ISOLATION, CHARACTERIZATION, SYMBIOTIC PERFORMANCE AND HOST RANGE OF INDIGENOUS SOYBEAN (*Glycine max* L.) NODULATING RHIZOBIA ISOLATED FROM SELECTED SMALL HOLDER FARMERS' FIELDS OF HAWELLA WOREDA, SIDAMA REGIONAL STATE

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This is to certify that the thesis entitled “ISOLATION, CHARACTERIZATION, SYMBIOTIC PERFORMANCE AND HOST RANGE OF INDIGENOUS SOYBEAN (*Glycine max* L.) NODULATING RHIZOBIA ISOLATED FROM SELECTED SMALL HOLDER FARMERS’ FIELDS OF HAWELLA WOREDA, SIDAMA REGIONAL STATE” submitted in partial fulfillment of the requirements for the degree of Master's with specialization in Applied Microbiology, the Graduate Program of the Department/School of Biology, and has been carried out by LEMI TESFA TAREKEGN Id. No GpApMir/0006/14, under my/our supervision. Therefore, I/we recommend that the student has fulfilled the requirements and hence hereby can submit the thesis to the department.

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We, the undersigned, members of the Board of Examiners of the open thesis defense by LEMI TESFA TAREKEGN have read and evaluated his work entitled “ISOLATION, CHARACTERIZATION, SYMBIOTIC PERFORMANCE AND HOST RANGE OF INDIGENOUS SOYBEAN (*Glycine max* L.) NODULATING RHIZOBIA ISOLATED FROM SELECTED SMALL HOLDER FARMERS’ FIELDS OF HAWELLA WOREDA, SIDAMA REGIONAL STATE” and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree.

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DECLARATION

I, the undersigned, author of this work, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

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LIST OF ABBREVIATIONS AND ACRONYMS

BNF	Biological Nitrogen Fixation
BTB	Bromothymol blue
CR	Congo red
CRD	Complete randomized design
CSA	Central Statistical Agency of Ethiopia
EPS	Exopolysaccharide
NDW	Nodule Dry Wieght
Nif	Nitrogen fixing genes
NN	Number of Nodules
NodD	Nodulation protein D
PGA	Peptone glucose agar
RSE	Relative symbiotic effectiveness
SDW	Shoot Dry Wieght
SE	Symbiotic effectiveness
Tg	Teragram
TGx	Tropical Glycine cross
YEMA	Yeast extract mannitol agar
YEMB	Yeast extract mannitol broth

ABSTRACT

Soybean (Glycine max L.), a protein and oil-rich crop, plays a significant role in Ethiopia's agriculture, contributing to nearly 18% of the country's total oilseed production. Despite its inherent ability for nitrogen fixation, soybeans often fail to reach their full potential when introduced to new regions, primarily due to the lack of compatible rhizobia strains or ineffective nodulation. This study aimed to address this issue by isolating, characterizing, and evaluating the symbiotic efficiency and host range of indigenous soybean nodulating rhizobia from smallholder farmers' fields in selected Kebeles of *Hawella woreda in the Sidama region where soybean was recently introduced. Six bulk soil samples were collected from soybean-grown fields for trapping the rhizobia using soybeans. Of these, 66.6% of the samples supported nodulation under greenhouse conditions. Eight rhizobia isolates were obtained from the root nodules of soybean plants. These isolates were found to be Gram-negative, and catalase-positive, and exhibited diverse physiological and biochemical characteristics. In the nodulation assays, all isolates formed nodules on the soybean variety and were successfully reisolated from the nodules, authenticating them as rhizobia. The relative symbiotic effectiveness of the isolates ranged from 48.31% to 102.24%, indicating their potential to enhance soybean productivity in the region. These isolates were also able to nodulate mung bean and haricot bean but not peanut, demonstrating their broad host range within tropical legumes except for specific nodulation of peanuts. The study concludes that these indigenous rhizobia strains could potentially play a significant role in sustainable agriculture by enhancing soybean production and contributing to food security in the region. The findings also suggest that further research is needed to explore the potential application of these indigenous rhizobia strains in biofertilizer production and sustainable agriculture practices.*

Key words*: Host range, Indigenous Isolates, Rhizobia, Soybean (Glycine max L.), Symbiotic efficiency, Sustainable agriculture,*

1. INTRODUCTION

1.1. Background

Soybean (*Glycine max* L), a protein and oil-rich crop, originated in East Asia and has been cultivated for millennia (Islam *et al.,* 2022). Introduced to Ethiopia in the 1950s, soybean cultivation has expanded across various agro-ecologies to meet local demand. Despite occupying only 6% of the oilseed-planted area, soybean now contributes to nearly 18% of Ethiopia's total oilseed production (Desalegn Teshale *et al.*, 2021). This growth is due to soybean's versatility in producing meals, milk, animal feed, and other products. Its high protein (40%), lipid (20%), carbohydrate (30%), and health-promoting phytochemical content (Kim *et al.,* 2021) underscore its importance for food security and economic value.

Despite the increase in soybeans production, Ethiopia struggles to meet the rising demand for soybean in terms of both quantity and quality. As per the Ethiopian Central Statistical Agency (CSA, 2020), soybean production on 54,543 ha yielded 125,623t, with a national average productivity of 2.30 t ha^{-1}, which is lower than the global average productivity of 2.79 t ha^{-1} (FAOSTAT, 2020). This is largely due to soil fertility issues, particularly nitrogen (N) deficiency (Anteneh Argaw, 2012; Abebe Zerihun & Derese Haile, 2017). While chemical nitrogenous fertilizers have been widely used to address this issue, they pose environmental risks and financial challenges for smallholder farmers and exacerbate nutrient imbalances in cultivated lands (Tilahun Abebe *et al.,* 2022). As a sustainable solution, there is growing interest in biological nitrogen fixation (BNF), a natural process facilitated by symbiotic relationships with rhizobia that form nodules on soybean roots and fix atmospheric nitrogen within these structures (Soumare *et al.,* 2020).

Like other legumes, soybeans establish symbiotic relationships with rhizobia, allowing them to fix a significant amount of atmospheric nitrogen annually (Hartman *et al.,* 2011). Rhizobia species that effectively nodulate soybeans are classified into four genera: *Bradyrhizobium, Rhizobium, Sinorhizobium,* and *Mesorhizobium* (Nakei *et al.*, 2022). The majority are slow-growing *Bradyrhizobium* species, such as *B. japonicum, B. elkanii, B. liaoningense, and B. yuanmingense* (Biate *et al.,* 2014). However, fast-growing species like *Sinorhizobium fredii* and *S. xinjiangense* as well as moderately slow-growing *Mesorhizobium tianshanense* can also nodulate soybeans (Herridge *et al.,* 2008).

Despite its potential for nitrogen fixation, soybeans often underperform when introduced to new areas due to the absence of compatible rhizobia strains or ineffective nodulation (Abaidoo *et al.,* 2007; Chianu *et al.,* 2011). The successful introduction of soybeans to new regions depends on inoculation with suitable exotic rhizobia. However, different soybean varieties respond differently to inoculation; some are incompatible with rhizobia while others selectively nodulate with specific groups of rhizobia (Van *et al.,* 2007). Moreover, the effectiveness of newly introduced rhizobial strains is often compromised by the presence of indigenous rhizobial strains that are well-adapted and competitive but ineffective in N_2 fixation (Mathu *et al.,* 2012).

Researchers across Africa have identified symbiotically effective rhizobia strains within indigenous populations (Musiyiwa *et al.,* 2005; Klogo *et al.,* 2015; Gyogluu *et al.,* 2016). Furthermore, these indigenous rhizobia have shown superior N_2 fixation capabilities compared to commercial inoculants previously used in soybean cultivation (Chibeba *et al.,* 2017; Nabintu *et al.,* 2019) indicating the potential of locally adapted strains for soybean cultivation.

In Ethiopia, previous trials involving the inoculation of soybeans with exotic rhizobia (*Bradyrhizobium japonicum*) yielded inconsistent results due to a lack of adaptation to local environmental conditions (Tamiru Solomon *et al.,* 2012; Workneh Beskere and Asfaw Hailemariam, 2012; Anteneh Argaw 2014). This highlights the need to identify specific indigenous rhizobia strains or their combinations for soybean production since such rhizobia isolates can adapt better to the local environmental and soil conditions (Ouma *et al.,* 2016). Thus, isolation and identification of symbiotically effective indigenous strains with wide host ranges could be useful in the development of inoculant strains that can survive longer in agricultural soils and hence reduce the need for inoculant application every growing season (Musiyiwa *et al.,* 2005).

1.2. Statement of the Problem

Efforts to address the low yield of soybeans in Ethiopia have primarily focused on the release of improved soybean varieties. To date, national and regional research centers have released 26 different soybean varieties aimed at enhancing yield potential, disease resistance, and stress tolerance (Deresse Hunde, 2019). However, the effectiveness of these varieties may be influenced by factors such as the availability and symbiotic effectiveness of rhizobia strains in the soil.

In addition to this, the use of exotic commercial inoculants and fertilizer applications has been emphasized to address the low yield of soybeans. While chemical fertilizers have led to significant crop yield improvements, their excessive use presents environmental challenges. These include contributions to greenhouse gas emissions, soil degradation, and water pollution through runoff, as well as contamination of underground water systems through leaching. Nitrate pollution in surface waters, a result of this contamination, leads to eutrophication, which compromises the sustainability of agricultural production systems and poses health risks to humans and animals (Patel *et al.*, 2017). Furthermore, the widespread use of chemical fertilizers results in energy loss and price inflation, which can be burdensome for smallholder farmers (Tilahun Abebe *et al.*, 2022).

Exotic commercial rhizobia inoculants have been successful in some regions (Tamiru Solomon *et al.*, 2012; Workneh Beskere and Asfaw Hailemariam, 2012) however, numerous studies also demonstrate that they failed to promote plant growth and final yield in many agricultural areas (Aregu Amsalu *et al.*, 2012). Failure of the commercial rhizobia in Ethiopian farms might be due to poor adaptation of the isolates to the local soil conditions. Most formulations contain strains isolated from other continents, which might not be sufficiently adapted to the local conditions. Therefore, it is necessary to isolate and investigate local rhizobial strains for use as effective inoculants.

To develop effective inoculants and maximize soybean productivity, it is essential to assess the symbiotic effectiveness of indigenous soybean-nodulating rhizobia strains. In this regard, limited studies were reported in Ethiopia in general (Aregu Amsalu *et al.*, 2012; Diriba Temesgen, 2017; Yifru Abera *et al.*, 2018; Diriba Temesgen & Fassil Assefa, 2020) but there remains a significant knowledge gap regarding the specific characteristics, host range, symbiotic effectiveness, and physiological adaptability of indigenous rhizobia strains nodulating soybeans from the current study area. Thus, this study was aimed at investigating the characteristics, host range and symbiotic effectiveness of indigenous soybean-nodulating rhizobia isolates from selected areas of Hawella woreda in Sidama Regional state, Ethiopia.

1.3. Objectives of the Study

1.3.1. General Objective

The overarching goal of this study was to isolate, characterize, and evaluate the symbiotic efficiency and host range of indigenous soybean (*Glycine max* L.) nodulating rhizobia from selected smallholder farmers' fields of Hawella woreda in Sidama Regional state, Ethiopia.

1.3.2. Specific Objectives

The study sets out to:

- Trap isolate rhizobia using soybeans and characterize them based on their morphological, physiological, and biochemical attributes.
- Authenticate rhizobia isolates and demonstrate their abilities to re-infect their hosts.
- Assess the symbiotic nitrogen fixation efficiency of these rhizobia under greenhouse conditions.
- Assess the host range of the isolates using different legume hosts.

1.4. Research Questions

This research seeks to answer:

1. Are there indigenous soybean nodulating rhizobia in the soils of selected smallholder farmers' fields of different Kebele's in Hawella woreda of Sidama regional state where soybean was recently introduced?

2. What are the morphological, physiological, and biochemical attributes of these native soybean nodulating rhizobia?

3. How efficient are these indigenous soybean nodulating rhizobia in symbiotic nitrogen fixation when tested under greenhouse conditions?

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

1.5. Significance of the Study

This study is significant as it addresses the existing knowledge gap concerning the characterization and utilization of indigenous soybean-nodulating rhizobia in the study area. Isolating and characterizing indigenous rhizobia from the study area may provide invaluable insights into their presence, physiological and biochemical properties, and symbiotic effectiveness. This information will be vital for selecting superior rhizobia strains to be recommended as potential inoculants, thereby leading to enhanced soybean production. Moreover, the result of this study will serve as a baseline for further investigation of the potential of indigenous rhizobia in the region capable of nodulating soybean and other legumes.

1.6. Scope of the Study

This study primarily focused on the isolation and characterization of rhizobia from soils of smallholder farmers' fields using soybean as a trapping host within the selected Kebeles' in Hawella woreda of Sidama regional state where soybean was recently introduced and there was no history of the application of commercial rhizobia inoculants. The isolated rhizobia were subjected to a comprehensive characterization process involving various techniques, including morphological analysis and biochemical tests. However, it should be noted that molecular characterization was not encompassed within the scope of this study. An integral part of this research involved assessing the symbiotic efficiency of the isolated rhizobia strains with soybean plants, specifically their performance in nitrogen fixation and potential to enhance plant growth.

The scope of this study was delineated along conceptual, geographical, methodological, and temporal dimensions as follows:

- Conceptual Delimitation: The conceptual framework of this study was confined to the isolation, characterization, and assessment of indigenous soybean nodulating rhizobia for their symbiotic performance and range of hosts they can nodulate.
- Geographical Delimitation: Geographically, this study was restricted to areas within the Sidama region where soybean was recently introduced.

- Methodological Delimitation: The methodological approach of this study was limited to morphological, physiological, and biochemical tests for characterizing rhizobia. Molecular characterization was explicitly excluded from the methodology due to financial and time constraints.
- Temporal Delimitation: This research was conducted over a defined period spanning from December 2022 to August 2023.

This delineation ensures a focused approach towards achieving the research objectives while acknowledging the inherent limitations of the study.

2. LITERATURE REVIEW

2.1. Biological Nitrogen Fixation (BNF)

2.1.1. Fundamentals of BNF

Nitrogen (N), which constitutes approximately 80% of earth's atmosphere, is a crucial constituent of amino acids (proteins), urea, nucleic acids, nicotinamide adenine dinucleotide, and adenosine triphosphate in all living cells. It is a primary component of the photosynthetic pigment (chlorophyll) that harvests light energy essential for plant growth and biomass production (Lindström & Mousavi, 2020; Simbine *et al.*, 2021). Despite its atmospheric abundance, it exists in biologically inaccessible forms, posing significant challenges to agriculture as it limits plant growth and crop production.

The conversion of inert atmospheric nitrogen (N_2) into biologically useful compounds is essential, and this is primarily achieved through two mechanisms: lightning-induced fixation and Biological Nitrogen Fixation (BNF) (Fisher & Newton, 2002). Lightning only makes ~1% ammonia of the net N fixed per year. N input through BNF is approximately 122 million tons of N per year of which 55 to 60 million tons is fixed by crops (Vitousek *et al.*, 2013).

BNF is the process by which atmospheric N_2 is reduced into biologically useful, combined forms of nitrogen, primarily ammonia (NH_3), by living organisms (Soumare *et al.*, 2020). This process is performed exclusively by prokaryotes: archaea and bacteria. For bacteria, different groups are involved, including free-living bacteria belonging to genera such as *Azotobacter*, *Azospirillum*, *Bacillus*, or *Clostridium*; symbiotic bacteria like *Rhizobium* associated with legumes; *Frankia* associated with actinorhizal plants; and cyanobacteria associated with cycads (Ravikumar *et al.*, 2007; Ininbergs *et al.*, 2011). For archaea, nitrogen fixation is still restricted to groups that produce methane, called methanogens (Deresa Welteji, 2018).

Nitrogen-fixing organisms can be classified into three categories: free-living N fixers, associative N fixers, and symbiotic N fixers. The last two groups can be found in the rhizosphere (the soil region that surrounds plant roots) of legume and non-legume plants (Santi *et al.*, 2013; Mus *et al.*, 2016). Nevertheless, root nodule symbiosis is one of the most studied mutualistic relationships of plants and nitrogen-fixing organisms. It is also the most effective in N-fixing and more important because it involves almost all food and fodder legumes (Soumare *et al.,* 2020).

The core of this mutualistic interaction unfolds within specialized plant structures known as nodules. Within these nodules, the host plant generously provides carbon compounds, synthesized through photosynthesis, to the associated bacteria. In return, the bacteria convert atmospheric nitrogen into ammonium, a form readily assimilable by the plant. Beyond its immediate benefits to leguminous crops, this symbiotic relationship carries profound implications for agricultural sustainability. It enables plants to colonize nitrogen-deficient soils naturally, effectively reducing the dependence on nitrogen-based synthetic fertilizers, and thereby mitigating the environmental consequences associated with their use (Bhattacharyya & Jha, 2012).

