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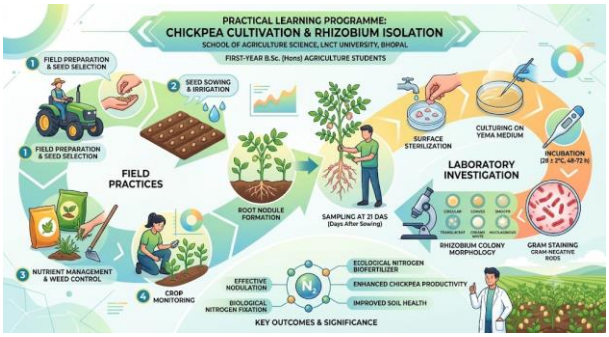
Isolation and Preservation of Native Rhizobium from Root Nodules of Chickpea (*Cicer arietinum* L.)

Aftarika Azmi Ahmed, Ankit Singh, and Mr Almas Ali

Abstract

This study outlines a practical learning program for first-year B.Sc. (Hons) Agriculture students at LNCT University, Bhopal, focusing on chickpea cultivation and *Rhizobium* isolation. Chickpea crops were managed from sowing to nodule formation, and active pink root nodules were harvested at 21 days after sowing (DAS). The nodules were surface-sterilized and cultured on Yeast Extract Mannitol Agar (YEMA). Isolated colonies were circular, convex, smooth, translucent, creamy white, mucilaginous, and confirmed as Gram-negative rods. This integrated field-to-laboratory approach effectively provided students with hands-on experience in sustainable agriculture and biofertilizer technology.

Infographic abstract



Keywords: Chickpea, Rhizobium, Biological nitrogen fixation, Root nodules, Yeast Extract Mannitol Agar (YEMA), Biofertilizer.

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Introduction

Chickpea (*Cicer arietinum* L.) is globally the third most important legume crop Wolde. Approximately 64% of the total chickpea production is from India, covering approximately 11.9 to 13.5 million tonnes annually, accounting for around 75% of global production**.** India's production has been increasing due to expanded acreage and favorable agricultural conditions. FAO. (2024). that play a key role in human diet, sustainable and eco-friendly agriculture (Yadav et al, 2023). Among vegetarian people, protein energy malnutrition is greatly reduced by this legume as it contains abundant carbohydrates, proteins and minerals. In addition to its not only play significant role in human diet even, chickpea can fix atmospheric nitrogen in symbiosis with rhizobia called "symbiotic nitrogen fixation" that improves fixed nitrogen content in environment is another attractive characteristic that separates it when compared to the cereal crops. This fixation by nodules in the form that is directly assimilated i.e., ammonia at an approximate rate of 140 kg/ha/year thereby improve fertility of soil and cereal crops productivity during crop rotation systems (Hungria et al, 2022).

Rhizobia having their ability to convert nitrogen into ammonia, and it can be used by the plants, also belong to PGPR. PGP bacteria colonize roots efficiently, enhance plants growth and yield.

Isolation and characterization of native *Rhizobium* strains are fundamental procedures in agricultural microbiology because the efficiency of biological nitrogen fixation depends on the compatibility between bacterial strains, host genotype, and environmental conditions (Giller, 2021). Isolation from healthy root nodules and using Agar medium for enables researchers to obtain pure cultures such as morphological, physiological, and biochemical characterization. Such studies facilitate the identification of efficient nitrogen-fixing strains that can be developed as biofertilizer for sustainable chickpea production. (Andrews et al. 2020).

The present investigation was isolate and characterization of *Rhizobium* from root nodules of chickpea, cultivated by first-year B.Sc. (Hons.) Agriculture students at the School of Agriculture Science, LNCT University, Bhopal. The study integrated field activities—from sowing, crop

management, and harvesting—with laboratory isolation, purification, and characterization of *Rhizobium*. This field-to-laboratory approach provided practical exposure to crop production, agricultural microbiology, and the role of beneficial microorganisms in sustainable agriculture while demonstrating the significance of biological nitrogen fixation in improving chickpea productivity and maintaining soil fertility.

