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ISOLATION, BIOCHEMICAL CHARACTERIZATION, AND GREENHOUSE AUTHENTICATION OF CHICKPEA RHIZOBIA COLLECTED FROM MAJOR CHICKPEA GROWING AREAS OF WOLDIA.NORTH WOLLO, ETHIOPIA.

By

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APPROVAL SHEET

This MSc Thesis entitled "**Isolation, Biochemical characterization and greenhouse authentication of chickpea rhizobia collected from some major chickpea growing areas of Woldia North Wollo, Ethiopia**" has been submitted by Kelemu Yimenu for evaluation. It has been reviewed and approved for presentation and defense in partial fulfillment of the degree of Master of Science in Biotechnology.

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DECLARATION

I hereby declare that I prepared this MSc thesis entitled "**Isolation, Biochemical characterization and greenhouse authentication of chickpea rhizobia collected from some major chickpea growing areas of Woldia North Wollo, Ethiopia**" with the guidance of my advisor. The work contained here is my own except where explicitly stated in the text, and this thesis has not been submitted, in whole or in part, for any other degree or professional qualification.

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ABBREVIATIONS

BNF	Biological Nitrogen Fixation
GPS	Global positioning system
LSD	Least significant difference
MBI	Menagesha Biotechnology Industry
NSTC	National Soils Testing Center
PGPB	Plant Growth Promoting Bacteria
SPSS	Statistical package for social sciences
LCO	Lipochitooligosaccharide
MLSA	Multilocus sequence analysis

ABSTRACT

Chickpea (Cicer arietinum L.) is a vital legume crop worldwide, valued for its high nutritional content and significant contribution to food security and soil fertility through biological nitrogen fixation. Despite its importance, chickpea yields remain suboptimal in many regions, including Ethiopia, primarily due to constraints such as poor soil fertility and inadequate use of effective rhizobial inoculants. Biological nitrogen fixation by symbiotic Rhizobium bacteria plays a crucial role in enhancing chickpea productivity, yet the diversity and effectiveness of native rhizobia in the Woldia area remain poorly understood. This study aimed to isolate and characterize native Rhizobium strains from chickpea root nodules collected from fields in the Woldia region and to assess their plant growth-promoting potential. A total of 41 bacterial isolates were obtained, of which 12 were presumptively identified as Rhizobium based on growth characteristics on Congo red and bromothymol blue media. These isolates were further characterized morphologically and biochemically. All confirmed isolates were Gram-negative, rod-shaped, motile, and exhibited positive reactions for oxidase, catalase, urease, starch hydrolysis, ammonia production, citrate utilization, and nitrogen fixation, while testing negative for indole production and gelatinase activity. Five biochemically promising isolates were selected for evaluation in a controlled 45-day greenhouse experiment under sterile conditions. Inoculation with these isolates significantly enhanced seed germination and early seedling growth compared to uninoculated controls. The symbiotic effectiveness of the isolates ranged from 74.3% to 121.9%, with isolates SFDG-23, SFMC-31, and SFMC-23 demonstrating high effectiveness. Particularly, isolate SFDG-23 markedly increased nodulation and biomass accumulation. Although morphological and biochemical characterization provided useful preliminary identification, it alone cannot confirm the taxonomic status of the isolates. Therefore, further molecular characterization is recommended. Moreover, field trials are necessary to validate the performance of the most promising isolates under natural growing conditions. In conclusion, this study highlights the potential of native Rhizobium isolates from Woldia chickpea fields, especially SFDG-23, as effective bio-inoculants to promote sustainable chickpea production and reduce dependence on chemical fertilizers around woldia.

Keywords: chickpea; green house; nodules; leguminous plants; *Rhizobia*,

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1. INTRODUCTION

1.1 Background of the study

Chickpea (*Cicer arietinum L.*) is a major pulse crop cultivated globally, including in Ethiopia, where it plays a vital role in household nutrition, food security, and income generation. It ranks as the third most important legume export in Ethiopia after faba bean and haricot bean, contributing approximately USD 61 million annually (Kumar *et al.*, 2022; FAOSTAT, 2024). Despite its economic and nutritional significance, chickpea productivity remains suboptimal in many Ethiopian regions such as Woldia, primarily due to low soil fertility and inadequate use of sustainable nutrient management practices (Yegrem, 2021; Mekonnen *et al*., 2023).

The widespread reliance on chemical nitrogen fertilizers to enhance crop yields has led to several agronomic and environmental challenges. Excessive fertilizer use contributes to soil acidification, nutrient imbalances, environmental pollution, and increased production costs, which disproportionately affect smallholder farmers (Kebede *et al*., 2020; Desta *et al*., 2023). These issues underscore the urgent need for environmentally sustainable and cost-effective alternatives to chemical fertilizers, such as plant growth-promoting bacteria (PGPB) and symbiotic nitrogen-fixing rhizobia (Bhattacharyya and Jha, 2017; Sharma *et al*., 2024).

Rhizobia are Gram-negative soil bacteria capable of establishing a symbiotic relationship with leguminous plants, including chickpea, by fixing atmospheric nitrogen (N_2) into ammonia (NH_3), a form directly usable by plants (Soumare *et al*., 2020; Rani *et al.,* 2024). This symbiosis is initiated when legume roots exude flavonoids and other chemical signals, which are recognized by rhizobia, triggering the production of Nod factors. These signaling molecules induce root hair curling, infection thread formation, and development of root nodulesspecialized organs where nitrogen fixation occurs under microaerobic conditions (Subrahmanyam *et al*., 2020; Liu *et al*., 2022).

Inside these nodules, rhizobia differentiate into bacteroids and express nitrogenase enzymes that catalyze the conversion of atmospheric nitrogen into ammonia. Although this process is energetically costly, it is supported by carbohydrates supplied by the host plant through photosynthesis, forming a mutually beneficial interaction (Kumar *et al*., 2015;Raza *et al*., 2020).

The fixed nitrogen is essential for synthesizing amino acids, proteins, nucleotides, and vitamins, supporting plant growth and productivity (Santoyo *et al*., 2021). Besides nitrogen fixation, rhizobial symbiosis improves soil structure and fertility, promoting sustainable agriculture and reducing the dependence on synthetic fertilizers (Soumare *et al*., 2020; Liu *et al*., 2022).

In Ethiopia, despite the potential benefits, the use of *rhizobial* inoculants is limited by the lack of commercial inoculant production facilities, poor farmer awareness, and the absence of effective native strains adapted to local agro-ecological conditions (Mekonnen *et al*., 2023; Desta *et al*., 2023). Native rhizobia often demonstrate superior symbiotic efficiency and environmental resilience compared to exotic strains, making them ideal candidates for developing biofertilizers tailored to Ethiopian soils and climates (Bhattacharyya and Jha, 2017; Rani *et al*., 2024).

This study therefore aims to isolate and characterize native *rhizobial* isolates from chickpea root nodules collected around Woldia. It also seeks to evaluate their symbiotic efficiency and potential as bio-inoculants to enhance chickpea growth and biological nitrogen fixation. By harnessing these adapted native strains, the research will contribute to sustainable, low-input agricultural practices, improving chickpea productivity while minimizing environmental impacts.

1.2 Statement of the problem

To supply plants with usable forms of nitrogen, chemical fertilizers are widely produced using the industrial Haber-Bosch process and applied across agricultural lands globally. This process synthesizes ammonia (NH_3) from atmospheric nitrogen (N_2) and natural gas under high pressure and temperature. While effective, it is highly energy-intensive, consuming over 1% of the world's annual energy and releasing approximately 1.87 tons of CO_2 per ton of NH_3 produced (Deng *et al*., 2018). In contrast, biological nitrogen fixation (BNF) offers a more sustainable alternative by converting atmospheric nitrogen into ammonia or nitrate under normal temperature and pressure conditions, mainly through symbiotic interactions between legumes and nitrogen-fixing bacteria such as *Rhizobium*.

BNF plays a critical role in ensuring optimal and sustainable crop productivity, especially in leguminous crops, as demonstrated by numerous studies across Ethiopia and other African countries (Argaw and Mnalku, 2017). Although chemical fertilizers can enhance short-term crop

yields, their long-term use poses serious environmental risks, including soil degradation, microbial imbalance, and groundwater contamination. These fertilizers alter soil microbial communities by directly affecting bacterial biomass and diversity and indirectly by acidifying the soil (Wang *et al*., 2017). Thus, biofertilizers microbial-based inputs are increasingly being promoted as sustainable alternatives (Wanore *et al*., 2023b).

In this context, symbiotic nitrogen fixers like *Rhizobia* are particularly important. These bacteria form nodules on legume roots and use nitrogenase enzymes to fix atmospheric nitrogen into forms that plants can absorb. This process is not only cost-effective and environmentally friendly but also helps preserve biodiversity and supports soil health (Hamza and Alebejo, 2017).

In the North Wollo zone, particularly around Woldia, agriculture remains the backbone of rural livelihoods, with chickpea (*Cicer arietinum*) being one of the key legume crops grown under rain-fed conditions. Despite the apparent presence of indigenous rhizobia in local soils, there is a lack of systematic research on isolating, characterizing, and utilizing native rhizobial strains for bio-inoculant development specific to this agroecological context. Given the increasing demand for sustainable agricultural practices and the need to reduce dependence on synthetic fertilizers, identifying effective native rhizobia strains is crucial for improving chickpea productivity and enhancing soil fertility in North Wollo and similar semi-arid regions of Ethiopia.

1.3 Objectives

1.3.1 General objective

- The main objective of this study was to isolate chickpea rhizobia and conduct biochemical characterization, and authentication under greenhouse conditions.

1.3.2 Specific objectives

- To isolate native the rhizobial isolates from chickpea root nodules,
- To conduct the presumptive test of the rhizobial isolates.
- To determine the morphological, cultural, and biochemical characterization of the isolates,
- To confirm the rhizobial isolates as chickpea rhizobia by authenticating them on chickpea plant under greenhouse conditions.

1.5 Significance of the study

The use of microbial inoculants as a bio-fertilizer is a great opportunity for smallholder farmers for sustainable crop production and improved soil fertility. From an economic and environmental point of view, the use of *rhizobium* inoculants can reduce environmental pollution and production costs. Therefore, using isolated nitrogen-fixing rhizobia as bio-fertilizer has a significant effect. This study has the potential to improve agricultural practices, promote environmental sustainability, and support economic development. Additionally, it will improve the pool of rhizobia culture collection that can be used to screen superior strains. Thus, the result of this study will serve as a foundation for the development and adoption of rhizobium inoculant technology to combat the urgent soil fertility problems and low feed production challenges. Moreover, improving chickpea productivity supports food security, farmer income, and soil health in the semi-arid areas of Ethiopia. The findings of this study also provide valuable scientific knowledge that can guide future research, policy-making, and the development of microbial inoculant technologies tailored to local agro-ecological conditions.

2. LITERATURE REVIEW

2.1 Chickpea (Cicer arietinum L.)

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes cultivated globally for its nutritional, agronomic, and economic value. It ranks third in global pulse production after common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*), contributing significantly to food security and soil fertility in many developing countries (FAO, 2020).

Globally, chickpea is cultivated on approximately 17 million hectares with a total production of over 17 million tonnes, primarily in India, which accounts for more than 70% of global production, followed by countries such as Australia, Turkey, Myanmar, and Ethiopia (FAOSTAT, 2023). In Ethiopia, chickpea is the second most important pulse crop after faba bean in terms of production and area coverage. According to the Central Statistical Agency (CSA, 2022), it is cultivated on over 250,000 hectares annually, mainly in the highland and sub-highland areas of the country including Wollo, Gojjam, and Shewa.

Nutritionally, chickpea is an excellent source of plant-based protein (20–25%), complex carbohydrates, dietary fiber, vitamins (especially B vitamins), and minerals such as iron, phosphorus, and magnesium (Jukanti *et al*., 2012). Due to its relatively low glycemic index and high protein content, chickpea plays an important role in addressing malnutrition and food insecurity, particularly in vegetarian and low-income populations.

Furthermore, chickpea cultivation is relatively resilient to drought and can be grown in marginal soils, making it well-suited to the semi-arid environments of northern Ethiopia. However, its productivity in Ethiopia remains lower than the global average due to factors such as poor soil fertility, pest and disease pressures, and limited use of improved varieties and bio-inoculants (Alemu *et al*., 2020).

In this context, the application of native rhizobia as bio-inoculants has gained attention as a sustainable approach to enhance chickpea productivity. Therefore, the identification and characterization of efficient local rhizobial isolates are crucial for improving chickpea yields in major production areas like Woldia, North Wollo.

2.2 Soil Nutrient Status for Chickpea Productivity

Chickpea thrives best in well-drained soils with moderate fertility, neutral to slightly alkaline pH (6.0–8.0), and adequate levels of organic matter (Yadav *et al.,* 2017). Nitrogen (N), phosphorus (P), potassium (K), sulfur (S), zinc (Zn), and boron (B) are among the key nutrients required for optimal chickpea growth. While legumes can fix atmospheric nitrogen through Rhizobium symbiosis, initial soil nitrogen levels still play a role in root development and early vegetative growth (Singh *et al*., 2016).

Phosphorus is critically important for nodule formation, energy transfer, and root development in chickpea. Studies have shown that phosphorus deficiency severely impairs nodulation and nitrogen fixation efficiency, leading to reduced biomass and seed yield (Reddy *et al.,* 2014). Similarly, potassium enhances water regulation, enzyme activation, and overall stress tolerance, which indirectly supports better nutrient uptake and microbial interactions in the rhizosphere (Ali *et al*., 2018).

In Ethiopia, particularly in the northern highlands such as Woldia and North Wollo, soil degradation, nutrient mining, and limited use of organic and inorganic fertilizers have contributed to declining soil fertility. Several studies have indicated that the soils in these regions are often deficient in available nitrogen, phosphorus, and organic matter (Tadesse *et al*., 2019). This nutrient limitation has not only reduced chickpea yields but also hindered effective rhizobial symbiosis, as nutrient-stressed plants are less capable of supporting nitrogen-fixing bacteria (Mesfin *et al*., 2020).

Moreover, the interaction between native rhizobia and soil nutrient levels is critical for the success of bio-inoculant strategies. Native Rhizobium strains may possess adaptations to local soil conditions, but their performance is often constrained by poor nutrient availability. Understanding and improving soil nutrient status is therefore a prerequisite for enhancing both chickpea productivity and the effectiveness of rhizobial inoculation (Bashir *et al*., 2021).

The productivity of chickpea in Woldia and similar agroecological zones is closely linked to the status of essential soil nutrients. Addressing nutrient deficiencies through integrated soil fertility

management, combined with the use of effective native rhizobia, holds promise for sustainable chickpea production and improved soil health in the region.

2.3 Effects of Chemical fertilizer application and draw backs

The fertilizer sector is heavily dependent on energy-demanding technologies for agricultural production, particularly in the creation of nitrogen fertilizers and pesticides. Overuse of nitrogen fertilizers can lead to serious environmental issues. A primary concern is nutrient runoff, where excessive nitrogen and phosphorus from synthetic fertilizers are carried into nearby water bodies, resulting in eutrophication, harmful algal blooms, oxygen depletion, and disturbances in aquatic ecosystems (Khan *et al*., 2018). Soil degradation is another issue. Synthetic fertilizers mainly target macronutrients such as nitrogen, phosphorus, and potassium, often overlooking other vital micronutrients. This unbalanced nutrient application can deplete soil organic matter, harm its physical structure, reduce beneficial microbial activity, and diminish overall fertility over time (Sainju *et al*., 2019).

Nutrient runoff causing eutrophication can damage aquatic life, leading to a decline in fish populations and other species. Furthermore, the reduction in soil fertility due to synthetic fertilizers can adversely affect soil organisms essential for maintaining healthy soil and biodiversity, including earthworms, beneficial insects, and microorganisms (Sud, 2020). The production and distribution of synthetic fertilizers also contribute to greenhouse gas emissions and air pollution. This energy-intensive process depends on fossil fuels and emits carbon dioxide (CO_2), nitrogen oxides (NOx), and methane (CH_4), worsening climate change (Ouikhalfan *et al*., 2022).Besides their environmental effects, synthetic fertilizers are costly to produce. The entire production cycle, encompassing raw material extraction, chemical production, transportation, and packaging, demands significant energy inputs and leads to high energy consumption and related environmental impacts (Chojnacka *et al*., 2020).

2.4 The Role of Plant Growth-Promoting Rhizobacteria and Rhizobia as Biofertilizers in Chickpea cultivation

Biofertilizers, particularly plant growth-promoting rhizobacteria (PGPR) and rhizobia, have emerged as key components in sustainable agriculture due to their ecological compatibility, role in nutrient cycling, and capacity to enhance crop productivity. These microbial inoculants act as

natural soil fertility enhancers by supporting nutrient acquisition, stimulating plant physiological functions, and improving stress tolerance functions especially critical in leguminous crops such as chickpea (*Cicer arietinum* L.) (Glick, 2012; Singh *et al*., 2022). In light of increasing environmental pressures and input constraints in smallholder systems, the integration of biofertilizers presents a viable strategy for sustainable intensification and climate-resilient agriculture.

