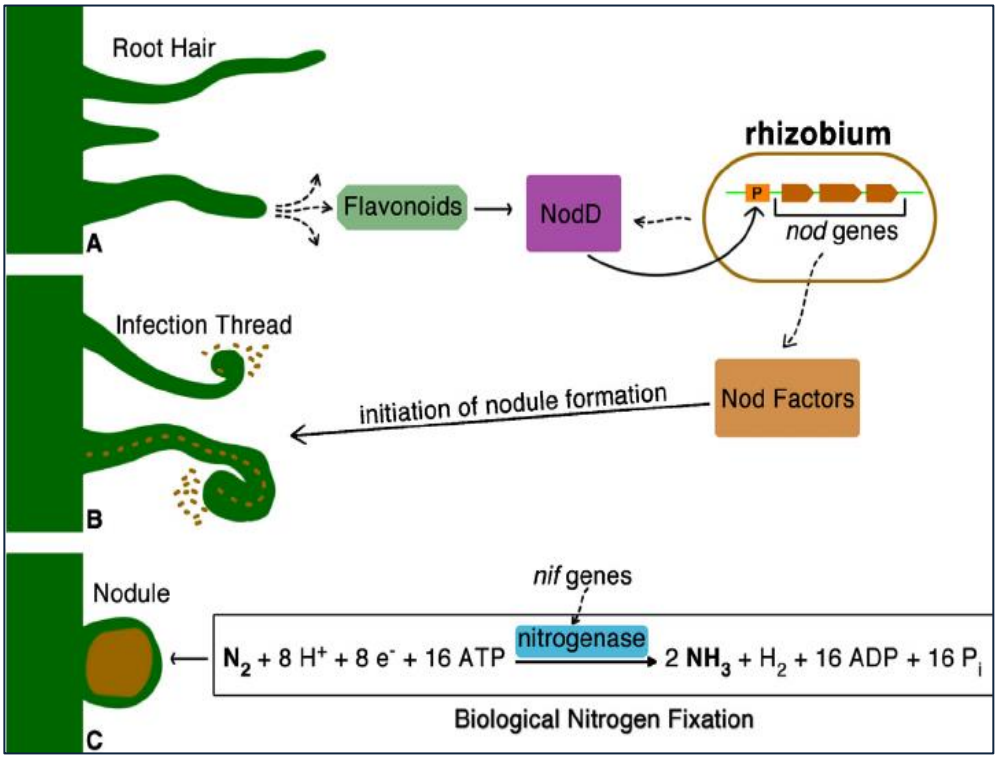

Figure 2. Schematic overview of the nodulation process and biological nitrogen fixation: Adopted from Laranjo *et al*. (2014).

2.10. Factors affecting the number of rhizobia in the soil

When rhizobia live in the soil, they are called saprophytes. Many soils contain rhizobia that live on soil organic matter, without legume partners and These are called native rhizobia, while those that farmers add as inoculants are called introduced rhizobia; the population of native rhizobia in any soil can be very diverse, including several species, and many distinct strains within each species (Slattery *et al.,* 2001). Numbers can range from zero to more than a million rhizobia per gram (g) of soil. Several factors affect the number of rhizobias in the soil. These include vegetation, cropping history, and environmental and soil conditions (Thies *et al.,* 2001).

Vegetation and cropping history means the presence of vegetation whether legumes or non-legumes appears to encourage large numbers of rhizobia. Rhizobia are found in especially large numbers in the region close to the roots of plants, known as the rhizosphere. While non-legume vegetation can encourage native rhizobia, the largest numbers of rhizobias are generally found in areas with wild or cultivated legumes (Slattery *et al.,* 2001; Thies *et al.,* 2001). The particular species of native rhizobia present in an area depends on the species of legume growing in the soil. For example, if a farmer grows peanuts (groundnuts) in a field for many years, you may expect to find a large number of native rhizobia that can form a symbiosis with peanuts. Remembering the cross-inoculation groups, these peanut rhizobia can also be expected to stimulate nodulation in mung bean, but not in soya bean (Slattery *et al.,* 2001)

Besides vegetation and cropping history, the population of rhizobia in the soil is influenced by the environment. One of the most important factors is rainfall. Areas with adequate rainfall often have large numbers of native rhizobia because the rhizobia themselves survive well in moist soils and also because more rainfall usually means that there are more legumes and other plants (Hungria *et al.,* 2006).

Rhizobia prefer soils that are moist but not waterlogged. Other conditions, such as soil temperature and acidity (pH), are also important The growth of rhizobia isolated from faba bean

was shown to grow at low and high temperature at 4 ^{o}C and 55 ^{o}C (Berrada *et al.,* 2012). High temperatures lead to increased drought intensity, due to enhanced transpiration water loss that can lead to reduction in nodule number, rhizobial growth, rate of colonization and infectious events, and can lead to delay in nodulation or restrict the nodule to the subsurface region (Gopalakrishnan *et al.*, 2014).

Large variations in tolerance to acidity factors are found both within and between Rhizobium species where fast growing rhizobia are generally considered more acid sensitive than *Bradyrhizobium,* but low pH- tolerant strains exist in many species. a pH of 6.0 to 6.8. Rhizobia are sensitive to low pH (acid soils), however Bacteria develop mechanisms to resist extremes of pH. Various physiological and biochemical mechanisms of rhizobial adaptation to acidic conditions are reported that include the exclusion and expulsion of H+, the increase of potassium and glutamate contents in the cytoplasm of stressed cells (Berrada and Fikri-Benbrahim, 2014), the change in the lipopolysaccharides composition, and the accumulation of polyamines/ glutamate concentrations in the cell (Berrada and Fikri-Benbrahim, 2014; Gopalakrishnan *et al.,* 2014).

Watkin *et al.,* (2003) reported the ability of acid tolerant Rhizobium *leguminosarum bv. trifolii* in accumulating higher level of potassium and phosphorous than an acid sensitive strain. The production of acid shock proteins (ASPs) is another common response contributing to this stress tolerance by conferring acid production on the bacteria with no alteration of the cellular pH ; Furthermore, several genes, such *as actA, actP, exoR, lpiA, actR, actS* and phrR were shown to be essential for rhizobia growth at low pH (Slattery *et al.*, 2001; Thies *et al.*, 2001).

2.11. Agricultural application of rhizobia

The ability of rhizobia to convert atmospheric nitrogen into a biologically available form has significant agricultural applications (benefits) in enhancing crop productivity, improving soil fertility, and promoting sustainable agricultural practices (Herridge *et al.,* 2008).

Inoculation with rhizobial strains has become a common agricultural practice to enhance nitrogen fixation and improve legume crop performance. Rhizobial inoculants, composed of selected strains of nitrogen-fixing bacteria, can be applied as seed treatments or soil amendments to establish symbiotic associations in cultivated fields; Inoculation ensures the presence of efficient and compatible rhizobia, particularly in areas where native rhizobial populations are limited or ineffective (Liu *et al.,* 2011).

Legume crops, in rotation with non-legume crops, can enhance soil fertility and reduce fertilizer requirements in subsequent crops by incorporating legumes into crop rotation systems, farmers can harness the nitrogen-fixing capabilities of rhizobia to replenish soil nitrogen levels naturally (Kumar *et al.,* 2013). Additionally, legume cover crops, grown during fallow periods or as living mulches, contribute to soil conservation, weed suppression, and improved nutrient cycling (Peoples *et al.,* 2009).

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nitrogen fixation contributes to the conservation of natural resources, improves soil structure, and enhances long-term soil fertility, ultimately supporting the sustainability of agricultural systems (Peoples *et al.*, 2009; Herridge *et al.*, 2008).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

Lokka Abaya and Boricha districts are located at 6°17′25″ N latitude and 37°49′44″ E longitude, 6°46′N and 38°04′E and 7°01′N and 38°24′E respectively. Boricha district, covers 588.05 km², and has a total population of 236,341 while Lokka Abaya district covers 1190 km² with 126,042 inhabitants (CSA, 2019). Both districts experience a bi-modal rainfall pattern, with the short rainy season occurring from March to May and the main rainy season from June to September. Annual rainfall ranges from 700 mm to 1,877 mm, while mean annual temperatures vary between 26°C to 35°C. The altitude of Lokka Abaya ranges from 1,500 m to 1,768 m, and Boricha lies below 2,000 m above sea level (Mellisse *et al.*, 2018). Agriculture is the primary livelihood in both districts, with rain-fed farming being common and they are characterized by the production of enset, coffee, pulses, tuber crops, vegetables, annual cereal crops and livestock keeping (Mellisse *et al.*, 2018).

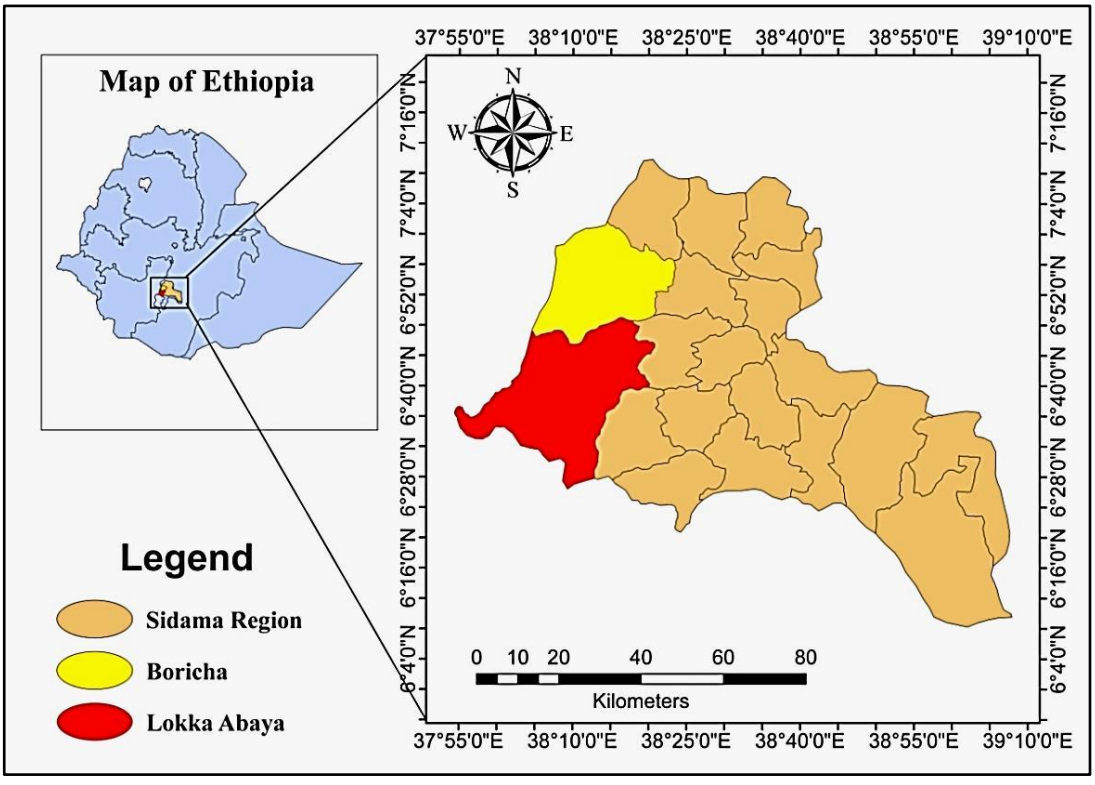

Figure 3. Map of the study area. The map was drawn using shape file sourced from (https://amu-geo.maps.arcgis.com) and processed with ArcMap software, version 10.4.

3.2. Soil sample collection sites

Thirteen Soil samples were collected from the Boricha and Lokka Abaya localities (kebeles), by randomly selecting farmer fields with a history of leguminous crop cultivation but no prior rhizobial inoculation and a fallow land. From each sampling sites, about 15 Kg of soil samples were collected from a depth of 10–15 cm, a composite was made and stored in alcohol-sterilized polyethylene plastic bags, adhering to the guidelines of Howieson and Dilworth (2016). The sampling locations were geo-referenced using a handheld GPS device (model: Garmin 48) for potential future use (Table 1.). The samples were then transported to the greenhouse at Hawassa University's main campus, where they were air-dried for 15 days.

Table 1. Soil sample collection site description

sample designation	District	kebele	Longitude	Latitude	Altitude
LAASS	Lokka Abaya	Aleta sodo	6° 40' 15.70" N	38° 17' 45.83" E	1665 m
LAASF	Lokka Abaya	Aleta sodo	6° 40' 5.69" N	38° 17' 44.33" E	1673 m
LAASC	Lokka Abaya	Aleta sodo	6° 40' 12.64" N	38° 17' 64.85" E	1669 m
LADGF	Lokka Abaya	Danshe Gambelle	6° 38' 40.61" N	38° 15' 39.50" E	1685 m
LADGMH	Lokka Abaya	Danshe Gambelle	6° 38' 55.59" N	38° 15' 38.84" E	1678 m
LASEF	Lokka Abaya	segeno	6° 44' 8.33" N	38° 15' 58.3" E	1783 m
BQAS	Boricha	Qonsor Arke	6° 54' 13.10" N	38° 21' 25.60" E	1992 m
BQAF	Boricha	Qonsor Arke	6° 54' 19.18" N	38° 21' 32.69" E	1987 m
BQCS	Boricha	Qonsore chafa	6° 55' 11.26" N	38° 19' 5.10" E	1873 m
BQCF	Boricha	Qonsore chafa	6° 55' 10.99" N	38° 19' 6.40" E	1877 m
BQCC	Boricha	Qonsore chafa	6° 55' 20.26" N	38° 19' 17.10" E	1867 m
BQOF	Boricha	Qorangoge	6° 57' 57.01" N	38° 18' 51.91" E	1855 m

BQOM	Boricha	Qorangoge	6° 57' 50.90" N	38° 18' 47.84" E	1858 m

The samples were designated using a systematic naming based on the first letters of the respective district and kebele, followed by an abbreviation indicating the field's legume history. The designations included: “F” for fallow land, “S” for soybean cultivation, “MH” for maize and haricot bean intercropping, and “C” for chickpea cultivation.

3.3. Soil analysis

After air-drying the collected soil samples, 300 grams of soil samples were taken for the analyses of various physicochemical properties. These included pH (soil acidity/alkalinity), electrical conductivity (EC), organic carbon (O/C), soil texture, total nitrogen (TN), and available phosphorus (P). The analyses were done by following the procedures outlined by Sahlemedhin and Taye (2000) at Hawassa University, college of agriculture soil laboratory.

3.4. Seed samples

Seeds of local landrace of chickpea (*Cicer arietinum*), groundnut (*Arachis hypogaea*), lentil (*Lens culinaris*), and faba bean (*Vicia faba*) were used. Meanwhile, seeds of soya bean (*Glycine max*) and the common bean (*Phaseolus vulgaris*) varieties "Nasir" and "Tafach" were obtained from the Sidama Agricultural Research Institute.