Materials and Methods

Study Location

The present study was conducted during the Rabi session (2025–2026) at the experimental farm and Microbiology Laboratory of the School of Agriculture (SOAG), LNCT University, Bhopal, Madhya Pradesh, India. The experiment was designed to provide first-year B.Sc. (Hons.) Agriculture students with practical exposure to chickpea cultivation and the isolation and characterization of *Rhizobium* from chickpea root nodules.

Sowing and Weather Conditions

Chickpea (*Cicer arietinum* L.) was sown on 31 October 2025 at 1:30 PM under favorable environmental conditions. At the time of sowing, the prevailing weather conditions were as follows:

Season	Rabi (2025–2026)
Crop	Chickpea (*Cicer arietinum* L.)
Date of Sowing	31 October 2025
Air Temperature	25–28°C
Relative Humidity	44%
Wind Speed	10 km h^{-1}
Probability of Rainfall	55%

The crop was cultivated following the recommended agronomic practices, including land preparation, seed sowing, irrigation, weed management, plant protection measures, and regular field observations until root nodule formation. (Bhattacharjee et al. 2021).

Collection of Chickpea Plants

The chickpea crop (*Cicer arietinum* L.) was sown on 31 October 2025 at the experimental farm of the School of Agriculture (SOAG), LNCT University, Bhopal, following recommended agronomic practices. Healthy plants were selected for laboratory analysis on 24 November 2025, 21 days after sowing (DAS), when active root nodules had developed. Plant sampling was carried out at 10:00 AM under favorable

weather conditions. At the time of sampling, the ambient temperature ranged from 25–28°C, the relative humidity was 41%, and the crop exhibited healthy vegetative growth with well-developed root systems. Plants were carefully uprooted using a hand hoe to preserve the root system and nodules intact. The collected plants were immediately placed in sterile polythene bags and transported to the Microbiology Laboratory for further processing.

Table 1. Field Observation at the Plant Collection

Parameter	Observation
Crop	Chickpea (*Cicer arietinum* L.)
Date of Sowing	31 October 2025
Date of Plant Collection	24 November 2025
Crop Age	21 Days After Sowing (21 DAS)
Time of Plant Collection	10:00 AM
Air Temperature	25–28°C
Relative Humidity	41%

Fig 1. Twenty-one-day-old chickpea plants collected for laboratory analysis

Selection of Healthy Root Nodules

After collection, the roots were washed gently under running tap water to remove adhering soil without damaging the nodules. Healthy, pink, firm, and well-developed root nodules were selected because the pink colour, resulting from the presence of leghemoglobin, indicates active nitrogen fixation and a successful symbiotic association between chickpea and *Rhizobium*. Immature, damaged, brown, black, or diseased nodules were excluded from the study. Selected nodules were processed immediately for surface sterilization and bacterial isolation under aseptic laboratory conditions to ensure maximum recovery of viable *Rhizobium* cells. Giller (2021).

Surface Sterilization of Root Nodules

Selected nodules were washed with sterile distilled water and surface sterilized by immersing them in 70% ethanol for 30 seconds, followed by 1% sodium hypochlorite solution for 2–3 minutes. The sterilized nodules were rinsed five times with sterile distilled water to remove traces of the sterilizing agents before further processing Andrews, M., & Andrews, M. E. (2020).

Crushing of Root Nodules

Surface-sterilized nodules were aseptically transferred into a sterile mortar and pestle and crushed gently with a small quantity of sterile distilled water to obtain a uniform bacterial suspension containing *Rhizobium* cells.

Serial Dilution and Isolation on Agar Medium

The bacterial suspension was serially diluted using sterile distilled water. Aliquots from appropriate dilutions were inoculated onto sterile Yeast Extract Mannitol Agar (YEMA) medium by the spread plate method. The inoculated plates were incubated under aseptic conditions for the development of isolated bacterial colonies. Hungria et al. (2022).

Fig 2. Healthy root nodules collected from chickpea roots

Fig 3. Washing and surface sterilization of nodules

Fig 4. Crushing of root nodules using a sterile mortar and pestle

Fig 5. Serial dilution of nodule suspension

Fig 6. Preparation and pouring of Agar medium.