2.4.1 Plant Growth-Promoting Rhizobacteria and their functional diversity

PGPR represent a taxonomically and functionally diverse group of beneficial bacteria that colonize the rhizosphere or plant endosphere and facilitate growth through multiple direct and indirect mechanisms. Their direct contributions include biological nitrogen fixation (BNF), solubilization of mineral nutrients such as phosphorus, potassium, and zinc, and synthesis of phytohormones like indole-3-acetic acid (IAA), gibberellins, and cytokinins (Bhattacharyya & Jha, 2012; Singh *et al*., 2022). These traits collectively enhance root architecture, nutrient uptake efficiency, and biomass accumulation.

PGPR also indirectly support plant health by antagonizing phytopathogens through the production of antibiotics, siderophores, hydrogen cyanide, and lytic enzymes such as chitinases and glucanases. Additionally, many PGPR can induce systemic resistance in host plants, priming them against future biotic stresses without causing disease (Backer *et al*., 2018). Such multifaceted traits make genera like *Pseudomonas*, *Bacillus*, *Azospirillum*, and *Serratia* particularly promising in biofertilizer development.

Chickpea is a critical source of dietary protein and soil fertility in Ethiopia, where limited access to synthetic fertilizers and recurrent climatic stresses challenge crop production. In regions like Woldia and North Wollo, where soils are often degraded and rainfall is erratic, biofertilizers offer a cost-effective and ecologically sound solution. Studies have demonstrated that inoculation with native rhizobial strains often more competitive and better adapted than exotic strains enhances nodulation, shoot and root growth, and grain yield under both greenhouse and field conditions (Argaw, 2014; Tsegaye *et al*., 2023). When combined with PGPR, these effects are further amplified, contributing to more resilient cropping systems.

2.4.2 Symbiotic relationship between *Rhizobia* and PGPR

Emerging evidence underscores the synergistic potential of co-inoculating rhizobia with compatible PGPR strains. Such consortia often result in enhanced nodulation, higher rates of nitrogen fixation, and improved nutrient uptake compared to single-strain inoculants (Goswami *et al*., 2016). This synergy is attributed to the complementary roles of rhizobia in BNF and of PGPR in nutrient solubilization, hormone production, and pathogen suppression. These interactions form the basis for dual or multi-strain biofertilizers, which are increasingly being tailored to specific crops and agroecological contexts.

Integrating PGPR and rhizobia into chickpea cultivation aligns with the broader goals of climate-smart agriculture by improving input use efficiency, enhancing soil biological health, and reducing greenhouse gas emissions associated with synthetic fertilizers (Rana *et al*., 2021). The use of indigenous microbial inoculants supports agroecological restoration, particularly in dryland and smallholder-dominated systems. Advancing the formulation, quality control, and field validation of such biofertilizers remains critical for scaling their use in sustainable agriculture.

2.5 Taxonomy and General Characteristics of Rhizobia

Rhizobia are a polyphyletic group of soil-dwelling, Gram-negative, rod-shaped bacteria renowned for their unique ability to form symbiotic relationships with leguminous plants. Through this mutualistic interaction, rhizobia colonize root nodules and convert atmospheric nitrogen (N_2) into ammonia (NH_3), a form of nitrogen readily utilized by plants. This biological nitrogen fixation (BNF) is critical to enhancing soil fertility, reducing dependence on chemical fertilizers, and promoting sustainable agricultural practices, particularly in nitrogen-deficient regions such as the highlands of Ethiopia (Mousavi *et al*., 2014).

Rhizobia were historically grouped under a single genus, *Rhizobium*. However, advances in molecular phylogenetics, including 16S rRNA sequencing and multilocus sequence analysis (MLSA), have led to significant revisions in their classification. Today, rhizobia are distributed among several genera within the class *Alphaproteobacteria*, including *Rhizobium*,

Bradyrhizobium, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Azorhizobium*, *Allorhizobium*, *Neorhizobium*, and others (Mousavi *et al*., 2014). These taxa vary widely in physiological traits, symbiotic host range, and ecological adaptability, reflecting the evolutionary complexity of legume-rhizobium interactions.

The taxonomic classification of rhizobia is based on a combination of phenotypic traits, biochemical tests, DNA–DNA hybridization, and increasingly, genomic approaches. For instance, the genus *Mesorhizobium*, which includes moderately slow-growing strains such as *M. ciceri* and *M. mediterraneum*, is commonly associated with chickpea (*Cicer arietinum L.*). These species are notable for their acid tolerance, efficient nodulation, and adaptation to diverse agro-ecological conditions (Rivas *et al*., 2007). In regions like Ethiopia, indigenous strains of *Mesorhizobium* have demonstrated considerable variability in nodulation capacity, nitrogen fixation efficiency, and resilience to abiotic stress (Argaw, 2012; Jida and Assefa, 2011).

Taxonomic identification is particularly important when selecting effective rhizobial inoculants for legume crops. Traditional methods such as colony morphology on Yeast Extract Mannitol Agar (YEMA), Gram staining, and biochemical assays (e.g., catalase, oxidase, urease activity, and carbohydrate fermentation) provide initial differentiation. However, modern approaches emphasize molecular techniques including 16S rRNA sequencing, MLSA, and whole-genome analysis, which offer high-resolution classification and help detect horizontal gene transfer events, such as the acquisition of symbiosis islands (Sullivan and Ronson, 2002; Sawada *et al*., 2003).

Importantly, the ability to nodulate legumes is not monophyletic but has evolved multiple times independently. The nodulation and nitrogen fixation capabilities are largely determined by symbiosis-related genes *nod*, *nif*, and *fix* which may be located on large plasmids or genomic islands and transferred horizontally among bacteria (Spaink, 2000). This genetic mobility contributes to the ecological flexibility and evolutionary success of rhizobia across diverse environments.

In chickpea production systems, particularly in developing countries like Ethiopia, the effective use of native rhizobia as biofertilizers offers a cost-effective and environmentally sustainable

alternative to chemical inputs. Several studies have documented the isolation and characterization of chickpea-nodulating *Mesorhizobium* strains from Ethiopian soils, highlighting their potential for inoculant development tailored to local agro-climatic conditions (Tena et al., 2016; Argaw, 2012).

The taxonomy of rhizobia reflects their ecological diversity and functional specialization. Accurate taxonomic classification, supported by molecular and biochemical tools, is fundamental to harnessing native rhizobial populations for the development of effective legume inoculants. Understanding the taxonomy and diversity of rhizobia, particularly in underexplored regions such as Woldia, North Wollo, provides a critical foundation for improving chickpea productivity and soil health through biological nitrogen fixation.

2.6 Biological nitrogen fixation

Nitrogen fixation is a critical biological process whereby atmospheric nitrogen gas (N_2), which is inert and unavailable to most plants, is converted into ammonia (NH_3), a form usable by plants for growth and development (Galloway et al., 2018). This process is primarily mediated by a specialized group of prokaryotes, known as diazotrophs, among which symbiotic nitrogen fixation carried out by rhizobia bacteria in leguminous plants is of paramount importance for sustainable agriculture (Peoples et al., 2019).

In legume-rhizobium symbiosis, rhizobia infect the roots of host legumes such as chickpea (*Cicer arietinum*), leading to the formation of root nodules where nitrogen fixation occurs (Oldroyd et al., 2011). The rhizobia reduce atmospheric N_2 to ammonia using the enzyme nitrogenase, in a highly energy-dependent process that requires ATP and a microaerobic environment (Poole et al., 2018). The fixed nitrogen is then assimilated into amino acids and other nitrogenous compounds essential for plant growth, thereby improving soil fertility naturally (Herridge et al., 2008).

Chickpea is one of the major legume crops cultivated in Ethiopia and is highly dependent on efficient symbiotic nitrogen fixation to achieve optimal yields, especially in the nitrogen-deficient soils of the Woldia region (Belete & Astatkie, 2021). However, the efficiency of biological nitrogen fixation (BNF) depends largely on the effectiveness and adaptability of the

rhizobial strains present in the soil (Temesgen et al., 2020). Thus, isolation and characterization of native rhizobia capable of efficient nitrogen fixation and symbiosis with chickpea are critical to developing bioinoculants that can improve productivity sustainably (Chen et al., 2019).

Recent advances have shown that indigenous rhizobia strains often outperform introduced strains due to their better adaptation to local environmental conditions, such as soil pH, temperature, and moisture (Tena et al., 2023). In the Ethiopian context, several studies have reported significant variability in the symbiotic efficiency of rhizobia strains associated with chickpea across different agroecological zones (Tesfaye et al., 2022; Yimer & Habte, 2020). The isolation and biochemical characterization of these native rhizobia provide insights into their physiological traits, stress tolerance, and nitrogen fixation potential (Mwenda et al., 2021).

2.6.1 Nodulation and Nitrogen Fixation Processes

Each *rhizobia* has a distinct range of hosts, and nodulation is a host-specific process (Goyal & Habtewold, 2023). The host plant and its rhizosphere symbionts are involved in a multi-step process. Legumes that are severely nitrogen starved produce exudates that contain flavonoids into the rhizosphere, where they interact with the rhizobia's nodulation protein D (NodD) (Wulandari et al., 2022). By binding to the nod operon, activated NodD causes the expression of nod genes. This results in the production and release of nod factors, which are signaling molecules based on lipochitooligosaccharide (LCO) that, when they interact with particular genes in the root hairs, cause cortical cell divisions that form root nodule primordia. At the same time, an infection process is started to introduce the bacteria into the nodule cells.

The development of plant-made infection threads by root hair branching, distortion, and curling is how the majority of legumes become infected (Goss *et al*., 2017). Nodules come in two varieties: determinate (found in common beans and soybeans) and indeterminate (found in Medicago, peas, and clover). Indeterminate nodules typically have a persistent apical meristem and are believed to result from cell divisions in the inner cortex. They are cylinder-shaped and have a developmental gradient that may split into distinct nodule zones from the apex to the base of the nodule (Wekesa *et al*., 2022). On the other hand, determinate nodules are spherical, lack a persistent meristem, and are the consequence of cell divisions in the middle/outer cortex of the root. Early in the developing process, a determinate nodule's cell divisions cease. Therefore, the

infected cells evolve more or less synchronously to the nitrogen-fixing stage, while the mature nodule develops by cell expansion (Goyal *et al*., 2021). Through an epidermal cell layer, the infection threads carrying the dividing bacteria proliferate and enter the nodule primordial cells (Figure 1). The cortical cells then release and internalize the bacteria in a mechanism akin to endocytosis. Individual bacteria in nodule cells are encased in a plant-derived membrane to create an organelle-like structure known as the symbiosomal. The bacteria continue to grow and develop into nitrogen-fixing bacteroids, which actively produce nitrogenase enzymes (Goyal *et al.*, 2021).The nodule must maintain low oxygen concentrations while maintaining an oxygen supply for bacterial metabolism since nitrogenase is highly sensitive to oxygen (Mamenko, 2021).

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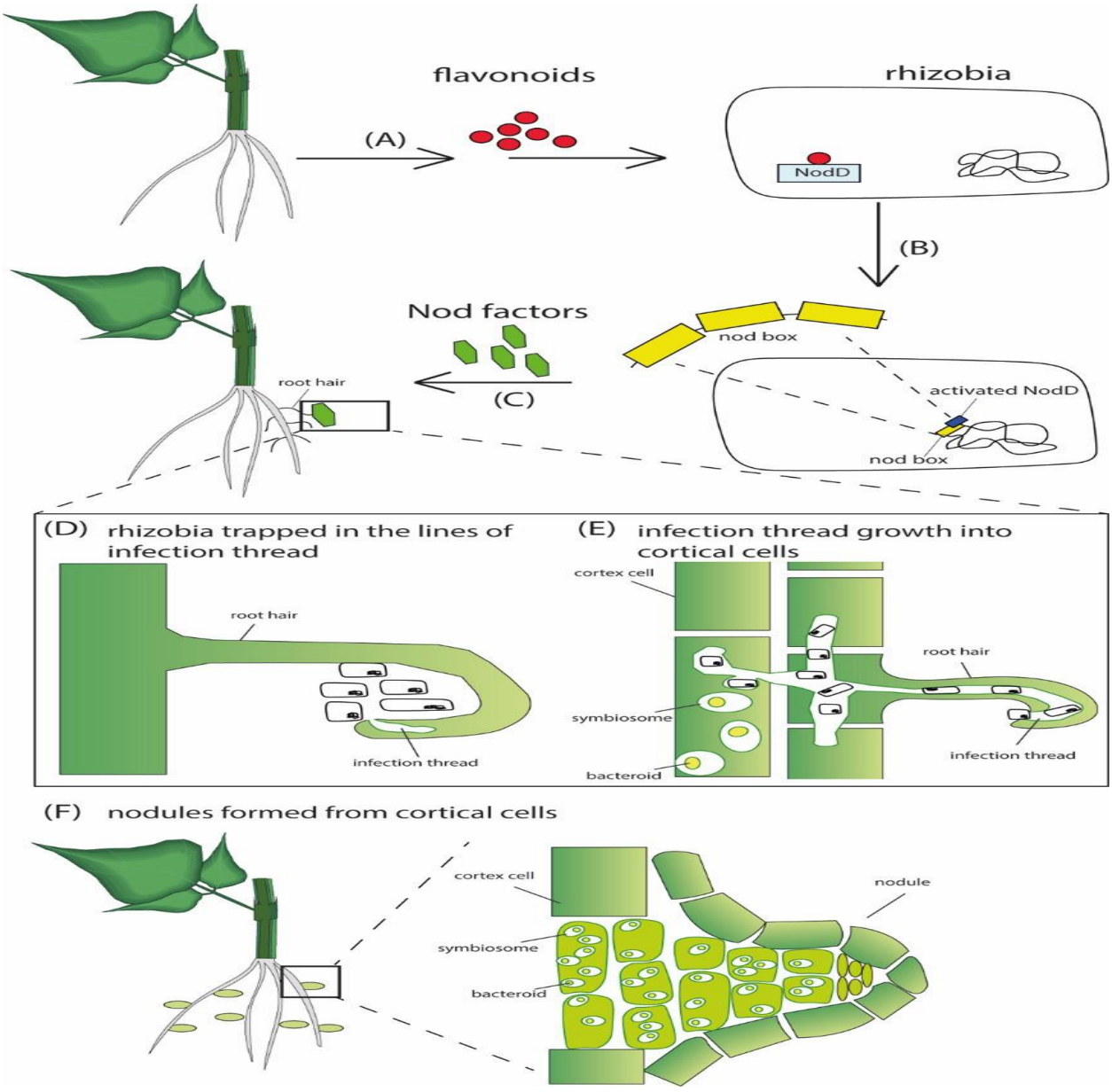


Figure 1 : An illustration showing the nodulation process (Wekesa *et al*., 2022)

(A) Certain rhizobia detect flavonoids released by the host plant into the rhizosphere. The plant believes that the flavonoids permit symbiotic infection of the root because they cause the transcription of the genes involved in the production of the *rhizobial* Nod factors. Following transcription, the nod box promoter (B) is bound by the activated NodD, triggering the production of Nod factors (C). The infection thread that ensnares rhizobia within the coiled surfaces is induced by the Nod factors (D). *Rhizobia* are released and internalized by the cortical

cells when the infection thread passes through the epidermal cells (E). Nodules occur as a result of the bacteria and the infected cortical cells' continued growth and differentiation (F).

2.7. Factors affecting biological nitrogen fixation

Environmental factors have a significant impact on *rhizobial* diversity, which is determined by how well microbial species adapt to the current environment (Koskey *et al.*, 2018). Environmental stress affects the *rhizobial* and host responses, resulting in a decrease in SNF efficiency.

2.7.1 Drought stress

Lack of soil moisture has an impact on the symbioses between legumes and rhizobia and is a major barrier to the growth, production, and spread of microorganisms. Although *rhizobial* populations have excellent nodulation and may thrive in extremely dry conditions, population densities have been found to decrease during drought stress (Basu *et al*., 2017). The *rhizobial* strains affect the microbial population's drought sensitivity. Therefore, drought-tolerant *rhizobial* strains must be chosen within the range of their legume host to maximize N_2 fixation and legume-rhizobium interaction. The *rhizobial* strains affect the microbial population's drought sensitivity. Therefore, drought-tolerant *rhizobial* strains must be chosen within the range of their legume host to maximize N_2 fixation and legume-rhizobium interaction.