3.5. Trapping of rhizobia

Nodulation was induced using the ‘plant trap’ method with chickpea (*Cicer arietinum*) and groundnut (*Arachis hypogaea*) as host plants in the greenhouse at Hawassa University’s main campus, under a 12-hour light/dark cycle. Soil samples were placed in 2 kg capacity plastic pots, which had been surface sterilized using 70% ethanol for 5 seconds. Seeds of chickpea and groundnut, selected for uniform size, were also surface-sterilized with 70% ethanol for 5 seconds, followed by a 3% (v/v) sodium hypochlorite solution for 3 minutes. The seeds were then thoroughly rinsed with five changes of sterile distilled water and placed on sterile Petri dishes containing moistened filter paper. They were incubated at 25 °C until germination.

After germination, six seedlings were planted in each pot, with the radicle facing downward into the soil samples. One week later, the seedlings were thinned to three per pot. The experiment was conducted in triplicate and the pots were randomly arranged in the greenhouse to minimize environmental bias (figure 4A). Plants were watered every two days for duration of 55 days.

At the end of the 55-day period, the plants were carefully uprooted and washed with tap water to remove soil. Pink, undamaged nodules were collected from the roots. The nodules were then stored in sealed vials containing a desiccant (silica gel), covered with 1 cm of cotton wool, (figure 4B) following the method described by Somasegaran and Hoben (1994)

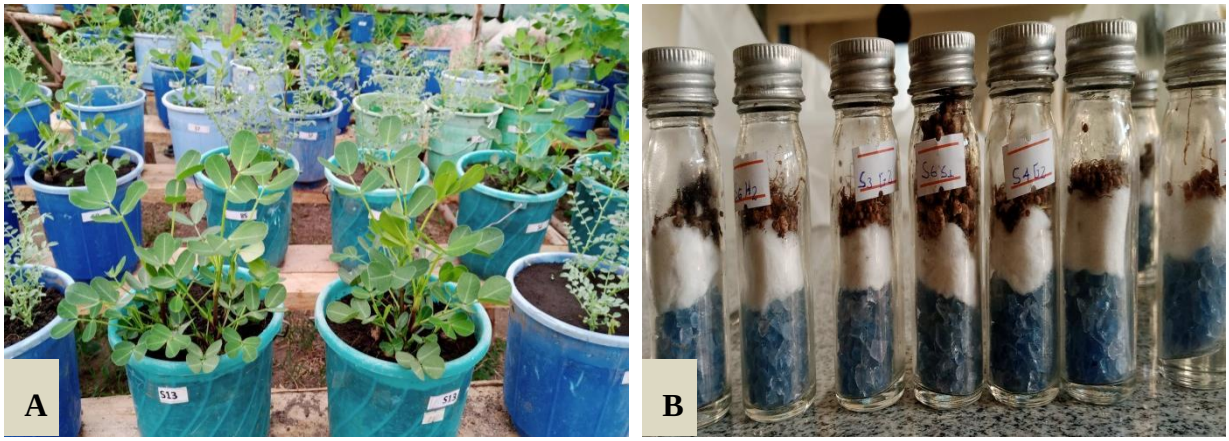

Figure 4. Trap culture. (A) Plants after 30 days in greenhouse. (B) Collected nodules on vial containing silica gel for isolation

3.6. Isolation of Rhizobia from Nodules

The desiccated nodules were rehydrated by soaking them in sterile distilled water overnight in 150 mL beakers. Once fully rehydrated, the nodules were surface sterilized by immersing them in 95% ethanol for 10 seconds, followed by treatment in a 3% (v/v) sodium hypochlorite solution for 3–4 minutes. After sterilization, the nodules were thoroughly rinsed with five changes of sterile distilled water to remove any residual sterilizing agents (Lupwayi and Haque, 1994).

The sterilized nodules were then transferred to sterile Petri dishes and crushed with a sterile, alcohol-flamed glass rod in a drop of normal saline solution (0.85% NaCl) under aseptic conditions in a laminar airflow hood (Somasegaran and Hoben, 1994).

Then 0.1ml (loop full) of the suspensions was streaked onto plates containing Yeast Extract Mannitol Agar with congo red (YEMA-CR) and incubated at 28 ± 2 °C for 3–7 days. The composition of YEMA, as described by Vincent (1970), includes mannitol (10g), dipotassium phosphate (K_2HPO_4) (0.5g), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (0.2g), sodium chloride (NaCl) (0.1g), yeast extract (0.5 g), and agar (15 g/L) in 1 liter of distilled water, with a pH of 6.8 ± 0.1. A stock solution of Congo red was prepared by dissolving 0.25 g of the dye in 100 mL of sterile distilled water. From this stock, 10 mL was added to 1 liter of YEMA. The prepared medium was sterilized by autoclaving at 121 °C for 15 minutes before use.

3.7. Purification and preservation of isolates

Individual colonies were carefully selected using a sterile inoculating loop and streaked onto fresh Yeast Extract Mannitol Agar (YEMA) plates, followed by incubation at 28 ± 2 °C for 3–7 days. To ensure the purity and uniformity of the colony morphology, the process of sub-culturing was repeated until pure and uniform colony types were obtained. Individual colony was then transferred to a YEMA slant containing 0.3% (w/v) calcium carbonate ($CaCO_3$) and incubated at 28 ± 2 °C until sufficient growth was observed. Once the culture had grown adequately, the slants were stored at 4 °C for preservation, following the method outlined by Somasegaran and Hoben (1994).

3.8. Designation of the isolates

The isolates were designated as NIC or NIG, where "NI" refers to native isolates. The letters "C" and "G" indicate the trapping host plants from which the isolates were obtained, "C" for chickpea and "G" for groundnut. Each isolate was assigned a unique number following these codes to distinguish between them.

3.9. Presumptive screening of pure cultures

All isolates were subjected to presumptive purity tests to preliminarily identify and differentiate rhizobial isolates from non-rhizobial bacteria. This was achieved through a series of methods, including Gram staining, the Peptone Glucose Test (PGT), and evaluating congo red absorption on Yeast Extract Mannitol Agar supplemented with Congo Red (YEMA-CR) (Somasegaran and Hoben, 1994).

3.9.1. Gram staining test

All isolates were subjected to Gram staining for the rapid identification of potential contaminants, following the method described by Lupwayi and Haque (1994). Using a sterile loop, a loopful of each sample was transferred onto slides, air-dried at room temperature, and then heat-fixed. The bacterial smears were stained using Gram's Method: first, crystal violet was applied for 1 minute, followed by gentle rinsing with tap water for 2–3 seconds. Next, Gram's iodine (I_2-KI) was applied for 1 minute and rinsed. Alcohol-acetone (95%) was then applied for 10 seconds, followed by another rinse. Finally, safranin (the counterstain) was applied for 20 seconds, washed with tap water, and the slides were air-dried.

The stained smears were examined under a compound light microscope using an oil immersion objective. Gram-negative bacteria appeared pink to red, while Gram-positive bacteria retained the primary dye and appeared purple.

3.9.2. Peptone-Glucose test

The isolates were streaked into a peptone-glucose medium prepared by dissolving 5 g of glucose, 10 g of peptone, 15 g of agar, and 10 mL of Bromocresol Purple (BCP) stock solution that is prepared by dissolving 1 g in 100 ml of ethanol, 1 liter of distilled water. The pH of the medium was adjusted to 6.8 using 1N NaOH and HCl, and then the medium was autoclaved at 121 ^{o}C for

15 minutes following the procedure described by Lupwayi and Haque (1994). After preparation, the inoculated plates were incubated at 28 ± 2 °C for 3 to 7 days.

3.9.3. Congo red absorption

To assess the purity of the isolates, Congo Red (CR) dye was incorporated into Yeast Extract Mannitol Agar (YEMA-CR) medium. A stock solution of CR was prepared by dissolving 0.25 g of the dye in 100 mL of sterile distilled water. From this stock, 10 mL was added to 1 liter of YEMA. The resulting medium was autoclaved at 121 °C for 15 minutes. After autoclaving, the medium was poured into sterile Petri dishes. Isolates were then streaked onto the solidified medium and incubated at 28 ±2 °C for duration of 3 to 7 days, for the observation of CR absorption (Samosagren and Hoben, 2012).

3.10. Authentication of isolates in sand culture

The ability of rhizobial isolates to form root nodules on their respective host chickpea (*Cicer arietinum*) and groundnut (*Arachis hypogaea*) was determined under greenhouse condition, according to Samosagran and Hoben (1994) and Howieson and Dilworth (2016).

3.10.1. Preparation of sterile sand

River sand was collected and all debris were manually removed. The cleaned sand was then soaked in sulfuric acid (1N) overnight. Following acid treatment, the sand was thoroughly rinsed with tap water until a neutral pH of 7 was achieved, and subsequently sun-dried. The prepared sand was then used to fill a modified Leonard jar, a two-part system constructed from commercially available cups. The lower compartment served as a reservoir for nutrient solution, while the upper compartment supported plant growth. A hole at the bottom of the upper compartment allowed a cotton wick to be positioned centrally, facilitating the movement of nutrient solution from the lower reservoir to the growth medium via capillary action. The upper compartment was covered with aluminum foil to maintain sterility and prevent contamination.

The entire setup was sterilized by autoclaving at 121 °C for 2 hours before use in the experiment (Howieson, and Dilworth, 2016).

Figure 5. Modified Leonard jars ready for autoclaving after filled with sand

3.10.2. Seed preparation and sowing

Seeds of chickpea (*Cicer arietinum*) and groundnut (*Arachis hypogaea*), selected for uniform size, were also surface-sterilized with 70% ethanol for 5 seconds, followed by a 3% (v/v) sodium hypochlorite solution for 3 minutes. The seeds were then thoroughly rinsed with five changes of sterile distilled water and placed on sterile Petri dishes containing moistened filter paper. They were incubated at 25 °C until germination. After germination, three seedlings were planted in each pot, with the radicle facing downward into the soil samples. One week later, the seedlings were thinned to one per pot.

3.10.3. Inoculant preparation and inoculation

The bacterial isolates were cultivated in 10 mL of Yeast Extract Mannitol (YEM) broth and incubated at 28 ± 2 °C until the broth reached turbidity, indicating bacterial growth. After one week of seedling germination, 1 mL of the bacterial suspension was directly inoculated at the base of each seedling to facilitate colonization and interaction with the plant roots. N+ and N-

Control groups were received no bacterial inoculation. However, the N+ receive 0.05% KNO_3 solution weekly as a nitrogen source

3.10.4. Greenhouse conditions, nutrient supply and watering

The pots were arranged in a randomized complete block design (RCBD) in the green house with the photoperiod of 12 hours day/night, at the temperature and humidity of the surrounding environment. Each pot was fertilized with 100 mL of nitrogen-free Jensen's medium to ensure consistent nutrient supply. The medium was composed of the following macronutrients: 1.0 g calcium phosphate ($CaPO_4$), 0.2 g dipotassium phosphate (K_2PO_4), 0.2 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.2 g sodium chloride (NaCl), and 0.1 g ferric chloride ($FeCl_3$).

Additionally, a trace element solution containing 0.5 g boric acid (H_3BO_3), 0.05 g sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$), 0.02 g copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 0.5 g manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$), and 0.05 g zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) was prepared. From this trace element stock solution, 5 mL was added to the medium, and distilled water was used to bring the final volume to 1000 mL, following the protocol by Howieson and Dilworth (2016). The pots were supplied with Jensen’s modified N-free nutrient solution twice per week and with sterilized, distilled water as necessary. The N+ controls were fertilized by 0.05% KNO_3 solution weekly as a nitrogen source

3.10.5. Harvesting the plants

After 55 days of growth in the greenhouse, the seedlings were carefully uprooted to avoid damage to the root system. The roots were gently washed with tap water to remove any adhering sand particles and inspected for the presence and number of nodulation.

3.11. Characterization of rhizobial isolates

3.11.1. Cultural characterization

3.11.1.1. Morphological study of the rhizobial isolates

The morphological study was done based on the shape, color, size, elevation, opacity, margin, and texture of colonies of Rhizobia on YEMA (Vincent, 1970). Then cultures were streaked on YEMA and incubated at 28 ± 2 °C for 3-7 days.

3.11.1.2. Acid-base production

The ability of the bacterial isolates to produce either acid or alkali was assessed using Yeast Extract Mannitol Agar supplemented with bromothymol blue (YEMA-BTB). The YEMA medium was prepared with 1 liter of YEMA and 5 mL of a 0.5% (w/v) bromothymol blue solution dissolved in 95% ethanol, the medium then adjusted to a pH of 6.8 and autoclaved at 121 °C for 15 minutes, culture was streaked onto the YEMA-BTB plates and incubated at 28 ± 2 °C for 3 to 7 days. The color change of the bromothymol blue indicator was used to determine acid or alkali production by the isolates, following the protocol of Somasegaran and Hoben (1994).

3.11.2. Biochemical studies of the bacteria

Catalase test: This test was performed by placing a drop of the isolated culture suspension on a clean glass slide, followed by the addition of a few drops of hydrogen peroxide (H_2O_2). The evolution of oxygen bubbles from the suspension indicated a positive catalase reaction, confirming the presence of the catalase enzyme in the isolates (Kohlerschmidt *et al.*, 2021).

Triple Sugar Iron (TSI) agar test: TSI medium was prepared and autoclaved at 121 ^{0}C for 15 minutes, poured into sterile test tubes, and allowed to solidify. The isolated cultures were inoculated into the solidified medium and incubated at 28±2 °C for 4 days. Results were recorded based on changes in color, along with the detection of gas or hydrogen sulfide (H_2S) production,

providing insights into sugar fermentation and sulfur reduction capabilities (Kohlerschmidt *et al.,* 2021).

Citrate utilization test: Simon's citrate agar medium was prepared, autoclaved at 121 ^{o}C for 15 minutes and distributed into sterile test tubes. The isolated cultures were inoculated into the test tubes and incubated at 28± 2 °C for 4 days. A color change from green to blue in the medium indicated a positive result, confirming the ability of the isolates to utilize citrate as their sole carbon source (Kohlerschmidt *et al.,* 2021).

Starch hydrolysis test: starch agar medium was prepared, autoclaved at 121 ^{0}C for 15 minutes and poured into sterile Petri plates to solidify. The isolated cultures were inoculated separately onto the medium and incubated at 28±2 °C for 4 days. After incubation, 5 mL of iodine solution was added to each plate, and the appearance of a clear zone around the bacterial growth indicated a positive result for starch hydrolysis, demonstrating the production of extracellular amylase by the isolates (Kohlerschmidt *et al.,* 2021).

3.11.3. Physiological studies of the bacteria

The Physiological study were done based on the salinity, pH, phosphate solubilization ability and temperature tolerance of Rhizobia isolates according to Amaresan *et al.,* (2022) and the result were designated as positive (+) or negative (-) for the presence or absence of growth under the tested conditions, respectively..