In soil, nitrogen is always found in two forms: inorganic (2% as mineral nitrogen) and organic (98%). Ammonia (NH_4+), nitrite (NO_2-), and nitrate (NO_3-) are inorganic forms, whereas organic forms are found in living organic matter (soil biota and fresh animal and plant debris) and non-living organic matter (humified and non-humified compounds). Plants can obtain mineral nitrogen in two forms: ammonium nitrogen (NH_4+-N) or nitrate-nitrogen (NO_3-N). Organic nitrogen is not directly available to plants and must be converted through a slow process (mineralization) to ammonium or nitrate (Liu *et al.,* 2014). Once available, nitrogen is subject to strong competition between plants and microorganisms. In addition, N is continually lost through soil erosion, denitrification, leaching, chemical volatilization, and perhaps most importantly, removal of N-containing crop residues from the land (Tamme *et al.,* 2010). In this respect, nitrogen is often in short supply in many croplands, limiting crop growth and productivity.

2.1.2. The Role of BNF in Sustainable Agriculture

Sustainability in agriculture hinges on the balance between inputs and outputs, encompassing the preservation of vital resources like soil, water, renewable energy, and environmental quality (Mukherjee & Sen, 2021). Sustainable agriculture entails resource management that meets evolving human needs while enhancing environmental quality and conserving natural resources. Hence, the quest for alternatives to nitrogen-based fertilizers is crucial, and BNF provides a viable solution. BNF leverages specific nitrogen-fixing bacteria's ability to convert atmospheric nitrogen into plant-usable ammonia (Rao, 2014).

Key Benefits of BNF in Sustainable Agriculture:

1. Increased Nitrogen Availability: Efficient rhizobia strains in legumes can fulfill a significant portion of their nitrogen needs through BNF. This heightened nitrogen availability leads to improved plant growth, increased biomass production, and ultimately, higher crop yields (Giller, 2001). Additionally, surplus nitrogen can enhance the nutritional quality of leguminous crops.

2. Improved Soil Fertility: BNF not only benefits legumes but also significantly enhances soil fertility. As leguminous plants grow and fix nitrogen, the organic matter left from their roots and nodules enriches the soil with nitrogen-containing compounds. This organic matter, along with nitrogen-rich root exudates, enhances soil structure, promotes microbial activity, and increases nutrient availability (Meena *et al.,* 2018). Over time, BNF contributes to improved soil health and sustainability.

3. Supporting Sustainable Practices: BNF supports sustainable agricultural practices such as crop rotation and intercropping. The nitrogen-enriched soil resulting from legume cultivation benefits subsequent crops in rotation, reducing the need for synthetic fertilizers. Intercropping legumes with other crops diversify agricultural systems, enhances overall yield stability, and promotes nutrient cycling within agroecosystems (Erana Kebede, 2021).

4. Environmental Benefits: Reduced reliance on synthetic nitrogen fertilizers results in fewer environmental concerns. BNF's environmental advantages include decreased greenhouse gas emissions, lower energy consumption, and reduced nitrogen runoff into water bodies (Alam *et al.,* 2023). In contrast to synthetic nitrogen fertilizers, which pose environmental and economic challenges, BNF offers a promising and sustainable alternative (Soumare *et al.,* 2020). BNF operates without fossil fuels, directly supplying ammonium to plants, minimizing nitrogen losses. Fixed nitrogen is less susceptible to denitrification, leaching, and volatilization (Braakhekke *et al.,* 2017; Mabrouk *et al.,* 2018). This inherent efficiency positions BNF as an environmentally responsible approach for enhancing crop yields, reducing dependence on external nitrogen inputs, and improving soil quality. BNF emerges as a compelling solution to the challenges posed by synthetic fertilizers, especially in regions like Africa, where

sustainable agricultural practices are vital for food security and environmental preservation (Massawe *et al.*, 2016).

2.2. Introduction to Rhizobia and Symbiotic Nitrogen Fixation

2.2.1. Rhizobia

Rhizobia are a group of gram-negative, rod-shaped bacteria that colonize the soil and the rhizosphere. They can form symbiotic associations with legumes and convert atmospheric nitrogen into ammonia, which is then assimilated by the plants. This process enhances the nitrogen availability in the soil and allows the legumes to grow in nitrogen-limited environments (Ormeño-Orrillo *et al.*, 2015). Under the microscope, they appear as short rods measuring 0.5-0.9 micrometers in width and 1.2 to 3 micrometers in length. They require oxygen for survival and move using thread-like structures called flagella. Unlike spore-forming bacteria, they multiply through cell division. Their life cycle consists of three phases: saprophytic, infective, and symbiotic (Somasegaran & Hoben, 1994).

Saprophytic rhizobia live in the soil without their legume host and are referred to as native rhizobia. The natural rhizobial population in the soil can be diverse, with various distinct strains. However, in cases where the native rhizobial population is too low or the strain is ineffective for the legume host, specific mixtures of appropriate rhizobial inoculants may be applied. The population of a particular rhizobial species is usually higher in soils where the host is present or has recently been grown, often due to bacteria released from senescent nodules. In the absence of the host, rhizobial numbers are typically low.

Most rhizobia weakly absorb Congo red dye, which is used in culture media when isolating rhizobia. If the culture medium is not buffered, acid-producing rhizobia can turn the dye purple. Most strains thrive at temperatures between 25-30°C and a pH range of 6.0-7.0 (Somasegaran and Hoben, 1994).

2.2.2. Symbiotic Nitrogen Fixation in Legumes

Symbiotic nitrogen fixation is best exemplified in legumes, which belong to the *Fabaceae* (*Leguminosae*) family. This family comprises nearly 770 genera and over 19,500 species, showcasing immense diversity. The *Faboideae* subfamily, the largest in legumes, includes economically important genera and species like (*Glycine max*), peanut (*Arachis hypogaea*), common bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), licorice (*Glycyrrhiza glabra*), cowpea (*Vigna unguiculata*), chickpea (Cicer *arietinum*) (Nadon & Jackson, 2020). These crops hold global agricultural significance, covering approximately 14% of total cultivated land (Sulieman & Tran, 2016). They play a vital role in food security, nutritional value, income generation for smallholder farmers, and environmental conservation (Peoples *et al.,* 2009; Yadav *et al.,* 2015; Guardia *et al.*, 2016).

Legumes fix atmospheric nitrogen through symbiotic relationships, supplying half of the nitrogen used in agriculture (Raza *et al.*, 2020). This ability enables them to thrive in nitrogen-poor soils without synthetic nitrogen fertilizers. Consequently, biologically fixed nitrogen remains sufficient for plant requirements, reducing reliance on synthetic nitrogen fertilizers and promoting soil health and sustainability. When integrated strategically into non-legume-based cropping systems (Meena & Lal, 2018), legumes serve as potent green manure, enriching the soil with nitrogen and organic matter, thereby enhancing soil quality and agricultural productivity (Mayer *et al.,* 2003; Peoples *et al.,* 2009; Dhakal *et al.,* 2016).This harmonious interplay between symbiotic nitrogen fixation and ecological contributions underscores the importance of sustainable agricultural practices, ensuring both current and future food needs while safeguarding the environment (Biswas & Gresshoff, 2014; Hajduk *et al.,* 2015).

In agroecosystems, symbiotic nitrogen fixation is pivotal, with an estimated annual nitrogen input of approximately 122 Tg N. This mutualistic relationship between legume crops, forage/fodder legumes, and rhizobia stands as the most significant agent of nitrogen fixation (Herridge *et al.,* 2008). Calculations reveal impressive nitrogen contributions, with oilseed legumes supplying 18.5 Tg N and pulses providing 2.95 Tg N. Notably, soybean alone contributes 16.4 Tg N annually, accounting for 77% of the nitrogen fixed by crop legumes.

The advantages of symbiotic nitrogen fixation in agriculture are manifold, aligning with sustainable practices. This ecological balance offers cost-effectiveness for farmers, exceptional nitrogen use efficiency, and minimal nitrogen leaching and denitrification in agricultural lands (Peoples *et al.,* 2009). Symbiotic nitrogen fixation thus emerges as an environmentally friendly cornerstone of modern agriculture, bridging science and sustainability seamlessly.

Symbiotic nitrogen fixation occurs through the collaboration of root nodule bacteria with legumes. This mutualistic relationship begins with a molecular dialogue between the host plant and the nitrogen-fixing organism, facilitated by flavonoids and isoflavonoids secreted by the host plant in its rhizosphere (Liu *et al.,* 2014; Jimenez-Jimenez *et al.,* 2019). The processes of nodulation and nitrogen fixation are explained in the next section.

2.2.3. Nodulation and Nitrogen Fixation Processes

Nodulation is a host-specific process, with each rhizobia having a specific range of hosts(Guan *et al.,* 2013). It is a multistep process involving the host plant and its rhizosphere symbionts. Under severe nitrogen deficiency, legume roots exude flavonoid-containing exudates into the rhizosphere, where they interact with the rhizobial protein Nodulation protein D (NodD) (Wulandari *et al.,* 2022). By binding to the nod operon, activated NodD stimulates the expression of nod genes. This causes the synthesis and release of nod factors, which are lipochitooligosaccharide-based signaling molecules that interact with specific genes in the root hairs to cause cortical cell divisions and the formation of root nodule primordia while also initiating an infection process to deliver bacteria into the nodule cells. Most legume infections involve the formation of plant-made infection threads via root hair branching, deformation, and curling (Goss *et al.,* 2017).

The infection threads harboring the dividing bacteria grow through an epidermal cell layer into the nodule primordial cells (Figure 2.1). The bacteria are then released and internalized in an endocytosis-like process by the cortical cells. In nodule cells, individual bacteria are enclosed by a membrane of plant origin, forming an organelle-like structure called the symbiosome. The bacteria further proliferate and differentiate into nitrogen-fixing bacteroids (Wang *et al.,* 2018) that actively produce nitrogenase enzymes. Because nitrogenase is extremely sensitive to oxygen, the nodule must maintain low oxygen concentrations while also supplying oxygen for bacterial metabolism (Mus *et al.,* 2016). Infected cells synthesize leghemoglobin that is thought to buffer free oxygen in the nanomolar range, avoiding the inactivation of oxygen-labile nitrogenase while maintaining high oxygen flux for respiration (Brear *et al.,* 2013).

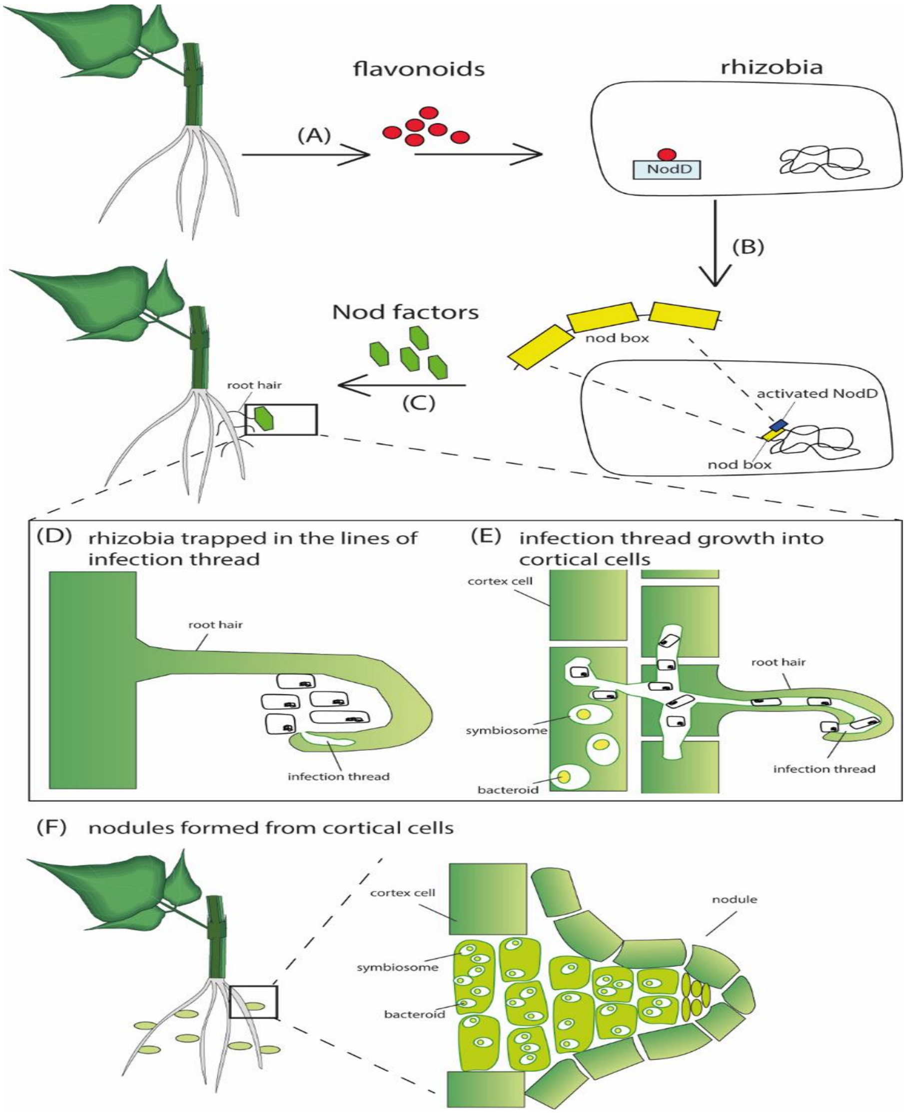

Figure 2.1: Schematic representation of nodulation process (adopted from Wekesa *et al.* (2022)). (A) Host plant releases flavonoids into the rhizosphere that are perceived by specific rhizobia. The flavonoids induce transcription of the genes for biosynthesis of the rhizobial Nod factors, which the plant perceives to allow symbiotic infection of the root. After transcription, the activated NodD binds to the nod box promoter (B), inducing the transcription and synthesis of Nod factors (C). The Nod factors induce the development of the infection thread that traps rhizobia within the curled surfaces (D). The infection thread grows through epidermal cells into the cortical cells, where rhizobia are released and internalized by the cortical cells (E).

Further proliferation and differentiation of both bacteria and infected cortical cells results in nodule formation (F).

The symbiosome membrane acts as the interface between eukaryotic and prokaryotic symbionts, and thus it possesses transporters for nutrient exchange between the symbiotic partners. The host plant provides carbon sources for bacteroid activities in the form of dicarboxylates, malate, and succinate. Phosphoenolpyruvate carboxylase and malate dehydrogenase convert the carbon flux from glycolysis to form malate, which can be taken directly by bacteroids (Liu *et al.,* 2018). Ammonia exported from the bacteroids diffuses into the cytosol of the infected host cells, where it is converted to glutamine (Gln) and glutamate (Glu) by the enzymes Gln synthetase and Glu synthase.

N_2 fixation is catalyzed by nitrogenase, which is quite similar in most of the nitrogen-fixing bacteria. Nitrogenase is an enzyme complex with two metal components: dinitrogenase MoFe (molybdenum-iron protein) serving as the catalytic component and dinitrogenase reductase (Fe protein). These two metal components are encoded by the *nif* genes, the *nif*D and *nif* K genes coding for MoFe dinitrogenase and the *nif* H gene coding for Fe dinitrogenase reductase. In addition to nitrogenase, several regulatory proteins involved in nitrogen fixation are encoded by *nif* genes. Each nitrogenase contains an active site for the reduction of the substrate and this site is composed of a complex metal group called FeV-cofactor, FeFe-cofactor, and FeMo-cofactor, for, respectively, V-nitrogenase, Fe-nitrogenase, and Mo-nitrogenase (Harris *et al.,* 2018).

2.3. Soybean Growth and Production in Ethiopia

2.3.1. Growth and Development of Soybean

Soybeans are cultivated over a wide range of latitudes, at least from 50° N to 35° S (Cao *et al.,* 2017), exhibiting resilience to extreme temperatures. Their growth rates diminish when exposed to temperatures exceeding 35°C or dropping below 18°C. While soybeans are considered short-day plants, their response to daylight duration varies depending on the specific variety and temperature. The growth and development of soybeans are strongly influenced by the length of the day. Short days result in early flowering, whereas long days delay flowering (Watanabe *et al.,* 2012).

Soybean seeds germinate within about 4 days under optimum soil temperature (28-29 °C.) but take 2 weeks or more in cold soil (10°C. or less) (Purcell *et al.*, 2014). They can be cultivated on a wide range of soils, except for excessively sandy ones. The ideal soil pH for optimal growth is between 6 and 6.5. Soybeans possess the ability to fix atmospheric nitrogen, fulfilling a significant portion of their nutrient needs. However, providing an initial dose of 10 to 20 kg/ha of nitrogen (N) is beneficial for promoting early growth.

Soybean requires the highest amount of nitrogen among agronomic crops, assimilating approximately 100 kg of nitrogen to produce a ton of seeds (Sinclair & Wit, 1975*)*. It utilizes soil mineral nitrogen as NO3- or NH4+, and atmospheric nitrogen fixed symbiotically in its root nodules. Early studies stressed the necessity of both symbiotic N-fixation and the application of nitrogen as nitrate for maximum yield of soybean. However, the relationship between nitrogen fertilization and soybean yield is complex, with factors such as soil acidity and moisture influencing the effectiveness of nitrogen fixation.