Incubation

The inoculated plates were incubated at 28 ± 2°C for 48–72 hours. Colony development was observed daily, and well-isolated colonies were selected for purification by repeated streaking.

Fig 7. Growth of *Rhizobium* colonies after incubation

Colony Morphology

Typical *Rhizobium* colonies were circular, smooth, convex, translucent to creamy white, glistening, and mucilaginous.

Fig 8. Isolation of bacterial colonies

Preservation of Isolates

Pure *Rhizobium* isolates were stored at **4°C** for future studies. Periodic sub-culturing was carried out to maintain culture viability and purity.

Results and Discussion

The present investigation successfully demonstrated the cultivation of chickpea (*Cicer arietinum* L.) under field conditions and the subsequent isolation, purification, and characterization of native *Rhizobium* isolates from healthy root nodules Hungria, M., Campo, R. J., & Mendes, I.

C. (2022) The study confirmed the presence of effective nitrogen-fixing bacteria associated with chickpea roots and validated the suitability of Yeast Extract Mannitol Agar (YEMA) medium for isolation of pure cultures (Somasegaran and Hoben, 2012; Hungria et al., 2022).

Field Establishment and Crop Growth

The chickpea crop was successfully established under the prevailing climatic conditions following sowing on 31 October 2025. Uniform seed germination was observed within the recommended period, resulting in healthy and vigorous crop establishment. During the crop growth period, no major incidence of insect pests or diseases was recorded, and the crop maintained a healthy green appearance throughout the vegetative stage.

Twenty-one days after sowing (24 November 2025), the plants exhibited active vegetative growth characterized by well-developed stems, expanded leaves, and an extensive root system. The environmental conditions prevailing during plant collection (25–28°C temperature and 41% relative humidity) were favourable for root development and nodulation (Merga and Haji, 2019; Bhattacharjee and Dey, 2021).

The healthy crop stand indicated that the agronomic practices adopted during cultivation, including irrigation, weed management, and nutrient management, provided suitable conditions for the establishment of a successful symbiotic association between chickpea and indigenous *Rhizobium* populations (Peoples et al., 2021).

Table 2. Field observations during chickpea cultivation

Parameter	Observation
Crop	Chickpea (*Cicer arietinum* L.)
Date of sowing	31 October 2025
Plant collection	24 November 2025
Crop age	21 DAS
Plant condition	Healthy and vigorous
Root development	Well developed
Nodulation	Abundant
Disease incidence	Nil

Temperature	25–28°C
Relative humidity	41%

Root Nodule Development

Healthy chickpea plants developed numerous root nodules distributed on both the primary and lateral roots. The nodules were spherical to oval in shape and firmly attached to the root surface. External examination showed that the nodules were cream colored, whereas transverse sections revealed a characteristic pink pigmentation. The pink colour observed inside the nodules indicated the presence of leghemoglobin, confirming active biological nitrogen fixation and effective symbiosis between chickpea plants and *Rhizobium* (Vincent, 1970; Giller, 2021). Damaged, immature, black, or brown nodules were not included for bacterial isolation because they generally represent inactive or senescent nodules (Jaiswal et al., 2022).

Each selected plant possessed several active nodules, suggesting that the native soil possessed an adequate population of compatible rhizobial strains capable of successful infection and nodulation (Somasegaran and Hoben, 2012).

Table 3. Morphological characteristics of root nodules

Character	Observation
Colour (external)	Cream
Colour (internal)	Pink
Shape	Spherical to oval
Texture	Firm
Position	Primary and lateral roots
Activity	Active nitrogen-fixing
Condition	Healthy

Isolation of *Rhizobium*

Surface sterilization using 70% ethanol followed by 1% sodium hypochlorite proved highly effective in eliminating surface contaminants. No fungal or bacterial contamination was observed during subsequent culture, indicating that aseptic techniques were successfully maintained throughout the isolation process. Crushed nodule suspension inoculated onto YEMA medium produced visible bacterial colonies after 48–72 hours of incubation at 28 ± 2°C. Colony development began approximately 48 hours after inoculation, while maximum colony growth was observed after 72 hours.