3.11.3.1 Salinity Tolerance

Yeast Extract Mannitol Agar (YEMA) plates were prepared with varying concentrations of sodium chloride (NaCl) ranging from 1% to 10% (w/v). The pure isolates were streaked onto the plates and incubated at 28±2 °C for 3–7 days. Growth of the isolates at different NaCl concentrations was recorded to assess their salinity tolerance.

3.11.3.2. pH Tolerance

YEMA plates were adjusted to different pH levels (4, 5, 8, 9, 10, and 11) using 1N HCl or NaOH. The isolated pure cultures were streaked on these plates and incubated at 28± 2 °C for 3–7 days. The ability of the isolates to grow under these varying pH conditions was then observed.

3.11.3.3. Temperature Tolerance

YEMA plates were prepared, and the isolated pure cultures were streaked onto the medium. The plates were incubated at different temperatures, including 4 °C, 10 °C, 15 °C, 20 °C, 35 °C, 40 °C, 45 °C, and 48 °C. The growth of the isolates was monitored at 24-hour intervals to assess their temperature tolerance.

3.11.3.4. Phosphate Solubilization Test

The ability of the isolates to solubilize tri-calcium phosphate was tested using the Basal Sperber Agar (BSA) medium, following the method of Lupwayi and Haque (1994). The BSA medium was prepared with the following ingredients: yeast extract (10 g/L), calcium chloride ($CaCl_2$) (0.1 g/L), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (0.25 g/L), tri-calcium phosphate ($Ca_3(PO_4)_2$) (5 g/L), glucose (10 g/L), and agar (10 g/L). After the components were mixed, the medium was autoclaved at 121 °C for 15 minutes. Forty-eight-hour-old isolates were streaked onto the BSA medium and incubated at 28±2 °C for 3–7 days. Phosphate solubilization was confirmed by the formation of a clear halo zone around the bacterial growth, indicating the solubilization of tri-calcium phosphate by the isolates.

3.11.3.5. Heavy metal resistance of the isolates

All isolates were tested for heavy metal tolerance on solid Yeast Extract Mannitol Agar (YEMA) medium. The YEMA medium was prepared and autoclaved at 121 °C for 15 minutes. After cooling, filter-sterilized heavy metals (using a 0.2 µm pore-size membrane) were added to the medium at the following concentrations (in µg mL^{-1}): aluminum potassium sulfate ($AlK_2(SO_4)_4$) (250), cobalt chloride ($CoCl_2$) (20), copper chloride ($CuCl_2$) (50), mercury chloride ($HgCl_2$) (10),

manganese chloride ($MnCl_2$) (500), and zinc chloride ($ZnCl_2$) (50) were added to The medium, and pH were adjusted to 6.8, as described by Maatallah *et al.,* (2002). The isolated cultures were streaked onto the prepared YEMA plates and incubated at 28± 2 °C for 3–7 days. Growth patterns were then observed.

3.12. Evaluation of host range and symbiotic effectiveness of rhizobia strains

To assess the host range and symbiotic performance of the rhizobial isolates, experiments were conducted in a greenhouse following the methods described by Howieson and Dilworth (2016). Seven leguminous crops were used as host plants: chickpea (*Cicer arietinum*), groundnut (*Arachis hypogaea*), lentil (*Lens culinaris*), faba bean (*Vicia faba*), soya bean (*Glycine max*), and two varieties of common bean (*Phaseolus vulgaris*), "Nasir" and "Tafach". Nitrogen-free sterile sand was used as the rooting medium.

The preparation of sterile sand, seed treatment, sowing, inoculant preparation, nutrient supply, watering and inoculation were conducted as previously described (**section 3.10. Authentication of isolates in sand culture**). The experiment was designed in a completely randomized manner, with three replications for each treatment. Three control treatments were included: a negative control (no nitrogen and no inoculation), a positive control (fertilized with nitrogen but no inoculation), and a reference strain for each legume except for groundnut (inoculated with a commercial inoculum designated as MBI but no nitrogen fertilizer). The positive control pots received 0.05% KNO_3 solution weekly as a nitrogen source. The plants were grown under greenhouse conditions with a 12-hour photoperiod for 45–60 days.

After the growth period, the plants were carefully harvested by uprooting, and the sand was gently removed from the roots by washing with tap water. Shoot biomass was collected by cutting the plants at the level of the sand surface, the height of the shoot was measured and then shoots from each growth unit were placed in paper bags and dried at 70 °C for 48 hours, following the protocol of Somasegaran and Hoben (1994).

Similarly, the roots were examined for nodules, which were collected, counted, and dried under the same conditions as the shoots. The dry weight of the nodules was also determined. The relative symbiotic effectiveness of each isolate was calculated based on shoot dry weight accumulation as follows:

$$\boldsymbol{SE} = \frac{\text{Inoculated plant shoot D.M.}}{\text{N}-\text{fertilized plant shoot D.M}} \text{ x } 100$$

Where, **SE**. = symbiotic effectiveness and **D.M**. = dry matter,

The nitrogen-fixing effectiveness of the isolates was ranked based on the symbiotic effectiveness (SE) values proposed by Date *et al.* (1993) as cited in Purcino *et al.* (2000). Isolates were rated as "highly effective" if SE values exceeded 85%, "effective" if SE values ranged between 55% and 85%," moderately effective" for SE values between 35% and 54%, and "ineffective" if SE values were below 35%.

3.13. Statistical Analysis

The Statistical analysis was performed using the SPSS software, Version 24. analysis for correlation between mean of shoot height, shoot dry weight, nodule number, and nodule dry weight per plant was conducted using Pearson's correlation coefficient at a significance level of $\alpha = 0.01$. And also when the ANOVA test indicated significant differences among means ($p < 0.05$), means was further evaluated using Tukey's HSD test at a significance level of $\alpha = 0.05$.

3.14. Numerical analysis

Phenotypic similarities among the rhizobial isolates were analyzed using numerical taxonomy, focusing on key phenotypic traits (37 traits) such as morphological characteristics, pH tolerance, temperature tolerance, salinity tolerance, and intrinsic resistance to heavy metals. The analysis was conducted using hierarchal clustering method using the SPSS software, Version 24.

4. RESULTS AND DISCUSSION

4.1. Soil analysis

The physiochemical analysis of the soil samples revealed variations in different parameters (Table 2). The pH of the soils ranged from 5.76 ± 0.04 (sample BQOF) to 7.24 ± 0.12 (sample LADGF), indicating slightly acidic to neutral conditions. Electrical conductivity (EC) values varied from 1.25 ± 0.01 dS/m (sample BQAS) to 4.94 ± 0.02 dS/m (sample LAASC). While, Organic carbon (O/C) content was above critical levels (0.40%), it ranged from 0.9 ± 0.1% (sample LAASS) to 3.8 ± 0.5% (sample BQAN), indicating a moderate to high level of organic matter content across most samples. However, total nitrogen (TN) was lowest at (0.5 ± 0.3) in sample LAASS and highest at 2.5 ± 0.2% in sample LAASF, reflecting a considerable variation in nitrogen content. The Soil texture varied across samples, with clay (C), sandy loam (S), loam (L), and clay loam (CL) being identified, indicating diverse soil types within the study area (Table 2).

Table 2 Physicochemical properties of collected soil samples

sample code	pH	EC (ds/m)	O/C (%)	TN (%)	P (mg/kg)	Texture
LAASS	6.54 ± 0.14	3.76 ±0.04	0.9 ±0.1	0.5 ±0.3	0.15 ±0.1	CL
LAASF	6.72 ±0.04	3.86 ±0.04	3.6 ±0.3	2.5 ±0.1	0.15 ±0.2	L
LAASC	7.02 ±0.10	4.94 ±0.02	1.1 ±0.3	1.83 ±0.04	0.24 ±0.01	C
LADGF	7.24 ±0.12	3.47 ±0.01	0.8 ±0.2	0.5 ±0.06	0.12 ±0.0	C
LADMH	6.50 ±0.01	2.57 ±0.01	1.4 ±0.6	0.3 ±0.1	0.12 ±0.01	C
LASEF	6.12 ±0.01	4.29 ±0.06	2 ±0.1	0.3 ±0.3	0.15 ±0.02	S
BQAS	6.74 ±0.02	1.25 ±0.01	1.3 ±0.3	1.6 ±0.6	0.16 ±0.0	C
BQAF	6.58 ±0.02	2.25 ±0.01	3.8 ±0.5	0.7 ±0.5	0.12 ±0.01	L
BQCS	6.86 ±0.04	2.43 ±0.02	1.5 ±0.4	1.5 ±0.7	0.11 ±0.0	L
BQCF	6.76 ±0.02	1.97 ±0.01	0.5 ±0.2	1.0 ±0.5	0.17 ±0.3	L

BQCC	6.93 ±0.03	3.92 ±0.02	1.2 ±0.2	2.0 ±0.1	0.21 ±0.4	CL
BQOF	5.76 ±0.04	2.95 ±2.43	0.9 ±0.2	1.1 ±0.5	0.07 ±0.01	C
BQOM	6.37 ±0.03	1.45 ±0.02	0.9 ±0.2	1.2 ±0.1	0.13 ±0.3	CL

The sample code used is derived from the designations listed in Table 1. EC = Electrical conductivity, O/C=Organic Carbon, TN=Total Nitrogen, P= Available Phosphorous, CL= Clay loam, C=clay, SCL= sand clay loam, L= Loam

4.2. Isolation and authentication of Rhizobia from Nodules

From 10 soil samples, a total of 18 bacterial isolates were obtained from trap culture experiments using two legume hosts, chickpea (*Cicer arietinum*) and groundnut (*Arachis hypogaea*). Of these, 6 isolates were derived from chickpea, and 12 isolates from groundnut. This result aligns with previous findings by Degefu *et al.,* (2018b), who similarly isolated 51 rhizobial strains from groundnut root nodules collected from various regions in Ethiopia. The isolates were initially screened through a presumptive test to evaluate their rhizobial characteristics. Four isolates (NiC13, NiG14, NiG16, and NiG18) were rejected after this screening process.

The exclusion of four isolates (NiC13, NiG14, NiG16, and NiG18) was due to traits such as being gram-positive, growing on glucose peptone agar, and absorbing Congo red dye aligning with established methods for distinguishing rhizobia from other soil bacteria. Rhizobia are typically gram-negative and do not grow on glucose peptone agar, as confirmed by earlier studies by Lupwayi and Hague (1994) who used these criteria to exclude contaminants and non-rhizobial species from initial screenings.

Moreover, the inability of rhizobia to absorb Congo red is well-documented, as this trait is linked to the cell wall composition of gram-negative bacteria, which differs from that of non-rhizobial, gram-positive species (Howieson and Dilworth, 2016). The remaining 14 isolates exhibited the opposite results: they were gram-negative, not grown on glucose peptone agar, and did not absorb Congo red, meeting the criteria for rhizobia (Table 3). These 14 isolates were

designated as rhizobia and were subsequently subjected to authentication tests in sand culture using their respective trapping host plants (chickpea or groundnut). All the 14 rhizobial isolates successfully induced pink nodule on their respective hosts further validating their identity as nitrogen-fixing bacteria (Table 4.) as described in Howieson and Dilworth (2016); and Somasegaran and Hoben (1994), where pink nodule formation is a reliable measure of symbiotic effectiveness in various legume-rhizobia interactions.

Table 3 Presumptive tests and authentication of the isolates

Isolates	Gram staining	Peptone-Glucose test		Congo red absorption
		Growth	Color	
NiC1	Negative	Not grown	purple	Not absorb
NiG2	Negative	Not grown	purple	Not absorb
NiG3	Negative	Not grown	purple	Not absorb
NiC4	Negative	Not grown	purple	Not absorb
NiG5	Negative	Not grown	purple	Not absorb
NiG6	Negative	Not grown	purple	Not absorb
NiC7	Negative	Not grown	purple	Not absorb
NiG8	Negative	Not grown	purple	Not absorb
NiG9	Negative	Not grown	purple	Not absorb
NiG10	Negative	Not grown	purple	Not absorb
NiC11	Negative	Not grown	purple	Not absorb
NiC12	Negative	Not grown	purple	Not absorb
NiC13	Positive	Grown	Yellow	Absorb CR
NiG14	Positive	Grown	Yellow	Absorb CR
NiG15	Negative	Not grown	purple	Not absorb
NiG16	Positive	Grown	Yellow	Absorb CR
NiG17	Negative	Not grown	purple	Not absorb
NiG18	Positive	Grown	Yellow	Absorb CR

Table 4. Authentication test of the isolates

Isolates	Authentication	
	Nodule	**Nodule Number**
NiC1	Present	30 ± 6.5
NiG2	Present	16 ± 1
NiG3	Present	17.3 ± 1.5
NiC4	Present	45 ± 8.7
NiG5	Present	32.3 ± 4.50
NiG6	Present	22.6 ± 3.5
NiC7	Present	36 ± 2
NiG8	Present	29.3 ± 2.5
NiG9	Present	20.3 ± 4.9
NiG10	Present	29 ± 7
NiC11	Present	24.3 ± 6.8
NiC12	Present	22 ± 4
NiG15	Present	17±4
NiG17	Present	16.6±4.5

4.3. Morphological study of the rhizobial isolates

The morphological characterization of 14 rhizobial isolates reveals diverse phenotypic and metabolic traits. In terms of colony size, the isolates exhibited variations, colonies of 8 isolates (NiC1, NiG3, NiG6, NiC7, NiG10, NiC12, NiG15 and NiG17) being large-sized (>2 mm), while 4 isolates (NiG5, NiG9, NiC4, and NiC11), produced medium colonies (1-2 mm). Isolates NiG8 and NiG2 formed small colonies (<1 mm). Colony margins also showed variations (Table 4) with 9 isolates having smooth entire margins, while others like NiG2 and NiG6 had filamentous edges. Some such as NiG9, NiG10 and NiG3 exhibited irregular margins (Table 4).

Regarding opacity, about 10 of the isolates displayed translucent colonies while the rest 4 isolates were opaque. Colony textures predominantly being mucoid as seen in 9 isolates, Although 5 isolates formed moist colonies (Table 4). Isolates Colony color ranged from milky white (MW)

in 5 isolates, light pink (LP) colony in 5 isolates, to yellowish white in 3 isolates. The colony shapes were predominantly circular (CI) in 9 isolates, while 4 isolates displayed an irregular (IR) shape, and 1 isolate had a concentric (CoN) shape. In terms of elevation, 8 isolates exhibited a dome-shaped elevation, while 6 isolates were flat.

The results of the morphological characterization closely aligned with the descriptions provided by Somasegaran and Hoben (1994), as well as findings from several related studies, including those by Jida and Assefa (2012), Muletta and Assefa (2012). These studies consistently highlight the phenotypic diversity observed in rhizobial isolates, particularly in colony size, shape, margin, and texture. The isolates in this study exhibited traits such as large and medium colony sizes, varied colony textures (mucoid and moist), and distinct margins (smooth, filamentous, undulate), all of which are characteristic of rhizobia, as described in earlier works.