2.7.2 Salt stress

Salt stress is one of the most important abiotic variables influencing plant development and productivity, particularly in arid and semi-arid regions (Bertrand *et al*., 2015). An estimated 30% of arable land and 6% of the world's total area are thought to be excessively salinized, rendering them unfit for conventional agriculture and rapidly degrading (Kirova &Kocheva, 2021). Some *rhizobial* strains can survive in saline soils, whereas others have varying degrees of sensitivity to salt stress. However, the majority of *rhizobia* cannot tolerate the negative consequences of high saline levels. In general, *rhizobia* that grow more quickly can withstand more salt than those that grow more slowly (Kirova &Kocheva, 2021). Because there are less *rhizobial* cells in saline environments, the effectiveness of the symbiotic connection is significantly reduced. This leads to lower activity, nodule growth inhibition, and root infection limitation.

2.7.3 Temperature stress

Temperature is one of the factors that hinders biological nitrogen fixation. High temperatures adversely affect the effectiveness of rhizobia and impede the growth and development of host legumes. Elevated soil temperatures lead to a delay in nodulation, which in turn reduces *rhizobia* populations and BNF in legumes (Hungria &Kaschuk, 2014). This also significantly influences nitrogen metabolism. Saha *et al*. (2017) found that warming facilitates microbial nitrogen fixation and the uptake of fixed gas, whereas low temperatures result in minimal activity. The study by Hungria &Kaschuk(2014) indicates that when temperatures exceed 40°C, the formation of inefficient nodules drastically decreases plant nitrogenase activity. Additionally, temperature affects the relative activity of *rhizobia*, meaning that *rhizobia* that are highly effective within a specific temperature range become less active outside of that range.

2.7.4 PH stress

Low pH soil has a lot of iron and aluminum oxides but little phosphorus. High or low soil pH can impede rhizobial activity in the rhizosphere surrounding legume roots. Low levels of phosphorus, calcium, and molybdenum are among the phytochemical traits of extremely acidic soils; *rhizobia* and plants are also impacted by the presence of manganese and aluminum toxicity (Ferreira *et al*., 2016). Thus, low soil pH has a more detrimental effect on nodulation and N fixation.

2.7.5 Climate change

This significant global event draws attention to environmental issues like erratic rainfall, frost occurrences (freezing temperatures), elevated temperatures (global warming), and the natural pollution of soil and water used in agriculture. Abiotic stresses such as water scarcity, salinity, heavy metals, and extreme temperatures can cause irreversible harm to cultivated plants, leading to cell and tissue death, nutritional and hormonal imbalances, reduced yields, and even plant mortality (He *et al.*, 2018). Water scarcity is a widespread issue, as farmers often resort to using poor-quality water meant for human consumption, which may be contaminated with salinity or heavy metals, to meet the needs of their crops (Oliveira *et al.*, 2022). To counteract the effects of drought, the agricultural sector has increased the areas under irrigation, enhancing both the quantity and quality of produce and preventing crop failures (Enebe and Babalola, 2018).

3. METHODOLOGY

3.1 Description of Study Area

This study was conducted in woldia university biotechnology laboratory, woldia. Woldia is approximately 521 km north of Addis Ababa and lies at an elevation of about 2,380 meters above sea level. The town is positioned at approximately 11°50′N latitude and 39°36′E longitude**.** Woldia is characterized by mountainous terrain and falls within the subtropical highland climate zone. The area experiences moderate temperatures ranging from 10°C to 25°C annually, with an average annual rainfall of 750 to 1,200 mm, predominantly occurring from June to September (Mekoya et al., 2024). The climate supports diverse agricultural practices and contributes significantly to the livelihoods of the local population.

The town has a population of over 90,000 residents and serves as a key administrative and commercial center in the North Wollo Zone. The local economy is predominantly agrarian, with mixed crop-livestock farming being the primary agricultural system. Major crops cultivated in the area include chickpea, wheat, barley, teff, sorghum**,** and maize**,** while common legumes include lentils and faba beans. Livestock such as cattle, sheep, goats, donkeys, and poultry are also widely reared.

The soils in and around Woldia are primarily Vertisols and Cambisols**,** which are moderately fertile and suitable for legume cultivation, particularly chickpeas. These soil types generally exhibit good moisture retention properties but may vary in texture and organic matter content depending on specific topographical and land use conditions.

The specific sampling sites for root nodule collection, along with their geographic coordinates and altitudes, are provided in Table 1 below:

Table 1: Geographic coordinates and altitudes of root nodule sampling sites

No.	Strain Designation	Zone	District	Latitude (N)	Longitude (E)	Altitude
1	WUSFME-1	N/Wollo	Mersa	11° 39' 53.84" N	39° 39' 34.41" E	1600 m
2	WUSFME-2	N/Wollo	Mersa	11° 40' 10.25" N	39° 39' 20.18" E	1610 m

3	WUSFME-3	N/Wollo	Mersa	11° 39' 35.70" N	39° 39' 45.50" E	1595 m
4	WUSFMC-1	N/Wollo	Mechare	11.8028° N	39.5729° E	1774 m
5	WUSFMC-2	N/Wollo	Mechare	11.8045° N	39.5742° E	1768 m
6	WUSFMC-3	N/Wollo	Mechare	11.8000° N	39.5705° E	1782 m
7	WUSFSI-1	N/Wollo	Sirinka	11.7560° N	39.6045° E	2106 m
8	WUSFSI-2	N/Wollo	Sirinka	11.7582° N	39.6060° E	2110 m
9	WUSFSI-3	N/Wollo	Sirinka	11.7548° N	39.6031° E	2098 m
10	WUSFDG-1	N/Wollo	Doro Gibir	11° 52' 0" N	39° 40' 0" E	1532 m
11	WUSFDG-2	N/Wollo	Doro Gibir	11° 51' 45" N	39° 40' 15" E	1526 m
12	WUSFDG-3	N/Wollo	Doro Gibir	11° 52' 20" N	39° 39' 50" E	1540 m

Where; SFDG -Sample from Doro Gibir, SFMC- Sample from Mechare, SFME -Sample from Mersa, SFSI- Sample from Sirina.

3.2 Sample collection

Twelve soil and root nodule samples were collected from chickpea-growing areas around Woldia, such as Sirinka, Mersa, Mechare, and Doro Gibir, using a random sampling method. Sampling points were located at least 5 meters away from the field edges to minimize potential edge effects. Soil and root nodules were collected at an approximate depth of 20 cm using a sterile soil and hand tools, such as spatulas, while wearing gloves to prevent contamination. Samples were placed into sterile polythene bags, labelled, and transported in a clean icebox to the laboratory. Upon arrival, samples were stored at 4ºC until processing. Fresh, healthy root nodules were selected based on their light brown or pinkish colour, indicating active nitrogen fixation between the rhizobia and chickpea plants (Pervin *et al*., 2017).

Figure2 : Rhizosphere soil and Root nodules Sample collection

3.3 Media preparation

The nutrient medium used for isolation of the *Rhizobia* was yeast extract mannitol (YEM) agar, with the following composition: mannitol 10 gm/l, anhydrous magnesium sulfate 0.2 gm/l, sodium chloride 0.1 0.1gm/l, di-potassium hydrogen phosphsate 0.5 0.5gm/l, anhydrous calcium chloride 0.2 0.2gm/l, anhydrous iron chloride 0.01 0.01gm/l, yeast extract 1.00 1.00gm/l, agar 20 gm/l, and distilled water1000 ml (Gyorgy *et al*., 2010). And the composition of Yeast Mannitol Broth (YMB) was 10 mg/l mannitol, 200 mg/l anhydrous magnesium sulfate, 100 mg/l sodium chloride, 500 mg/l di-potassium hydrogen phosphate mg/l yeast extract, and 1000 ml distilled water (Aung & Oo, 2020). Each of the components was measured with a weighing balance and put into a conical flask containing distilled water and then covered with aluminum foil. The pH of the medium was adjusted to 7 using a pH meter after this process. The medium was autoclaved at a temperature of 121ºC for 15 min and allowed to cool and was poured into Petri dishes (approx. 20 ml in each) aseptically in the hood. This solidified medium can be used for the enumeration of symbiotic *Rhizobia* microbes by using spread plate techniques.

3.4 Isolation of *rhizobial* isolates

Initially, chickpea root nodules were detached and washed under running tap water to remove adhering soil particles. Intact and undamaged nodules were then carefully excised from the roots using sterile forceps. To ensure surface sterilization, the nodules were immersed in 95% ethanol for 5–10 seconds to break the surface tension, followed by soaking in a 3% hydrogen peroxide (H_2O_2) solution for 2–3 minutes. The sterilized nodules were then rinsed thoroughly in five successive changes of sterile distilled water.

Sterilized nodules were aseptically crushed using a sterile mortar and pestle to obtain a milky suspension of bacteroids (Pervin et al., 2017). The suspension was streaked onto yeast extract mannitol agar (YEMA) medium containing ketoconazole (20.0 µg/ml) to reduce fungal contamination. Petri plates were incubated at 28 °C for 48 hours. After incubation, well-isolated, typical *rhizobial* colonies characterized by their white, circular, convex, and mucoid appearance were selected and subcultured onto fresh YEMA plates using the streak plate method to obtain pure cultures.

All isolation and subculturing steps were carried out in a laminar airflow cabinet to maintain aseptic conditions (Makkar and Jangra, 2017). Only morphologically distinct and purified colonies consistent with *rhizobial* characteristics were selected for further biochemical and physiological characterization.

3.5 Preliminary Tests of *Rhizobia*

Two different preliminary tests (growth on YEMA with Congo red and bromothymol blue) were performed to confirm the isolate as *Rhizobia* and to differentiate them from other microbes (Somasegaran and Hoben, 2012).

3.5.1 Bromothymol Blue Test

Rhizobia were cultivated on YEM agar medium supplemented with 0.025% bromothymol blue for this test, and the culture was kept at 28°C for 48 hours. In this test sample, YEM medium with BTB was allowed to grow. The formation of acid caused the positive sample to turn yellow after 48 hours of incubation at 28°C (Makkar & Jangra, 2017).

3.5.2 Congo Red Absorption Test

This was done to confirm the purity of *rhizobial* isolates. Congo red stock solution was prepared by dissolving 2.5 g Congo red in 1000 mL sterilized distilled water. Ten (10) milliliters of Congo red was added to a liter of YEMA media and sterilized. Finally, a loop full of test isolates was streaked on the medium, covered with aluminum foil, and incubated at 28 ± 2°C for 72 hours. The absorption of Congo red by the *rhizobial* isolates, which indicated contaminant growth, was recorded. Isolates that did not absorb the Congo red were selected for future purification work (Menelih *et al*., 2022).

3.6 Purification and preservation of Isolates

Using a sterile inoculating loop, isolated colonies were selected, streaked on sterile YEMA plates, and then incubated at 28±2ºC. Through serial re-streaking and the selection of a single well-isolated colony to a YEMA slant containing 0.3% (w/v) $CaCO_3$ in a culture tube and incubation at 28 °C, the purity and homogeneity of the colony type were meticulously investigated. The culture slant was kept at 4°C after it had grown sufficiently (Mekonnen & Debebe, 2021). The isolates were streaked on YMA slants for long-term storage, and sterile glycerol was added to the media (Pervin *et al*., 2017).

3.7 Morphological characterization

Each isolate was inoculated on YEMA with a loopful of a 48-hour broth culture (Yeast Extract Mannitol Broth (YEMB)) and incubated for 48 hours at 28 °C. Cell shape, Gram staining, and other colony traits were used to define individual colonies. Type of growth, colony color, elevation, borders, texture, and colony size were the morphological observations (Asrat et al., 2023).

3.7.1 Gram staining of the *rhizobia* isolates

The tests were conducted by using a loopful of pure culture grown on YEMA was put in for Gram staining for more specific identification of the colonies. The Gram staining was done in a laminar air flow hood. Using a clean slide and sterile wire loop, a loopful of normal saline was dropped at the center of the slide, and the loop was sterilized again. The specimen was collected with the wire loop. A smear was made circularly. After making the smear, it was heat-fixed on the slide by passing it gently over the Bunsen flame. Crystal violet was put on the smear for 30 seonds and poured off. Iodine solution was poured on and left for 1 min. It was then washed off with tap water. Then, ethanol was added for decolonization purposes, and subsequently, the ethanol was poured off, and water was poured immediately. Counterstaining using Safranin was done with water within a minute. After drying the slide surface, observe it through a compound microscope. Finally, immersion oil was added and viewed with a 100x oil immersion objective lens (Hamza *et al*., 2017).

3.8 Biochemical Characterization of *Rhizobia*

3.8.1 Motility test

Using a sterilized straight wire, *rhizobia* isolates were injected into a test tube with a SIM medium containing 0.5% YEMA. The test tube was then incubated temperature at 28°C, pH 7.0,

and incubation for 48 hours. If the isolates moved away from the line of inoculation, it was a positive result, suggesting motility; if not, it was a negative result, indicating non-motility (Asrat *et al*., 2023).

3.8.2 Starch hydrolysis

These experiments were conducted to ascertain *Rhizobia* capacity to use starch as a source of carbon. The test microorganisms in the starch hydrolysis test were cultivated on agar plates that contained 1% starch. The bacteria were cultivated on the hood's supplied media and incubated at 32 °C,pH 7.0,and for 48 hours. Following the appearance of bacterial colonies, a drop of iodine (0.1N) was applied to a culture that had been incubated for 48 hours and had a distinct zone of inhibition surrounding the colonies. Following the incubation period, a drop of iodine solution was added; the development of a clear zone signifies a successful starch hydrolysis test (Wanore *et al*., 2023b).

3.8.3 Catalase Test

The purpose of this test was to determine whether the examined organism contained the catalase enzyme. One loopful of the *rhizobial* isolates was suspended in one milliliter of 3% hydrogen peroxide on a glass slide to test for catalase activity. According to usual procedure, these tests were conducted (Pervin *et al.*, 2017). A positive catalase test result is indicated by the formation of bubbles.

3.8.4 Oxidase test

The presence of the oxidase enzyme in several *rhizobia* isolates was assessed using the oxidase test. Kovac's reagent (1% N, N, N.N-tetramethyl-phenylene diamine) was dissolved in warm water and kept in a dark bottle. One-day-old Rhizobial colonies from agar plates were placed on a strip of filter paper that had been dipped in this reagent and allowed to air dry. Within five minutes, oxidase-positive colonies changed from lavender to dark purple to black (Makkar & Jangra, 2017).

3.8.5 Triple sugar iron (TSI) agar test

Triple sugar iron agar medium was used to inoculate test tubes with cultures of *rhizobial* isolates that were actively growing. The test was conducted using the stab-and-streak method by incubating at 28°C, pH 7.0, for 48 hours. Through a shift in the butt and slant colours, the test was conducted to determine the isolates' capacity to ferment different sugars, such as lactose, glucose, and sucrose. Following incubation, colour was seen on the slant and butt. Three

observations might be made based on the bacterial isolate's capacity to consume carbohydrates. An alkaline/alkaline (red slant/red butt) shows the absence of carbohydrate fermentation, an acid/acid (yellow slant/yellow butt) indicates the fermentation of lactose and sucrose, and an alkaline/acid (red slant/yellow butt) indicates the fermentation of dextrose exclusively (Mir *et al*., 2020).

3.8.6 Citrate Utilization Test

The purpose of this test was to confirm whether or not the test organism used citrate as a carbon source. The test was conducted on Simmons' citrate agar slants by streaking the isolates onto Simmons' citrate agar slants and incubated at 28°C, pH 7.0, for 48 hours. According to (Hamza *et al.*, 2017), a noticeable shift in colour from green to blue indicates a positive citrate utilization test result.

3.8.7 Gelatin hydrolysis

The purpose of the test was to ascertain whether the *rhizobial* isolates could use gelatin as a media source and create the proteolytic enzyme gelatinase. The presence of the enzyme gelatinase is shown by the degradation of gelatin. The nutritional gelatin stab method is the most widely used and standard technique. After being stab-inoculated at 28 °C for 48 hours at pH of 7.0, in nutritional gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin, 1000 ml of distilled water). The culture was put at a low temperature of 4°C for 30 minutes (Aung and Oo, 2020).

3.8.8 Urease test

The *rhizobial* isolates were inoculated individually into test tubes with urea agar Slant (K_2HPO_4 2 g. L^{-1}, urea 20 g. L^{-1}, phenol red 0.012 g. L^{-1}, agar 15 g. L^{-1}, pancreatic digest of gelatine 1 g. L^{-1}, dextrose 1 g. L^{-1}, and 1 L dis. H_20) and incubated at 28°C for 48 to 48 hours. Following the incubation period, the isolates' positive urease activity is shown by the emergence of a deep pink colour (Mir *et al.*, 2020).