Serial dilution facilitated the production of well-separated colonies,

allowing easy purification by repeated streaking. All selected colonies remained morphologically uniform after purification, indicating successful isolation of pure cultures. The successful isolation confirmed that healthy chickpea nodules contained viable native *Rhizobium* populations capable of growth under laboratory conditions (Somasegaran and Hoben, 2012; Hungria et al., 2022).

Colony Morphology

The purified bacterial colonies exhibited typical morphological characteristics of *Rhizobium* when cultured on YEMA medium. Colonies were circular with smooth margins and convex elevation. The colony surface appeared smooth, shiny, and glistening due to the production of extracellular polysaccharides. The colonies were creamy white to translucent and exhibited a distinctly mucilaginous texture (Masson-Boivin and Sachs, 2018; Andrews and Andrews, 2020). No pigmentation or irregular colony forms were observed during purification. The uniform colony morphology suggested that the isolates belonged to a single dominant rhizobial population associated with chickpea root nodules (Mir et al., 2021).

Microscopic Characteristics

Microscopic examination following Gram staining revealed that all purified isolates consisted of Gram-negative rod-shaped bacterial cells. Individual cells appeared short, slender rods occurring singly or occasionally in pairs. No spore formation was observed. The bacterial cells retained the characteristic pink colour following Gram staining, confirming their Gram-negative nature and agreeing with the typical morphology of *Rhizobium* species. The microscopic observations corresponded closely with the colony characteristics observed on YEMA medium and further confirmed the identity of the isolated bacteria as *Rhizobium* (Vincent 1970).

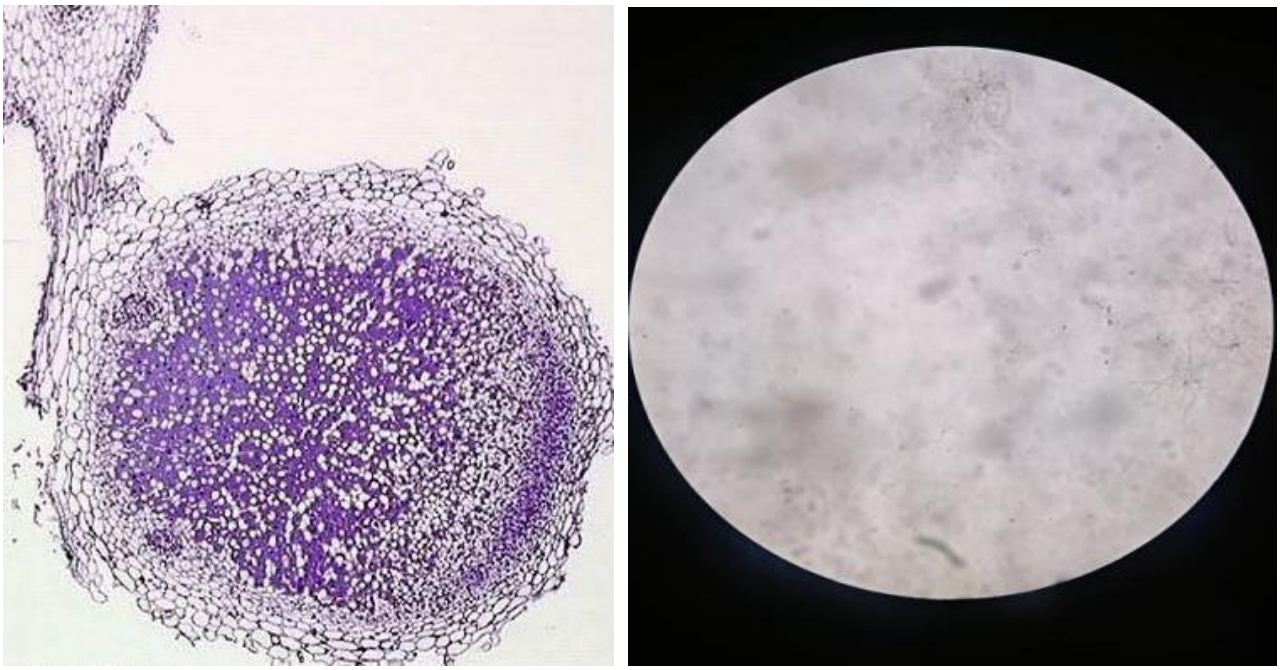

Fig 9. Observation of Rhizobium under a microscope

Table 4. Colony characteristics of isolated *Rhizobium*

Character	Observation
Colony colour	Creamy w
Shape	Circular
Margin	Entire
Elevation	Convex