Table 5. Morphological characterization of the rhizobial isolates

	Morphological studies						
Isolates	**Size**	**Margin**	**Opacity**	**Texture**	**Color**	**Shape**	**Elevation**
NiC1	L	E	TL	Mo	YW	CI	Dome
NiG2	S	Fil	TL	MU	LP	IR	Dome
NiG3	L	IR	Op	Mo	LP	CI	flat
NiC4	M	E	TL	Mu	MW	CI	Dome
NiG5	M	E	TL	MU	LP	CI	Dome
NiG6	L	Fil	OP	Mo	MW	IR	flat
NiC7	L	E	TL	MU	LP	CI	Dome
NiG8	S	E	TL	Mo	YW	IR	flat
NiG9	M	IR	OP	MU	MW	CoN.	flat
NiG10	L	IR	TL	MU	LP	IR	Dome
NiC11	M	E.	TL	Mo	W	CI	Dome
NiC12	L	E	TL	MU	MW	CI	flat

NiG15	L	E	TL	MU	YW	CI	flat
NiG17	L	E	OP	MU	MW	CI	Dome

Legend:- M=medium (1-2 mm), L=Large (>2mm), S= Small (<1mm), E=Entire, Fil= filamentous, IR=irregular, TL=translucent, Op=opaque, MU= mucoid, Mo=moist, YW= yellowish white, LP=Light pink, MW=milky white, W=white, CI=circular, IR=irregular, CoN.=concenteric,

4.4. Acid- base production and biochemical test

All the isolates demonstrated a change in the color of bromothymol blue (BTB) medium from green to yellow, indicating their acid production capability, a well-established trait of rhizobia (Jordan, 1984). Acid production in BTB medium is commonly used as an indicator of rhizobial activity, particularly in symbiotic nitrogen-fixing bacteria. Several studies have reported similar observations, where rhizobial isolates produced acidic reactions in growth media. For instance, studies by Amsalu *et al.,* (2012), Seid (2010), and Baye *et al.,* (2015) reported comparable acid production patterns in rhizobial isolates, reinforcing the consistency of this trait across different rhizobial species.

The biochemical test results underscore significant metabolic diversity among the isolates (Table 5). All isolates tested positive for catalase activity, indicating their ability to decompose hydrogen peroxide into water and oxygen. In the Triple Sugar Iron (TSI) test, the isolates exhibited varying fermentation profiles. Six isolates (NiC1, NiG3, NiG8, NiC11, NiC12, and NiG15) showed a K/A reaction (alkaline over acid), suggesting glucose fermentation with no gas production, while eight isolates displayed a K/K reaction (alkaline over alkaline), indicating no fermentation of glucose, lactose, or sucrose.

In the citrate utilization test, all isolates were consistently positive, demonstrating their capability to utilize citrate as the sole carbon source. Regarding the starch hydrolysis test, all isolates tested positive except for NiC1 and NiC4, suggesting that most isolates can hydrolyze starch to produce reducing sugars, as shown in (Table 5).

These findings align with previous research which reported positive catalase activity in rhizobial isolates. Wani and Khan (2013) and Gauri *et al.,* (2012) also observed that Mesorhizobium isolates exhibited positive results for catalase and citrate utilization. In the starch hydrolysis assay, Deora and Singhal, (2010) demonstrated that Rhizobium strains could utilize starch from different sources with clear zone formation, reflecting their variable starch-degrading capacities.

Table 6. Acid- base production and biochemical test result of the rhizobial isolates

Isolates	Acid-base production		Biochemical tests			
	Color	Result	Catalase test	TSI	Citrate utlzn.	Starch hydrolysis
NiC1	Y	+	+	K/A	+	-
NiG2	Y	+	+	K/K	+	+
NiG3	Y	+	+	K/A	+	+
NiC4	Y	+	+	K/K	+	-
NiG5	Y	+	+	K/K	+	+
NiG6	Y	+	+	K/K	+	+
NiC7	Y	+	+	K/K	+	+
NiG8	Y	+	+	K/A	+	+
NiG9	Y	+	+	K/K	+	+
NiG10	Y	+	+	K/K	+	+
NiC11	Y	+	+	K/A	+	+
NiC12	Y	+	+	K/A	+	+
NiG15	Y	+	+	K/A	+	+
NiG17	Y	+	+	K/K	+	+

Y= yellow, "+" =positive, "-"= Negative, K/A= Alkaline over Acid, K/k=Alkaline over Alkaline

4.5. Physiological Characterization

4.5.1. Salt tolerance

The isolates demonstrated varying degrees of tolerance to different NaCl concentrations when grown on Yeast Extract Mannitol Agar (YEMA) medium. All isolates exhibited robust growth at

1% and 2% NaCl, but their growth progressively declined as salt concentrations increased (Table 6). A total of twelve isolates were able to grow at NaCl concentrations ranging from 3% to 5%, while ten isolates managed to sustain growth at 6% NaCl. Among these, isolates NiG5, NiG6, NiC7, NiG8, NiG9, and NiC12 exhibited the highest salt tolerance, thriving at NaCl concentrations of 7% and 8%. In contrast, isolates NiC4 and NiC11 were the most salt-sensitive, only growing at 2% NaCl. None of the isolates could grow at NaCl concentrations of 9% or 10%.

This pattern of salt tolerance aligns with prior studies. Jordan (1984) reported that rhizobia can generally grow at NaCl concentrations as high as 2%, which matches the baseline for most isolates in the present study.

Maatallah *et al.*, (2002) found that only 3 out of 56 chickpea rhizobial isolates could tolerate up to 5% NaCl, showing that higher salt tolerance is rare but not unprecedented. Additionally, Seid (2010) observed that 12% of field pea isolates were able to grow at 7% NaCl, while all isolates thrived at 1% NaCl, consistent with the performance of the majority of isolates in this study. In contrast, other studies have reported more limited salt tolerance.

Amsalu *et al.*, (2012) found that 80% of their isolates were sensitive to salt, growing only at 0.1% NaCl, while only 20% were resistant to 0.52%. Baye *et al.*, (2015) reported that all test isolates grew only at 0.1% NaCl and were sensitive to higher concentrations (0.5%, 1%, and 1.5% NaCl). In line with these findings, Degefu *et al.*, (2018b) observed wide variations in salt tolerance among rhizobial isolates, with growth sharply declining as NaCl concentration increased. All 81 rhizobial isolates in their study grew at 0.5% and 1% NaCl, but the proportion that could tolerate higher concentrations rapidly diminished.

Table 7. Tolerance of the isolates to different salt concentrations

Isolates	Salt (NaCl) concentration (%)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
NiC1	+	+	+	+	+	-	-	-	-	-
NiG2	+	+	+	+	+	-	-	-	-	-
NiG3	+	+	+	+	+	+	+	-	-	-
NiC4	+	+	-	-	-	-	-	-	-	-
NiG5	+	+	+	+	+	+	+	+	-	-
NiG6	+	+	+	+	+	+	+	+	-	-
NiC7	+	+	+	+	+	+	+	+	-	-
NiG8	+	+	+	+	+	+	+	+	-	-
NiG9	+	+	+	+	+	+	+	+	-	-
NiG10	+	+	+	+	+	+	+	-	-	-
NiC11	+	+	-	-	-	-	-	-	-	-
NiC12	+	+	+	+	+	+	+	+	-	-
NiG15	+	+	+	+	+	+	-	-	-	-
NiG17	+	+	+	+	+	+	-	-	-	-

"+" = Grown, "+" "-" = Not grown

4.5.2. pH and Temperature tolerance

All isolates demonstrated tolerance to an alkaline pH of 8, with 10 isolates also capable of growing at pH 4, indicating significant acid tolerance **(Table 7)**. At pH 5, the majority of isolates exhibited growth, except for NiG2, NiG8, and NiC12. In more alkaline conditions, 9 isolates were able to tolerate pH 10. However, none of the isolates grew at pH 11, suggesting a threshold for their pH tolerance.

These findings contrast with the study by Kucuk and Kivanc (2008), which reported rhizobia could grow on YEMA medium at both pH 5 and 8, highlighting potential variability among rhizobial strains. Similarly, Maatallah *et al.,* (2002) found that rhizobia exhibited moderate

tolerance to both acidic and alkaline pH conditions, consistent with the moderate acid and alkali tolerance observed in this study.

In the temperature tolerance test, none of the isolates demonstrated growth at 4 °C, indicating that this temperature is below their viable range **(Table 7)**. Growth was initiated at 10 °C, with all isolates growing except NiC1, NiG8, and NiC12. At temperatures between 20 °C and 42 °C, all isolates exhibited robust growth, which defines their optimal temperature range. At 47 °C, a slight decline in growth was observed in certain isolates, particularly NiC1, NiG8, NiC12, and NiG17, suggesting a near-threshold tolerance for some. At 52 °C, no growth was detected in any of the isolates, confirming that this temperature exceeds the upper thermal tolerance limit for all tested rhizobia.

These findings align with the temperature tolerance ranges reported by Maatallah *et al.,* (2002), which indicated that rhizobial isolates were tolerant to temperatures between 20 °C and 30 °C. However, Kenasa *et al.,* (2014) found that rhizobial isolates from fava beans could tolerate temperatures as high as 45 °C, suggesting strain-specific thermal adaptations. Similarly, Degefu *et al.,* (2018b) reported that about 7% of cowpea isolates and 53% of groundnut isolates could grow at 40 °C, with one groundnut isolate (GH026) tolerating up to 45 °C. These findings indicate considerable variability in temperature tolerance across rhizobial strains from different host plants and environments.

Table 8. Tolerance of the isolates to different pH levels and temperature

Isolates	pH						Temperature(^{o}C)						
	4	5	8	9	10	11	4	10	20	37	42	47	52
NiC1	-	+	+	-	-	-	-	-	+	+	+	-	-
NiG2	-	-	+	+	-	-	-	+	+	+	+	+	-
NiG3	+	+	+	+	-	-	-	+	+	+	+	+	-
NiC4	+	+	+	+	+	-	-	+	+	+	+	+	-
NiG5	+	+	+	+	+	-	-	+	+	+	+	+	-
NiG6	+	+	+	+	+	-	-	+	+	+	+	+	-
NiC7	+	+	+	+	+	-	-	+	+	+	+	+	-
NiG8	-	-	+	+	+	-	-	-	+	+	+	-	-
NiG9	+	+	+	+	+	-	-	+	+	+	+	+	-
NiG10	+	+	+	+	+	-	-	+	+	+	+	+	-
NiC11	+	+	+	+	+	-	-	+	+	+	+	+	-
NiC12	-	-	+	+	+	-	-	-	+	+	+	-	-
NiG15	+	+	+	+	-	-	-	+	+	+	+	+	-
NiG17	+	+	+	+	-	-	-	+	+	+	+	-	-

"+" = Grown, "+" "-" = Not grown

4.5.3. Phosphate solubilization test

All isolates, except for NiG8, were unable to solubilize tri-calcium phosphate when cultured on basal Sperber agar medium. In contrast, isolate NiG8 exhibited a clear phosphate-solubilizing ability, as evidenced by the distinct clear zone surrounding its colony, indicative of its capacity to utilize tri-calcium phosphate. Supporting this observation, a study by Degefu *et al.,* (2018b) identified 53 isolates capable of solubilizing phosphate, confirmed by the formation of clear zones around their colonies. Similarly, Getaneh (2016) reported isolates with phosphate-solubilizing potential, exhibiting solubilization indices of 1.4 and 2.22. Furthermore, Kenasa *et al.,* (2014) isolated phosphate-solubilizing rhizobia from Faba bean (*Vicia faba L.*) nodules in acidic soils of Wollega, Ethiopia

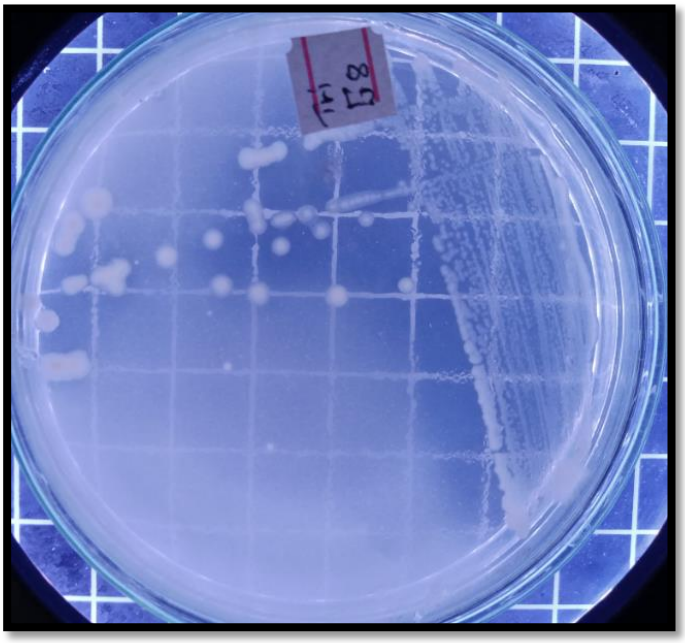

Figure 6. isolate NiG8 on basal Sperber agar medium forming a clear zone around the colony.

4.5.4. Heavy metal resistance

Most of the rhizobial isolates demonstrated growth on yeast extract mannitol agar (YEMA) medium supplemented with aluminum potassium sulfate (250 µg mL^{-1}), copper chloride (50 µg mL^{-1}), and zinc chloride (50 µg mL^{-1}). Among these, ten isolates showed tolerance to each of these metals. Additionally, eight isolates exhibited resistance to manganese chloride (500 µg mL^{-1}), while only six isolates were resistant to cobalt chloride (20 µg mL^{-1}). Notably, most isolates were sensitive to mercury chloride (10 µg mL^{-1}), with the exception of NiG5, NiG8, and NiC12, which displayed tolerance **(Table 8)**.

These findings align with earlier research by Amsalu *et al.*, (2012), who reported that rhizobial isolates exhibited resistance to manganese chloride but were sensitive to copper chloride, zinc chloride, and mercury chloride. Conversely, Baye *et al.*, (2015) found that all tested isolates showed high resistance to both zinc chloride and manganese chloride, suggesting variability in resistance patterns across different isolates and environments. Miressa (2018) further demonstrated that nearly all isolates were resistant to aluminum potassium sulfate, while most

exhibited resistance to manganese chloride. However, only about half of the isolates were resistant to zinc chloride, and fewer were resistant to cobalt chloride and copper chloride, with nearly all being sensitive to mercury chloride except for three isolates.