Soybeans exhibit two distinct growth phases: the vegetative phase, occurring before flowering, and the reproductive phase, which begins with the onset of flowering. The vegetative phase encompasses various stages, from cotyledons emerging above the soil surface to the development of leaves at multiple nodes on the main stem. Soybeans can be classified as determinate or indeterminate in terms of their growth habit. Determinate varieties have a shorter flowering period, distinct leaf characteristics, and a terminal raceme that can produce pod clusters. Indeterminate varieties have a more prolonged flowering period, a lack of terminal raceme, and a zigzag pattern in the top nodes (Purcell *et al.,* 2014).

2.3.2. Soybean Production Trends in Ethiopia

In Ethiopia, soybean is mainly cultivated in the southern and western regions, with production increasing significantly from 1,620 t in 2002 to 61,000 t in 2014 (Urgessa Tilahun, 2015). The demand for soybean has risen due to its diverse use in producing various soybean meals, processed soybean milk, and animal feed. Ethiopia has witnessed the rise of soy-based enterprises and agro-industrialization. Soybean oil is actively extracted for human consumption and industrial purposes, while defatted soymeal undergoes processing to create protein-rich foods and feed products (Desalegn Teshale *et al.,* 2021).

Despite the fact that it is a recent introduction in Ethiopia, records obtained from 2008 to 2016 show that soybean area, production, and yield have increased at a rate of 30.8%, 45.4%, and 11.2% per annum, respectively, and reached 38,166 ha of land to produce 812,420 quintals of soybean with a national average yield of 21.3 qt/ha (CSA, 2016). The total area coverage under the production and whole volume of production of soybean has been increasing over years. According to Mekonnen Hailu and Kaleb Kelemu (2014), the significant wellspring of increment in the total production of soybean has been principally come about because of increment in area of land allocated for its production. The average productivity level of soybean during the last 15 year were 1.4 ton/ha. This level is additionally low contrasted with the potential which could go up to 4 ton/ha if improved varieties are used. The last 15 years trend in the productivity level has grown from 0.92 ton/ha in 2001/02 to 2.22 ton/ha in 2016/17 (Gebre-egziabher Fentahun, 2019)

2.4. Soybean Nodulating Rhizobia

2.4.1. Taxonomy and Classification of Rhizobia

Rhizobia is made up of various species that are taxonomically classified as proteobacteria, specifically in the classes α-proteobacteria, and in β-roteobacteria (Gyaneshwar *et al.,* 2011). The taxonomic classification of rhizobia has witnessed significant advancements in recent years, primarily through molecular techniques such as DNA sequencing. These techniques have revolutionized our understanding of rhizobia diversity, helping identify distinct strains with varying levels of efficiency in nitrogen fixation (Nakei *et al.,* 2022).

There are 17 genera of rhizobia, which are categorized into different families (Lindström & Mousavi, 2020). However, soybean forms symbiotic nodules with a narrow range of rhizobia species. Rhizobia species that nodulate effectively with soybean are taxonomically placed in four genera, or groups as summarized in Figure 2.2, including *Bradyrhizobium*, *Rhizobium, Sinorhizobium*, and *Mesorhizobium*. Most of soybean nodulating rhizobia are slow growing *Bradyrhizobium* species: *B. japonicum*, *B. elkanii*, *B. liaoningense* and *B. yuanmingense* (Biate *et al.,* 2014).The other symbionts include fast growers classified into *Sinorhizobium fredii* and *S. xinjiangense* while moderately slow-growing rhizobia belong to *Mesorhizobium tianshanense* (Man *et al.,* 2008).

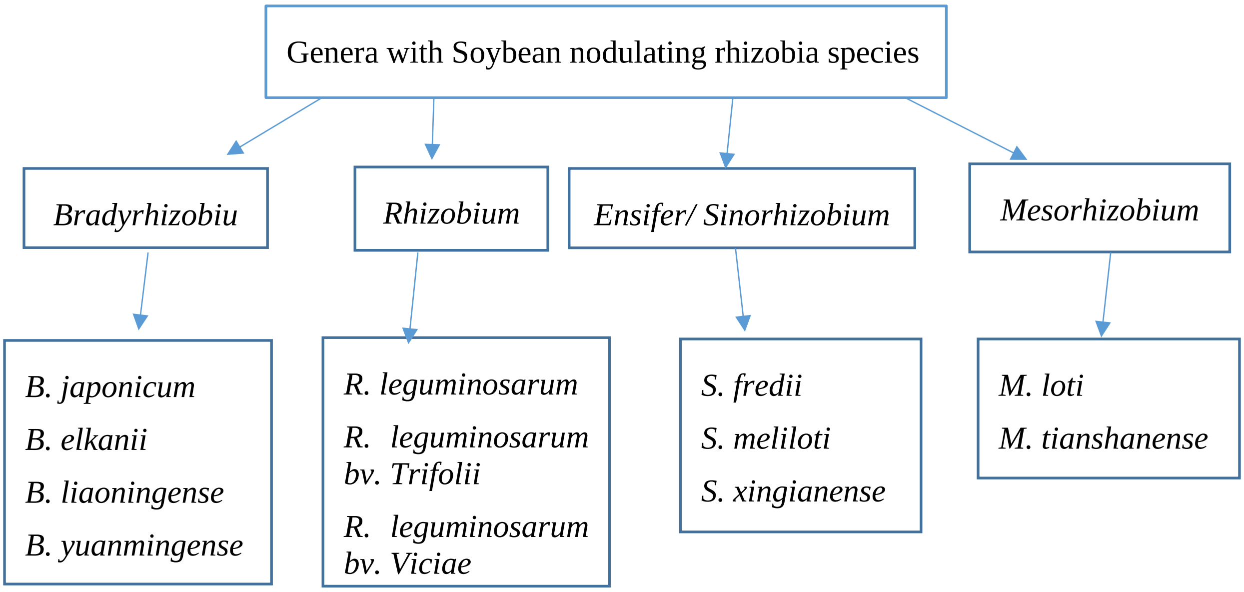

Figure 2.2: Classification of soybean nodulating rhizobia

The diversity of soybean nodulating rhizobia is affected by host specificity and the narrow range of rhizobia species, which forms an effective symbiosis with soybean (Xu *et al.*, 1995). Climatic conditions and soil properties (Adhikari *et al.*, 2012; Suzuki *et al.*, 2008) and stress conditions such as acidity/alkalinity, salinity, and high temperature (Chibeba *et al.*, 2017) are other factors that affect the effectiveness of soybean nodulating rhizobia. Different land uses and management of crops (Yan *et al.*, 2014) geographical location (Shiro *et al.*, 2013) as well, influence the diversity of soybean nodulating rhizobia.

Understanding the diversity and taxonomic classification of rhizobia is crucial for sustainable agriculture. It allows for the selection of optimal rhizobia strains that can thrive in specific soil and environmental conditions, maximizing nitrogen fixation and crop yield improvement (Herridge *et al.*, 2008).

2.5. Impact of Soybean-Nodulating Rhizobia on Plant Growth and Crop Production

Soybean is a protein-rich legume that plays a vital role in agriculture and food security. One of the main advantages of soybean is its ability to meet its nitrogen (N) requirements through a dual strategy: inorganic N uptake and symbiotic nitrogen fixation with rhizobia strains (Hungria & Mendes, 2015). Symbiotic nitrogen fixation is a process that involves a chemical interplay between soybean and rhizobia, mediated by signaling molecules such as isoflavones and lipochitooligosaccharides. This process leads to the formation of root nodules, where rhizobia transform atmospheric nitrogen into ammonia through nitrogenase enzymes. This symbiotic relationship not only protects these enzymes from oxygen damage but also fosters a microaerobic nodule environment conducive to efficient nitrogen fixation (Nakei *et al.,* 2022).

The symbiotic relationship between soybean and nodulating rhizobia has a significant impact on plant growth and crop production. This relationship provides several benefits for agriculture, such as:

- Supplying a significant portion of the plant's nitrogen needs, reducing the need for synthetic nitrogen fertilizers and saving costs for farmers. Soybeans can fulfill up to 94% of their nitrogen needs through symbiotic nitrogen fixation (Hungria & Mendes, 2015).

- Improving crop yields by enhancing the availability of nitrogen, which directly affects the growth and productivity of plants. Studies have shown that inoculation with rhizobia can increase soybean yields by 10-30% (Hungria & Mendes, 2015).

- Contributing to soil health by enriching the soil with nitrogen and organic matter after the legume crops are harvested, improving soil fertility and structure. This can also benefit subsequent crops grown in rotation with soybean (Hungria & Mendes, 2015).

- Promoting sustainability by reducing dependence on synthetic fertilizers, which can have negative environmental impacts such as water pollution from fertilizer runoff (Hungria & Mendes, 2015).

- Enhancing the plant's resilience to stress conditions such as drought, which could be beneficial in regions where water availability is a limiting factor for crop production. Some studies suggest that the symbiotic relationship between soybean and rhizobia can improve the plant's water use efficiency and drought tolerance (Hungria & Mendes, 2015).

However, several factors, including environmental variables and the absence of suitable rhizobia populations, can limit the efficiency of nitrogen fixation. Therefore, for smallholder farmers facing financial constraints particularly in regions like Ethiopia, cost-effective methods like rhizobia inoculation are gaining prominence. Careful selection of competitive inoculant strains becomes crucial, maximizing nitrogen fixation, enhancing soil nitrogen content, and ultimately influencing crop productivity and sustainability (Hungria & Mendes, 2015).

2.5.1. Rhizobia Inoculation Effects on Soybean Growth and Yield

When soybean is cultivated outside Southeast Asia for the first time, it exhibits poor yield, likely due to the absence of co-evolved rhizobial strains in soils abroad (Abaidoo *et al.,* 2007; Chianu *et al.,* 2011). Therefore, successful introduction of soybean to new regions relies on inoculating it with appropriate exotic rhizobia.

Numerous scientific studies consistently support the positive impact of rhizobia inoculation on soybean growth and yield. van Heerwaarden *et al.* (2018) conducted extensive field trials in sub-Saharan Africa from 2010 to 2015, revealing a substantial yield increase from 1,227 to 1,343 kg/ha following soybean seed inoculation with rhizobia. Similarly, Nyaguthii (2017) reported a remarkable grain yield of 2 tons ha^{-1} resulting from soybean inoculation with rhizobia. Ulzen *et al.* (2016) demonstrated enhanced grain yields for both soybeans and cowpeas in Ghana through the application of *Bradyrhizobium* inoculants. Highlighting the crucial role of rhizobia in soybean nitrogen fixation, Kassam *et al.,* (2019) emphasized soybeans' potential to fix nitrogen in the range of 88-188 kg N/ha/year. Under conditions of low soil nitrogen, soybeans exhibited an impressive nitrogen fixation capacity of up to 300 kg of N/ha when inoculated with effective *Bradyrhizobium* species (Ntambo *et al.*, 2017).

Tamiru Solomon *et al.* (2012) reported a significant 53% increase in soybean yield through the inoculation of *Bradyrhizobium* japonicum TAL 379 compared to the un-inoculated control. This positive response was consistent with findings reported by Workneh Bekere & Asfaw Hailemariam (2012). Furthermore, Kumaga and Ofori (2004) noted improved nodulation and plant growth in both promiscuous and non-promiscuous soybean varieties when highly competitive rhizobial inoculants were employed.

2.5.2. Indigenous Soybean Nodulating Rhizobia: Efficiency and Implications

Indigenous rhizobia, naturally occurring within specific soil regions, play a vital role in BNF and legume growth due to their diverse characteristics (Jaiswal *et al.,* 2021). These rhizobial communities exhibit substantial variability, primarily driven by environmental factors. Despite this variability, the genes responsible for BNF remain relatively stable across these indigenous populations (Moura *et al.,* 2020). The efficiency of indigenous rhizobia strains is pivotal in governing BNF, particularly concerning native or introduced legumes within a given area. The competitiveness and efficiency of the native rhizobia community directly impact host plants and environmental conditions. In cases where compatible rhizobia are scarce or the native community is deemed inefficient, it is generally recommended to inoculate with rhizobia strains known for their effectiveness.

Utilizing native Rhizobium strains as inoculants serves as an environmentally sustainable approach in agriculture. These strains enhance legume production due to their growth-promoting traits and adaptability to local soil and environmental stresses. Moreover, native rhizobia strains contribute to soil health by producing exopolysaccharides, sustaining root systems during drought conditions. These strains also enhance the soil's biological activity by bolstering microbial populations and enzyme activity. The ability of native strains to interact synergistically with resident soil microbiota and adapt to local agro-ecological climatic conditions underscores their superior performance compared to exotic commercial strains (Mendoza-Suárez *et al.*, 2021).

Research, such as the meta-analysis conducted by Thilakarathna and Raizada (2017), reveals that indigenous rhizobia strains occasionally outperform non-local, improved counterparts under field conditions. This superiority is evident in terms of nodule occupancy and overall yield, suggesting the promise of indigenous rhizobia for local-level commercial inoculant production, particularly in regions marked by challenging environmental stressors. Research conducted by Waswa *et al.* (2014) provides compelling evidence for the advantages of using local strain *Bradyrhizobium* species in soybean inoculation, demonstrating significant increases in grain yields compared to the commercial strain USDA110. In addition, Sharma and Kumawat (2011) found that indigenous *Bradyrhizobium japonicum* strains surpassed commercial inoculant in promoting crop height and dry weight in earthen potted soil. Furthermore, indigenous rhizobia have exhibited exceptional efficiency in nitrogen fixation compared to previously used commercial inoculants for soybean, as evidenced by studies conducted by Nabintu *et al.* (2019) which demonstrated the superior performance of indigenous strains of *Bradyrhizobium japonicum*, which increased soybean yields by 68.7% and 70.8% under greenhouse and field conditions, respectively, surpassing the commercial strain B. diazoefficiens USDA110. Similarly, Chibeba *et al.* (2017) observed effective nodulation in 85 indigenous soybean nodulating rhizobia isolates, with 10 isolates outperforming the best commercial reference strain, *B. diazoefficiens* USDA 110, in greenhouse conditions. These strains outperformed commercial strains in terms of effective nodulation and soybean yield increase.

2.5.3. Research on Indigenous Rhizobia in Ethiopia

In Ethiopia, several studies have focused on indigenous soybean-nodulating rhizobia and their impact on soybean growth. Aregu Amsalu *et al.* (2012) isolated phylogenetically diverse groups of *Bradyrhizobium* from soybean nodules in Ethiopia, indicating the presence of either promiscuous nodulation by indigenous symbionts of other legume hosts or local rhizobia specific to soybean in Ethiopian soils. Moreover, fast- and slow-growing rhizobia capable of effective nodulation were also isolated and characterized, suggesting that native legumes could serve as a source of novel ecologically adapted and symbiotically effective rhizobia for Ethiopia's recently introduced soybean crop (Diriba Temesgen, 2017).

In addition, Yifru Abera *et al.* (2018) reported effective nodulation of soybean by indigenous slow-growing *Bradyrhizobium* species, which commonly nodulated cross-nodulation cowpea and pigeon hosts. According to Aregu Amsalu *et al.* (2019), rhizobial inoculation improved drought tolerance, biomass, and grain yields of common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) in Halaba and Boricha, Southern Ethiopia. Furthermore, Kedir Woliy *et al.* (2019) identified various strains of *Bradyrhizobium* bacteria, demonstrating their potential for producing legume inoculants with the dual capacity of effective nitrogen fixation and reduction of nitrous oxide (N_2O) emissions. N_2O is a potent greenhouse gas and contributes to the depletion of the stratospheric ozone layer, primarily stemming from excessive fertilizer usage (Butterbach-Bahl *et al.,* 2013). Diriba Temesgen and Fassil Assefa (2020) conducted greenhouse and field trials, demonstrating that inoculation with native symbiotically effective *Sinorhizobium* species enhanced soybean (*Glycine max* (L.) Merr.) grain yield in Ethiopia.

In addition to soybeans, research has also been conducted on other legumes. For instance, a study by Erana Kebedea *et al.* (2021) found that the abundance of native rhizobia nodulating cowpea in major production areas of Ethiopia was high, ranging from 3.1×10^4 to 1.0×10^7 rhizobia cells g^{-1} of soil. This suggests that the soils of cowpea production areas in Ethiopia harbor adequate levels of rhizobia capable of nodulating cowpea, which are passable to provide satisfactory nitrogen fixation and nodulation.

Another study on the phylogeographic distribution of rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia revealed that *Rhizobium etli* and *Rhizobium phaseoli* were the predominant strains of bean-nodulating rhizobia in Ethiopia (Hailu Gunnabo *et al.,* 2021). This indicates the presence of diverse rhizobia capable of nodulating different legume species in the country. A study on the symbiotic effectiveness of rhizobia nodulating chickpea (*Cicer arietinum* L.) in Ethiopia found that most of the new isolates belonged to a clade related to *M. plurifarium*, with very few sequence differences. The study also found that all Ethiopian strains had nearly identical symbiotic genes that grouped them in a single cluster with *M. ciceri*, *M. mediterraneum* and *M. muleiense*, but not with *M. plurifarium.* (Hailu Gunnabo *et al.,* 2021).