3.8.9 Nitrogen Fixation Test

To qualitatively assess the nitrogen-fixation potential of the *rhizobia* isolates, a nitrogen-free (N-free) medium was prepared based on the formulation described by Rodrigues *et al*. (2016), with slight modifications. The medium contained the following components per liter of distilled water: 0.2 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.2 g sodium chloride (NaCl), 0.1 g ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 5 g sodium molybdate dihydrate

($Na_2MoO_4 \cdot 2H_2O$), 1 g calcium carbonate ($CaCO_3$), 1 g dipotassium hydrogen phosphate (K_2HPO_4), and 15 g agar. The pH of the medium was adjusted to 7.0 using 1 N NaOH a HCl before sterilization by autoclaving at 121 °C for 15 minutes and incubated at 28 °C for five days.

3.8.10 Indole test

Tryptophan broth medium was prepared and poured into 10 mL test tubes and then autoclaved at 121°C for 15 min. *Rhizobium* was inoculated at 28°C with a pH of 7.0 for 48 hours. An un inoculated broth served as the control. After incubation, 1 mL of Kovac's reagent (isoamyl alcohol, *para*-dimethyl amino benzaldehyde and concentrated hydrochloric acid) was added to each test tube, including the control. Tubes were shaken gently and allowed to stand until the reagent surfaced. The formation of a red ring indicated a positive result, but a yellow ring indicated a negative result (Hossain *et al*., 2019).

3.8.11 Ammonia (NH3) production

A loopful of newly developed cells was inoculated into 10 ml of sterile peptone water (pH 7.0) and the isolates were then incubated at 28°C for 72 hours to test for the production of ammonia. To identify the formation of a brown to yellow colour as a positive test for ammonia generation, the tubes were treated with 0.5 ml of Nessler's reagent (potassium iodide 50 g, saturated mercuric chloride 35 mL, distilled water 25 mL, potassium hydroxide (40%) 400 mL) (Manasa et al., 2017b).

3.9 Effect of *Rhizobia* on seed germination

Seed germination studies were conducted using the water agar method. Chickpeas were surface sterilized with 70% ethanol and then treated with 1% calcium hypochlorite for 2 min, followed by repeated washing with sterile water. After this, the seeds were soaked in the *rhizobial* culture broth, while seeds that were soaked in normal nutrient broth were kept as a control. A final concentration of 10^8 CFU mL^{-1}, then coated onto the surface-sterilized chickpea seeds and allowed to air dry. Ten seeds of each treatment were kept equidistant in sterilized Petri plates containing moist filter paper with three replicates for each treatment, and the Petri plates were incubated at room temperature (25°C). Germination counting began after 24 hours of sowing and continued until the 15th day. Seed germination and percent seedling emergence were calculated using the following formula: % seed emergence = number of emerged seedlings/number of seeds sown × 100 (Mia *et al*., 2012). After 2 weeks, the germination, secondary root number and

primary root length were measured and recorded (Xu *et al*., 2022). Seedling vigour index = % germination × (seedling dry weight) (Mesele *et al*., 2024).

3.10 Symbiotic effectiveness screening at the Greenhouse

The experiment was conducted in a greenhouse at Wolda University Botanical Garden. A total of 24 pots were set up in a completely randomized design (CRD), each containing eight different treatments: five rhizobial inoculants, namely SFSI (from Sirinka), SFDG (from Doro Gibir), SFME (from Mersa), SFMC (from Mechare); CP17 (a commercial inoculum from Desse soil science); N fertilizer (20 kg N ha^{-1}, without inoculant); and dH2O (neither fertilizer nor inoculation). The study was organized with three replications in a completely random design within the greenhouse. 3 kg of steralized sand soil was placed into surface-sterilized plastic pots (Somasegaran& Hoben, 1985) with a capacity of five kg. The chickpea (Cicer arietinum L.) cultivar "Metek," sourced from the Sirinka Agricultural Institute, was surface sterilized using 95% ethanol for 10 minutes and 3% sodium hypochlorite for 3 minutes, followed by rinsing with sterile water. The seeds were allowed to dry in a laminar airflow for a few minutes. Five surface-sterilized seeds were germinated on sterilized water agar plates (7.5 g of agar in 1,000 ml of distilled water) at room temperature(25°C) for 48 hours. Five uniform, undamaged seeds were selected and transferred into each pot containing sterilized sand using sterile forceps, and later thinned to three after one week of germination. Three days post-germination, each seedling received 1 ml of a 72-hour YEMB culture (approximately 1 ml of each Rhizobium isolate at 1×10^{8}cfu/ml) applied around the root with a sterile pipette and covered with a layer of sterile sand soil. One week after germination, the seedlings were thinned to maintain three healthy plants per pot. All plants were given 100 ml of quarter-strength N-free nutrient solution(with the composition of :Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) 0.025 g/L, Magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$) 0.025 g/L, Monopotassium phosphate (KH_2PO_4) 0.025 g/L, Dipotassium phosphate (K_2HPO_4) 0.025 g/L, Boric acid (H_3BO_3) 0.000715 g/L, Manganese sulphate monohydrate ($MnSO_4{\cdot}H_2O$) 0.0005575 g/L, Zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$) 0.000055 g/L, Copper sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$) 0.00002 g/L, Sodium molybdate dihydrate ($Na_2MoO_4{\cdot}2H_2O$) 0.0000975 g/L, Iron (Fe) as Fe-EDTA 0.0025 g/L.) weekly, as described by Somasegaran and Hoben (1985). The pots were watered every two days (Wubie and Adal, 2021).

3.10.1 Symbiotic Effectiveness of the *Rhizobia* Isolates

Plants were uprooted forty-five days post-planting to measure shoot length, nodule number, nodule dry weight, and shoot dry weight. Before measurement, roots and nodules were carefully cleaned using running tap water to remove adhering soil particles, ensuring accurate assessment of biomass. The symbiotic effectiveness was calculated using the following formula: % SE = Inoculated plants SDW/N fertilized plants SDW × 100, where SDW = shoot dry weight, N = nitrogen, and SE = symbiotic effectiveness. High efficacy (≥80%), low effectiveness (35–49%), effective (50–79%), and ineffective (<35%) were the rates of nitrogen-fixing effectiveness (Mulongoy, 1992; Somasegaran and Hoben, 2012).

3.11 Data analysis

All experimental data were collected in triplicate, and the results were presented. Statistical analyses were conducted with statistics 10, and SPSS (version 27.0.1). The mean values comparison was performed following the significance test results from One-way ANOVA using least significant difference (LSD) at 5% level of probability. The LSD test also provides clearer interpretability when assigning statistical groupings using letter notations. Differences between means were considered statistically significant at a 5% probability level ($p \leq 0.05$). Significant variations between treatments were indicated by different lowercase letters assigned to each mean in the data tables and scatter plot figures. The e correlation between different parameters was evaluated by using the Pearson correlation coefficient.

4. RESULTS AND DISCUSSION

4.1 Results

4.1. 1. Isolation, screening, and morphological characterization of Rhizobium

In this study, *rhizobia* isolates were obtained from root nodules of chickpea plants collected from different chickpea-growing areas. A total of forty-one isolates were successfully cultured on Yeast Extract Mannitol Agar (YEMA) medium. All isolates were subjected to preliminary morphological examination based on colony and cell characteristics. Across all samples, the colonies demonstrated remarkably consistent morphological traits. The colonies appeared white in color, with a circular shape and entire margins. They exhibited a mucoid texture, which is a typical feature of *rhizobial* exopolysaccharide production, and were transparent to translucent in optical appearance. The colony surface was uniformly smooth, indicating healthy, non-filamentous bacterial growth. Microscopic examination using Gram staining revealed that all 41 isolates were Gram-negative (-ve) and rod-shaped, further supporting their classification within the *Rhizobium* genus. This uniformity in colony and cell morphology across all isolates suggests that the isolates are likely to be members of the same or closely related species of *Rhizobia*. However, further physiological and biochemical tests were conducted to support this preliminary identification and to assess potential variability in functional traits.

Table 2: Morphological Characteristics of 41 Rhizobia Isolates

Isolate Code	CC	CF	CT	O	CM	A	GS	SB
WUSFSI-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-22	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod

WUSFSI-31	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-33	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-22	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-31	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-31	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-32	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-33	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod

WUSFDG-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-13	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-22	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-31	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-32	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-33	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod

Where; CC – Colony Color, CF – Colony Form, CT – Colony Texture, O – Opacity, CM – Colony Margin, A – Appearance, GS – Gram Staining, SB – Shape of Bacterium.

As presented in Table 3, twelve *rhizobial* isolates were selected for further characterization based on their responses to Congo Red (YEMA-CR) and Bromothymol Blue (YEMA-BTB) tests. In the YEMA-CR medium, these isolates did not absorb the Congo red dye after three days of incubation at 28°C, forming creamy-colored colonies. This behavior is typical of Rhizobium species, which are known to resist dye absorption, thereby helping differentiate them from other fast-growing soil bacteria such as Agrobacterium spp., which tend to absorb the dye and appear red. In the YEMA-BTB test, all twelve isolates changed the deep green color of the medium to yellow, indicating acid production from carbohydrate metabolism. This is a key trait of fast-growing rhizobia (e.g., *Rhizobium* spp.), in contrast to slow-growing strains such as *Bradyrhizobium*, which generally maintain an alkaline or neutral pH in this medium.

Microscopically, all isolates were confirmed as Gram-negative and rod-shaped, further supporting their tentative classification as *Rhizobia* species. Colonies on YEMA were transparent to white, circular with entire margins, and mucoid in texture all consistent with morphological

characteristics typical of rhizobia. To ensure culture purity, each isolate was sub-cultured three times by streaking on fresh YEMA plates. Overall, the results of dye-based tests, combined with colony morphology and microscopy, strongly suggest that these isolates belong to the genus *Rhizobia*, and more specifically, to the fast-growing group. This preliminary categorization sets the stage for further biochemical and plant growth-promoting assessments.

Table 3: Morphological characterization of selected Rhizobia isolates grown on YEMA at 28 °C

Isolates	CC	CF	CT	O	CM	A	GS	SB
WUSFSI-11	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFSI-32	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-21	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFME-32	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-31	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFMC-32	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-12	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod
WUSFDG-23	White	Circular	Mucoid	Transparent	Entire	Smooth	-ve	Rod

Where; CC is colony colour, CF is colony form, CT is colony texture, A is appearance, O is opacity, CM is colony margin, GS is Gram staining, and SB is the shape of the bacterium.

4.1.2 Biochemical characterization of rhizobium isolates

4.1.2.1. Motility test

All twelve *rhizobial* isolates tested SFSI-11, SFSI-21, SFSI-32, SFME-12, SFME-21, SFME-32, SFMC-12, SFMC-23, SFMC-31, SFMC-32, SFDG-12, and SFDG-23 exhibited positive

motility, as evidenced by uniform turbidity and diffused growth throughout the SIM medium. The pattern of dispersion was comparable to the motile positive control, indicating active flagellar movement in all isolates. None of the isolates showed restricted or localized growth, and all were categorized as motile (+).

The observation that 100% of the tested isolates demonstrated motility highlights their potential ecological fitness and adaptability in the soil environment. Motile behavior is advantageous for rhizosphere colonization, root surface attachment, and effective nodule formation, suggesting these isolates possess favorable characteristics for development as bio-inoculants. Detailed results are summarized in Table 4 and visually illustrated in Figure 5.

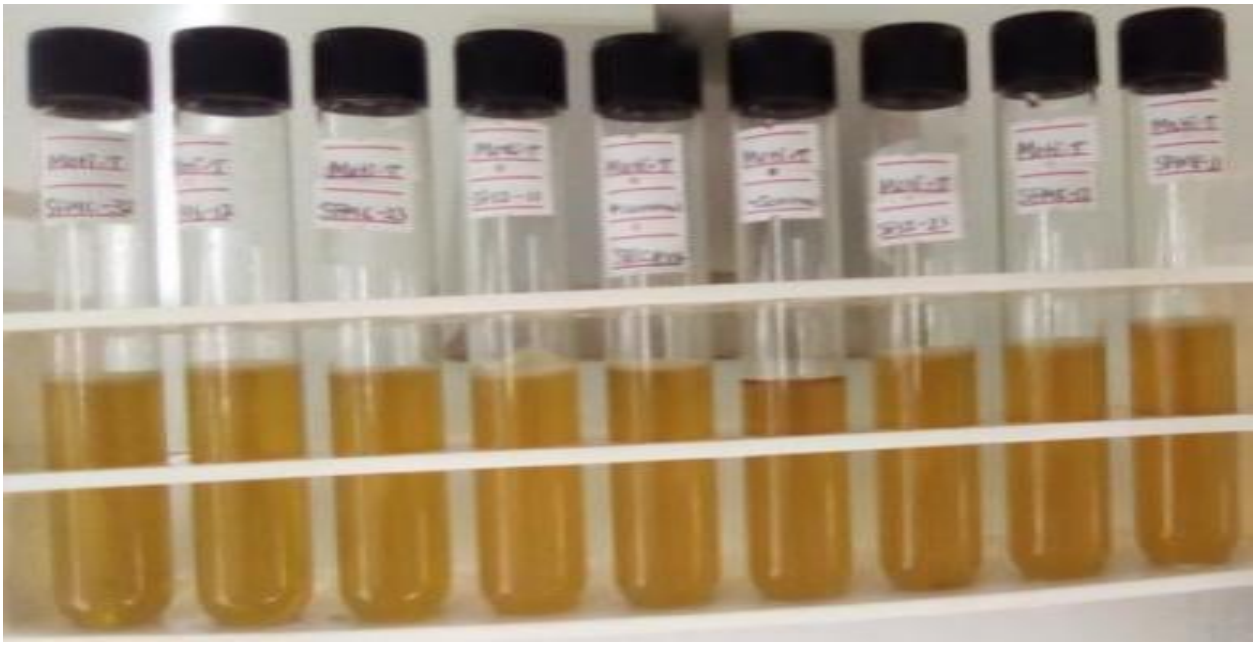


Figure 3: Motility test of *rhizobia* isolates

4.1.2 .2.Triple sugar agar test

Based on the quantitative results of the Triple Sugar Iron (TSI) agar test (Figure 6 and Table 4), 80% of the tested *rhizobial* isolates (SFDG-12, SFME-21, SFME-12, and SFMC-31) exhibited a red slant and yellow butt, indicating glucose-only fermentation without gas or hydrogen sulfide (H_2S) production. These results were consistent with the positive control, suggesting a conserved glucose fermentation pathway among the majority of isolates. In contrast, 20% of the isolates (specifically SFMC-23) displayed a yellow slant and yellow butt, indicating the ability to

ferment multiple sugars glucose, lactose, and/or sucrose. This broader carbohydrate fermentation profile reflects greater metabolic versatility, which may enhance the isolate's adaptability and effectiveness in diverse soil environments. Importantly, 0% of the isolates produced black precipitate, confirming the absence of H_2S production in all tested strains. The higher sugar utilization efficiency observed in isolate SFMC-23 suggests its strong potential as a bio-inoculant candidate for improving plant growth and soil fertility.

Figure 4: Triple sugar agar test of the *rhizobia* isolates

4.1.2.3 Citrate utilization test

All *Rhizobia* isolates tested SFSI-11, SFSI-21, SFSI-32, SFME-12, SFME-21, SFME-32, SFMC-12, SFMC-23, SFMC-31, SFMC-32, SFDG-12, and SFDG-23 exhibited a distinct color change from green to blue on Simmons' citrate agar, confirming positive citrate utilization (Fig. 6; Table 4). This color change is a clear indication of citrate metabolism, which raises the pH of the medium, leading to the observed shift. Overall, 100% of the isolates (12 out of 12) tested positive for citrate utilization.

Figure 5: Citrate utilization test of *rhizobia* isolates

4.1.2.4 Starch hydrolysis test

All tested *rhizobial* isolates SFSI-11, SFSI-21, SFSI-32, SFME-12, SFME-21, SFME-32, SFMC-12, SFMC-23, SFMC-31, SFMC-32, SFDG-12, and SFDG-23 demonstrated starch hydrolysis ability, as indicated by clear zones formed around their colonies. These clear zones represent the enzymatic breakdown of starch by extracellular amylase produced by the isolates.

Based on the diameter of the clear zones relative to the positive control (considered 100%), isolates SFME-21, SFMC-31, and SFDG-23 showed high amylase activity with clear zone sizes reaching approximately 95–100% of the positive control, indicating strong starch degradation potential. Isolate SFMC-32 exhibited moderate activity, with clear zones about 60–70% of the positive control. The isolates SFSI-32, SFME-12, SFME-32, SFDG-12, and SFSI-21 displayed smaller clear zones, representing lower amylase activity ranging from 30–50% compared to the control.

This quantitative variation reflects the diverse biochemical capabilities of the rhizobial isolates, which may impact their effectiveness in nutrient cycling and plant growth promotion. The detailed measurements are summarized in Table 4 and illustrated in Figure 8.