Table 9. Heavy metal resistance of the isolates

Isolates	Heavy metal (µg mL^{-1})					
	($AlK_2(SO_4)_4$) (250)	**($CoCl_2$) (20)**	**($CuCl_2$) (50)**	**($HgCl_2$) (10)**	**($MnCl_2$) (500)**	**($ZnCl_2$) (50)**
NiC1	+	-	+	-	+	+
NiG2	+	-	-	-	-	+
NiG3	+	+	+	-	-	+
NiC4	+	-	+	-	+	+
NiG5	+	+	-	+	-	-
NiG6	-	-	+	-	+	+
NiC7	+	-	+	-	+	-
NiG8	+	-	+	+	-	+
NiG9	-	+	-	-	-	+
NiG10	+	-	+	-	+	+
NiC11	+	-	+	-	+	+
NiC12	-	+	+	+	-	-
NiG15	+	+	+	-	+	-
NiG17	-	+	-	-	+	+

"+" = Grown, "-"= Not grown

4.6. Evaluation of host range

The cross-inoculation ability test of the rhizobial isolates reveals significant host specificity and variability in their ability to nodulate different legume species **(Table 9)**. A total of eight isolates were able to nodulate soya bean, six isolates successfully nodulated the Tafach variety of common bean, seven isolates nodulated the Nasir variety, and five isolates showed effective nodulation on fava bean. Importantly, isolates obtained using groundnut as the host trap failed to

nodulate chickpea, and vice versa, indicating a clear specificity between these two legumes. And notably no isolate form nodulation with lentil.

Table 10. Host range of the 14 rhizobial isolates tested on seven legume crops.

Isolates	Groundnut	chickpea	Soya bean	Tafach	Nasir	Fava bean	Lentil
NiC1	-	√	-	-	-	-	-
NiG2	√	-	√	√	√	-	-
NiG3	√	-	√	-	√	√	-
NiC4	-	√	-	-	-	-	-
NiG5	√	-	√	√	√	√	-
NiG6	√	-	√	√	√	-	-
NiC7	-	√	-	-	-	-	-
NiG8	√	-	√	√	√	-	-
NiG9	√	-	√	-	-	√	-
NiG10	√	-	√	√	√	√	-
NiC11	-	√	-	-	-	-	-
NiC12	-	√	-	-	-	-	-
NiG15	√	-	√	-	√	√	-
NiG17	√	-	-	√	-	-	-

The checkmarks (√) indicate that the respective rhizobial isolate was effective in nodulating the host, while dash (-) indicate a lack of nodulation**.**

Several isolates, including NiG5, NiG6, NiG2, NiG8, NiG9, and NiG15, exhibited a broader host range by nodulating multiple legume species. For instance, NiG5 effectively nodulated soya bean, fava bean, Tafach, and Nasir, while NiG6 demonstrated a similar pattern except for fava bean. This broader host compatibility is indicative of more promiscuous nodulation ability in these isolates compared to others that showed limited host specificity.

According to Somasegaran and Hoben (1994), the concept of a cross-inoculation group refers to a collection of legume species that develop effective nodules when inoculated with rhizobia

isolated from any member of that legume group. While this classification is practical, it is not perfect, as there are cases of cross-inoculation with rhizobia from outside the assigned group and failures to cross-inoculate within a group. This system is not taxonomic but has utility in understanding legume-rhizobial interactions. Certain legume-rhizobial associations are highly specific, while others exhibit promiscuity. For example, the symbiosis between *Cicer arietinum* (chickpea) and *Rhizobium sp*. is highly specific, as *Cicer arietinum* nodulate effectively only when inoculated with rhizobia isolated from its own nodules. This pattern of high specificity is consistent with the observations in this study, where isolates from chickpea failed to nodulate groundnut, and vice versa.

Woliy *et al.,* (2019) also observed that an isolate may fail to induce nodulation, even when the host plant belongs to the same cross-inoculation group or is the trapping host. They attributed this failure to several factors, including the specificity of the host genotype to particular rhizobial strain and cultivar variability. The interaction between legume host and rhizobia can be highly specific, meaning that not all strains are compatible with all cultivars, even within the same species or inoculation group. Furthermore, they emphasized that nodulation alone does not guarantee an effective symbiosis capable of fulfilling the nitrogen (N) requirements of the host. Ineffective nodules may form despite successful rhizobial infection, but these nodules may not contribute significantly to nitrogen fixation. Therefore, it is crucial to conduct careful screening and selection of elite rhizobial strains that can form effective nitrogen-fixing nodules with their legume hosts, ensuring a symbiosis that meets the plant's nitrogen demands for optimal growth and productivity. This highlights the need for not only identifying rhizobia capable of nodulation but also evaluating their symbiotic efficiency in nitrogen fixation.

4.7. Symbiotic effectiveness

On chickpea (*Cicer arietinum*), the isolate NiC4 demonstrated the highest shoot height, measuring 52.4 ± 5.32 cm, surpassing all other isolates. In comparison, the positive control (N+) reached a shoot height of 37.8 ± 3.72 cm, while the negative control (N−) exhibited a substantially lower shoot height of 26.7 ± 3.48 cm. Similarly, shoot dry weight (SDW) varied significantly across isolates ($P< 0.05$) **(Table 10).** NiC4 recorded the highest SDW at 1.46 ± 0.45 g/plant, whereas NiC11 had the lowest at 0.69 ± 0.17 g/plant. The negative control (N−) displayed a markedly lower SDW of 0.48 ± 0.17 g/plant, while the reference strain MBI showed SDW of 0.83 ± 0.17 g/plant. These results align with those of Talukdar *et al.*, (2008), who found that rhizobium inoculation significantly affected shoot dry weight, their study reported that inoculated plants achieved a 70% higher shoot dry weight than uninoculated controls. Nodule number (NN) also varied significantly ($P< 0.05$) among treatments. NiC4 produced the highest mean nodule number of 45 ± 8.72 nodules/plant, while NiC11 had the lowest at 18.7 ± 3.51 nodules/plant. The reference strain MBI formed 22.7 ± 3.79 nodules/plant.

Nodule dry weight (NDW), NiC7 achieving the highest NDW at 0.08 ± 0.01 g/plant, the reference strain MBI, which recorded an NDW of 0.05 ± 0.03 g/plant. The significant correlation between SDW and NN ($r = 0.606$, $p < 0.01$) suggests that increased shoot growth is linked to greater nodule formation. However, no significant correlations were found between NDW and other parameters **(appendix 1).**

The nitrogen-fixing effectiveness of the isolates varies significantly ($P< 0.05$) between the different treatments (Table 10). It ranged from 61.1% in NiC11 to 129.2% in NiC4. Isolate NiC4 was ranked as "highly effective" with an SE of 129.2%, alongside NiC7 (99.1%), all of which outperformed the commercial inoculant MBI. The MBI strain, with an SE of 73.5%, was ranked as "effective."

These results are consistent with previous studies, which have reported a wide range of symbiotically effective chickpea rhizobial isolates. For example, Muleta (2009) found that 17% of isolates were effective, while Laranjo *et al.,* (2008) observed 38% effectiveness. Other studies reported higher rates, with Maatallah *et al.,* (2002) identifying 77% effective isolates, Rhitu *et al.,* (2012) 83%, and Jida and Assefa (2012) were reporting the highest rate of 89%. These variations reflect differences in the effectiveness of rhizobial isolates across regions and conditions.

Table 11. Influence of isolated indigenous rhizobia on growth parameters of Chickpea

Isolates	**SH (cm plant^{-1})**	**SDW (g plant^{-1})**	**NN**	**NDW (g plant^{-1})**	**SE (%)**
NiC1	34.7 ± 3.20^{ab}	0.83 ± 0.07^{ab}	30 ± 6.56^{b}	0.06 ± 0.03^{a}	73.5
NiC4	52.4 ± 5.32^{c}	1.46 ± 0.45^{c}	45 ± 8.72^{c}	0.05 ± 0.01^{a}	129.2
NiC7	48.1 ± 6.38^{bc}	1.12 ± 0.13^{bc}	36 ± 2^{bc}	0.08 ± 0.01^{a}	99.1
NiC11	40.4 ± 4.40^{bc}	0.69 ± 0.17^{ab}	18.7 ± 3.51^{b}	0.06 ± 0.02^{a}	61.1
NiC12	43.7 ± 6.12^{bc}	0.78 ± 0.15^{ab}	22 ± 4^{b}	0.05 ± 0.02^{a}	69.0
N+	37.8 ± 3.72^{ab}	1.13 ± 0.18^{bc}	Na	Na	Na
N-	26.7 ± 3.48^{a}	0.48 ± 0.17^{a}	Na	Na	Na
MBI	35.8 ± 4.27^{ab}	0.83 ± 0.17^{ab}	22.7 ± 3.79^{b}	0.05 ± 0.03^{a}	73.5
Average	39.5 ± 8.18	0.89 ± 0.30	27.9 ± 8.92	0.09 ± 0.14	Na
F – Statistics	8.71**	6.13**	30.2**	2.9^{ns}	Na

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectivenes. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. MBI= commercial inoculant. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "**" significant at 0.05.

Inoculated groundnut (*Arachis hypogea)* plants showed significant variations at $p< 0.05$ in mean nodule number ranging from the lowest (NiG2) to the highest (NiG5), with 16 ± 1 – 32.3 ± 4.5 mean nodule number respectively (Table 11). The mean shoot dry weight (SDW) also showed significant difference at $P<0.05$ ranging from 0.31 g/plant to 0.9 g/plant from N- and plants inoculated with NiG3 and NiG8, respectively. There was correlation between nodule number and nodule dry weight at (Pearsons correlation r=0.42** at 0.01 significant level. there was also association between the nodule number and the shoot dry weight (r=0.59**, at α =0.01) (Appendix 1).

The plants inoculated with the isolates (NiG10 and NiG6) displayed the maximum mean shoot height (SH) of 39.7 ±2.3 and 37.2 ±4.1 cm /plant respectively. With respect to mean shoot height, the inoculated plants showed significant difference within each other and with respect to the +N and ‑N control at $p<0.05$

The symbiotic effectiveness expressed as percentage of shoots dry mass of inoculated plants over positive control. Accordingly, 5 isolates were highly effective, 4 isolates were effective, 3 isolates were moderate effective. And four isolates (NiG3, NiG5, NiG8 and NiG10) show greater symbiotic effectiveness percentage (Table 11).

In terms of nodule number, the observed variation ($p<0.05$) in this study is consistent with previous reports that have shown similar variability across different rhizobial isolates. Such variability in nodule formation has been linked to the genetic diversity of rhizobia, as well as environmental factors (Simbine *et al.*, 2021).

Positive association between nodule number and plant biomass has been well-documented on groundnut (Jain *et al.*, 2020). Studies like those by Mintah *et al.*, (2020) have found that higher nodule numbers, resulting from effective inoculation, tend to drive greater nitrogen fixation, ultimately increasing shoot and root biomass.

The symbiotic effectiveness observed, where isolates such as NiG3, NiG5, NiG8, and NiG10 is particularly noteworthy. This is consistent with findings from Simbine *et al.,* (2021), who highlighted that indigenous strains often show better performance in specific soil conditions due to better adaptation. Moreover, research has shown that symbiotic effectiveness can vary widely, with some isolates contributing significantly more to plant growth and yield compared to standard inoculants (Wekesa *et al.,* 2022). Also in study by Anbessa *et al.,* (2022) where indigenous rhizobial isolates, such as Dibate 2, have shown significantly higher nodulation, yield and symbiotic effectiveness value compared to uninoculated controls and some reference strains on groundnut.

Figure 7. The appearance of nodules on groundnut and Relative comparison of shoot phenotypes received isolates NiG10 and NiG6, along with N+ and N- controls

Table 12. Influence of isolated indigenous rhizobia on growth parameters of Groundnut

Isolates	SH (cm plant^{-1})	SDW (g plant^{-1})	NN	NDW (g plant^{-1})	SE (%)
NiG2	24.4 ± 1.5^{ab}	0.54 ± 0.05^{ab}	16 ± 1^{b}	0.04 ± 0.01^{a}	62.1
NiG3	33.5 ± 1.3^{abc}	0.9 ± 0.07^{c}	17.3 ± 1.5^{b}	0.15 ± 0.18^{a}	103.4
NiG5	34.5 ± 2.8^{bc}	0.87 ± 0.09^{c}	32.3 ± 4.5^{d}	0.12 ± 0.01^{a}	100
NiG6	37.2 ± 4.1^{bc}	0.77 ± 0.10^{bc}	22.7 ± 3.5^{bcd}	0.12 ± 0.02^{a}	88.5
NiG8	29.9 ± 4.3^{abc}	0.9 ± 0.02^{c}	29.3 ± 2.5^{cd}	0.14 ± 0.02^{a}	103.4
NiG9	27.6 ± 10.1^{abc}	0.6 ± 0.13^{abc}	20.3 ± 4.9^{bc}	0.03 ± 0.01^{a}	69
NiG10	39.7 ± 2.3^{c}	0.87 ± 0.17^{c}	29 ± 7^{bcd}	0.14 ± 0.03^{a}	100
NiG15	29.7 ± 8.4^{abc}	0.54 ± 0.20^{ab}	17 ± 4^{b}	0.03 ± 0.01^{a}	62.1
NiG17	29 ± 7.2^{abc}	0.37 ± 0.06^{a}	16.7 ± 4.5^{b}	0.06 ± 0.04^{a}	42.5
N+	35.3 ± 2^{bc}	0.87 ± 0.12^{c}	Na	Na	Na
N-	18.6 ± 2^{a}	0.31 ± 0.05^{a}	Na	Na	Na
Average	29.80 ± 7	0.65 ± 0.2	21.5 ± 6.2	0.08 ± 0.07	
F - Statistics	4.19**	12.4**	24.6**	3.512**	Na

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectiveness. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "**" significant at 0.05.

On soya bean (*glycine max*) plants, all parameters showing highly significant differences ($p < 0.05$). Isolate NiG3 exhibited the highest shoot height, measuring 52.3 ± 4.10 cm, significantly surpassing other isolates. This was followed by NiG2 (49.6 ± 2.91 cm) and NiG10 (48.0 ± 4.91 cm), while the lowest shoot height was recorded in plants inoculated with the negative control (N−) at 30.6 ± 3.63 cm. The reference strain MBI showed a mean shoot height of 45.8 ± 3.75 cm, which was comparable to the overall average of the isolates (Table 12).

In terms of shoot dry weight (SDW), NiG3 again outperformed others with a value of 1.01 ± 0.19 g/plant, while the reference strain MBI had an SDW of 0.87 ± 0.06 g/plant. The negative control (N−) recorded a notably lower SDW of 0.41 ± 0.3 g/plant, highlighting the importance of rhizobial inoculation for plant growth.

Regarding nodule number (NN), NiG3 produced the highest number of nodules, with an average of 32 ± 4.5 nodules per plant. NiG2 and NiG6 followed closely with 27 ± 5 and 27.7 ± 6 nodules, respectively. The lowest nodule number was observed in NiG9, which formed 12.7 ± 4.04 nodules per plant. The reference strain MBI produced an intermediate nodule number of 24.3 ± 4.61 nodules per plant. Nodule dry weight (NDW) also varied among the isolates, with NiG3 achieving the highest NDW of 0.135 ± 0.054 g/plant, followed by NiG2 with 0.065 ± 0.021 g/plant. In contrast, NiG6 showed the lowest NDW at 0.039 ± 0.033 g/plant. The reference strain MBI recorded an NDW of 0.091 ± 0.04 g/plant. A correlation was observed between SH and SDW ($r = 0.534$, $p < 0.05$), as well as between SDW and NN ($r = 0.640$, $p < 0.01$) and NN and NDW ($r = 0.522$, $p < 0.05$) (appendix 1) These results suggest that increased nodule formation positively influences both shoot growth and nodule mass, reflecting the close relationship between effective nodulation and plant biomass accumulation.