Despite the development of promiscuous cultivars that can nodulate with indigenous rhizobial strains, it is important to search for indigenous strains that are capable of nodulating a wide range of soybean cultivars. This is because the effectiveness of nitrogen fixation and the subsequent impact on crop yield can vary depending on the specific rhizobial strain and its compatibility with the host legume. The indigenous rhizobia in Ethiopia hold promise for sustainable agriculture practices, contributing to enhanced legume production, soil health, and resilience in the face of environmental challenges.

3. MATERIALS AND METHODS

3.1. Study Area Description

The Hawella woreda, a recent addition to the administrative divisions within the Sidama National Regional State, is located about 20 km from Hawassa, the capital city of the Sidama National Regional State in south-central Ethiopia. Although the woreda is newly established, it shares the diverse landscape and rich natural resources that are typical of the Sidama region. The region covers an area of approximately 6,806.231/6,981.8 square kilometers and includes altitudes that range from 1,148 to 3,368 meters above sea level (Sidama Region, 2023).

This study is particularly focused on smallholder farmers’ fields in the Hawella woreda due to the recent introduction of soybean cultivation in the area. Soil samples were systematically collected from accessible locations within the woreda for this study (Table 3.1 and Figure 3.1). These kebeles were selected based on their recent soybean introduction and absence of prior inoculation. Suitable sampling sites were pinpointed in collaboration with agricultural extension officers and farmers involved in soybean production.

Table 3.1: Altitude and geographic coordinates of soil sampling locations across selected kebeles

Kebele	Latitude	Longitude	Altitude (m)
Dobe Negasha	6° 55’ 54.372" N	38° 25’ 54.073" E	1894.29
Gaaluko Horo	6° 53’ 40.228" N	38° 29’ 6.907" E	1956.35
Gala Galo	6° 54’ 47.181" N	38° 32’ 21.181" E	2158.56
Haweela Lida	6° 55’ 47.834" N	38° 27’ 58.734" E	1914.34
Muranicho Quxala	6° 54’ 47.268" N	38° 27’ 45.677" E	1924.07
Nure Dullacha	6° 57’ 34.282" N	38° 26’ 6.658" E	1840.19

The latitude and longitude are in degrees (°), minutes ('), and seconds ("). The altitude is in meters above sea level. Locations were extracted from Google Maps after using GPS application.

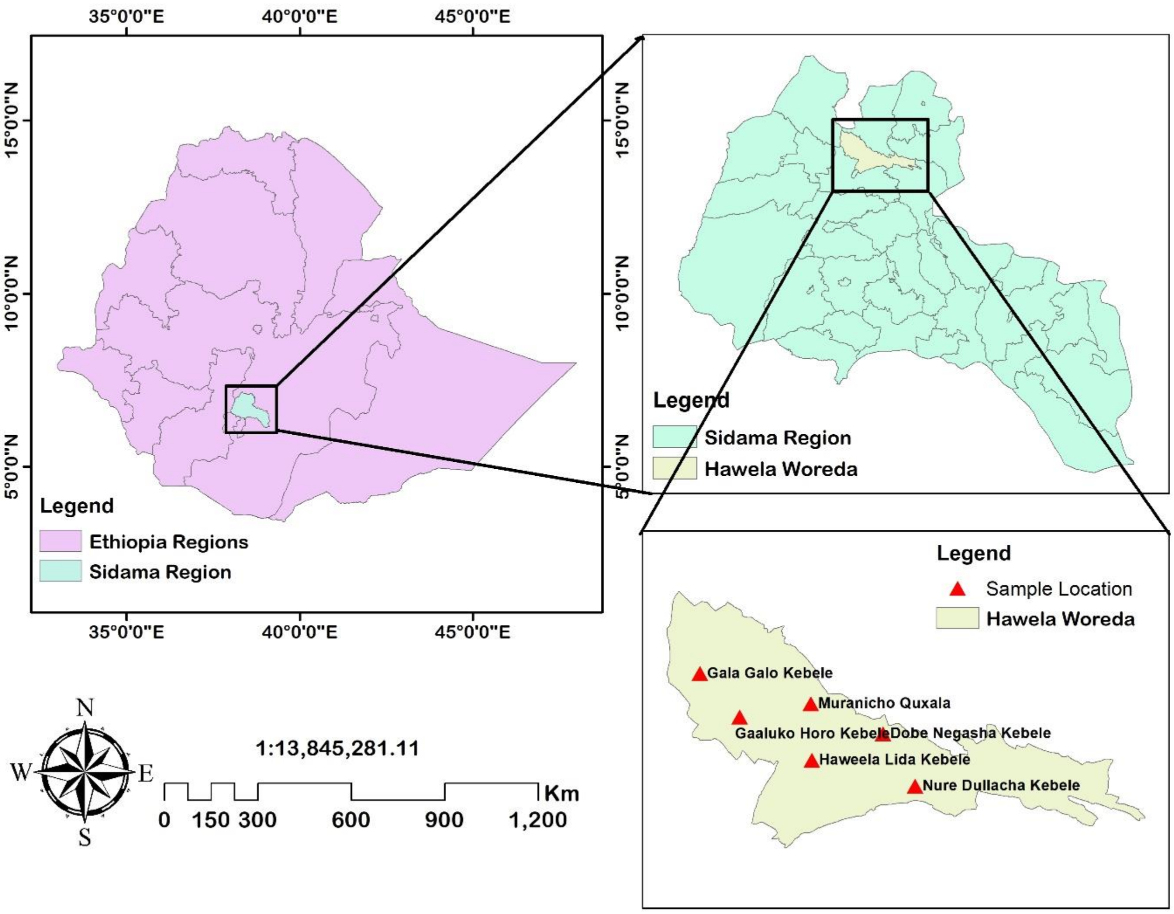

Figure 3.1: Geographic distribution of sampling sites in Hawella, Sidama, Ethiopia.

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

3.2. Soil Sampling Procedure

Soil samples were randomly gathered from three smallholder farmers' fields in each selected Kebele's during April, 2023 G.C. The sampling was conducted in a zigzag pattern from the soil surface to a depth of 20 cm in fields that had no previous exposure to exogenous rhizobia associated with soybeans. The samples, collected from different spots within the field, were thoroughly mixed to create a homogeneous soil sample for each field, each weighing approximately 3 kg as described by Erana Kebede *et al.* (2020). These samples were individually packed in sterile plastic bags and labelled accordingly. All six samples were preserved at their original moisture levels and transported to soil microbiology laboratory of

Hawassa University’s College of Agriculture for further experimentation involving nodule trapping and rhizobial isolation.

3.3. Greenhouse Nodulation Induction and Assessment

The 'plant trap' method, as per Vincent (1970), was used to induce rhizobia nodulation on a promiscuous soybean genotype, the Tropical Glycine cross (TGx) variety, which were obtained from Alation Manufacturing Company, in a greenhouse at Hawassa University's College of Agriculture. The soybean seeds were surface sterilized using a process involving 70% ethanol and 3% sodium hypochlorite solution, then sown into sterilized 3-kg capacity plastic pots filled with soil samples. Post-planting care included thinning of seedlings to two per pot and regular watering with approximately 100 ml of water daily. After 45 days, plants were uprooted, and the presence of nodules was assessed to measure the soil's nodulation potential.

3.4. Isolation of Rhizobia from Root Nodules

The isolation of rhizobia was conducted using the protocols established by (Vincent, 1970) and (Somasegaran & Hoben, 1994). Initially, nodules were placed in a Petri dish and immersed in sterilized distilled water for rehydration over a period of four hours. Following this, the nodules were surface sterilized using sterile forceps to immerse them in 70% (v/v) ethanol for one minute, followed by a three-minute immersion in 3% (v/v) sodium hypochlorite solution.

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

3.5. Purification, Designation, and Preservation of Isolated Rhizobia

The purification of rhizobial isolates involved selecting a single well-grown colony and transferring it to a fresh YEMA plate with Congo Red (CR). This plate was incubated at

28±2°C for 7-10 days, with sub-culturing repeated until purity and uniformity were achieved. For preservation, isolated colonies were transferred to YEMA slants containing 0.3% (W/V) Calcium carbonate ($CaCO_3$) and stored at 4°C for subsequent characterization (Vincent, 1970). Isolates were labelled as 'ISR' (Indigenous Soybean Rhizobia), followed by unique numbers.

3.6. Morphological, Physiological, and Biochemical Characterization of the Isolates

3.6.1. Assessment of Growth and Cultural Characteristics

The isolates were cultured on Yeast Extract Mannitol Agar (YEMA) plates to assess a variety of characteristics. These characteristics encompassed the colony's size, which was determined by calculating the average diameter of five distinct colonies. Additionally, the colony's shape, margin, and texture were evaluated. The colony's appearance was also noted, specifically whether it was translucent or opaque. The production of gum, whether dry or gelatinous, was another characteristic assessed. This evaluation was conducted in accordance with the methodologies detailed by Lupwayi and Haque (1994), as well as Aneja (2007)).

3.6.2. Gram Staining

As indicated in Lupwayi and Haque (1994), all the isolates were tested in gram reaction (gram-negative or not) for rapid means of contaminants identification. Pure cultures grown on YEMB for 3 days were used for the staining. A loopful of each isolate was spread on a slide, air-dried, and heat-fixed. The slide was then flooded with crystal violet for 1 minute, followed by Gram's iodine for 1 minute. After rinsing with water, ethanol was applied for 10 seconds, followed by safranin for 1 minute. The color development was recorded after washing off the excess safranin and air-drying the smear. Gram staining was followed by a microscopic examination to differentiate rhizobia from other Gram-positive bacteria. All isolates were observed microscopically for shape and Gram reaction to confirm their identities.

3.6.3. Growth on the peptone-glucose agar (PGA) medium

The peptone glucose agar assay was conducted to determine whether the isolates could utilize peptone as their primary carbon source (Lupwayi & Haque, 1994). The PGA medium comprised glucose (5g), peptone (10g), agar (10g), bromocresol purple (10ml), and distilled water (1,000ml), with a pH of 6.8 (Somasegaran & Hoben, 1994). After streaking the isolates onto this medium and incubating them at 28±2°C for 5-7 days, growth and color changes around the colonies were observed. Rhizobial cells typically exhibited minimal to no growth on the PGA medium, as they lack the ability to utilize peptone as a carbon source. On the other hand, if heavy growth is observed, particularly accompanied by noticeable alterations in the medium's color, it may indicate the presence of contaminants such as *Agrobacterium*, which can utilize and grow rapidly on this medium.

3.6.4. Congo Red Dye Absorption Test

The Congo Red (CR) dye absorption test was used to evaluate the purity of rhizobial isolates and their capacity to absorb dye. Rhizobia typically exhibit weak dye absorption, while contaminants such as *Agrobacterium* absorb it strongly. A stock solution of CR was prepared by dissolving 0.25g in 100 ml of sterile distilled water, from which 10 ml was then added to a litre of YEMA and autoclaved. The isolates were streaked onto this medium and incubated in darkness at 28±2°C for 3 to 7 days to observe CR absorption. When strains of nodule bacteria were plated on YEMA+CR, rhizobia stood out as white, translucent, glistening elevated colonies, and comparatively small colonies with entire margins in contrast to stained colonies of other contaminants (Somasegaran & Hoben, 1994).

3.6.5. Keto-lactose test

The keto-lactose test differentiates Rhizobia from other bacteria such as *Agrobacterium*, which can produce a ketolactase enzyme that converts lactose into keto-lactose (Holt, 1994). The isolates were streaked at the center of yeast extract lactose agar plates (Composition: lactose 10 g; K_2HPO_4 0.52 g; $MgSO_4 \cdot 7\ H_2O$ 0.2 g; NaCl 0.12 g; $CaCO_3$ 3 g; yeast extract 1 g; agar 15 g; per litre). The medium is similar to YEMA except replacement of mannitol with lactose. A loop full of the pure culture was streaked on yeast extract lactose medium and incubated for 5–7 d at 28±2 ˚C. After incubation, the plates were flooded by pouring Benedict’s reagent (Solution A-sodium citrate 17.3 g; sodium carbonate (anhydrous) 10 g; distilled water 60 ml; Solution B-copper sulphate 1.73 g; distilled water 10 ml mixed both and make up 1 l with water) and further incubated for 1 hour. The color change was observed to assess the presence or absence of contaminants. A yellowish zone surrounds the growth of contaminants, indicating the production of 3-ketolactose whereas no such yellow zone was observed around the growth of rhizobia, as Lupwayi and Haque (1994) have described.

3.6.6. Acid/Base production

Bromothymol blue (BTB) test was conducted to determine the acid/alkali production of each rhizobial isolate by inoculating them onto freshly prepared YEMA medium plates (pH 6.8) containing 25 mg/l BTB. All inoculated plates were incubated at 28 °C for 7 days and results were observed after every 24 h of incubation. The isolates were classified as acid producer/fast growers and alkali producer/slow growers based on color change of medium from green to yellow and/or from green to blue, respectively (Sharma *et al.,* 2010).

3.6.7. Assessment of Tolerance to pH Extremes

The ability of rhizobial isolates to tolerate extremes of pH was evaluated using YEMA agar media with pH adjusted to 4.0, 6.8, and 9.0 using 1 M HCl and 1 M NaOH as indicated by Kapembwa (2016). All isolates were tested for the development of distinct colonies at each pH level by incubating at 28±2°C for 5-7 days (Somasegaran & Hoben,1994).

3.6.8. Assessment of Sodium Chloride Tolerance

The ability of rhizobial isolates to tolerate sodium chloride stress was evaluated using YEMA plates containing 2% NaCl, a concentration that represents a significant salt stress for most bacteria (Paudyal *et al.,* 2021). The isolates were streaked onto these plates and then incubated for a period of 7 days. This duration allows sufficient time for the bacteria to react to the saline conditions and for observable growth patterns to emerge. Observations were made regarding the presence or absence of growth, as well as any changes in the growth patterns compared to control conditions. The ability to grow under these conditions indicates a level of salt tolerance, which is an important trait for rhizobia functioning in saline environments (Cardoso *et al.,* 2015).

3.6.9. Catalase Test

Catalase test was performed to study the presence of the enzyme catalase, which hydrolyzes hydrogen peroxide (H_2O_2) into H_2O and O_2 in bacterial strains. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell; because of this, any cell that uses O_2 or can live in the presence of O_2 must have a way to get rid of the peroxide. The cultures were streaked upon YEMA agar plates and incubated for 3 days at 30°C. After incubation, the cultures were flooded over by

H_2O_2. Appearance of bubbles (effervescence) upon the addition of hydrogen peroxide, indicates a positive catalase test (Paudyal *et al.*, 2021).

3.6.10. Carbon Source Screening of Indigenous Rhizobia Isolates

The study evaluated indigenous rhizobia isolates from soybean nodules for their ability to utilize different carbon sources. The carbon sources tested were D-, glucose, sucrose, fructose, lactose, arabinose, starch and sodium citrate, each at a concentration of 1% (w/v). The basal medium used contained 1g K_2HPO_4, 1g KH_2PO_4, 0.01g $FeCl_3.6H_2O$, 0.2g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 1g $(NH_4)_2SO_4$, and 15g agar per liter. The carbon sources were autoclaved with the basal medium. The isolates were inoculated in triplicate on basal medium plates supplemented with individual carbon sources. Plates with D-mannitol and without a carbon source served as controls. Incubation was done at 28°C for 10 days. Growth was scored as no visible growth (-) or measurable colony formation (+) denoting substrate utilization.

3.6.11. Utilization of Various Nitrogen sources

The ability of rhizobia isolates to utilize different organic nitrogen compounds was evaluated. The nitrogen sources tested included L-alanine, L-arginine and L-phenylalanine, added individually to basal medium at a concentration of 0.5 g/L. Basal medium was modified from the carbohydrate utilization medium by omitting ammonium sulfate and supplementing with D-mannitol at 1 g/L as the sole carbon source (Amarger *et al.,* 1997). Each isolate was inoculated in triplicate on basal medium plates containing individual nitrogen sources. Plates without a supplemental nitrogen source served as the negative control. Incubation was carried out at 28°C for 10 days. Colony growth indicating the metabolism of the nitrogen source was quantified by measuring the diameter of surface colonies. Growth was scored as no visible growth (-) or measurable colony formation (+) denoting substrate utilization.

3.6.12. Methyl Red (MR) Test

The Methyl Red (MR) test, used to detect mixed acid fermentation, was conducted. In this test, a red color indicates a positive result (pH below 4.4 due to glucose fermentation into organic acids), while a yellow color indicates a negative result (pH above 6.0 due to conversion of pyruvic acid into neutral products). A Methyl Red-Voges Proskauer (MR-VP) broth medium was prepared with 7.0 g buffered peptone, 5.0 g glucose, and 5.0 g dipotassium phosphate

(K_2HPO_4) in 1000 ml distilled water. About 5ml of this broth was transferred into sterile test tubes and incubated for 24 to 48 hours (Agarwal *et al.*, 2013). Post incubation, Methyl Red indicator was added to each tube. The color change in the broth indicated the result: red for positive (acidic end products from glucose fermentation) and yellow for negative (no acidic end products) (Shanmugaraj *et al.* 2021).