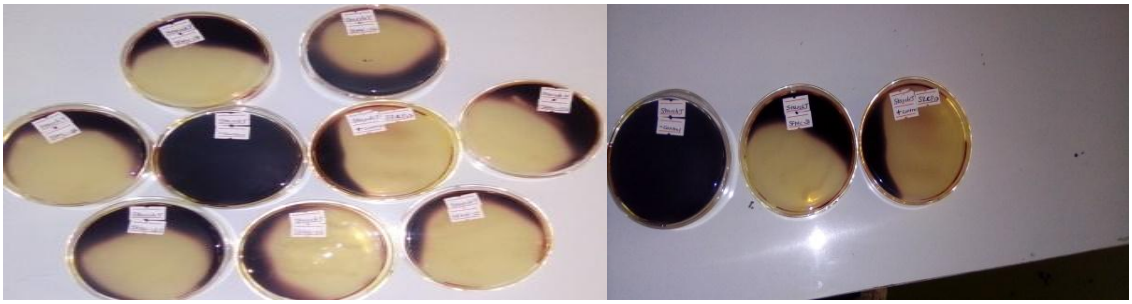


Figure 6: Starch hydrolysis test of *rhizobia* isolates

4.1.2.5 Urease test

All *rhizobial* isolates (100%) exhibited a pink slant and yellow butt in the urease test, indicating a positive urease reaction comparable to the positive control. The distinct color change from yellow to deep pink confirmed significant ammonia production, suggesting effective enzymatic hydrolysis of urea by urease, which subsequently increased the pH of the medium. This

enzymatic activity highlights the strong metabolic potential of the isolates to utilize urea as a nitrogen source.

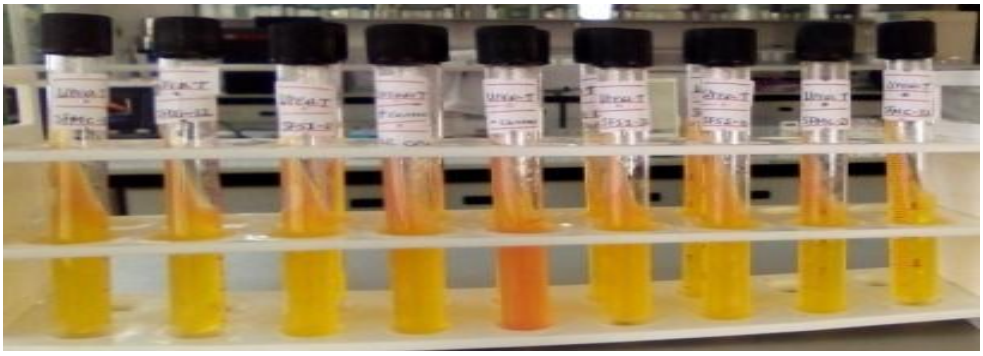


Figure 7: Urea test of *rhizobia* isolates

4.1.2.6 Gelatinase test

In the gelatin hydrolysis test, all bacterial isolates exhibited a negative reaction. Following incubation, the cultures were subjected to low-temperature treatment at 4°C for 30 minutes. The medium remained solidified, indicating the absence of gelatin hydrolysis and confirming negative gelatinase activity in all isolates.

Figure 8: Gelatin test of *rhizobia* isolates

4. 1.2.7 Oxidase test

Upon contact with the reagent-impregnated strips, all *Rhizobia* isolates exhibited a rapid oxidase reaction, demonstrated by a color change from dark purple to black within five minutes. Quantitatively, isolates SFMC-23, SFMC-31, and SFDG-23 showed the highest oxidase activity,

with color change intensities reaching approximately 95–98% relative to the positive control. Other isolates displayed oxidase activity ranging from 75% to 90% of the control's intensity. The sequential progression of color from dark purple to black confirmed the presence of cytochrome c oxidase activity in all tested isolates, underlining their strong potential for efficient electron transport and metabolic activity important for effective symbiotic nitrogen fixation.

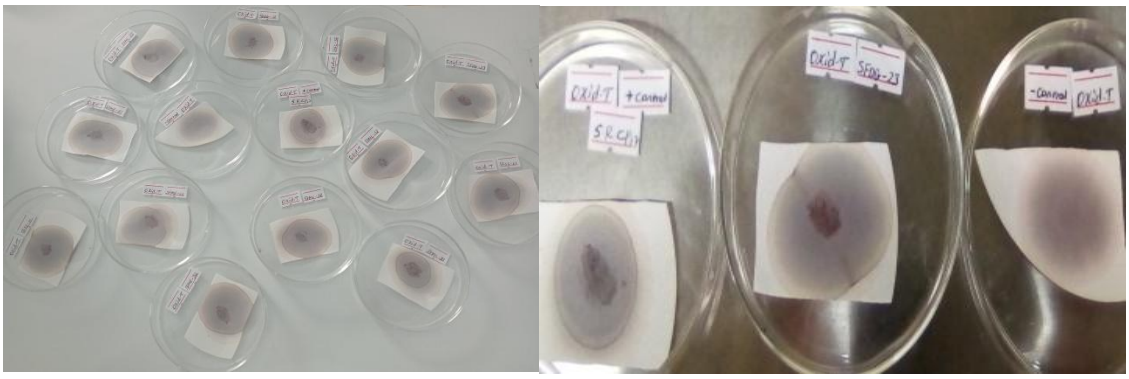


Figure 9: Oxidase test of *rhizobia* isolates

4.1.2.8 Indole test

All *Rhizobial* isolates (SFSI-11, SFSI-21, SFSI-32, SFME-12, SFME-21, SFME-32, SFMC-12, SFMC-23, SFMC-31, SFMC-32, SFDG-12, and SFDG-23) showed negative results for indole production. Specifically, no red color or red ring developed in any of the test tubes after the addition of Kovac's reagent, indicating the absence of indole in all isolates. Instead, the medium retained a yellowish color, consistent with negative results. These observations were validated by comparison with the positive control (which showed a distinct red ring) and the negative control (which showed no color change), as illustrated in Figure 12.

Figure 10: Indole test of *rhizobia* isolates

4.1.2.9 Catalase test

All tested *rhizobial* isolates showed positive catalase activity, indicated by oxygen bubble formation. Quantitative analysis revealed that isolates SFDG-23, SFMC-32, and SFMC-23 exhibited catalase activities of approximately 135%, 128%, and 125%, respectively, relative to the positive control (set at 100%). This significant increase in bubble production reflects a higher enzymatic breakdown of hydrogen peroxide, suggesting these isolates have enhanced oxidative stress tolerance and greater potential for survival under stressful conditions.

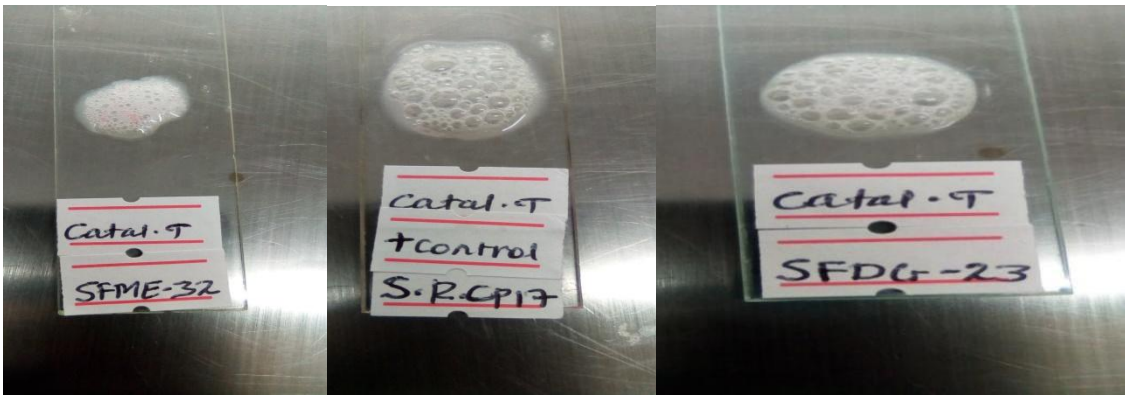


Figure 11: Catalyst test of *rhizobia* isolates

4.1. 2.10 Ammonia production

All tested *rhizobia* isolates showed positive ammonia production. Among them, 58.3% (7/12) were strong ammonia producers, exhibiting a brown-yellow color, while 41.7% (5/12) were moderate producers, indicated by a light yellow coloration. This variation in color intensity corresponds to the range of ammonia produced, reflecting the metabolic capacity of the isolates to deaminate amino acids or utilize nitrogenous compounds in peptone water. These results indicate the metabolic capacity of the isolates to deaminate amino acids or utilize nitrogenous compounds present in peptone water.

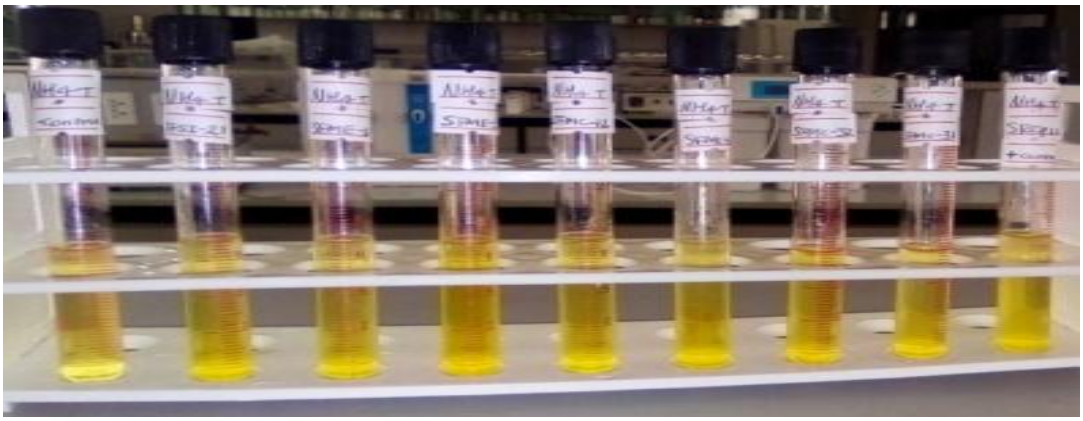


Figure 12: Ammonia production test of *rhizobia* isolates

4.1.2.11. Nitrogen fixation test

100% of the tested *Rhizobia* isolates (i.e., all isolates) formed a conspicuous pellicle just beneath the surface of the nitrogen-free medium after 5 days of incubation at 28 °C. This indicates that all isolates possess the ability to fix atmospheric nitrogen and grow in the absence of an external nitrogen source. All isolates (100%) showed positive pellicle formation in nitrogen-free medium, suggesting active nitrogenase systems.

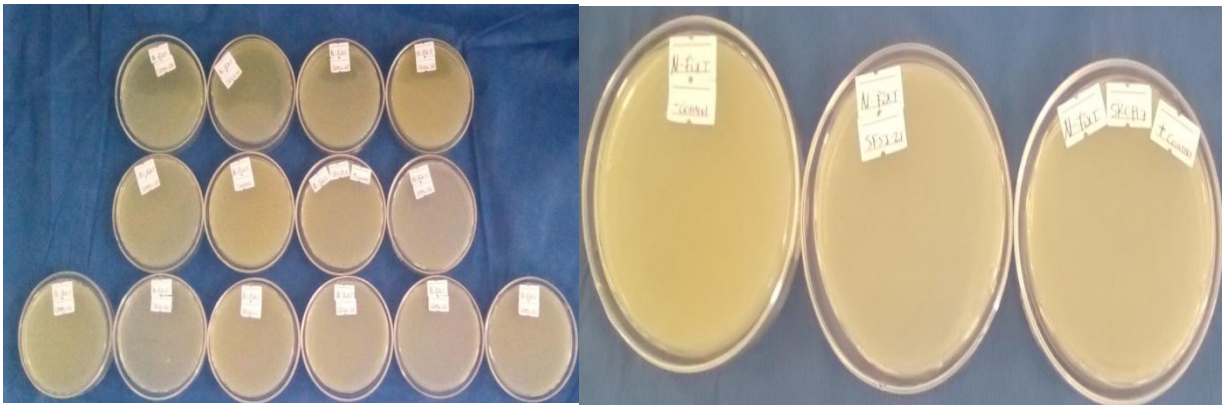


Figure 13: Nitrogen fixing test of *rhizobia* isolates

Table 4: Biochemical characterization of rhizobia isolates from different geographical locations

Isolates	Biochemical characteristics										
	MT	UT	OT	SHT	TSIAT	IT	GT	CT	CUT	APT	NFT

$SRCP_{17}$	+	+	+	+	RSYB	-	-	+	+	+	+
SFSI-11	+	+	+	+	RSYB	-	-	+	+	+	+
SFSI-21	+	+	+	+	RSYB	-	-	+	+	+	+
SFSI-32	+	+	+	+	RSYB	-	-	+	+	+	+
SFME-12	+	+	+	+	RSYB	-	-	+	+	+	+
SFME-21	+	+	+	+	RSYB	-	-	+	+	+	+
SFME-32	+	+	+	+	RSYB	-	-	+	+	+	+
SFMC-12	+	+	+	+	RSYB	-	-	+	+	+	+
SFMC-23	+	+	+	+	YSYB	-	-	+	+	+	+
SFMC-31	+	+	+	+	RSYB	-	-	+	+	+	+
SFMC-32	+	+	+	+	RSYB	-	-	+	+	+	+
SFDG-12	+	+	+	+	RSYB	-	-	+	+	+	+
SFDG-23	+	+	+	+	RSYB	-	-	+	+	+	+

Key: (+) positive result, (-) negative result, UT: urea test, MT: motility test, OT: oxidase test, SHT: starch hydrolysis test, IT: indole test, GT: gelatin test, CT: catalase test, CUT: citrate utilization test, APT: ammonium production test, NFT: nitrogen fixation test, TSIAT: triple sugar iron agar test; RSRB: red slant/red butt; YSYB: yellow slant/yellow butt.

From the twelve *Rhizobia* isolates, five were identified as potential candidates based on their efficiency in selective biochemical tests, including the oxidase test, starch hydrolysis test, and catalase test. Those isolates were further screened for their ability to promote seed germination and their symbiotic effectiveness under sterile conditions to confirm their symbiotic performance.

4.1.3. Effect of *rhizobia* inoculation on seed germination and early seedling growth

Germination was first observed within 24 hours and continued until day 15, with *rhizobial*-treated seeds showing a faster and more uniform emergence pattern. After 48 hours, all seeds in

both groups had germinated, but the *Rhizobium*-treated seeds exhibited a higher germination percentage compared to the non-treated control. After two weeks of incubation at 25°C, treated seeds showed statistically significant improvements ($p < 0.05$) in primary root length (PRL), number of secondary roots (NSR/P), and seedling dry weight (SDW).

The Pearson correlation analysis among various seedling traits revealed significant and positive relationships, indicating strong interconnections that contribute to early seedling development.

Primary root length showed a very strong correlation of 89.2% with secondary root number, meaning that as the primary root grows longer, there is almost a 90% likelihood that the number of secondary roots will also increase. Similarly, it had a 76.9% positive association with seedling dry weight and a 74.3% correlation with the vigor index, suggesting that increased root length supports both greater biomass accumulation and seedling vigor. In addition, primary root length was 60.7% correlated with shoot length and 64.4% with germination percentage, indicating its broader influence on both root and shoot development.

Shoot length also exhibited strong relationships with other traits, showing an 85.3% correlation with vigor index, 78.7% with secondary root number, and 76.4% with seedling dry weight. These values demonstrate that longer shoots are consistently associated with better root development and greater biomass, reflecting the coordinated nature of above- and below-ground seedling growth.

The strongest correlation observed was between secondary root number and seedling dry weight at 90.9%, indicating a very tight relationship. This suggests that increases in secondary root formation are directly and highly related to the accumulation of dry matter, which is essential for healthy seedling establishment.

Germination percentage was also highly associated with vigor index, showing a correlation of 80.9%, which highlights the critical role of rapid and uniform germination in promoting strong early growth and seedling vigor.

Overall, the results indicate that most of the seedling traits are highly interconnected, with correlations ranging from 60.7% to 90.9%. These findings emphasize the importance of selecting

for integrated traits such as root and shoot growth, germination rate, and biomass in breeding programs aimed at improving seedling performance and establishment under different conditions.