Surface	Smooth
Texture	Mucilaginous
Appearance	Glistening
Opacity	Translucent

Table 5. Microscopic characteristics of isolated *Rhizobium*

Character	**Observation**
Gram reaction	Negative
Cell shape	Rod-shaped
Arrangement	Single or paired
Spore formation	Absent
Cell morphology	Typical of *Rhizobium*

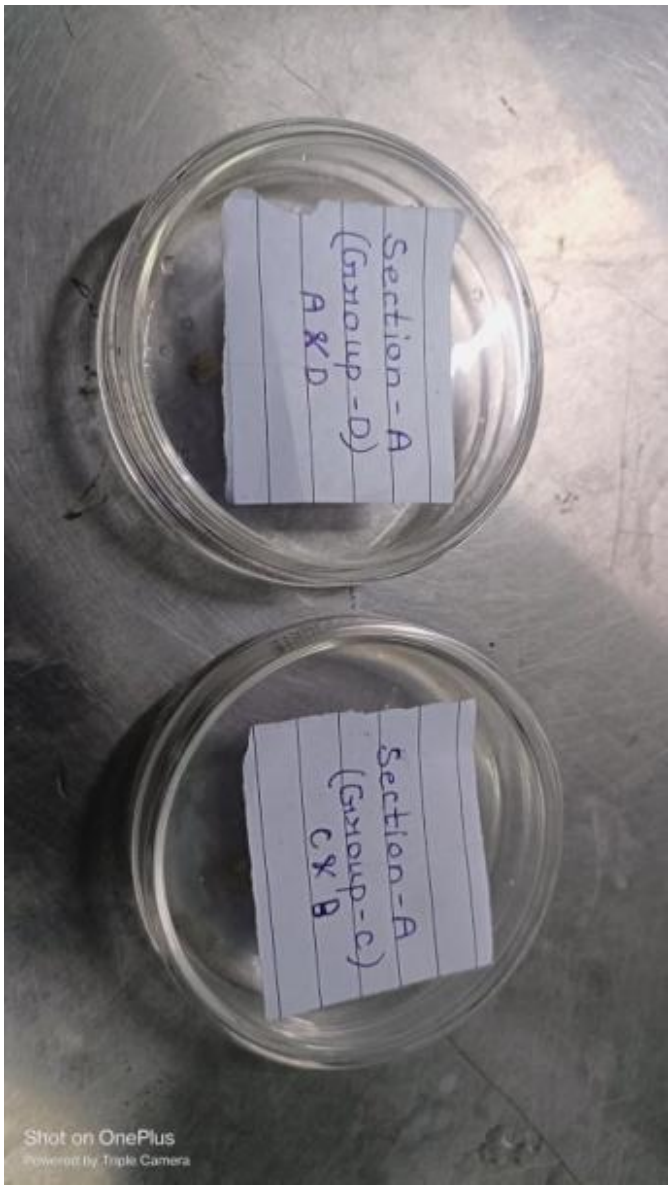

Fig 10. Preservation of Pure Cultures

Purification and Preservation of Isolates

Repeated streaking successfully produced pure bacterial cultures free from contamination. The purified isolates maintained identical colony morphology throughout repeated subculturing, demonstrating culture stability. Pure cultures preserved on YEMA agar slants at 4°C remained viable during the storage period without any visible contamination. The preserved isolates are suitable for future investigations involving biochemical characterization, molecular identification, assessment of nitrogen-

fixing efficiency, and evaluation as potential biofertilizer strains (Somasegaran and Hoben, 2012; Hungria et al., 2022).

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