3.6.13. Urea Hydrolysis Test

The Urea Hydrolysis Test was conducted to assess the ability of the rhizobia isolates to hydrolyze urea. The test began with the inoculation of exponential phase culture into tubes containing Yeast Extract Mannitol (YEM) medium, supplemented with 2% (W/V) urea and 0.012% phenol red, a pH indicator. The inoculated tubes were then incubated at a constant temperature of 30°C for a period of 7 days. The presence of urease activity, an enzyme that hydrolyzes urea to produce ammonia and carbon dioxide, was indicated by a color change in the medium from yellow to pink due to the increase in pH (Paudyal., 2021).

3.6.14. Indole production test

The Indole production test was conducted using a Tryptone broth medium, prepared by dissolving 0.50 grams of tryptophan in 50 ml of distilled water. The rhizobia culture was inoculated into this medium and incubated at 30°C for 48 hours. Post-incubation, 1 ml of Kovac's reagent was added to each tube, including a control tube with uninoculated broth. After a reaction time of about 10 minutes, a red ring indicated a positive result (Hossain *et al.*, 2019).

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

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

3.7.1. Setup of Growth on the Sterile Sand medium

Soybeans were grown in sterilized (with 95% ethanol) plastic cups which had two sections: a base for nutrient solution and a top filled with sterile river sand. The sections were connected by a cotton wick (Yates *et al.,* 2016). The sand was treated with sulfuric acid, washed, dried, and autoclaved at 121°C for 30 minutes, then cooled for 24 hours before use.

3.7.2. Culturing of Isolates and Seed Preparation

Isolates were cultured in YEMB for 5-7 days before planting. Seeds were surface sterilized by immersing in 95% alcohol for 10 seconds, then immersed in a 3% sodium hypochlorite solution for 3-5 minutes. They were washed six times in distilled sterile water and left in the final change of water for one hour until fully imbibed. Afterwards, they were washed twice more and transferred to a 1% water agar petri dish to incubate at 25°C (Bala *et al.,* 2019).

3.7.3. Planting and Inoculating

Pre-germinated seeds were planted one centimetre below the surface of the rooting medium in the plastic cups. Each seedling was inoculated with 1 ml of a broth culture containing about of 10^8 the isolate a concentration determined using a technique of serial dilution and counting. The cups were then placed in the greenhouse. Five days after emergence, the plants were thinned to one per cup. During thinning, the rooting medium was not disturbed; instead, the shoot of the unwanted plant was cut using scissors (Bala *et al.,* 2019).

3.7.4. Experimental Design and Conditions

Greenhouse experiments were conducted using a completely randomized design (CRD) with three replications across 10 treatments. These treatments included eight indigenous rhizobia isolates and non-inoculated plants both with and without the mineral Nitrogen (N). The negative control consisted of treatments without inoculation and no chemical nitrogen fertilizer, while the positive control involved treatments without inoculation but with nitrogen fertilizer applied as a 0.05% KNO_3(w/v) solution weekly (Somasegaran & Hoben, 1994).

Seedlings were irrigated with Nitrogen free nutrient solution prepared according to Broughton and Dilworth (1971) (see Appendix 1 for composition) as described by Somasegaran and Hoben (1994). Moisture levels were regularly checked and adjusted. After 45 days, plants were harvested, and nodules in the roots were assessed. Shoots were dried at 70°C for 48 hours, and shoot dry weights (SDW) were recorded as described by Solomon Legesse (2016). Nodules were cleaned, dried, and weighed (NDW). The number of nodules (NN) was also counted.

3.8. Determination of symbiotic effectiveness indices of rhizobia isolates

The relative symbiotic effectiveness percentage (RSE %) of the isolates for atmospheric nitrogen fixation was calculated using the methods of Purcino *et al.* (2000) by comparing the inoculated plant with the N-fertilized positive control by using the following formula:

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

The nitrogen-fixing efficiency of the isolates was rated as highly effective (SE % > 80%), effective (SE % = 50- 80%), poorly effective (SE % = 35-50% and ineffective (SE % <35%) (Purcino *et al.,* 2000).

3.9. Host Range Evaluation

Seven isolates were tested for their ability to nodulate peanut *(Arachis hypogaea,* mung bean *(Vigna radiata),* haricot bean (*Phaseolus vulgaris*) and (*Glycine max* L.) soybean variety. Seeds were surface sterilized, pre-germinated, and transplanted into sterilized plastic cups (Somasegaran & Hoben, 1994). After three days, seedlings were thinned and inoculated with a specific rhizobial isolate. Controls for each legume species included treatments without inoculation and a positive N treatment. Plants were irrigated weekly with an N-free nutrient solution prepared according to Broughton and Dilworth (1970) cited in Somasegaran and Hoben (1994) and harvested after 45 days for NN, NDW, and SDW assessment.

The method to determine the Relative symbiotic effectiveness percentage (RSE %) for these isolates was described in section 3.8. This involves comparing the shoot dry weight of plants inoculated with rhizobia isolates to the shoot dry weight of N-fertilized plants and expressing the result as a percentage. The nitrogen-fixing efficiency of the isolates was then rated as described in section 3.8.

3.10. Data Analysis

The data collected (nodule numbers, nodule dry weight and shoot dry weight) were subjected to the analysis of variance (ANOVA) using the Generalized Linear Model procedure of SAS JMP pro software Version 17.0. The data were checked for the assumptions of ANOVA, such as normality and homogeneity of variance, before performing the ANOVA. Post hoc test such as Tukey’s HSD was used to compare the means of different treatments. Correlation analysis was carried out to study the nature and degree of relationship between nodule numbers, nodule dry weight, shoot dry weight and symbiotic effectiveness. Symbiotic effectiveness was calculated as the ratio of shoot dry weight of inoculated plants to that of uninoculated positive control plants.

4. RESULTS

4.1. Rhizobia Trapping and Nodulation Potential

Four out of the six soil samples (66.66%) collected from different sampling sites demonstrated the capacity to induce and support nodulation in soybean (*Glycine max* L.) (Table 4.1). This suggests that these soils contain compatible nodulating rhizobia strains for soybeans. The average number of nodules (NN) observed per plant ranged from 2 to 16 nodules.

Table 4.1: Nodulation potential and rhizobia isolates in different Kebeles

Kebele	NN plant^{-1}	Isolate obtained
Dobe Negasha	nno	-
Gaaluko Horo	2	ISR7w
Gala Galo	9	ISR5 ISR6
Haweela Lida	13	ISR4 ISR4c
Muranicho Quxala	16	ISR1 ISR2 ISR2w
Nure Dullacha	nno	-

Note: nno=nodulation not obtained, NN=Number of Nodules, ISR= Indigenous Soybean Rhizobia

4.2. Morphological and Cultural Characteristics of Indigenous Soybean Rhizobia

Eight rhizobia isolates were successfully obtained from the root nodules of TGx Soybean variety plants that were cultivated under controlled greenhouse conditions.

4.2.1. Growth and Cultural Characteristics of Indigenous Soybean Rhizobia Isolates

The indigenous soybean rhizobia isolates exhibited diverse colony characteristics. Predominantly, the isolates were mucoid and translucent, with variations in the density and consistency of mucus production, exopolysaccharide (EPS). An exception was ISR2, which displayed a watery and translucent phenotype. ISR6 and ISR2w, while mucoid, were opaque. All isolates formed circular colonies with entire margins. Post-incubation (7–10 days), colony diameters ranged from 2.0 to 5.7 mm. Most isolates formed raised colonies, with ISR2 and ISR7w forming flat colonies. These morphological characteristics are detailed in Table 4.2. and see Appendix 2 for pictures.

Table 4.2: Morphological characteristics of indigenous soybean rhizobia isolates from selected small holder farmers' fields of Hawella woreda, Sidama regional state

Soybean Rhizobia Isolates	Colony shape	Colony margin	Colony texture	Colony Size (mm)	Colony Opacity	Elevation
ISR1	Circular	Entire	Mucoid	2.0	Translucent	Raised
ISR2	Circular	Entire	Watery	2.5	Translucent	Flat
ISR2w	Circular	Entire	Mucoid	4.0	Opaque	Raised
ISR4	Circular	Entire	Mucoid	5.7	Translucent	Raised
ISR4c	Circular	Entire	Mucoid	3.0	Translucent	Raised
ISR5	Circular	Entire	Mucoid	3.4	Translucent	Raised
ISR6	Circular	Entire	Mucoid	3.0	Opaque	Raised
ISR7w	Circular	Entire	Mucoid	3.7	Translucent	Flat

4.2.2. Gram Staining

Following Gram staining, microscopic examination conclusively revealed that all isolates exhibited a Gram-negative staining pattern, further confirming their identity as rhizobia (Table 4.3 and Appendix 3).

4.2.3. Growth Response of Rhizobia Isolates on YEMA medium containing CR

Post a 7-day incubation period on YEMA medium containing Congo Red (CR), all eight isolates formed visible colonies, with diameters spanning 1-3 mm (Table 4.3 and Appendix 4). Notably, isolates ISR2w, ISR4, and ISR4c exhibited no CR absorption, resulting in a contrasting whitish appearance against the medium's red background. ISR1 demonstrated minimal absorption, rendering it almost colorless against the medium. The remaining four isolates, while absorbing the dye, only displayed a slightly pale pink hue, indicating minor absorption.

4.2.4. Growth Response of Rhizobia Isolates on Glucose Peptone Agar (PGA) Medium

Of the eight Rhizobia isolates grown on Peptone Glucose Agar (PGA) medium, ISR1 and ISR6 exhibited negligible growth. In contrast, the remaining six isolates demonstrated growth on the PGA medium. Notably, despite this growth, no color change was observed in the medium (Table 4.3 and Appendix 5).

4.2.5. Acid-Base Production Test

Most isolates exhibited an acidic reaction, as indicated by the yellow coloration surrounding their colonies within 3 days of incubation, suggesting rapid growth and metabolic activity (Table 4.3 and Appendix 6a). In contrast, ISR1 demonstrated an alkaline reaction, with a slower growth rate indicated by a colony diameter of 2 mm after 5 to 7 days of incubation. This isolate produced white colonies with translucent opacity on the YEMA medium containing BTB (Table 4.3 and Appendix 6b).

4.2.6. Ketolactose Test Results of Rhizobia Isolates

In the Keto Lactose test, none of the Rhizobia isolates produced a yellowish zone when flooded with Benedict's reagents, suggesting the absence of ketolactase-producing contaminants (Table 4.3 and Appendix 7).

Table 4.3: Overview of the result of various tests

Test	Rhizobia Isolates							
	ISR1	ISR2	ISR2w	ISR4	ISR4c	ISR5	ISR6	ISR7w
YEMA + CR	Min. abs.	No abs.	Pale pink	No abs.	No abs.	Pale pink	Pale pink	Pale pink
Growth on PGA	Neg. growth	+	+	+	+	Neg. growth	Neg. growth	+
Acid-base production	Alk.	Acid.	Acid.	Acid.	Acid.	Acid.	Acid.	Acid.
Ability to produce 3-ketolactase	-	-	-	-	-	-	-	-
Gram Reaction	-	-	-	-	-	-	-	-

Key: Min. abs.: Minimal absorption, No abs.: No absorption, Pale pink: Slight pale pink, Neg. growth: Negligible growth, +: Growth or positive result, Alk.: Alkaline, Acid.: Acidic, -: No growth or negative result.

4.3. Physiological and Biochemical Characteristics of the Isolates

4.3.1. pH Tolerance Profile of Rhizobia Isolates

The pH tolerance test revealed that ISR1 and ISR4 exhibited robust growth across all tested pH levels, suggesting a broad pH tolerance. ISR2 showed sensitivity to both acidic and alkaline conditions, indicating a narrow pH tolerance range. ISR2w and ISR4c displayed moderate tolerance to alkaline conditions, while ISR7w showed consistent growth across all tested pH levels (Table 4.4 and Appendix 8).

4.3.2. Sodium Chloride Stress Response of Rhizobia Isolates

The sodium chloride stress response test revealed that ISR1, ISR4, ISR4c, and ISR2 showed growth in the presence of 2% NaCl, indicating a level of tolerance to saline conditions. However, ISR7w displayed poor tolerance to 2% NaCl. The remaining isolates did not show discernible tolerance to the elevated salt concentration (Table 4.4 and Appendix 9).

4.3.3. Catalase Test Results for Rhizobia Isolates

The catalase test results revealed that all isolates were catalase-positive, suggesting a shared ability to decompose hydrogen peroxide into water and oxygen (Table 4.4 and Appendix 10).

4.3.4. Alternative Carbon Source Utilization by Indigenous Rhizobia Isolates

The rhizobia isolates demonstrated robust growth on plates containing glucose, fructose, arabinose, lactose, and sucrose, forming visible colonies within the incubation period. Most of the isolates also utilized sodium citrate as a sole carbon source, reaching an average colony diameter of 2 mm after 7 days. However, some isolates formed smaller colonies on the medium containing sodium citrate. On starch plates, most of the isolates produced slight growth. These results indicate that while the soybean rhizobia isolates can utilize a range of carbon sources, preferences exist, with glucose, fructose, lactose, arabinose, and sucrose supporting maximal growth across all isolates tested (Table 4.4 and Appendix 11A).

4.3.5. Metabolic Capabilities of Rhizobia Isolates in Utilization of Nitrogen Sources

All rhizobia isolates were capable of metabolizing the tested organic nitrogen compounds or amino acids as sole nitrogen sources, as indicated by visible colonies on basal agar plates. Specifically, all isolates exhibited growth comparable to control conditions when L-alanine, L-arginine, or L-phenylalanine replaced ammonium sulfate in the basal medium, with colonies appearing post a 10-day incubation at 28°C. The lack of growth on nitrogen-devoid basal medium plates confirmed the necessity of a nitrogen compound for growth. No significant differences were observed in growth rates or extents among isolates on individual amino acid supplements. All isolates utilized L-alanine, L-arginine, and L-phenylalanine with equal efficiency, as per surface colonization scores. (Table 4.4 and Appendix 11B).

4.3.6. Methyl Red Test Results for Rhizobia Isolates

The MR test indicated that all rhizobia isolates, except ISR6, can ferment glucose into acid end products within 48 hours, as evidenced by the development of red color in the MR reagent. (Table 4.4 and Appendix 12).

4.3.7. Urea Hydrolysis Test Results for Rhizobia Isolates

The urea hydrolysis test results indicated that all rhizobia isolates can produce urease, an enzyme that decomposes urea into ammonia and carbon dioxide. This was evidenced by the color change of the urea agar medium from yellow to pink within 3-7 days, suggesting an additional nitrogen source for growth (Table 4.4).

4.3.8. Indole Production Test Results for Rhizobia Isolates

The indole production test results showed that most rhizobia isolates can break down the amino acid tryptophan to produce indole, as evidenced by the pink color development in the Kovac’s reagent layer within 48 hours. This suggests the likely functionality of the metabolic pathways required for nitrogen fixation in these isolates (Table 4.4 and Appendix 13).

Table 4.4: Physiological and biochemical test result of the rhizobia

Test		Rhizobia Isolates							
		ISR1	ISR2	ISR2w	ISR4	ISR4c	ISR5	ISR6	ISR7w
Growth on 2% NaCl		+	+	Poor	+	+	-	-	Poor
pH tolerance	pH4	+	-	+	+	+	-	-	+
	pH6.8	+	+	+	+	+	+	+	+
	pH9	+	-	+	+	+	-	-	+
Carbon source utilization		6/7	6/7	6/7	6/7	6/7	5/7	5/7	5/7
Nitrogen source utilization		All	All	All	All	All	All	All	All
Catalase activity		+	+	+	+	+	+	+	+
Methyl Red test		+	+	+	+	+	+	-	+
Urea hydrolysis test		+	+	+	+	+	+	+	+
Indole production test		+	+	+	+	+	-	-	+

Note: In the table, “+” indicates positive growth or a positive test result, while “-” indicates no growth was observed. “Poor” indicates poor growth, “All” refer to the types of sources utilized.

4.4. Authentication and Symbiotic Effectiveness of Rhizobia Isolates in Sand Culture

4.4.1. Authentication of Rhizobia Isolates in Sand Culture

The rhizobia isolates, initially collected from soybean plants in greenhouse conditions, were reinoculated in a sterile sand culture experiment, resulting in nodule formation on soybean roots. All indigenous rhizobia isolates formed nodules on the variety, while the uninoculated control showed no nodules (Table 4.5). See also Appendix 16 for bar chart for the effects of rhizobia isolates on the nodule numbers, nodule dry weights and shoot dry weights per plant of soybean.