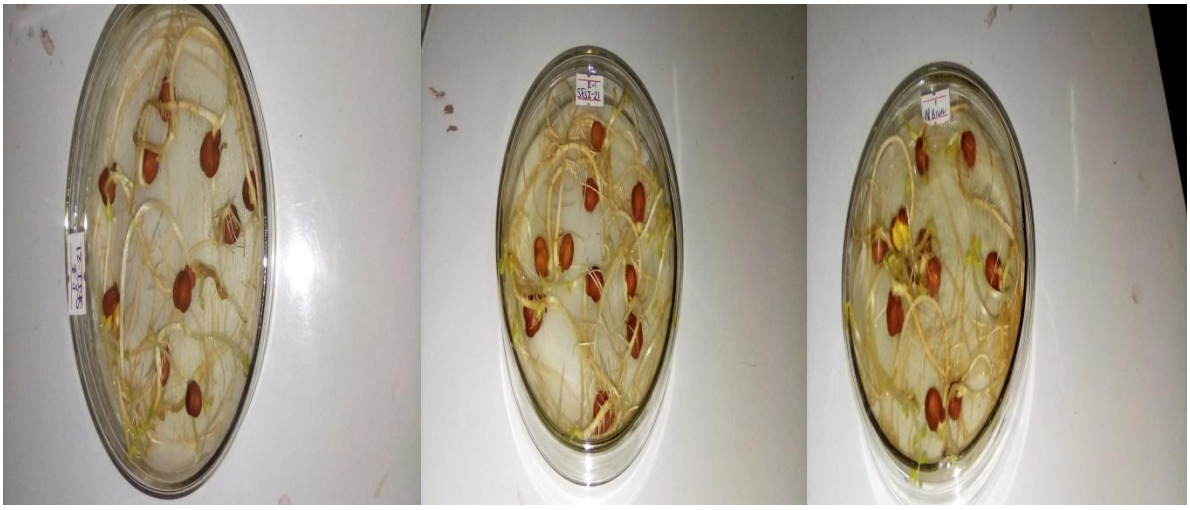


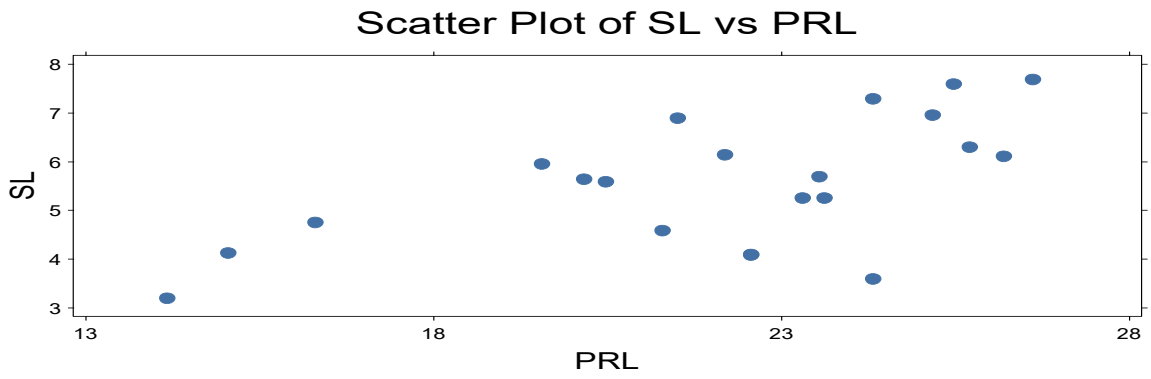
Figure 14: Germination tests of *Rhizobia* isolates

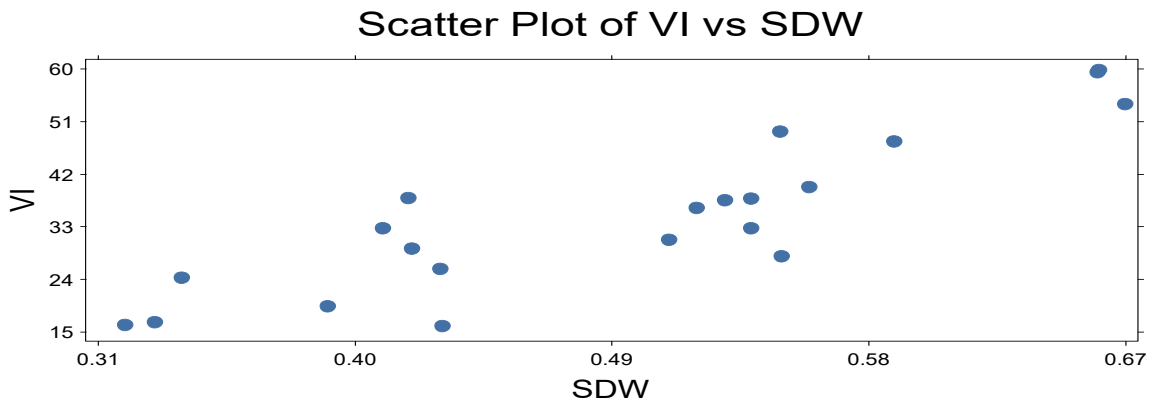
Table 5: Effect of Rhizobia on seed germination

Treatments	PSG	PRL (cm)	SL (cm)	SDW (g)	NSR/P	VI
SRCP17	86.667±3.33^{a}	25.933±0.338^{a}	7.20±0.4509^{a}	0.6633±3.333^{a}	45.66±0.8090^{a}	57.46±1.933^{a}
SFDG-23	80.000±5.77^{ab}	25.233±0.548^{a}	6.76±0.352^{a}	0.5667±0.012^{b}	44.40±0.4359^{a}	45.40±3.023^{b}
SFMC-31	73.33± 8.819^{abc}	22.433±0.617^{b}	6.23± 0.352^{ab}	0.5000±0.040^{cd}	39.93±0.8762^{b}	36.00±1.80^{bc}
SFSI-21	63.33±8.819^{c}	22.233±0.913^{b}	4.96±0.448^{bc}	0.4100±0.011^{e}	32.13±0.5487^{d}	26.03± 3.841^{cd}
SFMC-23	70.000±000^{abc}	23.400±0.493^{b}	4.30±0.472^{cd}	0.4567± 0.0318^{de}	36.70±0.2517c	27.26±5.987^{cd}
SFME-21	60.00±5.773^{bc}	20.367±0.497^{c}	5.36±0.393^{bc}	0.5300± 0.011^{bc}	36.20±0.4726^{c}	31.73±2.828^{c}
N broth	56.667±6.66^{c}	15.200±0.60	4.00± 0.435^{d}	0.3300±5.7	25.20±0.5196e	18.76±2.520^{d}

		8[d]		74[f]		
GM	70	22.114	5.5476	0.4938	37.176	34.66
CV	15.59	4.67	13.04	7.34	2.77	17.01
LSD	19.108	1.8099	1.2669	0.0635	1.8018	10.323

Where, PSG is the percentage of seeds germinated, PRL is primary root length (cm), SL is shoot length (cm), SDW is seedling dry weight (g), NSR/P is the number of secondary roots/plant, GM is the grand mean, and VI is the vigor index.





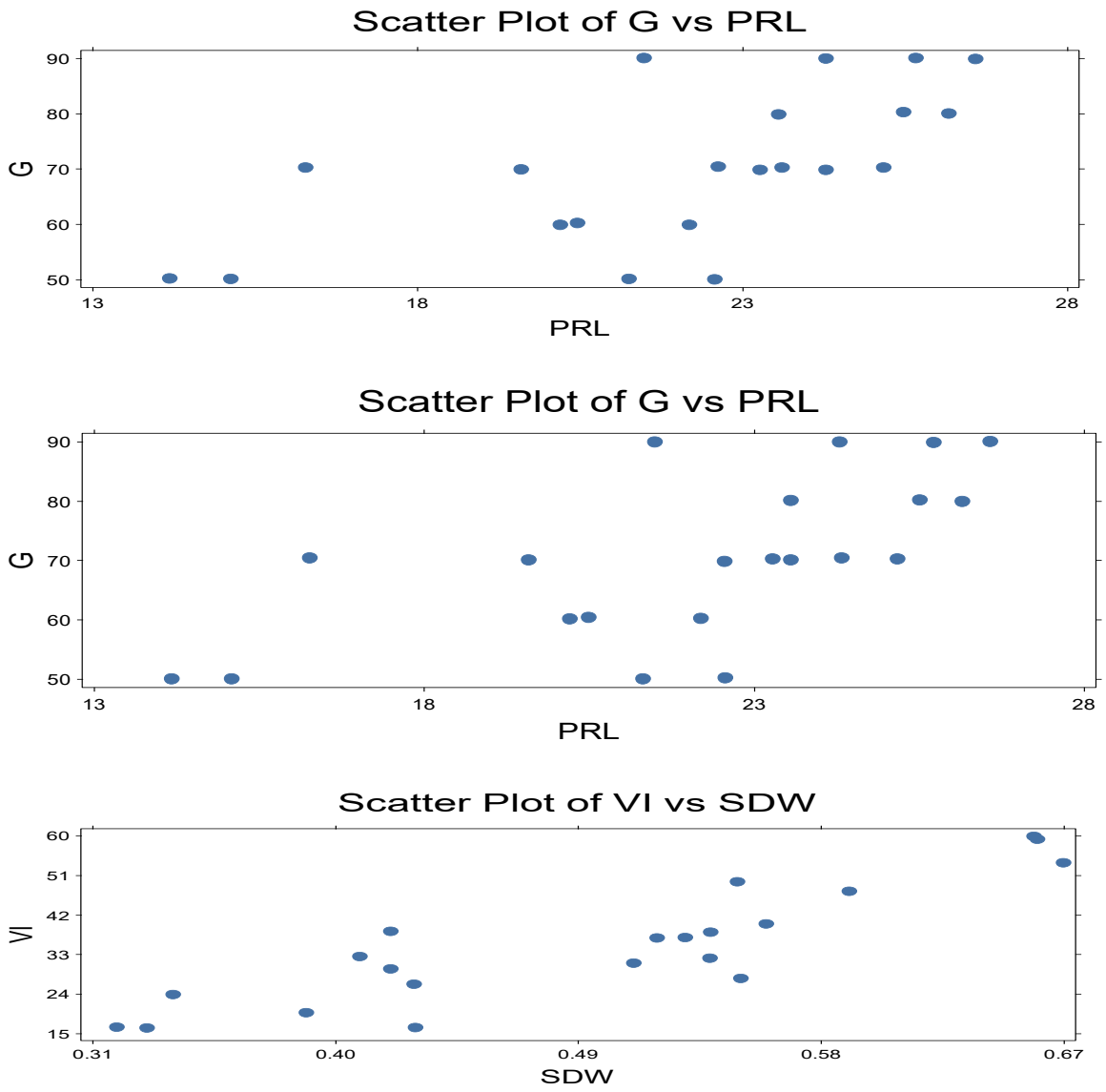


Figure 15: Presents a scattered plot of the marginal means of seed germination across the experimental treatments

Table 6: Pearson correlation coefficient comparisons for primary root length, seedling dry weight, secondary root number, shoots length, germination percentage, and vigor index

Correlations

	PRL		SDW	SRN	GP	VI
PRL	1	.607**	.769**	.892**	.644**	.743**

SL	.607**	1	.764**	.787**	.720**	.853**
SDW	.769**	.764**	1	.909**	.537*	.878**
SRN	.892**	.787**	.909**	1	.674**	.852**
GP	.644**	.720**	.537*	.674**	1	.809**
VI	.743**	.853**	.878**	.852**	.809**	1

∗∗Correlation is significant at the 0.01 level (2-tailed). ∗Correlation is significant at the 0.05 level (2-tailed). PRL-Primary root length, SL- SL SDW- seedling dry weight, SRN- Secondary root number, GP- Germanton percentage, VI- Vigor index.

4.1.4 Symbiotic Effectiveness of the *Rhizobial* Isolates in the greenhouse

After 45 days of growth, greenhouse evaluation of shoot height, nodule number, and dry matter was conducted (Table 7; Figure 17). The results revealed statistically significant differences ($p < 0.05$) among Rhizobia isolates in terms of nodule number, nodule dry weight, and shoot dry weight. Isolate SFDG-23 was the most effective, producing the highest number of nodules (41.6 per plant) and the greatest nodule dry weight (0.1667 g per plant). These values were markedly higher than both control treatments: the urea-treated control, which did not form nodules due to the absence of rhizobial inoculation, and the negative control, which also showed no nodulation at all. This confirms that nodulation in chickpea plants strictly depended on inoculation with effective Rhizobia isolates.

Among the inoculated treatments, SFMC-23 had the lowest nodule count (33 nodules per plant) and nodule dry weight (0.123 g per plant), though still significantly better than either control treatment. The complete absence of nodules in both controls further highlights the importance of symbiotic interaction introduced through inoculation.

Shoot dry weight (SDW) varied significantly among treatments. The highest SDW (0.6667 g per plant) was recorded in plants inoculated with SFDG-23, which was 21.9% higher than the urea-treated control (0.5467 g) and 190% greater than the negative control (0.23 g). Even the lowest-performing inoculated isolate, SFME-21, with 0.4067 g SDW, still performed 74.6% better than the negative control, though it was 25.6% lower than the urea control.

These results demonstrate that all Rhizobium inoculated treatments improved shoot dry matter accumulation compared to the controls, indicating the benefit of biological nitrogen fixation. Importantly, some isolates like SFDG-23 and SFMC-31 even surpassed the urea control, showing that they could potentially replace chemical nitrogen fertilizers.

Symbiotic effectiveness (SE), calculated based on shoot dry weight relative to the urea control, ranged from 74.3% to 121.9%. Isolates SFDG-23 (121.9%), SFMC-31 (109.7%), and SFMC-23 (103.6%) were classified as highly effective, meaning they stimulated shoot growth beyond that achieved by nitrogen fertilization. In contrast, SFSI-21 (76.8%) and SFME-21 (74.39%) were effective, performing better than the negative control but lower than the urea control.

These results clearly highlight that certain native Rhizobia isolates, especially SFDG-23, possess strong potential as bio-inoculants for enhancing chickpea growth and biological nitrogen fixation in place of chemical fertilizers.

Additionally, Pearson correlation analysis (Table 8) revealed significant positive associations between key traits. Shoot dry weight per plant was strongly correlated with nodule number ($r = 0.533$, $p < 0.01$), nodule dry weight ($r = 0.531$, $p < 0.01$), root length ($r = 0.769$, $p < 0.01$), root dry weight ($r = 0.826$, $p < 0.01$), and symbiotic effectiveness ($r = 0.794$, $p < 0.01$). This suggests that higher shoot biomass is consistently associated with enhanced root development and nodulation performance.

Interestingly, shoot dry weight showed no significant correlation with shoot length ($r = 0.072$, $p = 0.737$), indicating that biomass accumulation is more dependent on root and symbiotic function than on plant height. Similarly, nodule number was highly correlated with nodule dry weight ($r = 0.976$), and both were positively associated with root traits and SE. Root parameters were strongly interrelated, with root length and root dry weight showing a very strong correlation ($r = 0.885$, $p < 0.01$), and both traits also contributing significantly to symbiotic effectiveness ($r = 0.624$ and $r = 0.674$, respectively). Shoot length showed a modest but significant correlation with SE ($r = 0.428$, $p < 0.05$), while its correlation with other parameters was not significant.