Symbiotic effectiveness (SE) ranged from 101% for NiG3, ranked as "highly effective," to 51% for NiG9, which was categorized as "moderately effective." The reference strain MBI, with an SE of 87%, was also ranked as "highly effective." Isolate NiG2 (84%) was ranked "effective," while NiG6 (76%) also performed well. Supporting these findings, studies such as Abera *et al.*, (2019), demonstrated that inoculation with indigenous rhizobial isolates like Jm-1-Bo, As-5-Aw, and Bk-3-Aw significantly enhanced soya bean growth, nodulation, and Nitrogen content. Similarly, Abawari and Hailu (2023) found that inoculation with the indigenous isolate Tgp-3 rhizobium isolate improved yield, nodule number, and biomass in soya bean. According to Abera

et al., (2019) Inoculation with indigenous rhizobial isolates also significantly increased soya bean plant height by 14-41%, shoot dry weight by 24-46%, and grain yield by 22-115.

Table 13. Influence of isolated indigenous rhizobia on growth parameters of Soya bean

Isolates	SH (cm plant^{-1})	SDW (g plant^{-1})	NN	NDW (g plant^{-1})	SE (%)
NiG2	49.6 ± 2.91^{bc}	0.84 ± 0.17^{bcd}	27 ±5.0^{cd}	0.065 ± 0.021^{abc}	84
NiG3	52.3 ± 4.10^{c}	1.01 ± 0.19^{d}	32 ±4.58^{d}	0.135 ± 0.054^{c}	101
NiG5	45 ± 3.20^{bc}	0.77 ± 0.11^{abcd}	18.3 ±3.21^{bc}	0.04 ± 0.050^{ab}	77
NiG6	39.5 ± 5.75^{bc}	0.76 ± 0.09^{abcd}	27.7 ±6.03^{cd}	0.039 ± 0.033^{ab}	76
NiG8	48.8 ± 2.40^{bc}	0.6 ± 0.13^{ab}	17.3 ±5.03^{bc}	0.044 ± 0.046^{ab}	60
NiG9	37.8 ± 3.78^{ab}	0.51 ± 0.21^{ab}	12.7 ±4.04^{b}	0.06 ± 0.013^{ab}	51
NiG10	48 ± 4.91^{bc}	0.56 ± 0.11^{ab}	19 ±3^{bc}	0.043 ± 0.02^{ab}	56
NiG15	38.8 ± 4.63^{ab}	0.62 ± 0.04^{abc}	25.7 ±3.2^{cd}	0.051 ± 0.018^{abc}	62
N+	46.7 ± 8.14^{bc}	1.0 ± 0.12^{cd}	Na	Na	NA
N-	30.6 ± 3.63^{a}	0.41 ± 0.03^{a}	Na	Na	NA
MBI	45.8 ± 3.75^{bc}	0.87 ± 0.10^{bcd}	24.3 ±6.81^{bcd}	0.091 ± 0.04^{bc}	87
Average	45.1 ±7.44	0.73 ± 0.22	22.7 ±7.10	0.059 ± 0.043	NA
F – Statistics	6.34**	7.17**	18.8**	5.0**	NA

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectivenes. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. MBI= commercial inoculant. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "**" significant at 0.05.

For the common bean variety Tafach, plants inoculated with isolate NiG6 exhibited the tallest shoot height (SH), reaching an average of 95.6 ± 5.3 cm, significantly exceeding the heights of plants inoculated with other isolates. This was followed by plants inoculated with NiG17 (89.3 ± 7 cm) and NiG8 (78.6 ± 8 cm). The shortest shoot height was observed in the negative control

(N−) at 57.8 ± 9.6 cm. The commercial reference strain (MBI) achieved a relatively high shoot height of 92.8 ± 3.9 cm, comparable to the positive control (N+) at 93.8 ± 3.5 cm (Table 13).

Significant variation ($P< 0.05$) was observed in shoot dry weight (SDW) across treatments. The highest SDW was recorded for plants inoculated with NiG6 (1.2 ± 0.2 g/plant), followed by NiG17 (0.93 ± 0.03 g/plant). In contrast, the lowest SDW was observed in plants inoculated with NiG2 (0.6 ± 0.06 g/plant) and in the negative control (N−) (0.6 ± 0.15 g/plant). The reference strain MBI produced a SDW of 1.0 ± 0.24 g/plant, which was close to the overall average across the treatments.

For nodule number (NN), plants inoculated with NiG6 formed the highest number of nodules (57.3 ± 8.5 nodules/plant), followed by plants inoculated with NiG10 (48.7 ± 8.6 nodules/plant). The lowest nodule number was recorded for plants inoculated with NiG5 (29 ± 8 nodules/plant). The reference strain MBI resulted in 34 ± 3 nodules/plant.

Nodule dry weight (NDW) also show significant variation across treatments ($p< 0.05$), isolate NiG10 exhibited the highest NDW at 0.166 ± 0.043 g/plant, followed by NiG8 (0.136 ± 0.016 g/plant) and NiG6 (0.14 ± 0.041 g/plant). The lowest NDW was observed in plants inoculated with NiG17 (0.093 ± 0.031 g/plant). The reference strain MBI had an NDW of 0.124 ± 0.081 g/plant.

Tafach shows a significant correlation between SH and SDW ($r = 0.678$, $p < 0.01$), but no other significant correlations are observed among the other parameters. Symbiotic effectiveness (SE) varied between isolates, with NiG6 ranked as "highly effective" with an SE of 120%, and the reference strain MBI also ranked as "highly effective" with an SE of 100%. Isolates NiG8 (86%) and NiG5 (83%) were ranked as "effective," while NiG10 was similarly ranked with an SE of 73%. NiG2, with an SE of 60%, was ranked as "moderately effective."

Table 14. Influence of isolated indigenous rhizobia on growth parameters of Tafach

Isolates	SH (cm plant^{-1})	SDW (g plant^{-1})	NN	NDW (g plant^{-1})	SE (%)
NiG2	58.1 ± 3.2^{a}	0.6 ± 0.06^{a}	40.7 ± 8.1^{bcd}	0.144 ± 0.061^{b}	60
NiG5	76.5 ± 7.4^{abc}	0.83 ± 0.09^{ab}	29 ± 8.0^{b}	0.102 ± 0.048^{ab}	83
NiG6	95.6 ± 5.3^{d}	1.2 ± 0.22^{b}	57.3 ± 8.5^{d}	0.14 ± 0.041^{b}	120
NiG8	78.6 ± 8^{bcd}	0.86 ± 0.11^{ab}	39 ± 6.0^{bc}	0.136 ± 0.016^{b}	86
NiG10	74.1 ± 7.7^{ab}	0.73 ± 0.06^{ab}	48.7 ± 8.6^{cd}	0.166 ± 0.043^{b}	73
NiG17	89.3 ± 7^{bcd}	0.93 ± 0.30^{ab}	37 ± 4^{bc}	0.093 ± 0.031^{b}	93
N+	93.8 ± 3.5^{cd}	1.0 ± 0.2^{ab}	Na	Na	Na
N-	57.8 ± 9.6^{a}	0.6 ± 0.15^{a}	Na	Na	Na
MBI	92.8 ± 3.9^{bcd}	1.0 ± 0.24^{ab}	34 ± 3^{bc}	0.124 ± 0.081^{ab}	100
Average	79.6 ± 15	0.87 ± 0.25	40.8 ± 10.7	0.129 ± 0.048	Na
F - Statistics	14.9**	3.46**	31.3**	5.8**	Na

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectivenes. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. MBI= commercial inoculant. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "**" significant at 0.05.

For Nasir, the isolates exhibited significant variation (P <0.05) in shoot height (SH), shoot dry weight (SDW), nodule number (NN), and nodule dry weight (NDW). NiG6 had the SH of 28 ± 6.13 cm, while NiG3 recorded the lowest SH at 16.2 ± 1.55 cm. The positive control (N+) achieved a shoot height of 33.1 ± 4.12 cm, whereas the negative control (N−) had a reduced SH of 17.3 ± 1.59 cm. NiG6 showing the highest SDW at 0.88 ± 0.12 g/plant, while NiG3 had the lowest at 0.33 ± 0.07 g/plant. The N+ control had a higher SDW of 0.74 ± 0.11 g/plant compared to the N− control at 0.38 ± 0.14 g/plant (Table 14).

Figure 8. Relative comparison of shoot phenotypes and the appearance of nodules on Nasir inoculated with isolate NiG6

In terms of nodule formation, NiG6 had the highest NN at 32.7 ±5.13 nodules/plant, while NiG3 exhibited lower NN 25.3 ± 4.93 nodules/plant. The reference strain MBI showed the highest NN at 43 ± 4.58 nodules/plant.

Symbiotic effectiveness (SE) varied between isolates, with NiG6 ranked as "highly effective" with an SE of 118.9%, while NiG10 (91.9%) and NiG15 (86%) were also ranked as "highly effective." The reference strain MBI, with an SE of 85 %, was classified as "effective." In contrast, NiG3, with the lowest SE of 44.6%, was ranked as "moderately effective." This ranking highlights NiG6, NiG10, and NiG15 outperforming the reference strain MBI.

Native rhizobia isolates have demonstrated substantial potential in enhancing legume growth, particularly in common bean varieties. According to Ouma *et al.*, (2016), the inoculation of common beans with native rhizobia resulted in a significant increase in both nodulation and shoot dry weight compared to the use of commercial inoculants. This highlights the effectiveness of indigenous rhizobial strains in improving plant growth, potentially due to their better adaptation

to local soil and environmental conditions and their enhanced symbiotic efficiency in nitrogen fixation.

Similarly, Pohajda *et al.,* (2016) reported that inoculation with indigenous rhizobial strains led to notable improvements in several agronomic traits, including nodulation, seed yield, plant nitrogen content, and seed protein levels. Among the strains tested, *Rhizobium leguminosarum* strain S21/6 exhibited the highest symbiotic efficiency, reinforcing the idea that native rhizobia can outperform commercial strains in enhancing legume productivity. These findings suggest that native strains may offer a more sustainable and effective solution for improving legume production, especially in regions with specific environmental conditions.

Furthermore, Abou-Shanab *et al.,* (2019) found that inoculating dry bean varieties 'Eclipse' and 'Redhawk' with indigenous *Rhizobium* strains OrgK9 and CIAT899 significantly boosted symbiotic performance and grain yield, particularly in organic farming systems. This further underscores the adaptability and efficiency of native rhizobia in promoting legume growth, even under low-input agricultural practices.

Table15. Influence of isolated indigenous rhizobia on growth parameters of Nasir

Isolates	**SH (cm plant^{-1})**	**SDW (g plant^{-1})**	**NN**	**NDW (g plant^{-1})**	**SE (%)**
NiG2	19.9 ±1.2 ^{ab}	0.38 ±0.04^{a}	23.7 ±7.64^{b}	0.066 ±0.01^{ab}	51.4
NiG3	16.2 ±1.55 ^{a}	0.33 ±0.07^{a}	25.3 ±4.93^{b}	0.07 ±0.02^{ab}	44.6
NiG5	23.7 ±4.52^{abc}	0.5 ±0.15^{ab}	31 ±3.61^{bc}	0.104 ±0.01^{ab}	67.6
NiG6	28 ± 6.13^{bc}	0.88 ±0.12^{c}	32.7 ±5.13^{bc}	0.113 ±0.05^{b}	118.9
NiG8	18.7± 4.30 ^{ab}	0.56 ±0.16^{ab}	28.7 ±8.02^{b}	0.052 ±0.03^{ab}	75.7
NiG10	23.9 ± 3.5 ^{abc}	0.71 ±0.06^{bc}	31.7 ±3.21^{bc}	0.151 ±0.06^{b}	95.9
NiG15	24.8 ± 4.3 ^{abc}	0.68±0.10^{bc}	30 ±5^{bc}	0.105 ±0.08^{ab}	91.9
N+	33.1 ± 4.12 ^{c}	0.74 ±0.11^{bc}	Na	Na	Na

N-	17.3 ± 1.59 ^{ab}	0.5 ± 0.14^{ab}	Na	Na	Na
MBI	27 ± 2.85^{bc}	0.63 ±0.11^{abc}	43 ±4.58^{c}	0.136 ±0.04 ^{b}	85.1
Average	23.3 ±5.99	0.59 ±0.19	3.8 ±7.23	0.1 ±0.05	Na
F - Statistics	6.08**	8.3**	24.0**	5.6**	Na

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectivenes. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. MBI= commercial inoculant. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "**" significant at 0.05.

Faba bean (*Vicia faba*) inoculated with different rhizobial isolates demonstrated notable variation in plant growth and nodulation efficiency. Isolate NiG5 showed superior growth performance, producing the tallest plants with a mean shoot height (SH) of 72.4 ± 6.10 cm, closely followed by NiG15 at 68.8 ± 2.41 cm. Conversely, NiG9 resulted in the shortest plants, with an SH of 58.1 ± 7.89 cm. The positive nitrogen control (N+), which received supplemental nitrogen, achieved an SH of 70.6 ± 3.29 cm, while the negative control (N−), uninoculated, showed a significantly lower SH of 52.7 ± 2.85 cm. In terms of shoot dry weight (SDW), the N+ control led with 1.03 ± 0.10 g/plant, followed by NiG5 with 0.81 ± 0.81 g/plant, and NiG15 with 0.79 ± 0.12 g/plant. The lowest SDW was observed in the N− treatment, at 0.57 ± 0.13 g/plant (Table 15).

Nodule number (NN), also varied significantly ($P<0.05$) among isolates. Isolate NiG15 produced the highest NN with an average of 37.8 ± 2.52 nodules per plant, followed closely by NiG3 with 36 ± 3.61 nodules. The reference strain MBI yielded an average NN of 27 ± 5.57 nodules per plant. A positive correlation was found between SH and SDW ($r = 0.530$, $p < 0.05$), indicating that increased shoot growth is often associated with higher dry weight (Appendix 1).

Symbiotic effectiveness (SE) differed across isolates, with NiG5 ranked as "effective," achieving an SE of 78.6% and surpassing the reference strain MBI, which recorded an SE of 72.8%. Other

isolates, including NiG3 (68.9%), NiG15 (76.6%), NiG9 (72.8%), and NiG10 (75.5%), were also ranked as "effective".

These findings align with previous studies. For instance, Mekonnen and Kebede (2021) reported that 52%, 35%, and 13% of rhizobial isolates were categorized as highly effective, effective, and lowly effective, respectively, in nodulating faba beans in Ethiopia's eastern Hararghe highlands. Similarly, Awlachew *et al.* (2018) found that inoculation with Rhizobium isolates significantly enhanced SH, SDW, and total nitrogen content in faba beans, with 80% of isolates demonstrating high effectiveness.