Table 4.5: Comparative analysis of the effects of various rhizobia isolates on nodule formation and plant growth in soybeans grown in sand culture under greenhouse conditions

	NN	NDW (mg)	SDW (g)	RSE (%)	SE rating
Isolates	Mean ± Std Err	Mean ± Std Err	Mean ± Std Err		
ISR1	5.67 ± 0.67^{b}	33.30 ± 3.61^{a}	0.75 ± 0.02^{b}	84.26	HE
ISR2	2.67 ± 0.33^{cde}	19.10 ± 3.06^{b}	0.61 ± 0.01^{cd}	68.53	E
ISR2w	3.67 ± 0.33^{bcd}	20.07 ± 1.80^{b}	0.74 ± 0.02^{b}	83.14	HE
ISR4	9.33 ± 0.88^{a}	41.03 ± 4.16^{a}	0.91 ± 0.02^{a}	102.24	HE
ISR4c	3.33 ± 0.88^{bcd}	18.77 ± 2.94^{b}	0.63 ± 0.03^{c}	70.78	E
ISR5	1.33 ± 0.33^{de}	9.83 ± 2.84^{bc}	0.43 ± 0.02^{ef}	48.31	PE
ISR6	1.67 ± 0.33^{cde}	8.80 ± 0.96^{bc}	0.52 ± 0.02^{de}	58.42	E
ISR7w	4.33 ± 0.88^{bc}	18.50 ± 2.39^{b}	0.61 ± 0.01^{cd}	68.53	E
- ctrl	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}	0.41 ± 0.02^{f}		
+ ctrl	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}	0.89 ± 0.01^{a}		
All	3.20 ± 0.52	16.94 ± 2.42	0.65 ± 0.03		
Cv	88.92	78.09	26.11		
F ratio	24.7663	26.1575	71.8998		
Prob>F	0.0001	0.0001	0.0001		

Note: Levels not connected by the same letter on superscripts are significantly different at ($p < .05$). CV = Coefficient of variation, NN= number of nodules, NDW=nodule dry weight, SDW=shoot dry weight, - ctrl= Negative control, + ctrl= positive control, SE= Relative symbiotic effectiveness percentage, SE= symbiotic effectiveness

4.4.2. Influence of Indigenous Soybean Rhizobia Isolates on Nodule Formation

A one-way ANOVA revealed a highly significant effect of the indigenous rhizobia isolates on the number of nodules per plant ($F(9, 20) = 24.77$, $p < .0001$) as shown in Table 4.5. This indicates that there is a significant difference in the number of nodules produced by different rhizobia isolates, with at least two of the isolates differing significantly in their ability to form nodules. The average nodule count was 3.20 per plant, with isolate ISR4 inducing the most nodules (9.33 per plant) and ISR2w the least (1.33 per plant). The control groups showed no nodule formation. Further analysis confirmed significant differences between isolate groups in their ability to induce nodule formation (Appendix 15A).

4.4.3. Influence of Indigenous Soybean Rhizobia Isolates on Nodule Dry Weight

A similar one-way ANOVA conducted to examine the impact of the isolates on nodule dry weight also revealed a highly significant effect ($F(9, 20) = 26.16$, $p < .0001$). The average nodule dry weight across all isolates was 16.94 mg. ISR4 yielded the highest average nodule dry weight (41.03 mg), while ISR6 exhibited the lowest (8.80 mg). The control groups displayed no nodule formation (Table 4.5). Post-hoc analysis delineated significant distinctions in nodule dry weight among isolate groups (Appendix 15B).

4.4.4. Influence of Indigenous Soybean Rhizobia Isolates on Shoot Dry Weight

A one-way ANOVA conducted to investigate the impact of indigenous soybean rhizobia isolates on shoot dry weight revealed a highly significant effect ($F(9, 20) = 71.90$, $p < .0001$). The average shoot dry weight across all isolates was 0.65 g. ISR4 induced the highest average shoot dry weight (0.91 g), a significant increase above the control (non-inoculated plants), while ISR5 generated the lowest average shoot dry weight (0.43 g) (Table 4.5). ISR4 were found to be the most effective in promoting higher shoot dry weights, while ISR2w and Negative control treatments resulted in the lowest shoot dry weights. Post-hoc analysis using Tukey’s HSD test delineated significant distinctions in shoot dry weight among isolate groups (Appendix 15C).

4.4.5. Symbiotic Effectiveness of Indigenous Rhizobia Isolates

The relative symbiotic effectiveness of these isolates was estimated by comparing the shoot dry matter accumulation of plants inoculated with each isolate to that of a nitrogen-fertilized control. The estimated values for relative symbiotic effectiveness exhibited a broad range, with isolate ISR5 at the lower end with 48.31% RSE, and isolate ISR4 at the higher end with 102.24% RSE (Table 4.5). Further classification of these isolates based on the criteria established by Purcino *et al.* (2000) revealed varying levels of effectiveness. Specifically, three of the isolates, representing 37.5% of the total, were categorized as highly effective. Four isolates, accounting for 50% of the total, were deemed effective. Lastly, one isolate, constituting 22.5% of the total, was classified as poorly effective.

4.4.6. Correlation Analysis of Selected Parameters in Sand Culture

A correlation analysis was conducted to examine the relationships between the number of nodules (NN), nodule dry weight (NDW), shoot dry weight (DW), and SE (%). The results indicated strong positive correlations between all pairs of variables, which were statistically significant ($P < 0.001$) as shown in Table 4.6. Specifically, the NN showed a strong positive correlation with NDW ($r = 0.9587$), SDW ($r = 0.9233$), and SE (%) ($r = 0.9227$). This suggests that as the NN increases, both the NDW and SDW, as well as SE (%), tend to increase. Similarly, NDW also exhibited strong positive correlations SDW ($r = 0.9346$) and SE (%) ($r = 0.9308$). This indicates that an increase in NDW is associated with increases in both SDW and SE (%). Finally, an extremely strong positive correlation was observed between SDW and SE (%) ($r = 0.9998$), suggesting that these two variables increase in tandem.

Table 4.6: Correlation coefficients among number of nodules, nodule dry weight, shoot dry weight, and symbiotic effectiveness

	NN	NDW (mg)	SDW (g)	SE (%)
NN	1.0000			
NDW (mg)	0.9587	1.0000		
SDW (g)	0.9233	0.9346	1.0000	
SE (%)	0.9227	0.9308	0.9998	1.0000

Note: The correlations are estimated by Row-wise method. NN= number of nodules, NDW=nodule dry weight, SDW=shoot dry weight, SE (%) = Symbiotic Effectiveness

4.5. The Host range of Soybean Rhizobia Isolates

All isolates were able to nodulate mung bean *(Vigna radiata)*, haricot bean (*Phaseolus vulgaris*) and soybean (*Glycine max* L.) but none were able to nodulate peanut *(Arachis hypogaea.* Significant variations were observed in the nodulation properties across different treatments for soybean, haricot bean, and mung bean. As shown in Table 4. 7, the most effective isolate for soybean was ISR4, which resulted in the highest number of nodules (3.00 plant^{-1}) and nodule dry weight (15.87 mg plant^{-1}). This isolate also led to a shoot dry weight of 0.86 g plant^{-1} and a symbiotic efficiency of 90.88%. For haricot bean, the ISR7w isolate was the most effective, leading to the highest number of nodules (30.00^{-1}) and nodule dry weight (41.93 mg plant^{-1}). This isolate also resulted in a shoot dry weight of 1.24 g plant^{-1} and a symbiotic efficiency of 83.99%. In the case of mung bean, the ISR7w isolate again proved to be the most effective, resulting in the highest number of nodules (36.00 plant^{-1}) and nodule dry weight (80.97 mg plant^{-1}). This isolate also led to a shoot dry weight of 1.31 g plant^{-1} and a symbiotic efficiency of 81.12%. See Appendix 14A, 14B and 14C for the pictures of nodule formed by rhizobia isolates in sand culture trial. In the table below, the results of tests on soybean, haricot bean, and mung bean are presented. Please note that while tests were also conducted on peanuts, none of the isolates formed nodules on the peanut plants. Therefore, nodulation data for peanuts is not included in this table.

Table 4.7: Differential in nodulation and symbiotic efficiency across legume hosts by the soybean rhizobial isolate

	Soybean				Haricot bean				Mung bean			
Isolate	NN	NDW (mg)	SDW (g)	SE	NN	NDW (mg)	SDW (g)	SE	NN	NDW (mg)	SDW (g)	SE
ISR1	1.67±0.67^{b}	7.63±4.22^{bc}	0.67±0.01^{d}	71	23.33±1.45^{bc}	36.93±1.54^{bc}	0.9±0.01^{ef}	60	19±1.53^{c}	39.83±3.44^{d}	1.2±0.02^{cd}	74
ISR2	1.33±0.33^{b}	7.53±3.71^{bc}	0.83±0.01^{bc}	87	17±1.15^{d}	25.13±1.94^{de}	0.87±0.01^{f}	58	18.33±2.03^{c}	48.47± 0.48^{c}	1.19±0.03^{d}	73
ISR2w	1.67±0.33^{b}	9.27±3.75^{b}	0.77±0.04^{c}	81	19±1.15^{cd}	30.17±1.70^{cd}	0.99±0.05^{d}	67	24.67±1.45^{c}	54.8± 2.14^{c}	1.24±0.01^{cd}	76
ISR4	3±0.58^{a}	15.87±2.96^{a}	0.86±0.01^{b}	90	28.33±1.76^{ab}	44.43±3.78^{ab}	1.11±0.05^{c}	75	21.33±1.45^{bc}	66.53± 3.17^{b}	1.25±0.01^{c}	77
ISR4c	1.33±0.33^{b}	6.6±1.55^{bc}	0.61±0.04^{e}	64	10.67±0.88^{e}	19.03±1.30^{e}	0.95±0.01^{de}	64	13.33±1.33^{d}	34.43± 5.82^{d}	0.88±0.01^{e}	54
ISR6	0.67±0.33^{bc}	4.17±3.65^{cd}	0.55±0.02^{e}	58	14.33±1.45^{de}	24.63±1.85d^{e}	0.73±0.02^{g}	49	11.33±1.86^{d}	35.93± 3.27^{d}	0.81±0.01^{f}	50
ISR7w	1.33±0.33^{b}	9.93±2.00^{b}	0.68±0.02^{d}	72	30±4.51^{a}	41.93±5.79^{a}	1.24±0.01^{b}	83	36±1.53^{a}	80.97± 1.13^{a}	1.31±0.01^{b}	81
- ctrl	0±0.00^{c}	0±0.00^{d}	0.37±0.01^{f}		0±0.00^{f}	0±0.00^{f}	0.53±0.01^{h}		0±0.00^{e}	0± 0.00^{e}	0.36±0.02^{g}	
+ ctrl	0± 0.00^{c}	0±0.00^{d}	0.94±0.01^{a}		0±0.00^{f}	0±0.00^{f}	1.48±0.02^{a}		0±0.00^{e}	0± 0.00^{e}	1.61±0.02^{a}	
Mean	1.22±0.20	6.77±1.03	0.69±±0.03		15.85±2.08	25.36±3.26	0.97±0.05		16±2.16	40.1±5.09	1.09±0.07	
CV	85.91	78.95	24.51		68.34	66.71	27.85		70.26	65.96	31.79	
F ratio	5.8125	9.0541	64.3487		34.5304	42.8020	97.5498		65.3735	91.9622	459.8473	
Prob>F	0.0010	0.0001	0.0001		0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	

Note: Levels not connected by same letter on superscripts are significantly different at ($p < .05$) CV = Coefficient of variation, NN= number of nodules, NDW=nodule dry weight, SDW=shoot dry weight, - ctrl= Negative control, + ctrl= positive control, SE= Relative symbiotic effectiveness percentage

5. DISCUSSIONS

5.1. Nodulation Potential of Study Area Soils

The results (Table 4.1) suggest the presence of rhizobia isolates compatible with soybeans in the Hawella woreda of the Sidama region, compared to other Ethiopian regions. This is particularly noteworthy when compared to the findings of Diriba Temesgen (2017), who reported that only 13% of soil samples from various parts of Ethiopia harboured root nodule bacteria nodulating soybeans. However, not all soil samples in this study demonstrated the capacity to induce and support nodulation, suggesting that there may be factors limiting rhizobia activity in certain areas. This aligns with another study by Yifru Abera *et al.* (2019), which found that 80% of soil samples induced nodulation on soybeans, indicating that most soils harbour compatible nodulating rhizobia for the crop. The variation in the number of nodules observed further highlights the diversity of rhizobia populations within these soils. This diversity might have important implications for the symbiotic efficiency of these rhizobia and their contribution to soybean growth and productivity.

Comparatively, the study by Erana Kebede *et al.* (2020) in Ethiopia on the other legume, cowpea, reported that 93.3% of soil samples collected from different sampling sites (Oromiya region, Southern Nations, Nationalities and Peoples region, and Gambella region) induced and supported nodulation on cowpea, suggesting that these soils are rich with rhizobia nodulating the cowpea. This difference in nodulation capacity between soybean and cowpea could be attributed to variations in legume varieties, soil properties, climate conditions, or agricultural practices. These comparisons provide a broader context for understanding the distribution and activity of legume-nodulating rhizobia in Ethiopia and highlight areas for further research. Further investigation into the factors contributing to the variation in nodulation capacity among different soil samples could provide valuable insights for obtaining diverse rhizobia compatible with soybeans and other pulses.

5.2. Characteristics of the Indigenous Soybean Nodulating Rhizobia Isolates

5.2.1. Morphological and Cultural Aspects

The isolation process yielded eight rhizobia isolates with diverse growth and morphological traits (Table 4.2). Most isolates formed mucoid and translucent colonies, produced abundant mucus, and had circular colonies with entire margins. Remarkably, one isolate displayed a unique watery and highly translucent morphology. Variations were also observed in mucus production levels and colony opacity between some isolates. These results demonstrate the phenotypic heterogeneity existing among indigenous rhizobia populations colonizing soybeans and possible genetic variations of such rhizobia in the study area.

The colony phenotypes of the local soybean nodulating rhizobia share similarities but also differ from isolates reported elsewhere. Many studies found that common morphologies include mucoid-raised colonies, matching the majority here (Gachande & Khansole, 2011; Youseif *et al.*, 2014). The isolates developed colonies with sizes ranging from 2.0 to 5.7 mm in diameter (Table 4.2). This variability in size hinted at differing growth rates and biomass production capacities among the isolates. Similar results were reported by Diriba Temesgen and Fassil Assefa (2020), where soybean rhizobial isolates had colony diameters ranging from 2.5 to 6 mm. Yifru Abera *et al.* (2018), reported the isolates' variations in colony size up to 5.0 mm in diameter after five days of incubation.

All isolates produced mucous extracellular polysaccharides (EPS) ranging from excessive to intermediate, with density/consistency variations. EPS facilitate rhizobial adaptation to environments, aiding processes like root nodule formation and nitrogen fixation (Skorupska *et al.*, 2006; De Sousa *et al.*, 2021). Isolates with high EPS ability have competitive advantages in infection/colonization (Ondieki *et al.*, 2017). Mucus produced by rhizobia may serve as a potential source of energy under conditions of nutrient scarcity (Kapembwa, 2016).

The isolates formed whitish to pale pink colonies on YEMA+CR, indicating no dye absorption. This inability of the isolates to absorb CR dye is a distinctive character of rhizobia (Somasegaran & Hoben, 1994). The same result was reported by Yifru Abera *et al.* (2018), who reported that all soybean isolates produced colonies of whitish to pale pink when cultured on a YEMA medium containing CR.

Six rhizobia isolates were found to grow on PGA medium without causing a color change, while two isolates showed minimal growth (Table 4.3). This result contradicts the earlier belief that rhizobia cannot grow on PGA, and aligns with recent studies. For instance, Sharma *et al.* (2010) found that out of 18 soybean rhizobia isolates, 14 showed poor or no growth on PGA, while 5 did grow. Similarly, Ansari and Rao (2014), and Yifru Abera *et al.* (2018) reported some soybean rhizobia isolates' growth in this medium. These findings challenge the previous understanding of rhizobia's growth capabilities on PGA, emphasizing the importance of continual research in this field. The ability of some rhizobia isolates to grow on PGA could have significant implications for future studies.

The results of the ketolactose test showed that none of the rhizobial isolates produced 3-ketolactose from lactose, which confirms the rhizobia colonies as no yellow zone of copper oxide was observed around the colonies, indicating an inability to ferment lactose under the test conditions (Table 4.3). This aligns with previous research on soybean nodulating rhizobia which also did not produce 3-ketolactose (Sharma et al., 2010; Harpreet *et al.,* 2012; Ansari & Rao, 2014). However, the rhizobia may be still able to metabolize lactose as a carbon source without utilizing the specific pathway leading to 3-ketolactose production. This alternative interpretation is consistent with both robust growth on lactose media and the negative 3-ketolactose results. Importantly, 3-ketolactose production is typically a feature of *Agrobacterium* species rather than rhizobia, highlighting the specificity and utility of this test in distinguishing bacterial species (Teresa *et al.,* 2021).