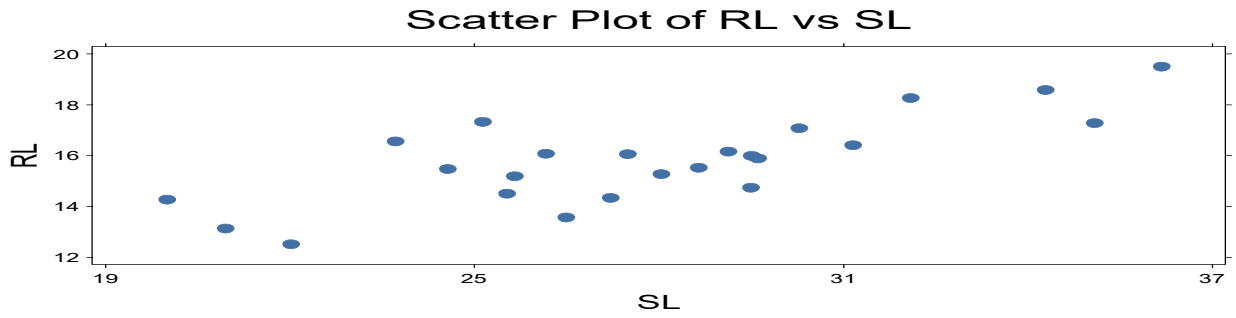
Figure 16: Effect of *Rhizobia* on growth of chickpea by pot assay

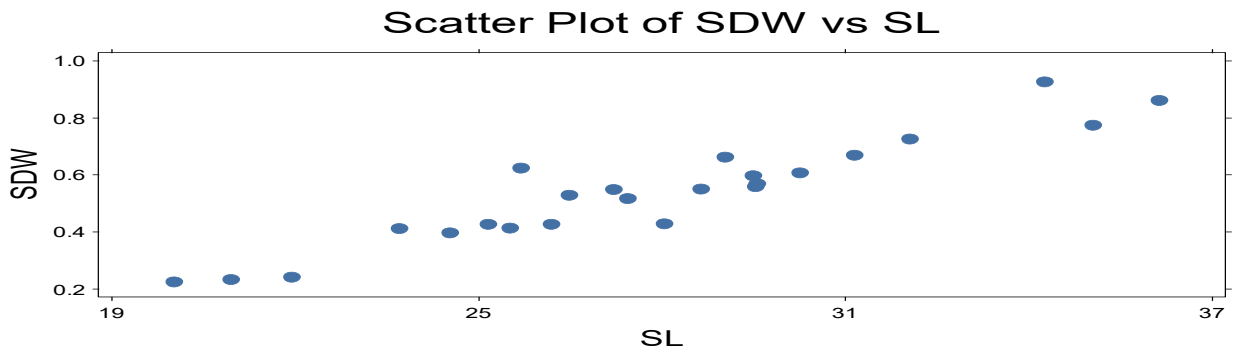
Table 7: Effect of Rhizobia isolates on growth parameters of chickpea on potted sterilized sand under greenhouse conditions 45 days after sowing

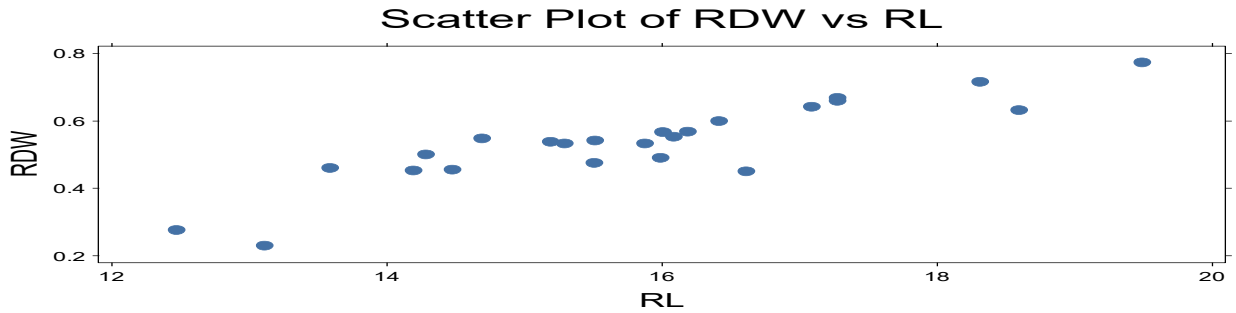
Treatments	NN/p	NDW/p	SL/P	SDW/p	RL/P	RDW/P	SE(%)	SE status
UREA	0.00±00^{dc}	0.00±00^{d}	28.86±0.6839^{c}	0.5467±0.0145^{c}	15.53±0.4177^{cd}	0.523±0.0176^{c}	100	-
SRCP17	42.53±0.4631^{a}	0.173±8.819^{a}	35.20±0.5508^{a}	0.8533±0.0463^{a}	18.46± 0.6386^{a}	0.690± 0.0416^{a}	156.08	HE
SFDG-23	41.63±0.811^{a}	0.166±0.0145^{ab}	31.20±0.5196^{b}	0.6667±0.0318^{b}	17.26±0.5548^{ab}	0.650±0.0321^{ab}	121.9	HE
SFMC-31	41.40±0.9018^{a}	0.160±5.77^{ab}	29.10±0.2309^{c}	0.6000±0.0321^{bc}	15.90±0.208^{bc}	0.560±1.00^{bc}	109.7	HE
SFSI-21	36.00±0.9165^{b}	0.133±0.0219^{b}	26.60±0.7767^{d}	0.4200±5.77^{d}	15.30±0.4619^{cd}	0.510±0.0306^{c}	76.8	E
SFMC-23	33.36±0.5608^{c}	0.123±8.819^{c}	26.46±0.4333^{d}	0.5667± 0.0273^{c}	14.36±0.4631^{de}	0.500±0.0231^{c}	103.6	HE
SFME-21	34.00±0.9292^{bc}	0.140±0.0173^{abc}	24.50± 0.4359^{e}	0.4067±8.819^{d}	16.46± 0.5239^{bc}	0.526±0.0669^{bc}	74.39	E
dH2O	0.00±00^{dc}	0.00±00^{d}	21.00±0.5774^{f}	0.2300±5.77^{e}	13.26±0.497^{e}	0.316±0.0677^{d}	42	-
Grand mean	28.617	0.1121	27.867	0.5363	15.821	0.5346	96.7	-
CV(%)	4.12	18.75	3.41	8.30	5.31	13.41	18.3	-
LSD(*P*< 0.05)	2.039	0.034	1.6451	0.0771	1.4546	0.1241	2.12	-

Mean values in columns and rows designated with similar letters are not significant each other while designed with different letters significantly different each other at p<0.05. LSD (5%) = Least significant difference at p<0.05 and CV (%) = percentage of coefficient of variation. Where SL/p = shoot length per plant, NN/p = nodule number per plant, NDW/p = nodule dry

weight per plant, SDW/p = shoot dry weight per plant, LSD = least significant difference, RE = relative effectiveness, E = effective, LE = lowly effective, HE = highly effective, HE > 80%, E 50–80%, LE 35–50%, IE < 35%, the same letters are not significantly different at the $P < 0.05$ level, and all the data are the means of triplicates. The unit for measuring SL/p is centimeters, and the other NDW/p and SDW/p are in grams.







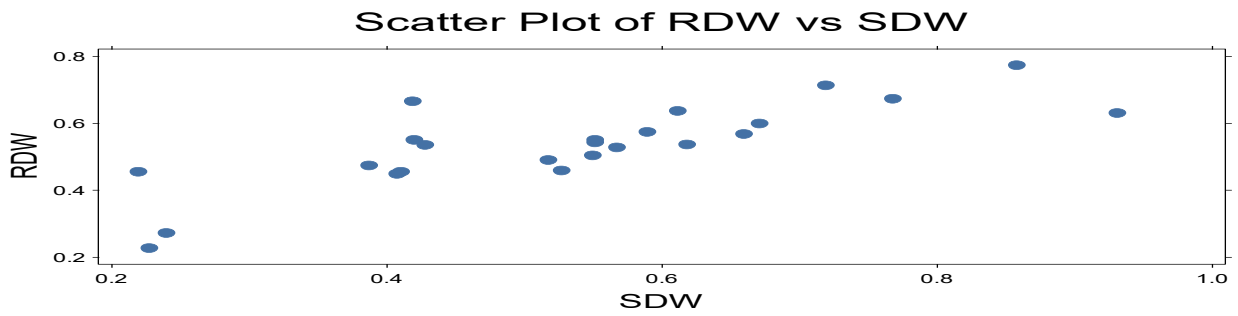


Figure 17: Marginal mean of symbiotic effectiveness of pot experiment scattered plot

Table 8: Pearson correlation coefficient comparisons for nodule number, nodule dry weight, shoot dry weight, shoots length, root length per plants, root dry weight per plants and symbiotic effectiveness.

Correlations

	SDW	NN/P	NDW/P	SL/P	RL/P	RDW/P	SE
SDW	1	.533**	.531**	.072	.769**	.826**	.794**
NN/P	.533**	1	.976**	.174	.580**	.629**	.554**
NDW/P	.531**	.976**	1	.151	.618**	.648**	.528**
SL/P	.072	.174	.151	1	.055	.070	.428*
RL/P	.769**	.580**	.618**	.055	1	.885**	.624**
RDW/P	.826**	.629**	.648**	.070	.885**	1	.674**
SE	.794**	.554**	.528**	.428*	.624**	.674**	1

∗∗Correlation is significant at the 0.01 level (2-tailed). ∗Correlation is significant at the 0.05 level (2-tailed). SDW- Shoot dry weight per plant, NN/P- Number of nodules per plant, NDW/P- Nodule dry weight per plant, SL/P- Shoot length per plant, RL/P- Root length per plant, RDW/P- Root dry weight per plant, SE- Symbiotic effectiveness.

4.2 Discussion

In this study, the bacterial isolates were obtained from chickpea root nodules to isolate and identify rhizobia, following protocols consistent with those of Menelih *et al.* (2022), who also utilized Yeast Extract Mannitol Agar (YEMA) and confirmatory media such as Congo Red (YEMA-CR) and Bromothymol Blue (YEMA-BTB). All (100%) isolates that did not absorb

Congo red dye and formed creamy colonies after three days at 28°C were presumed to be authentic *rhizobia*, aligning with the criteria established by Hamza *et al*. (2017). The yellow color shift observed in YEMA-BTB medium indicated acid production, classifying all selected isolates as fast-growing *rhizobia*, in agreement with the findings of (Gebremedhin, 2016; Nagalingam *et al 2020;*Al-Shakarchi, (2023). Microscopic analysis revealed that all isolates were gram-negative, rod-shaped bacteria, while colony morphology was transparent to white, mucoid, circular with entire margins matching typical *Rhizobia* characteristics as described by Nagalingam *et al*. (2020). A rigorous purification process involving three successive streaking ensured the isolation of pure cultures.

The biochemical characterization of *Rhizobia* isolates revealed a consistent profile of enzymatic and metabolic capabilities, reflecting both conserved traits and isolate-specific adaptations. All isolates exhibited motility, as evidenced by diffuse growth in SIM medium. This finding aligns with those of Asrat *et al*. (2023), confirming active motility in *rhizobial* populations from legume nodules. Our observation of 100% motility surpasses the 85% motility reported by Al-Shakarchi (2023), possibly due to strain-specific factors or regional soil conditions influencing flagellar gene expression. The association of motility with symbiotic efficiency, as suggested by De *et al*. (2022), underscores the potential of these isolates in bio-fertilizer development.

Carbohydrate fermentation profiles, assessed via the TSI test, revealed that most isolates primarily fermented glucose, consistent with Rhizobium strain behavior (Mir *et al*., 2020;Wanore *et al*., 2023). The uniform absence of H_2S production across isolates matches findings by Verma *et al*. (2022), reaffirming that this trait is rare among agriculturally relevant rhizobia. Starch hydrolysis was variably expressed, with some isolates (SFME-21, SFMC-31, and SFDG-23) producing large clearing zones indicative of high amylase activity. In contrast, isolates such as SFSI-32, SFME-12, and SFME-32 formed smaller and less distinct clear zones, indicating comparatively lower amylase production or activity. This variability is in line with (Wanore *et al*., 2023a) reflects strain-specific enzymatic capabilities. Differences from the more uniform activity reported by Mir *et al*. (2020) highlight the influence of ecological origin on enzyme expression. The quantitative and qualitative hydrolysis results are summarized in Table 4 and visually represented in Figure 7. All isolates showed positive citrate utilization, demonstrated by the color changes on Simmons' citrate agar. This confirms a widespread

capacity for organic acid metabolism, supporting findings by Makkar & Jangra. (2017). Contrasts with Hossain *et al*. (2019), who observed partial citrate utilization in acidic soils, suggest environmental modulation of metabolic gene expression.

Universal urease positivity among isolates supports the role of Rhizobium in nitrogen cycling, as described by Mir *et al.* (2020) and Pawar *et al*. (2014). This trait's consistency suggests adaptation to rhizosphere environments with high nitrogen turnover. All isolates tested negative for gelatinase, indicating a lack of extracellular protease production. This is consistent with Mir *et al*. (2020) but contrasts with the positive gelatinase activity reported by Aung and Oo. (2020), possibly due to differences in protein availability or ecological pressures in marine vs. terrestrial environments. Oxidase activity varied across isolates but was universally present, suggesting aerobic metabolism supported by cytochrome c oxidase. These results corroborate Hossain *et al*. (2019) and reinforce oxidase testing as a useful indicator of respiratory potential in rhizobia. Furthermore, oxidase-positive reactions suggest that the tested isolates possess active aerobic respiratory pathways, which may influence their ecological behavior, symbiotic performance, and potential suitability for bioinoculant development.

The complete absence of indole production in all isolates implies a lack of tryptophanase activity. This result aligns with Shihab and Alkurtany (2023) but differs from Hossain *et al*. (2019), who observed weak indole production in some strains. The lack of indole biosynthesis may limit auxin production via the tryptophan-dependent pathway under laboratory conditions.

The catalase activity observed in all *Rhizobia* isolates confirms their ability to detoxify hydrogen peroxide, a reactive oxygen species commonly encountered in soil environments and during symbiotic interactions. The vigorous bubbling, particularly in isolates SFDG-23, SFMC-32, and SFMC-23, indicates elevated catalase expression, suggesting enhanced oxidative stress tolerance. These findings corroborate those of Pervin *et al*. (2017), who linked high catalase activity in Rhizobium spp. to environmental resilience and symbiotic efficiency. Conversely, the lower catalase activity reported by Pawar *et al*. (2014) in saline and arid regions highlights the influence of ecological factors on gene regulation. The strong activity in our isolates may reflect adaptation to microenvironments with high organic matter turnover. Moreover, Al-Shakarchi (2023) reported a positive correlation between catalase expression and nitrogen fixation

efficiency, supporting the potential of catalase as a functional biomarker for selecting elite bio-inoculant strains.

Ammonia production was uniformly positive across all 100% isolates, as evidenced by the characteristic color change in Nessler's reagent. This result is consistent with previous studies (Manasa *et al*., 2017a; Richard *et al*., 2018), which link ammonia production to plant growth promotion, especially under nutrient-limited conditions. Notably, the consistently strong color development suggests robust metabolic activity across all isolates, warranting further quantitative analysis for differentiation. Additionally, all (100%)isolates formed visible pellicles in nitrogen-free medium, a hallmark of nitrogenase activity. This aligns with reports by Rodrigues *et al*. (2016) and Purwaningsih *et al*. (2024), emphasizing pellicle formation as an indicator of nitrogen-fixing potential. The uniform response may reflect optimal assay conditions that favor nitrogenase expression. Collectively, the catalase activity, ammonia production, and nitrogen fixation capabilities of these isolates underscore their promise as multifunctional plant growth-promoting rhizobacteria for sustainable agriculture. Overall, the biochemical profiles presented here affirm both core metabolic competencies and ecological adaptations of the tested rhizobial isolates, supporting their potential for use in targeted bio-fertilization strategies.

The present study demonstrated that *Rhizobia* inoculation significantly enhanced seed germination and early seedling development in treated seeds compared to uninoculated controls. Germination was initiated within 24 hours, with maximum emergence observed by 72 hours. Inoculated seeds exhibited both higher germination percentages and more uniform emergence, confirming earlier reports on the beneficial role of *rhizobial* isolates in promoting early plant growth (Pawar et *al.*, 2014; Khaitov *et al*., 2016). These improvements are not solely attributed to nitrogen fixation but are also linked to a suite of biological mechanisms through which *Rhizobium spp*. enhance plant growth. *Rhizobial* isolates are known to synthesize and secrete various phytohormones, including indole-3-acetic acid (IAA), cytokinin, and gibberellins, which modulate root architecture and stimulate cell division and elongation (Spaepen and Vanderleyden, 2011; Egamberdieva et al., 2017). The observed increase in PRL and NSR/P in inoculated seeds, particularly with strain SFDG-23, may be a direct outcome of such hormonal stimulation, which enhances water and nutrient absorption in the early growth stages.

Significant improvements ($p < 0.05$) in primary root length (PRL), number of secondary roots per plant (NSR/P), and seedling dry weight (SDW) were observed in inoculated treatments, particularly with strain SFDG-23. The resulting increase in seedling vigour index (VI) highlights *Rhizobium*'s potential in boosting early-stage biomass accumulation. This supports findings by Soboka *et al*. (2024), who emphasized strain-specific variability in plant-growth promotion due to mechanisms such as nitrogen fixation and phytohormone production. Although SFDG-23 showed superior performance across most parameters. These results align with Xu *et al*. (2022), who emphasized that differences in plant response may depend on specific host-microbe interactions and environmental compatibility. Such strain-dependent variations suggest the need for targeted bio-inoculant selection based on crop and site conditions.

Strong positive correlations among seedling traits further validate the importance of integrated early development. The high correlation between NSR/P and SDW ($r = 0.909$) echoes findings by Ali *et al*. (2024), who demonstrated that enhanced root branching improves nutrient uptake and biomass accumulation. Additionally, the correlation between germination percentage and VI ($r = 0.809$) reinforces the idea that rapid, uniform germination is critical for competitive seedling establishment, as observed by (Walia *et al*., 2020).

Moreover, multivariate trait relationships emphasize the importance of holistic selection strategies in breeding. Studies like Kumar *et al*. (2023) have effectively applied such methods in legume improvement programs. Deeper and faster-growing roots, as highlighted by Meza *et al*. (2022), further contribute to early vigor by improving access to water and nutrients key for resilience under field conditions. The data suggest that *Rhizobium* inoculation, particularly with high-performing strains like SFDG-23, holds promise not only for enhancing nodulation and nitrogen fixation in later stages but also for promoting early vigor, an essential trait for successful crop establishment.