Melak (2018) also reported significant improvements in nodule dry weight, SDW, and total nitrogen accumulation upon inoculating faba beans with effective rhizobia. Additionally, Legesse and Assefa (2014) observed that 74% of indigenous rhizobia isolates were effective in nodulating faba beans, with SH dry matter production ranging from 0.47 to 1.5 g/plant.

Table 16. Influence of isolated indigenous rhizobia on growth parameters of Fava bean

Isolates	SH (cm plant^{-1})	SDW (g plant^{-1})	NN	NDW (g plant^{-1})	SE (%)
NiG3	62.4 ±3.63 ^{abc}	0.71 ±0.07^{a}	36 ±3.61^{bc}	0.108 ±0.01^{c}	68.9
NiG5	72.4 ±6.10^{c}	0.81 ±0.12^{ab}	34.3 ±4.04^{bc}	0.107 ±0.01^{c}	78.6
NiG15	68.8 ±2.41^{abc}	0.79 ±0.16^{ab}	37.8 ±2.52^{bc}	0.032 ±0.03^{bc}	76.6
NiG9	58.1 ±7.89^{bc}	0.75 ±0.10^{ab}	33.7 ±5.02^{c}	0.066 ±0.03^{ab}	72.8
NiG10	60.6 ±2.85 ^{ab}	0.78 ±0.12^{ab}	33.7 ±2.52^{bc}	0.053 ±0.01^{bc}	75.7
N+	70.8 ±3.29^{bc}	1.03 ±0.10^{b}	Na	Na	Na
N-	52.7 ±2.85^{a}	0.57 ± 0.09^{a}	Na	Na	Na
MBI	65.8 ±6.19 ^{abc}	0.75 ±0.13^{ab}	27 ±5.57^{bc}	0.067 ± 0.04^{bc}	72.8
Average	64.2 ±7.24	0.76 ±0.15	33.8 ±4.81	0.096 ± 0.14	Na
F - Statistics	5.9**	3.97**	62.6**	16.4**	Na

Average values are given (n = 3) ± standard deviation. SH=Shoot height, SDW=Shoot dry weight, NN=Nodule number, NDW=Nodule dry weight, SE=Symbiotic effectivenes. N+ = control fertilized uninoculated; N-= control non-fertilized uninoculated. MBI= commercial inoculant. Average values in the same column, followed by the same letter, are not significantly different at the 5% probability level by Tukey's test. Na = not applicable; ns = non-significant at 0.05; "∗∗" significant at 0.05.

4.8. Numerical analysis

The analysis of physiological traits revealed considerable diversity among the 14 rhizobial isolates. A dendrogram generated from the numerical assessment of their phenotypic characteristics classified the isolates into four distinct clusters, (Figure 9).

Cluster I was the largest, comprising 7 isolates (NiG6, NiG10, NiC7, NiC4, NiC11, NiG2, and NiG9) originating from diverse locations. These isolates exhibited common traits such as the ability to tolerate alkaline conditions (pH 8–9) and extreme temperatures, growing at both 10 °C and 47 °C. However, all isolates in this cluster were sensitive to mercury chloride and, with the exception of NiG9, were sensitive to cobalt chloride. Their shared tolerance to high pH and temperature suggests a degree of environmental adaptability, although their vulnerability to specific chemical stressors indicates limitations under heavy metal exposure.

Cluster II included NiC1 and NiG17, which shared similarities in colony morphology, including medium-sized colonies with entire margins, mucoid texture, and circular shapes. These isolates were sensitive to high salt concentrations (7% NaCl) but exhibited a broad temperature tolerance (10 °C–47 °C). They were also resistant to manganese chloride and zinc chloride, suggesting their ability to withstand metal toxicity while displaying uniform colony morphology.

Cluster III comprised 3 isolates (NiG3, NiG15, and NiG5) characterized by entire colony margins and circular shapes. These isolates displayed resilience to both low and high pH (4–9), as well as temperature extremes (10 °C–47 °C). They demonstrated resistance to aluminum potassium sulfate and cobalt chloride but were sensitive to mercury chloride, with the exception

of NiG5, which showed some tolerance. This group's resistance to a range of stress factors highlights their potential robustness in diverse environments.

Cluster IV consisted of NiG8 and NiC12, which were distinct from the other clusters. These isolates exhibited resistance to mercury chloride and copper chloride but were sensitive to manganese chloride. They tolerated salt concentrations up to 8%, yet were unable to grow in highly acidic conditions (pH 4). Additionally, these isolates displayed unique colony morphology, with milky white, flat colonies. Their resistance to heavy metals and salt concentrations, along with sensitivity to low pH, distinguishes them from the other groups.

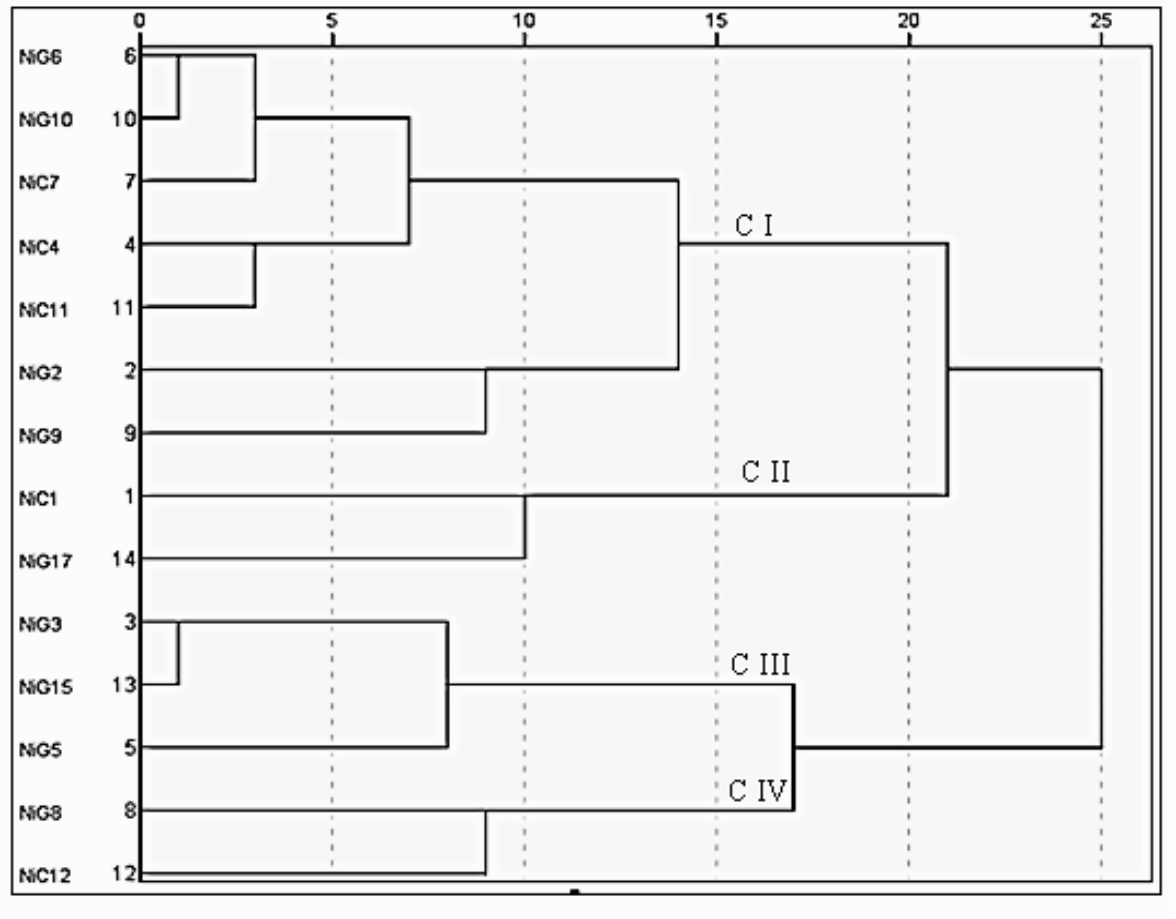

Figure 9. Dendrogram highlighting the phenotypic similarities among the rhizobial isolates

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

In conclusion, this study's comprehensive analysis of the cultural, physiological, and symbiotic characteristics of rhizobial isolates has demonstrated the presence of diverse indigenous rhizobia capable of effectively nodulating several leguminous host plants, including chickpea (*Cicer arietinum*), groundnut (*Arachis hypogaea*), faba bean (*Vicia faba*), soya bean (*Glycine max*), and two common bean (*Phaseolus vulgaris*) varieties, "Nasir" and "Tafach." Characterization of 14 isolates based on their cultural, physiological, and biochemical traits also revealed substantial diversity among the rhizobial strains, with notable differences in symbiotic effectiveness across different legume species. For example, isolates NiC4 and NiC7 demonstrated high effectiveness on chickpea, with values of 129% and 99.1%, respectively. On groundnut, NiG3 and NiG8 reached 103.4% effectiveness, with NiG6 also achieving 120% on the "Tafach" variety. For variety Nasir NiG6 showed 118.9% effectiveness, and NiG10 recorded 95.9%. These findings underscore the considerable potential of indigenous rhizobia to improve legume productivity through effective biological nitrogen fixation, with the variation in symbiotic performance highlighting the importance of selecting appropriate rhizobial strains for different legume species and localities.

5.2. Recommendations

Based on the study, the following recommendations are provided:

- Molecular characterization of rhizobial isolates should be performed using advanced techniques, such as 16S rRNA sequencing and multilocus sequence analysis, to complement phenotypic traits and uncover the true genetic diversity of the collection.
- Isolates with high symbiotic effectiveness, initially tested in sterile sand pot experiments under controlled conditions, should be evaluated in natural soil samples or directly in field environments to assess their real-world performance.
- Further characterization of the isolates is recommended, including antibiotic sensitivity testing and carbon source utilization profiling, to better understand their adaptability and resilience under different environmental conditions.
- The total nitrogen content of plants inoculated with the isolates should be analyzed to gauge the nitrogen-fixing efficiency of each strain, enabling the identification of the most effective isolates for agricultural use.
- Detailed taxonomic studies, including genomic sequencing, should be carried out to identify and characterize potentially novel rhizobial strains in the collection.
- Additional studies should be conducted across different agroecological zones of Ethiopia to fully explore the potential of indigenous rhizobial isolates. These studies will help identify strains with good symbiotic performance and environmental adaptability under diverse agricultural conditions.
- Isolates NiG6, NiG3, NiC4, NiC7, NiG8, and NiG5 are strongly recommended for field application due to their high symbiotic effectiveness observed in controlled experiments. These isolates are promising candidates for improving nitrogen fixation and legume productivity in field conditions.

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APPENDIXES

Appendix 1. Pearson correlation for different parameters

Host plant		SH	SDW	NN	NDW
Groundnut	SH	1			
	SDW	.652**	1		
	NN	.480**	.591**	1	
	NDW	.512**	.650**	.420**	1
Chickpea	SH	1			
	SDW	.617**	1		
	NN	.577**	.606**	1	
	NDW	-0.235	-0.204	-0.091	1
Soya bean	SH	1			
	SDW	.534**	1		
	NN	0.313	.438*	1	
	NDW	0.239	.640**	.522**	1
Tafach	SH	1			
	SDW	.678**	1		
	NN	0.214	0.287	1	
	NDW	-0.214	0.258	0.335	1
Nasir	SH	1			
	SDW	.645**	1		
	NN	.550**	.435*	1	
	NDW	0.297	0.336	0.276	1
Fava bean	SH	1			
	SDW	.530**	1		
	NN	-0.164	-0.032	1	
	NDW	-0.081	-0.034	-0.282	1

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).SH=shoot height, SDW =Shoot dry weight, NN= Nodule number, NDW=Nodule dry weight.

Appendix 2. Description of soil sample collection sites

No.	sample code	wored a	kebele	Coordinates	Altitude	Field type	Used inputs	Obtained isolates
1	**LAASS**	Lokka Abaya	Aleta sodo	6° 40' 15.70" N 38° 17' 45.83" E	1665 m	Soya bean cropped land	NPS, and liquid commercial biofertilizer called eco green	NiG5, NiG3
2	**LAASF**	Lokka Abaya	Aleta sodo	6° 40' 5.69" N 38° 17' 44.33" E	1673 m	No history of cropping	–	NiC11
3	**LAASC**	Lokka Abaya	Aleta sodo	6° 40' 12.64" N 38° 17' 64.85" E	1669 m	chickpea cropped land		NiC12
4	**LADGF**	Lokka Abaya	Danshe Gambelle	6° 38' 40.61" N 38° 15' 39.50" E	1685 m	No history of croping	–	-
5	**LADGMH**	Lokka Abaya	Danshe Gambelle	6° 38' 38.59" N 38° 15' 38.84" E	1678 m	Haricot bean intercropped with maize	No use of chemical fertlizers and inoculant	NiG6, NiG9
6	**LASEF**	Lokka Abaya	segeno	6° 44' 8.33" N 38° 15' 58.3" E	1783 m	No history of cropping		NiG15
7	**BQAS**	Borich a	Qonsor Arke	6° 54' 19.18" N 38° 21' 32.69" E	1987 m	Soya bean cropped land		-
8	**BQAF**	Borich a	Qonsor Arke	6° 54' 13.10" N, 38° 21' 25.60" E,	1992 m	No history of cropping	–	NiC7
9	**BQCS**	Borich a	Qonsor e chafa	6° 55' 11.26" N 38° 19' 5.10" E	1873 m	Soya bean cropped land	NPS, and cow dung were used and TGX seed variety	NiG2, NiG17
10	**BQCF**	Borich a	Qonsor e chafa	6° 55' 10.99" N 38° 19' 6.40" E	1877 m	No history of cropping	–	NiG8, NiC1
11	**BQCC**	Borich a	Qonsor e chafa	6° 55' 20.26" N 38° 19' 17.10" E	1867	chickpea cropped land		NiC4
12	**BQOF**	Borich a	Qorang oge	6° 57' 57.01" N 38° 18' 51.91" E	1855 m	No history of cropping	–	-
13	**BQOM**	Borich a	Qorang oge	6° 57' 50.90" N 38° 18' 47.84" E	1858 m	maize intercropped with Common bean	Urea and Dap were used	NiG10