Most isolates were identified as fast-growing soybean rhizobia, based on colony development duration on YEMA, colony diameter (Table 4.2), and BTB-YMA color changes (Table 4.3). This is consistent with earlier reports by Hungria *et al.* (2001). However, one isolate was considered a slow-growing rhizobia, producing white colonies with translucent opacity. This finding aligns with the results of Ondieki *et al.,* (2017). Purwaningsih *et al.* (2020) reported similar results, with all isolates being fast-growing rhizobium nodulating soybean. Kapembwa (2016) also found a predominance of fast-growing rhizobia, with 59 out of 61 isolates being classified as such. Despite previous research in Africa and Ethiopia reporting a dominance of slow-growing soybean rhizobia, this study found a predominance of fast-growing rhizobia in the Sidama region of Ethiopia. This aligns with the findings of Diriba Temesgen and Fassil Assefa (2020), who reported a dominance of fast-growing rhizobia among soybean isolates in Ethiopia. Specifically, they found that 10 out of 12 soybean rhizobia isolates were fast-growing. This consistency in findings strengthens the evidence for the dominance of fast-growing rhizobia in the study area and potentially other parts of Ethiopia. It also suggests that these fast-growing strains could belong to the *Sinorhizobium* genus, known for its symbiotic efficiency and adaptability to various environmental conditions. These findings underscore the importance of regional studies in contributing to our understanding of rhizobial population dynamics.

5.2.2. Physiological and Biochemical Aspects

The physiological and biochemical characteristics of soybean-nodulating rhizobia isolates revealed in this study provide nuanced insights into their adaptability and potential effectiveness under in vitro conditions. The pH tolerance of the isolates, as observed in this study, offers valuable information about their potential adaptability in varying conditions. Isolates ISR1, ISR4, and ISR7w demonstrated robust growth across all tested pH levels, indicating broad pH tolerance (Table 4.4). This suggests that the isolates possess versatile pH tolerance, which could enhance their survival and nodulation efficiency in diverse soil pH environments. The ability to grow under these conditions indicates a level of pH tolerance, which is an important trait for rhizobia functioning in various soil environments (Zhang *et al.*, 2020). In contrast, ISR2 showed sensitivity to both acidic and alkaline conditions, with no observable growth at pH 4.0 and 9.0 (Table 4.4). This narrow pH tolerance range indicates ISR2 may be more suited to neutral pH soils. Isolates ISR2w and ISR4c exhibited moderate tolerance to alkaline conditions, suggesting potential to survive in slightly alkaline soils. However, their limited growth at pH 9.0 implies performance could be hindered in highly alkaline conditions.

These pH tolerance findings align with previous in vitro research. Yifru Abera *et al.* (2018) reported nearly all isolates grew on YEMA at pH 5.0-9.5, regardless of taxonomic grouping. Diriba Temesgen and Fassil Assefa (2020) also found soybean rhizobia isolates tolerant to a wide pH range (5-10). This study further revealed many isolates that were pH 4 tolerant, consistent with findings by Youseif *et al.* (2014) and Mulama *et al.* (2020) on acid tolerance in some fast-growing Egyptian soybean rhizobia. This pattern of tolerance to both acidic and alkaline conditions, also noted by Ali *et al.* (2019), suggests a common trait among soybean-nodulating rhizobia isolates. Their survival at acidic pH could stem from physiological and biochemical adaptation mechanisms like proton exclusion, polyamine accumulation, high potassium and glutamate levels, and altered lipopolysaccharide composition (Vriezen *et al.*, 2007). These findings highlight the adaptability of soybean-nodulating rhizobia isolates under varying soil conditions, which could enhance survival and nodulation efficiency across diverse pH environments.

Some isolates thrived in a 2% NaCl medium, demonstrating their salt tolerance, unlike most isolates in Yifru Abera *et al.* (2018). However, consistent with this study, Yifru Abera *et al.* (2018), also found fast-growers that were more salt resilient (up to 4% NaCl) than slow-growers. Tulu Degefu *et al.* (2018) reported southern Ethiopian rhizobia tolerated up to 5.5% NaCl, suggesting the potential for effective nitrogen fixation in saline soils. Supporting these findings, Paudyal *et al.* (2021) showed good rhizobial strain growth and tolerance in 2% NaCl, indicating fast-growers may be well-suited for saline soils. Ali *et al.* (2019) similarly reported ~57% of soybean nodulating rhizobia strains tolerated 2.0% salt, with one (SB-452) growing at 4.0%. These observations demonstrate certain rhizobia isolates' adaptability under saline conditions, potentially enhancing competitiveness and nitrogen fixation efficiency.

The positive catalase test results for all isolates suggest they possess the ability to neutralize hydrogen peroxide's bactericidal effects, which could be key for rhizobia survival and function in various environments (Table 4.4). These findings align with Paudyal *et al.* (2021), who reported 100% positive catalase tests in soybean nodulating rhizobia. Similarly, Abrar Hamza and Letebo Alebejo, 2017) found comparable results in rhizobia from cowpea, elephant, and lablab root nodules in Ethiopia. These consistent findings across studies highlight the importance of catalase activity in rhizobia survival and function.

Most isolates (75%) produced indole by breaking down tryptophan, a trait common in many nitrogen-fixing legume root nodulating rhizobia species, suggesting these isolates likely possess pathways required for nitrogen fixation (Table 4.4). This highlights the potential adaptability, competitiveness, and nitrogen fixation efficiency of these rhizobia isolates under varying conditions. These findings concur with Yifru Abera *et al.* (2018), who found most soybean rhizobial isolates were indole producers with L-tryptophan supplementation. Additionally, Lebrazi *et al.* (2020) found Rhizobium species produced the highest indole levels with L-tryptophan. This suggests that these isolates likely possess pathways required for nitrogen fixation.

The ability of the rhizobia isolates to ferment glucose into acid end products, as indicated by the red color in the MR reagent, suggests that they possess a key characteristic of effective nodule-forming rhizobia. The result aligns with Soundarya *et al.* (2022).

The isolates showed strong growth on various carbon sources (Table 4.4), enhancing their adaptability in different soils. Some could also utilize sodium citrate, but less efficiently, and starch led to minimal growth. This ability to use diverse carbon sources could improve biofertilizer production. According to Tulu Degefu *et al.* (2018), effective biofertilizer production requires stress-tolerant isolates that can compete for nutrients. Isolates that utilize more carbon sources have an advantage. Fast-growing soybean rhizobia were found to be more efficient in utilizing a broader spectrum of carbon sources and amino acids (Diriba Temesgen and Fassil Assefa 2020). Yifru Abera *et al.* (2019) reported that fewer isolates could assimilate sodium citrate and propionate. All isolates also demonstrated robust growth on different nitrogen sources, indicating an ability to utilize a wide nitrogen range. Mullisa Jida and Fassil Assefa (2011) reported this broader nitrogen use provides survival and competitive benefits in soil.

5.3. Authentication and Symbiotic Effectiveness of Soybean Nodulating Rhizobia

The rhizobial isolate's ability to form a nodulation relationship with a legume and its nitrogen fixation outcome can vary significantly (Terpolilli *et al.,* 2008). Hence, it's essential to evaluate the infectivity, nodulation, and symbiotic effectiveness of native rhizobial populations for inoculant production. This study revealed that all indigenous isolates could form nodules on the tested soybean Tgx variety, affirming their status as true rhizobia capable of establishing a symbiotic relationship with their host. The absence of nodules in the uninoculated control indicates that aseptic conditions were maintained in the experimental setup.

The average nodule count across all isolates was 3.20 nodules per plant, with considerable variation among the different isolates (Table 4.5). For instance, isolate ISR4 induced the highest average number of nodules per plant (9.33), while ISR2w showed the lowest average nodule count (1.33 nodules per plant). This suggests that different rhizobia strains may vary in their ability to induce nodule formation, which could be attributed to differences in their genetic makeup or environmental adaptation. This suggests that different rhizobia strains may vary in their ability to induce nodule formation, which could be attributed to differences in their genetic makeup or environmental adaptation (Yifru Abera *et al.,* 2019; Erana Kebede *et al.,* 2020). The enhancing effects of soybean inoculation on the number of nodules per plant, as evidenced by a significant increase in root nodules and nodulation, were corroborated by the findings of

Hungria *et al.* (2006) and further supported by Manish and Kumawat (2011), who observed similar results when inoculating soybean varieties with native *Bradyrhizobium* isolates compared to uninoculated treatments.

In terms of nodule dry weight, the average across all isolates was 16.94 mg. ISR4 yielded the highest average nodule dry weight (41.03 mg), indicating that it was not only effective at inducing nodule formation but also promoted the development of larger and potentially more active nodules (Table 4.5).

The significant difference observed in the number of nodules and nodules' dry weight indicates the different abilities of rhizobia in forming symbiotic nodules (Zerihun Getachew and Lijalem Abeble, 2020). Nodulation, particularly the dry weight of nodules, is a crucial factor in assessing the ability of rhizobia to fix nitrogen. The dry weight of nodules is determined by their composition, which includes bacteroids (N_2 fixing rhizobia), proteins, enzymes, and nutrients, as evidenced by different isolates in this study. These various components each perform unique roles to ensure a successful and efficient N_2 fixation process (Schwember *et al.,* 2019). However, the number of nodules may not precisely indicate the extent of N_2 fixation if they lack certain components such as leghemoglobin, which is responsible for supplying oxygen to bacteroids inside the nodules (Ott *et al.*, 2005; Thilakarathna and Raizada, 2017).

A large number of nodules with a higher dry weight typically indicate effective symbiotic nodulation and host specificity (Alam *et al.,* 2015; Zerihun Getachew and Lijalem Abeble, 2020). In this study, for instance, plants inoculated with some isolates, including ISR4, had many nodules with the highest nodule dry weight (Table 4.5). Moreover, the highest shoot dry weight and shoot length for a specific isolate suggest its effectiveness in N_2 fixation (Hungria and Vargas, 2000; Alam *et al.,* 2015; Schwember *et al.,* 2019).

The variation between the nodule dry weight per plant obtained from inoculated plants may be attributed to the size and number of the nodules. Isolates having better infective capacity and effectiveness formed a significantly larger number of nodules ($p < .0001$) than those having the least effectiveness since effective rhizobia isolates are competitive and able to initiate nodulation with soybean roots which agrees with the report of Chiamaka (2014). The shoot dry weight of soybean plants was also significantly influenced by the rhizobia isolates ($p < .0001$). The average shoot dry weight across all isolates was 0.65 g, with ISR4 inducing the highest average shoot dry weight (0.91 g). This suggests that the benefits of rhizobia inoculation extend beyond nodule formation and nitrogen fixation to promote overall plant growth and productivity. While ISR4 consistently excelled in all measures, other isolates like ISR1 and ISR2w, despite producing fewer nodules, still achieved substantial nodule dry weights, indicating effective nitrogen fixation (Table 4.5).

The study’s findings indicate a range of symbiotic effectiveness among the 8 soybean rhizobia isolates, with values from 48.31% (isolate ISR5) to 102.24% (isolate ISR4) on the basis relative shoot dry matter accumulation of inoculated plants with nitrogen-fertilized control (Table 4.5). Notably, 3 of the isolates were highly effective, contributing to 37.5% of the total, while 4 isolates (50%) were effective, and only 1 isolate (22.5%) was poorly effective based on Purcino *et al.* (2000) classification. The occurrence of rhizobial isolates that are highly effective in nitrogen fixation indicates the potential benefits of native isolates from natural environments.

These findings are consistent with previous research demonstrating the variability in symbiotic efficiency among different rhizobia isolates in Ethiopia. Selected isolates from Ethiopian soils were found to be either effective or highly effective, indicating that the indigenous rhizobia were capable of establishing highly effective symbiosis with soybean (Diriba Temesgen, 2017; Yifru Abera *et al.,* 2019). Furthermore, Diriba Temesgen and Fassil Assefa (2020) found that native rhizobia formed effective nodules on soybean roots, significantly increasing total dry matter and nitrogen content. Field trials showed an 18-35% increase in grain yield with rhizobia inoculation. These findings highlight the potential of fast-growing strains in enhancing soybean cultivation in Ethiopia.

The study revealed strong correlations between the symbiotic effectiveness of indigenous soybean nodulating rhizobia and parameters such as the number of nodules, nodule dry weight, and shoot dry weight (Table 4.6). This aligns with accepted criteria for nitrogen fixing effectiveness in systems that are free of mineral nitrogen, which is the shoot dry weight of plants harvested after significant plant biomass accumulation (Howieson & Dilworth, 2016) and underscores the significant influence of rhizobial isolates on soybean growth and symbiotic interactions.

5.4. Host range of Soybean Rhizobia Isolates

The major finding of this study was the isolation and characterization of predominantly fast-growing rhizobia, from the root nodules of soybean. This is a significant observation as it expands our understanding of the diversity of rhizobia capable of forming symbiotic relationships with soybeans. Fast-growing rhizobia are known for their ability to form effective nodules on a wide range of legume hosts (Laguerre *et al.,* 2007). In this study, all the isolated rhizobia strains were able to nodulate haricot bean (*Phaseolus vulgaris*), mung bean (*Vigna radiata*), and another variety of soybeans (Table 4.7). This suggests that these rhizobias have a broad host range and can form effective symbiotic relationships with multiple legume species. This broad host range is consistent with previous studies that have reported the ability of fast-growing rhizobia to nodulate multiple legume species (Zahran, 1999). However, the specificity of legume-rhizobia symbiosis was highlighted by the inability of the isolated strains to nodulate peanut (*Arachis hypogaea*). This is consistent with previous studies that have shown that peanuts form specific symbiotic relationships with *Bradyrhizobium* species (Moreira *et al.,* 2006). This specificity is thought to be driven by co-evolution between the host plant and its symbiotic rhizobia, leading to adaptations that enhance the efficiency of nitrogen fixation.

The results indicate that the ISR4 isolate for soybean and the ISR7w isolate for haricot bean and mung bean were the most effective in terms of nodulation and symbiotic efficiency. However, it’s important to note that these results were obtained under greenhouse conditions, and further studies are needed to validate these findings under field conditions.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The research has successfully isolated and characterized indigenous rhizobia strains from root nodules of Soybean plants that were cultivated on soil collected from smallholder farmer fields of selected kebele's in Hawella woreda of Sidama region where soybean was recently introduced.

The result of this study revealed the presence of rhizobia compatible with different legume crops. Mostly the rhizobia isolated from the study area were fast-growing. These isolates exhibited morphological and physiological heterogeneity and the ability to use varied carbon sources and tolerate different pH levels and salinity indicating their adaptability.

All isolates formed nodules on soybean upon reinoculation, confirming their identity as true rhizobia. Isolate ISR4 was the most effective in inducing nodule formation and growth as measured by nodule number, dry weight and shoot dry weight.

The relative symbiotic effectiveness of isolates ranged from 48.31-102.24%, with 3 isolates classified as highly effective and 4 as effective in promoting soybean growth. Strong positive correlations were found between symbiotic effectiveness and nodulation/growth parameters.

All isolates could nodulate soybean, mung bean and haricot bean but not peanut, demonstrating their broad host range within tropical legumes except for specific nodulation of peanut. Isolate ISR4 and ISR7w were the most effective for soybean and mung bean/haricot bean respectively.

6.2. Recommendation

Given these findings, it is recommended to:

1. Further characterize the top-performing isolates, particularly ISR4, through genetic fingerprinting and whole genome sequencing to better understand their nodulation traits and symbiotic mechanisms.

2. Conduct multi-location field trials to validate the effectiveness of isolates under different agro-climatic conditions and soil types representative of soybean growing regions in Ethiopia.

3. Evaluate the mass culture and formulation techniques for selected rhizobia isolates to develop effective and low-cost inoculants suited for smallholder farming systems.

4. Assess the adaptability and symbiotic effectiveness of isolates under different management practices such as fertilization, tillage, and cropping systems commonly used by farmers.

5. Explore the commercial potential of top isolates as biofertilizers through a partnership with the inoculant industry and agricultural extension agencies. Conduct demonstrations and training to promote their use.

6. Investigate the genetic basis of host specificity and symbiotic efficiency of isolates through genomics and mutant studies to select superior strains.

7. Further surveys are needed to explore rhizobia diversity across more regions and soil types to isolate new efficient strains adapted to diverse agro-ecologies.

8. Future studies could also characterize local rhizobia from other important legumes in Ethiopia like the common bean, chickpea and faba bean for enhanced crop productivity.