The greenhouse pot experiment conducted at Woldia University Botanical Garden provides compelling evidence for the efficacy of native *Rhizobium* isolates in enhancing chickpea growth and symbiotic nitrogen fixation. Inoculated treatments, particularly those involving strains SFDG-23 and SFMC-31, showed significantly higher shoot dry weight, nodule number, and nodule biomass compared to un inoculated and nitrogen-fertilized controls. These results are in

line with previous findings demonstrating that well-adapted native *rhizobial* isolates can outperform commercial inoculants and synthetic nitrogen sources due to their ecological compatibility and host-specific effectiveness (Zahran, 2001; Wubie and Adal, 2021).

Notably, SFDG-23 achieved the highest shoot dry weight (0.6667 g) and nodule dry weight (0.1667 g), resulting in a symbiotic effectiveness (SE) of 121.9%, which exceeded that of the nitrogen-fertilized control. According to Somasegaran and Hoben (1985), SE values exceeding 100% are indicative of highly efficient biological nitrogen fixation. This reinforces the sisolates promise as a viable alternative to chemical nitrogen fertilizers. The complete absence of nodulation in the sterile water control confirms the dependence of nodulation and nitrogen fixation on effective *rhizobial* inoculation, as also noted by Tena *et al*. (2016).

The Symbiotic effectiveness (SE) among the isolates ranged from 74.3% to 121.9%. Isolates SFDG-23 (121.9%), SFMC-31 (109.7%), and SFMC-23 (103.6%) were classified as highly effective, while SFSI-21 (76.8%) and SFME-21 (74.39%) were categorized as effective. Among isolates suggests that rhizobial strain origin and host-strain computability play crucial roles in determining symbiotic performance, as indicated in Table 7. Isolates SFDG-23 and SFMC-31, sourced from Doro Gibir and Mechare, respectively, were classified as highly effective. This supports the argument by Howieson & Dilworth (2016) for the screening and utilization of native *rhizobial* isolates specifically adapted to native agro-ecological conditions. Their promising performance in enhancing both nodulation and biomass production makes them strong candidates for development into region-specific bio-inoculants for sustainable chickpea production in Ethiopia.

Correlation analysis highlighted strong positive relationships between shoot dry weight and root dry weight ($r = 0.826$), root length ($r = 0.769$), and symbiotic effectiveness ($r = 0.794$), indicating that effective symbiosis promotes balanced shoot-root development. These findings are consistent with those of (Kumar *et al*., 2023), who reported that improved root systems resulting from rhizobial inoculation enhance nutrient acquisition and plant growth. Furthermore, the strong correlation between nodule number and nodule dry weight ($r = 0.976$) suggests that nodule count may serve as a reliable phenotypic marker for assessing inoculant effectiveness, echoing observations by da Silva *et al.* (2024).

Interestingly, shoot length demonstrated only a moderate correlation with symbiotic traits (r = 0.428), indicating that above-ground elongation may be influenced by other physiological or hormonal factors beyond nitrogen fixation, such as the plant's endogenous growth regulator profile (Rathjen *et al*., 2024). Nevertheless, the strong correlation between symbiotic effectiveness and shoot dry weight (r = 0.794) reinforces the role of SE as a key integrative metric of rhizobial performance (Wubie & Adal, 2021).

From a sustainable agriculture perspective, these findings have important implications. First, the use of native, highly effective Rhizobium strains such as SFDG-23 and SFMC-31 as bio-inoculants can reduce dependence on costly and environmentally harmful chemical fertilizers. By enhancing biological nitrogen fixation, these bio-inoculants promote soil health and reduce nutrient runoff, thereby contributing to ecological sustainability(Zaidi *et al*., 2017;Maitra *et al*., 2021) The promotion of native inoculants tailored to local conditions supports low-input, cost-effective chickpea cultivation, a critical factor for smallholder farmers in resource-limited settings. Such innovations can directly contribute to improving yield stability, crop productivity, and food security in Ethiopia and other legume-dependent regions. The superior performance of selected native *rhizobia* strains, especially SFDG-23 and SFMC-31, highlights their potential as bio-fertilizers in sustainable chickpea production. These findings support the integration of native microbial resources into soil fertility management strategies, offering an eco-friendly alternative to chemical fertilizers while addressing regional challenges in legume productivity and food security.

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study successfully isolate rhizobial isolates from chickpea root nodules collected across major chickpea-growing areas around Woldia. Through a series of rigorous presumptive tests, morphological observations, cultural characteristics, and detailed biochemical characterizations including motility, carbohydrate utilization, enzyme activities, and other key biochemical assays authentic rhizobial isolates were identified. These isolates exhibited diverse phenotypic traits consistent with efficient chickpea rhizobia.

Greenhouse authentication trials further validated the symbiotic effectiveness of these indigenous rhizobial isolates, demonstrating significant enhancements in seed germination, nodulation, plant height, and overall biomass accumulation compared to un inoculated controls. These results confirm the capability of native rhizobia to form effective symbioses with chickpea, thereby promoting plant growth and productivity under controlled conditions.

Collectively, the findings highlight the potential of these locally adapted rhizobial isolates as eco-friendly bio-inoculants that can contribute to sustainable chickpea production systems in Woldia and similar agro-ecological zones. The application of these native rhizobia can reduce reliance on synthetic nitrogen fertilizers, improve soil fertility, and enhance crop yield, aligning with sustainable agricultural practices. This study thus provides a comprehensive foundation for the development and use of indigenous rhizobial inoculants tailored to the local environment, supporting efforts toward improved chickpea productivity and environmental sustainability in the region.

5.2 Recommendation

Based on the findings of this study, it is recommended that the most promising native *rhizobial* isolates particularly SFDG-23 and SFMC-31 undergo multi-location field trials to validate their effectiveness under diverse agro-ecological conditions. To support taxonomic clarity and facilitate standardized inoculant development, molecular identification using techniques such as 16S rRNA gene sequencing and BOX-PCR should be conducted.

In addition, the isolates should be assessed for abiotic stress tolerance (e.g., salinity, drought, pH, and temperature extremes) to identify isolates capable of thriving in marginal environments. Further physiological and biochemical analyses, including nitrogen fixation efficiency, phosphate solubilization, siderophore production, and IAA synthesis, are essential to fully characterize their plant growth-promoting traits.

The development of stable, carrier-based formulations with good shelf life should also be prioritized to support practical application and commercialization. Finally, integrating these native isolates into sustainable agricultural systems can significantly reduce reliance on synthetic nitrogen fertilizers, improve soil health, and enhance chickpea productivity in Woldia and similar regions.

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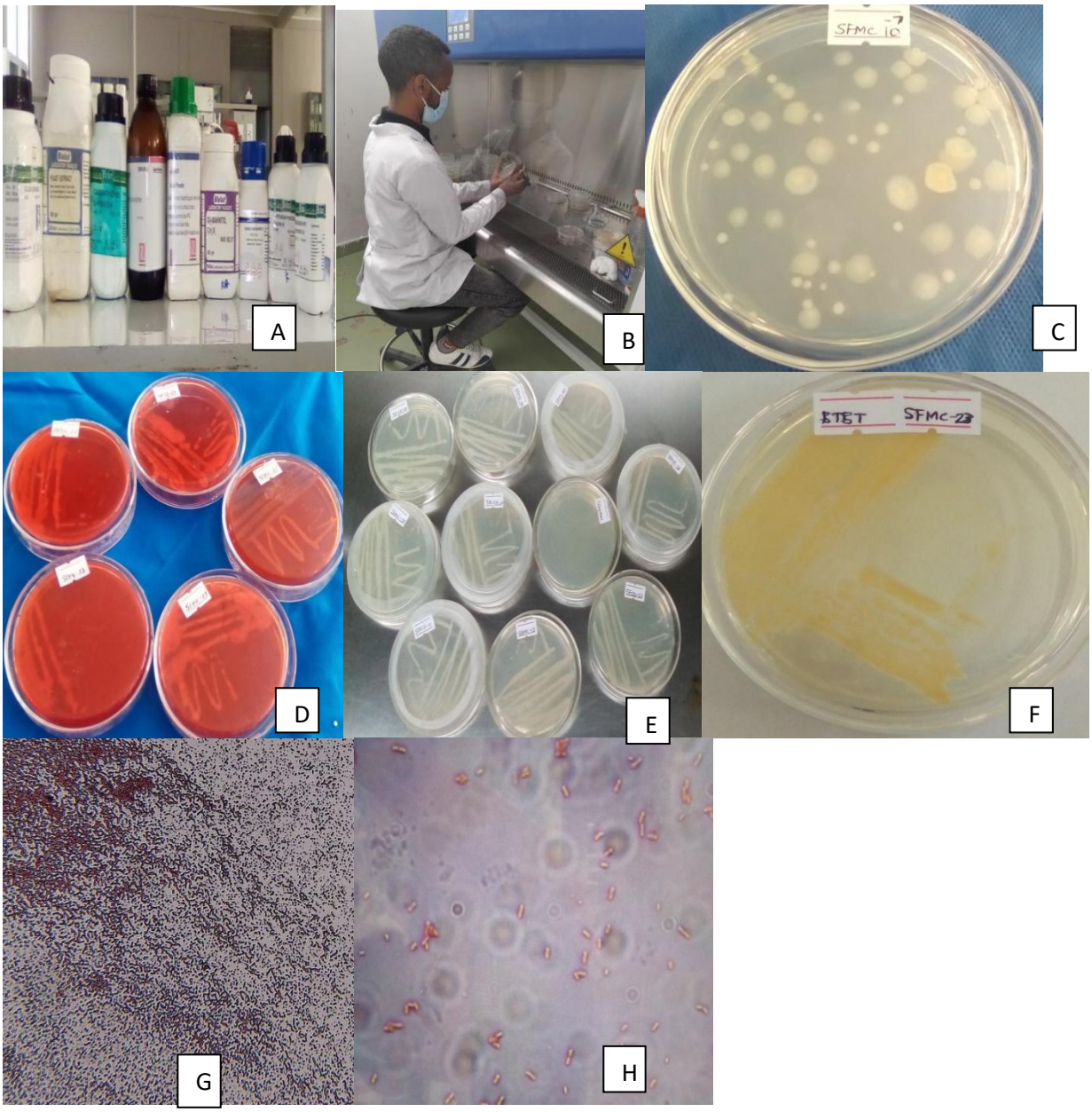
7. LIST OF APPENDICES

Appendix1: Colonies and cultures of rhizobia on YEMA medium



Where; A. Reagents of isolation; B. Aseptic techniques of culturing;C. Rhizobium colonies observed on YEMA; D. screening of cultures on CR-YEMA E. subculture; F. Bromothymoleblue-yeast extract mannitol agar test; G. Gram stain; H. Cell morphology in microscope (100×).

Appendix2: Results of biochemical characterization of rhizobial isolates

Where: A. catalyst test; B. Citrate utilization test; C. Gelatin test; D. indole test; E. Triple sugar agar test; F. Motility test; G. Nitrogen fixing test; H. Ammonia production test; l. oxidase test; J. Starch hydrolysis test; K. urea test.

Appendix 3: Analysis of variance for seed germination

ANOVA						
		Sum of Squares	Df	Mean Square	F	Sig.
Prmary root length	Between Groups	230.832	6	38.472	36.019	.000
	Within Groups	14.953	14	1.068		
	Total	245.786	20			
Shoot length	Between Groups	27.026	6	4.504	8.607	.000
	Within Groups	7.327	14	.523		
	Total	34.352	20			
Seedlng dry weght	Between Groups	.212	6	.035	26.871	.000
	Within Groups	.018	14	.001		
	Total	.230	20			
Secondary root number	Between Groups	905.738	6	150.956	142.604	.000
	Within Groups	14.820	14	1.059		
	Total	920.558	20			
Germnaton percentage	Between Groups	2133.333	6	355.556	2.987	.043
	Within Groups	1666.667	14	119.048		

	Total	3800.000	20			
Vigour index	Between Groups	3082.741	6	513.790	14.785	.000
	Within Groups	486.519	14	34.751		
	Total	3569.260	20			

Appendix4: Analysis of variance for Symbiotic Effectiveness of Rhizobia under Greenhouse Conditions

ANOVA						
		Sum of Squares	Df	Mean Square	F	Sig.
Shoot length/plant	Between Groups	388.380	7	55.483	61.420	.001
	Within Groups	14.453	16	.903		
	Total	402.833	23			
Number of nodules per plants	Between Groups	6811.213	7	973.030	700.652	.001
	Within Groups	22.220	16	1.389		
	Total	6833.433	23			
Nodule dry weight per plants	Between Groups	.107	7	.015	34.457	.001
	Within Groups	.007	16	.000		
	Total	.114	23			

Shoot dry weight per plants	Between Groups	.740	7	.106	53.318	.001
	Within Groups	.032	16	.002		
	Total	.772	23			
Root length per plants	Between Groups	55.520	7	7.931	11.230	.001
	Within Groups	11.300	16	.706		
	Total	66.820	23			
Root dry weight per plants	Between Groups	.263	7	.038	7.307	.001
	Within Groups	.082	16	.005		
	Total	.345	23			

Appendix5: Pearson correlation coeficient comparisons for Prmary root length, Seedlng dry weght, Secondary root number, shoots length, Germnaton percentage, and Vigour index

Correlations							
		Prmary root length	Shoot length	Seedlng dry weght	Secondary root number	Germnaton percentage	Vigour index
Prmary root length	Pearson Correlation	1	.607**	.769**	.892**	.644**	.743**
	Sig. (2-tailed)		.004	.000	.000	.002	.000
	N	21	21	21	21	21	21
Shoot length	Pearson Correlation	.607**	1	.764**	.787**	.720**	.853**
	Sig. (2-tailed)	.004		.000	.000	.000	.000
	N	21	21	21	21	21	21
Seedlng dry weght	Pearson Correlation	.769**	.764**	1	.909**	.537*	.878**
	Sig. (2-tailed)	.000	.000		.000	.012	.000
	N	21	21	21	21	21	21
Secondary root number	Pearson Correlation	.892**	.787**	.909**	1	.674**	.852**
	Sig. (2-tailed)	.000	.000	.000		.001	.000
	N	21	21	21	21	21	21
Germnaton percentage	Pearson Correlation	.644**	.720**	.537*	.674**	1	.809**
	Sig. (2-tailed)	.002	.000	.012	.001		.000
	N	21	21	21	21	21	21
Vigour index	Pearson Correlation	.743**	.853**	.878**	.852**	.809**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	
	N	21	21	21	21	21	21
**. Correlation is significant at the 0.01 level (2-tailed).							
*. Correlation is significant at the 0.05 level (2-tailed).							

Appendix 6: Pearson correlation coefficient comparisons for nodule number, nodule dry weight, shoot dry weight, shoots length, Root length per plants, Root dry weight per plants and symbiotic effectiveness

Correlations								
		Shoot dry weight per plants	Number of nodules per plants	Nodule dry weight per plants	Shoot length per plant	Root length per plants	Root dry weight per plants	Symbotc effectiveness
Shoot dry weight per	Pearson Correlation	1	.533**	.531**	.072	.769**	.826**	.794**

plants	Sig. (2-tailed)		.007	.008	.737	.000	.000	.000
	N	24	24	24	24	24	24	24
Number of nodules per plants	Pearson Correlation	.533**	1	.976**	.174	.580**	.629**	.554**
	Sig. (2-tailed)	.007		.000	.417	.003	.001	.005
	N	24	24	24	24	24	24	24
Nodule dry weight per plants	Pearson Correlation	.531**	.976**	1	.151	.618**	.648**	.528**
	Sig. (2-tailed)	.008	.000		.482	.001	.001	.008
	N	24	24	24	24	24	24	24
Shoot length per plant	Pearson Correlation	.072	.174	.151	1	.055	.070	.428*
	Sig. (2-tailed)	.737	.417	.482		.798	.746	.037
	N	24	24	24	24	24	24	24
Root length per plants	Pearson Correlation	.769**	.580**	.618**	.055	1	.885**	.624**
	Sig. (2-tailed)	.000	.003	.001	.798		.000	.001
	N	24	24	24	24	24	24	24
Root dry weight per plants	Pearson Correlation	.826**	.629**	.648**	.070	.885**	1	.674**
	Sig. (2-tailed)	.000	.001	.001	.746	.000		.000
	N	24	24	24	24	24	24	24
Symbotceffect veness	Pearson Correlation	.794**	.554**	.528**	.428*	.624**	.674**	1
	Sig. (2-tailed)	.000	.005	.008	.037	.001	.000	
	N	24	24	24	24	24	24	24

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).