Appendix 3. Raw data from soil physicochemical analysis

sample code	pH	Electrical conductivity (EC)	Organic carbon (O/C)	Total nitrogen (TN)	Available phosphorus (P)	Soil texture		% of clay	silt +clay (%)	silt (%)	sand %
						first read B (23^oC)	second read A (23^oC)				
LAASS	6.5	3.77	0.9	0.7	0.151	30.3	17.3	33.6	59.6	26	40.4
	6.42	3.72	0.8	0.2	0.149	29.3	18.3	35.6	57.6	22	42.4
	6.7	3.79	1	0.6	0.153	30.3	17.3	33.6	59.6	26	40.4
LAASF	6.77	3.86	3.8	2.4	0.153	30.3	12.3	23.6	59.6	36	40.4
	6.71	3.82	3.2	2.6	0.15	28.3	11.3	21.6	55.6	34	44.4
	6.69	3.89	3.7	2.5	0.149	32.3	13.3	25.6	63.6	38	36.4
LAASC	7.04	4.92	0.8	3.6	0.243	33.3	25.3	49.6	65.6	16	34.4
	6.91	4.94	1.3	4	0.242	31.3	24.3	47.6	61.6	14	38.4
	7.11	4.96	1.2	3.3	0.243	31.3	25.3	49.6	61.6	12	38.4
LADGF	7.33	3.47	0.6	0.5	0.124	36.3	25.3	49.6	71.6	22	28.4
	7.11	3.48	0.8	0.7	0.123	36.3	24.3	47.6	71.6	24	28.4
	7.29	3.47	0.9	0.2	0.123	34.3	26.3	51.6	67.6	16	32.4
LADGMH	6.5	2.57	1.8	0.33	0.122	32.3	26.3	51.6	63.6	12	36.4
	6.5	2.57	1.6	0.3	0.123	32.3	25.3	49.6	63.6	14	36.4
	6.51	2.58	0.7	0.4	0.122	31.3	24.3	47.6	61.6	14	38.4
LASEF	6.11	4.32	2.1	0.6	0.149	22.3	15.3	29.6	43.6	14	56.4
	6.12	4.32	1.9	0.3	0.146	21.3	17.3	33.6	41.6	8	58.4
	6.13	4.22	2	0.1	0.145	23.3	15.3	29.6	45.6	16	54.4
BQAS	6.75	1.25	1.6	0.9	0.154	28.3	22.3	43.6	55.6	12	44.4
	6.72	1.24	1.3	1.9	0.159	27.3	21.3	41.6	53.6	12	46.4

sample code	pH	Electrical conductivity (EC)	Organic carbon (O/C)	Total nitrogen (TN)	Available phosphorus (P)	Soil texture		% of clay	silt +clay (%)	silt (%)	sand %
						first read B (23^oC)	second read A (23^oC)				
	6.76	1.25	1.1	2	0.155	29.3	22.3	43.6	57.6	14	42.4
BQAF	6.56	2.24	4.2	1.1	0.12	30.3	13.3	25.6	59.6	34	40.4
	6.58	2.26	3.9	0.8	0.13	30.3	13.3	25.6	59.6	34	40.4
	6.59	2.25	3.3	0.1	0.12	31.3	14.3	27.6	61.6	34	38.4
BQCS	6.89	2.41	1.1	2.3	0.107	30.3	12.3	23.6	59.6	36	40.4
	6.88	2.43	1.9	0.9	0.107	29.3	12.3	23.6	57.6	34	42.4
	6.81	2.44	1.4	1.2	0.104	30.3	12.3	23.6	59.6	36	40.4
BQCF	6.77	1.97	0.6	0.9	0.171	26.3	10.3	19.6	51.6	32	48.4
	6.76	1.98	0.7	0.5	0.171	25.3	9.3	17.6	49.6	32	50.4
	6.74	1.96	0.3	1.5	0.172	26.3	10.3	19.6	51.6	32	48.4
BQCC	6.94	3.91	1	1.9	0.205	32.3	18.3	35.6	63.6	28	36.4
	6.9	3.91	1.1	1.9	0.207	31.3	19.3	37.6	61.6	24	38.4
	6.95	3.95	1.4	2.1	0.206	31.3	18.3	35.6	61.6	26	38.4
BQOF	5.73	1.57	1.1	0.9	0.07	31.3	21.3	41.6	61.6	20	38.4
	5.81	1.52	0.7	0.7	0.06	32.3	22.3	43.6	63.6	20	36.4
	5.75	5.75	0.8	1.7	0.07	31.3	21.3	41.6	61.6	20	38.4
BQOM	6.39	1.43	0.9	1.2	0.126	30.3	14.3	27.6	59.6	32	40.4
	6.38	1.45	0.77	1.1	0.127	31.3	13.3	25.6	61.6	36	38.4
	6.34	1.46	1.1	1.2	0.128	30.3	15.3	29.6	59.6	30	40.4

Appendix 4. Raw data from symbiotic effectiveness tests

Groundnut				
Isolates	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
Ni G2	25.7	0.541	15	0.029
Ni G2	24.8	0.487	17	0.034
Ni G2	22.7	0.582	16	0.045
Ni G3	33.7	0.84	17	0.037
Ni G3	32.1	0.88	19	0.066
Ni G3	34.8	0.97	16	0.357
Ni G5	34.4	0.89	32	0.127
Ni G5	37.3	0.78	37	0.111
Ni G5	31.7	0.95	28	0.134
Ni G6	32.9	0.85	23	0.118
Ni G6	37.5	0.79	19	0.119
Ni G6	41.2	0.66	26	0.122
Ni G8	28.8	0.91	27	0.137
Ni G8	34.7	0.877	29	0.167
Ni G8	26.3	0.923	32	0.122
Ni G9	28.7	0.69	18	0.019
Ni G9	37.1	0.45	26	0.022
Ni G9	16.9	0.66	17	0.037
Ni G10	37.4	0.94	29	0.132
Ni G10	39.7	0.99	36	0.124
Ni G10	42.1	0.67	22	0.171
Ni G15	29.9	0.43	13	0.016
Ni G15	38.1	0.77	17	0.028
Ni G15	21.2	0.432	21	0.038
Ni G17	27.9	0.39	12	0.012
Ni G17	36.7	0.42	17	0.087
Ni G17	22.3	0.31	21	0.091
N+	35.6	0.98	NA	NA
N+	37.1	0.88	NA	NA
N+	33.1	0.74	NA	NA
N-	19.7	0.25	NA	NA
N-	16.2	0.34	NA	NA
N-	19.9	0.33	NA	NA

Soya bean				
Host	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
Ni G2	49.3	0.88	27	0.085
Ni G2	52.7	0.65	32	0.066
Ni G2	46.9	0.99	22	0.044
Ni G3	53.9	1.25	31	0.197
Ni G3	47.6	0.99	37	0.11
Ni G3	55.3	0.87	28	0.099
Ni G5	44.8	0.78	17	0.012
Ni G5	48.3	0.66	22	0.011
Ni G5	41.9	0.87	16	0.098
Ni G6	49.7	0.84	22	0.031
Ni G6	55.1	0.77	27	0.011
Ni G6	43.6	0.67	34	0.075
Ni G8	47.6	0.66	12	0.011
Ni G8	51.6	0.45	22	0.025
Ni G8	47.3	0.69	18	0.096
Ni G9	38.7	0.47	9	0.017
Ni G9	41.1	0.32	17	0.041
Ni G9	33.7	0.74	12	0.021
Ni G10	48.9	0.55	16	0.027
Ni G10	52.4	0.67	19	0.037
Ni G10	42.7	0.45	22	0.066
Ni G15	39.8	0.62	22	0.056
Ni G15	42.9	0.66	27	0.066
Ni G15	33.8	0.59	28	0.031
N+	47.4	1.12	NA	NA
N+	58.7	1.01	NA	NA
N+	42.9	0.877	NA	NA
N-	31.1	0.42	NA	NA
N-	26.7	0.38	NA	NA
N-	33.9	0.44	NA	NA
MBI	45.7	0.83	19	0.092

MBI	49.6	0.79	22	0.087
MBI	42.1	0.98	32	0.094

Tafach				
Host	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
Ni G2	58.2	0.55	42	0.192
Ni G2	61.2	0.58	32	0.075
Ni G2	54.9	0.67	48	0.165
Ni G5	79.4	0.84	29	0.137
Ni G5	81.9	0.74	37	0.047
Ni G5	68.1	0.91	21	0.121
Ni G6	100	1.42	67	0.178
Ni G6	89.7	1.21	54	0.147
Ni G6	97.2	0.98	51	0.096
Ni G8	76.9	0.96	39	0.132
Ni G8	87.4	0.88	45	0.122
Ni G8	71.6	0.74	33	0.154
Ni G10	77.7	0.75	41	0.193
Ni G10	65.2	0.67	47	0.117
Ni G10	79.3	0.78	58	0.189
Ni G17	88.7	1.21	37	0.111
Ni G17	96.6	0.62	33	0.057
Ni G17	82.7	0.97	41	0.112
N+	89.9	1.22	NA	NA
N+	94.7	1.11	NA	NA
N+	96.7	0.68	NA	NA
N-	68.4	0.74	NA	NA
N-	55.3	0.44	NA	NA
N-	49.7	0.61	NA	NA
MBI	91.1	1.32	31	0.217
MBI	97.2	0.99	37	0.087
MBI	90	0.85	34	0.069

Nasir				
Host	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
Ni G2	19.7	0.39	17	0.057
Ni G2	21.2	0.41	22	0.066
Ni G2	18.8	0.33	32	0.074
Ni G3	14.6	0.32	22	0.091
Ni G3	17.7	0.27	23	0.055
Ni G3	16.3	0.41	31	0.064
Ni G5	19.9	0.41	27	0.111
Ni G5	22.5	0.42	32	0.087
Ni G5	28.7	0.67	34	0.114
Ni G6	27.3	0.91	37	0.162
Ni G6	34.5	0.75	34	0.067
Ni G6	22.3	0.98	27	0.111
Ni G8	16.7	0.48	21	0.025
Ni G8	23.6	0.74	28	0.078
Ni G8	15.7	0.46	37	0.054
Ni G10	22.7	0.681	28	0.188
Ni G10	27.9	0.66	33	0.178
Ni G10	21.1	0.78	34	0.088
Ni G15	24.5	0.673	30	0.147
Ni G15	29.3	0.78	35	0.012
Ni G15	20.7	0.59	25	0.155
N+	31.1	0.85	NA	NA
N+	37.8	0.74	NA	NA
N+	30.3	0.63	NA	NA
N-	15.5	0.41	NA	NA
N-	18.6	0.47	NA	NA
N-	17.7	0.52	NA	NA
MBI	23.9	0.62	42	0.166
MBI	27.6	0.74	48	0.097
MBI	29.5	0.52	39	0.144

Fava bean				
Host	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
NiG3	61.1	0.71	37	0.112
NiG3	66.5	0.77	32	0.117
NiG3	59.6	0.64	39	0.096
NiG5	72.7	0.87	32	0.113
NiG5	66.2	0.67	39	0.095
NiG5	78.4	0.88	32	0.112
NiG15	69.1	0.77	36	0.017
NiG15	71.1	0.67	39	0.011
NiG15	66.3	0.98	41	0.069
NiG9	56.4	0.73	29	0.098
NiG9	66.7	0.85	33	0.063
NiG9	51.2	0.66	39	0.036
NiG10	60	0.78	31	0.057
NiG10	58.1	0.66	34	0.066
NiG10	63.7	0.89	36	0.037
N+	71.7	1.14	NA	NA
N+	67.2	1.01	NA	NA
N+	73.6	0.95	NA	NA
N-	52.7	0.55	NA	NA
N-	49.9	0.67	NA	NA
N-	55.6	0.49	NA	NA
MBI	66.6	0.89	28	0.055
MBI	59.3	0.74	32	0.067
MBI	71.6	0.63	21	0.079

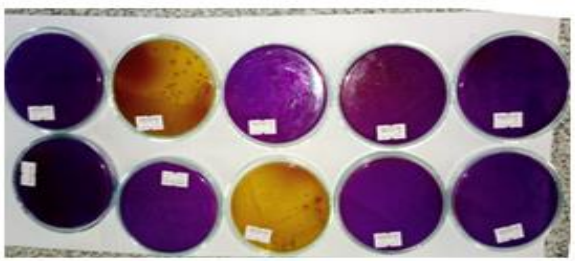
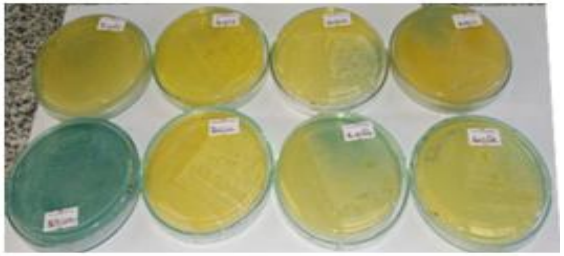
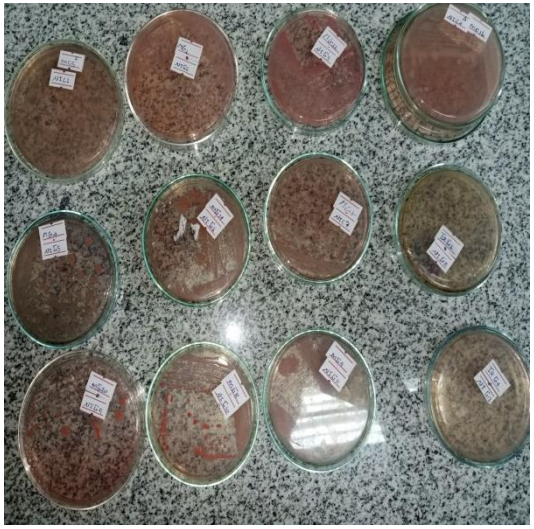
chickpea				
Host	**Height(cm)**	**SDW(g)**	**NN**	**NDW (g)**
Ni C1	34.7	0.82	29	0.062
Ni C1	37.9	0.77	37	0.026
Ni C1	31.5	0.91	24	0.087
Ni C4	51.9	1.28	41	0.041
Ni C4	47.3	1.12	55	0.037
Ni C4	57.9	1.97	39	0.057
Ni C7	49.2	1.17	36	0.078
Ni C7	53.8	1.22	34	0.067
Ni C7	41.2	0.97	38	0.095
Ni C11	39.1	0.64	22	0.033
Ni C11	45.3	0.55	32	0.127
Ni C11	36.8	0.87	19	0.011
Ni C12	44.2	0.71	18	0.022
Ni C12	49.5	0.67	22	0.035
Ni C12	37.3	0.95	26	0.066
N+	41.3	1.32	NA	NA
N+	38.3	0.97	NA	NA
N+	33.9	1.11	NA	NA
N-	29.7	0.47	NA	NA
N-	27.6	0.32	NA	NA
N-	22.9	0.66	NA	NA
MBI	36.7	0.97	20	0.036
MBI	31.2	0.88	27	0.078
MBI	39.6	0.64	21	0.022

Appendix 5. sample photos taken throughout the experiments

1. **pictures during sample collection, seed preparation, sowing, thinning and plants after 25 days at greenhouse**

2. **pictures during nodule collection, and picture of isolates NiC4, NiC7 and NiG8**

3. **sample pictures from peptone-glucose, Acid-base production, starch utilization and citrate utilization tests; pH tolerance**

4. **sample pictures from seeds for host range, authentication test and plants after 20 days**



5. **sample photos from symbiotic effectiveness test, plant height comparison of some efficient isolates along with their control(soya bean, Nasir and chickpea) and nodules from the test**