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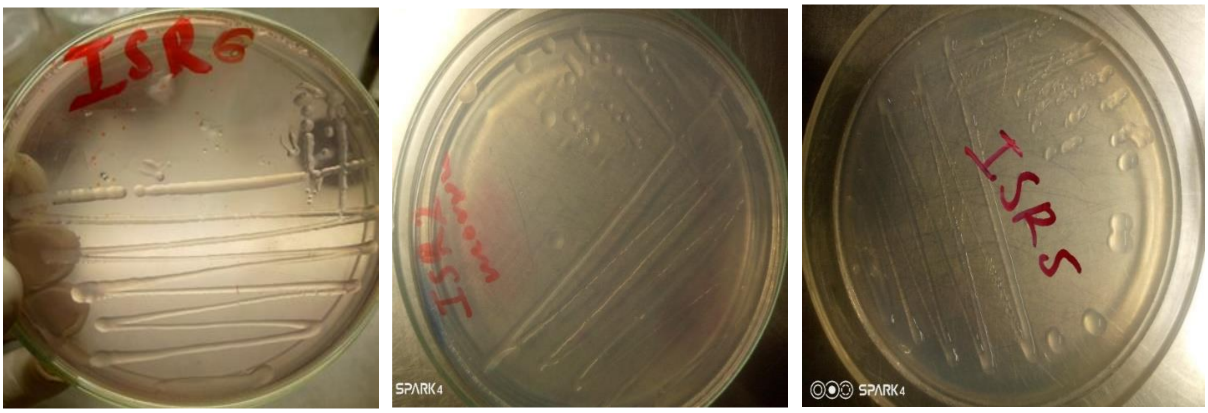
LIST OF APPENDICES

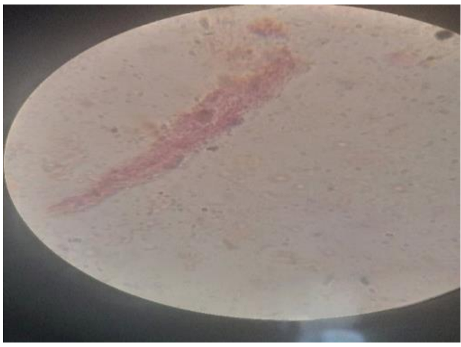
Appendix 1: Composition of N-free nutrient solution prepared according to Broughton and Dilworth (1970)

Stock Solution	Composition (g/L)
Nutrient	0.1 $CaCl_2$
	0.12 $MgSO_4.7H_2O$
	0.1 KH_2PO_4
	0.15 $Na_2HPO_4.2H_2O$
	0.005 Ferric Citrate
Trace Elements	2.86 H_3BO_3
	2.03 $MnSO_4.7H_2O$
	0.22 $ZnSO_4.7H_2O$
	0.08 $CuSO_4.5H_2O$
	0.14 $NaMoO_2.2H_2O$

Appendix 2: Colony morphology of soybean rhizobia isolates on YEMA



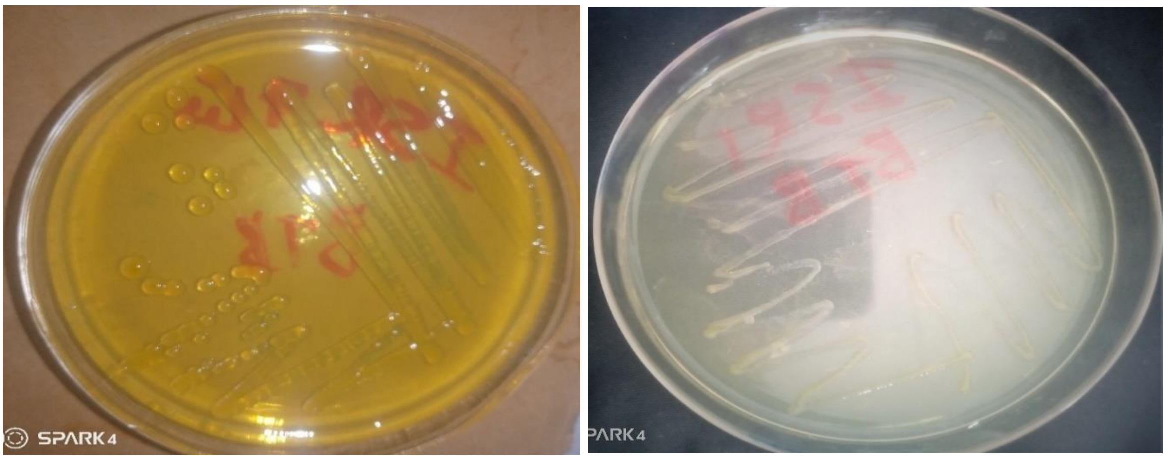
Appendix 3: Gram staining of rhizobia isolates under the microscope


ISR4

Appendix 4: Congo Red absorption by rhizobia isolates on YEMA

Appendix 5: Growth responses of rhizobia isolates on PGA medium

Appendix 6: Colonies of rhizobia isolates on YEMA+BTB. a. Yellow with creamy and smooth margins, medium sized, round. b. white



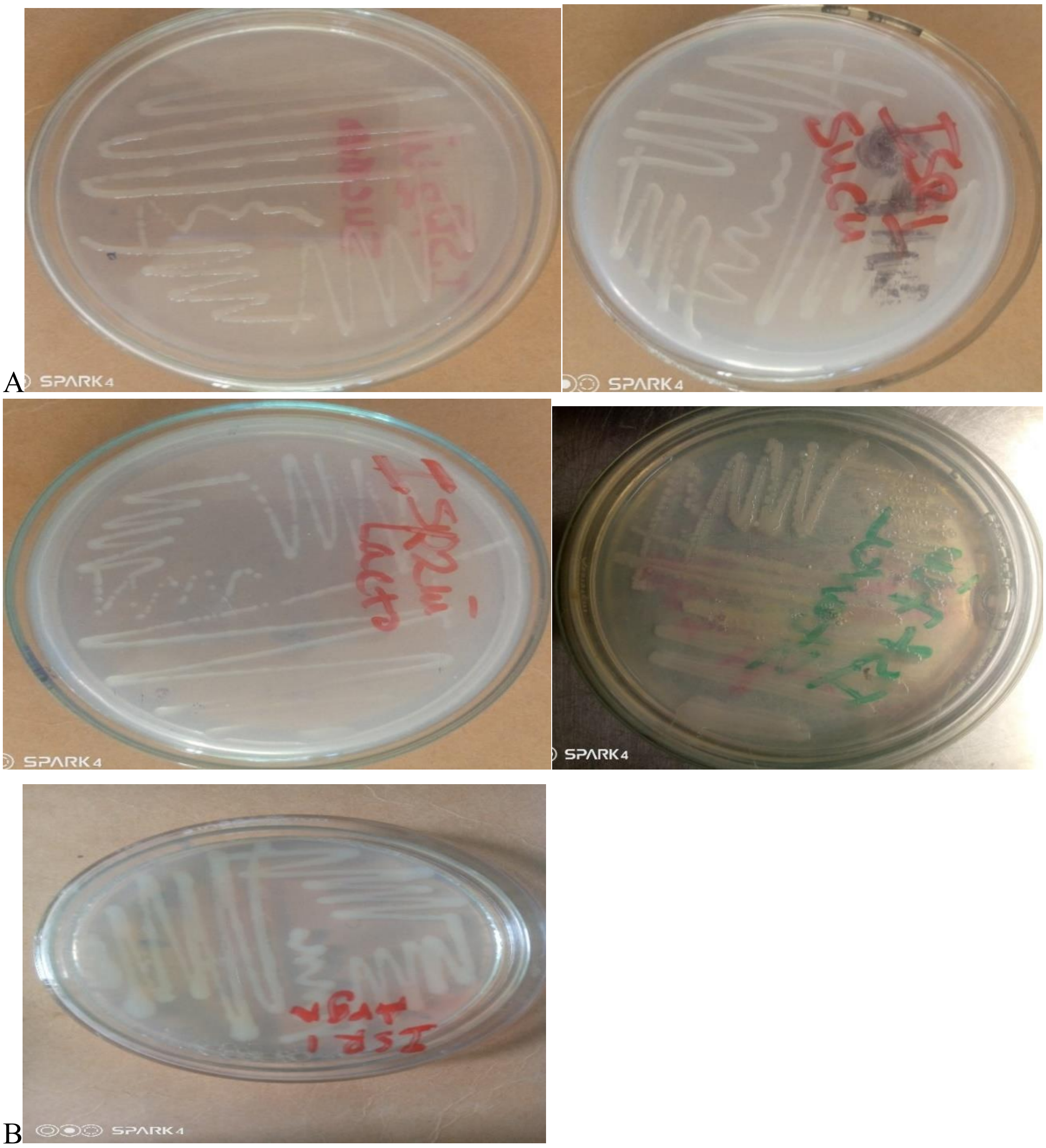
Appendix 7: Keto lactose test results of rhizobia isolates

Appendix 8: pH tolerance profile of rhizobia isolates

Appendix 9: Salt tolerance of rhizobia isolates in NaCl amended media

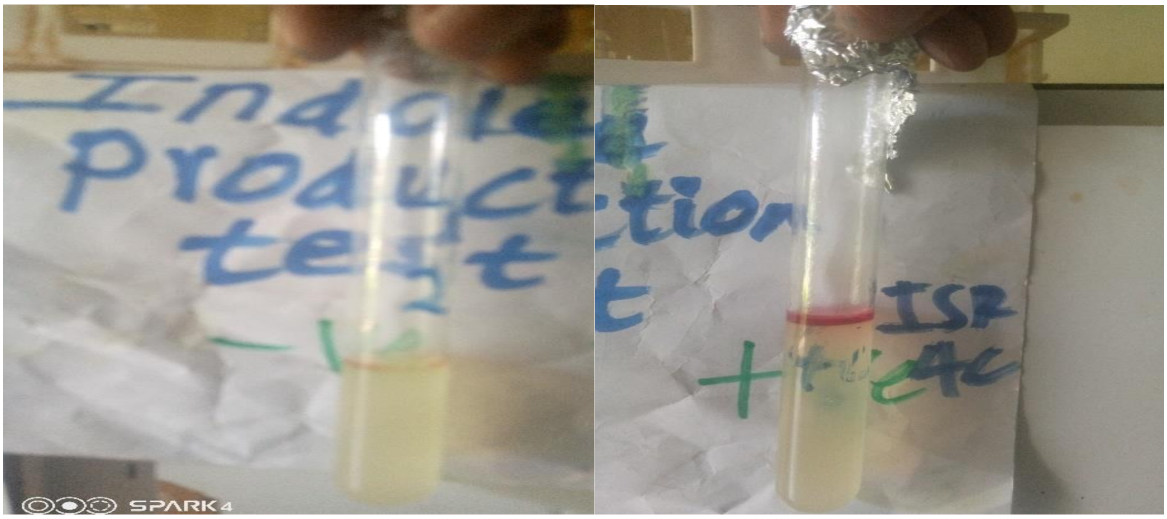
Appendix 10: Catalase activity of the rhizobia isolates

Appendix 11: Carbon and Nitrogen source utilization of the rhizobia isolates



Appendix 12: Methyl red test result

Appendix 13: Indole production test



Appendix 14: Nodule formation by rhizobia isolates in sand culture trial

A: Soybean inoculated with isolates

a. Positive Control

b. Negative Control

Appendix 14B. Mung bean inoculated with isolates

Negative Control

Appendix 14C. Haricot bean inoculated with isolates

Appendix 15: One-way ANOVA for effect of isolates on number of nodules, nodule dry weight, shoot dry weight and post hoc Tukey HSD test

A. One-way ANOVA for effect of isolates on number of nodules

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Rhizobia Isolates	9	215.46667	23.9407	24.7663	<.0001*
Error	20	19.33333	0.9667		
C. Total	29	234.80000			

Confidence Quantile

q*	Alpha
3.54111	0.05

HSD Threshold Matrix

Abs(Dif)-HSD

	ISR4	ISR1	ISR7w	ISR2w	ISR4c	ISR2	ISR6	ISR5	Negative Ctrl	Positive Ctrl
ISR4	-2.8427	0.8240	2.1573	2.8240	3.1573	3.8240	4.8240	5.1573	6.4906	6.4906
ISR1	0.8240	-2.8427	-1.5094	-0.8427	-0.5094	0.1573	1.1573	1.4906	2.8240	2.8240
ISR7w	2.1573	-1.5094	-2.8427	-2.1760	-1.8427	-1.1760	-0.1760	0.1573	1.4906	1.4906
ISR2w	2.8240	-0.8427	-2.1760	-2.8427	-2.5094	-1.8427	-0.8427	-0.5094	0.8240	0.8240
ISR4c	3.1573	-0.5094	-1.8427	-2.5094	-2.8427	-2.1760	-1.1760	-0.8427	0.4906	0.4906
ISR2	3.8240	0.1573	-1.1760	-1.8427	-2.1760	-2.8427	-1.8427	-1.5094	-0.1760	-0.1760
ISR6	4.8240	1.1573	-0.1760	-0.8427	-1.1760	-1.8427	-2.8427	-2.5094	-1.1760	-1.1760
ISR5	5.1573	1.4906	0.1573	-0.5094	-0.8427	-1.5094	-2.5094	-2.8427	-1.5094	-1.5094
Negative Ctrl	6.4906	2.8240	1.4906	0.8240	0.4906	-0.1760	-1.1760	-1.5094	-2.8427	-2.8427
Positive Ctrl	6.4906	2.8240	1.4906	0.8240	0.4906	-0.1760	-1.1760	-1.5094	-2.8427	-2.8427

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level						Mean
ISR4	A					9.3333333
ISR1		B				5.6666667
ISR7w		B	C			4.3333333
ISR2w		B	C	D		3.6666667
ISR4c		B	C	D		3.3333333
ISR2			C	D	E	2.6666667
ISR6			C	D	E	1.6666667
ISR5				D	E	1.3333333
Negative Ctrl					E	0.0000000
Positive Ctrl					E	0.0000000

Levels not connected by same letter are significantly different.

B. One-way ANOVA for effect of isolates on nodule dry weight

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Rhizobia Isolates	9	4677.1253	519.681	26.1575	<.0001*
Error	20	397.3467	19.867		
C. Total	29	5074.4720			

Confidence Quantile

q*	Alpha
3.54111	0.05

HSD Threshold Matrix

Abs(Dif)-HSD

HSD Threshold Matrix

Abs(Dif)-HSD

	ISR4	ISR1	ISR2w	ISR2	ISR4c	ISR7w	ISR5	ISR6	Negative Ctrl
ISR4	-12.887	-5.154	8.079	9.046	9.379	9.646	18.313	19.346	28.146
ISR1	-5.154	-12.887	0.346	1.313	1.646	1.913	10.579	11.613	20.413
ISR2w	8.079	0.346	-12.887	-11.921	-11.587	-11.321	-2.654	-1.621	7.179
ISR2	9.046	1.313	-11.921	-12.887	-12.554	-12.287	-3.621	-2.587	6.213
ISR4c	9.379	1.646	-11.587	-12.554	-12.887	-12.621	-3.954	-2.921	5.879
ISR7w	9.646	1.913	-11.321	-12.287	-12.621	-12.887	-4.221	-3.187	5.613
ISR5	18.313	10.579	-2.654	-3.621	-3.954	-4.221	-12.887	-11.854	-3.054
ISR6	19.346	11.613	-1.621	-2.587	-2.921	-3.187	-11.854	-12.887	-4.087
Negative Ctrl	28.146	20.413	7.179	6.213	5.879	5.613	-3.054	-4.087	-12.887
Positive Ctrl	28.146	20.413	7.179	6.213	5.879	5.613	-3.054	-4.087	-12.887

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean
ISR4	A	41.033333
ISR1	A	33.300000

Level			Mean
ISR2w	B		20.066667
ISR2	B		19.100000
ISR4c	B		18.766667
ISR7w	B		18.500000
ISR5	B	C	9.833333
ISR6	B	C	8.800000
Negative Ctrl		C	0.000000
Positive Ctrl		C	0.000000

Levels not connected by same letter are significantly different.

C. One-way ANOVA for effect of isolates on shoot dry weight

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Rhizobia Isolates	9	0.81103000	0.090114	71.8998	<.0001*
Error	20	0.02506667	0.001253		
C. Total	29	0.83609667			

HSD Threshold Matrix

Abs(Dif)-HSD

	ISR4	Positive Ctrl	ISR1	ISR2w	ISR4c	ISR2	ISR7w	ISR6	ISR5
ISR4	-0.10236	-0.08569	0.05431	0.06764	0.18097	0.19764	0.20097	0.29097	0.37431
Positive Ctrl	-0.08569	-0.10236	0.03764	0.05097	0.16431	0.18097	0.18431	0.27431	0.35764
ISR1	0.05431	0.03764	-0.10236	-0.08903	0.02431	0.04097	0.04431	0.13431	0.21764
ISR2w	0.06764	0.05097	-0.08903	-0.10236	0.01097	0.02764	0.03097	0.12097	0.20431
ISR4c	0.18097	0.16431	0.02431	0.01097	-0.10236	-0.08569	-0.08236	0.00764	0.09097
ISR2	0.19764	0.18097	0.04097	0.02764	-0.08569	-0.10236	-0.09903	-0.00903	0.07431
ISR7w	0.20097	0.18431	0.04431	0.03097	-0.08236	-0.09903	-0.10236	-0.01236	0.07097
ISR6	0.29097	0.27431	0.13431	0.12097	0.00764	-0.00903	-0.01236	-0.10236	-0.01903
ISR5	0.37431	0.35764	0.21764	0.20431	0.09097	0.07431	0.07097	-0.01903	-0.10236
Negative Ctrl	0.39431	0.37764	0.23764	0.22431	0.11097	0.09431	0.09097	0.00097	-0.08236

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level							Mean
ISR4	A						0.91000000
Positive Ctrl	A						0.89333333
ISR1		B					0.75333333
ISR2w		B					0.74000000
ISR4c			C				0.62666667
ISR2			C	D			0.61000000
ISR7w			C	D			0.60666667
ISR6				D	E		0.51666667
ISR5					E	F	0.43333333
Negative Ctrl						F	0.41333333

Levels not connected by same letter are significantly different.

Appendix 16: Bar graphs depicting the effect of rhizobia isolates on soybean plants