Isolation and Characterization of *Agrobacterium rhizogenes* from the Root Nodules of Leguminous Plants

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Isolation and Characterization of *Agrobacterium rhizogenes* from the Root Nodules of Leguminous Plants" is based on the results of investigations carried out by me in the Botany Discipline at the School of biological sciences and biotechnology, Goa University under the supervision of Dr. Siddhi K. Jalmi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Ms. Gautami Mukesh Jalmi

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Date: 8 April 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "Isolation and Characterization of *Agrobacterium rhizogenes* from the Root Nodules of Leguminous Plants" is a bonafide work carried out by Ms. Gautami Mukesh Jalmi under my supervision in partial fulfilment of the requirements for the award of the degree of M.Sc. in the Discipline of Botany at the School of biological sciences and biotechnology, Goa University.

08/04/2024

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PREFACE

The study of plant-microbe interactions has long been a fascinating area of research due to its significance in agriculture and environmental sustainability. Among these interactions, the symbiotic relationship between leguminous plants and nitrogen-fixing bacteria is of particular interest. These bacteria, collectively known as rhizobia, form nodules on the roots of legumes and fix atmospheric nitrogen into a form that plants can utilize, thus enhancing soil fertility.

Agrobacterium rhizogenes is a soil-borne bacterium closely related to *Agrobacterium tumefaciens*, which is well-known for its ability to induce crown gall disease in plants. However, *A. rhizogenes* exhibits a different phenotype, causing the formation of hairy roots in susceptible plant species. Despite its unique characteristics, relatively little is known about the prevalence and diversity of *A. rhizogenes* in natural environments.

This study aims to isolate and characterize *A. rhizogenes* strains from the root nodules of leguminous plants namely *Arachis hypogaea, Vigna radiata* and *V. unguiculata*. By conducting this research, I aim to broaden my understanding of the ecological significance and potential agricultural applications of this bacterium.

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Ms. Gautami Mukesh Jalmi

TABLES

PLATES

ABBREVIATIONS USED

Entity	Abbreviation
Degree	°
Grams per liter	g/L
Micrometer	µm
Percent	%
Potato Dextrose Agar	PDA
Root inducing	Ri
Transferred DNA	T-DNA
Tumor inducing	Ti

ABSTRACT

Agrobacterium rhizogenes also called *Rhizobium rhizogenes* is a Gram-negative, rod-shaped soil bacterium belonging to the genus Rhizobium. It is widely known for its ability to induce hairy root formation in various plant species. A total of 24 gram negative bacterial colonies were isolated from both root nodules and roots of *Arachis hypogaea, Vigna radiata and V. unguicula* by using yeast extract mannitol agar (YEMA). The isolated bacteria were characterized and identified as *Agrobacterium rhizogenes* based on cultural (Congo red Test, Growth on Glucose Peptone Agar and Lactose Agar Test) and *Agrobacterium*-specific tests (Growth on Potato Dextrose Agar, Ketolactose Test and Citrate utilization Test). *A. rhizogenes* is a bacterium used to study plant-microbe interactions, as well as for producing secondary metabolites and genetic transformation. By transferring genes into plant cells via *A. rhizogenes*, researchers can enhance traits like disease resistance, herbicide tolerance, and stress resilience. *A. rhizogenes*-mediated hairy root culture in bioreactors is employed to produce valuable secondary metabolites from plants. Thus, *A. rhizogenes*-mediated transformation is a powerful tool in agricultural research, biotechnology, and pharmaceutical industries. Despite its unique characteristics, relatively little is known about the prevalence and diversity of *A. rhizogenes* in natural environments.

KEY WORDS

Agrobacterium rhizogenes, Agrobacterium-specific tests, Cultural tests, Leguminous plants, Root nodules, Roots

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Leguminous plants are of great interest in agriculture. They play central roles at the food system level, both for human and animal consumption, as a source of plant proteins and are increasingly important in improving human health. These plants possess the unique ability to fix atmospheric nitrogen through a symbiotic relationship with nitrogen-fixing bacteria, which primarily belong to the genus *Rhizobium*. Legumes introduced into a cropping system can enhance the fertility of the soil by their nitrogen fixing ability and additional benefits to improve soil organic matter, nutrient recycling, soil porosity and soil structure. While *Rhizobium* has been extensively studied for its role in nitrogen fixation, the presence of *Agrobacterium rhizogenes* in root nodules of various leguminous species has also been documented. Moreover, a better understanding of plant-microbe interactions such as symbiotic nitrogen fixation, mycorrhizal associations, and legume-pathogen interactions can be possible with legume studies (Chilton *et al.,* 1982; Christey, 2001).

1.1.1 Characteristics of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes also called *Rhizobium rhizogenes* is a Gram-negative, rod-shaped soil bacterium belonging to the genus *Rhizobium*. The species name "*rhizogenes*" originates from the Greek words "rhiza" meaning a root and "gennao" to make, thus resulting in a root-producing bacteria (Strobel *et al.,* 1985). It is a non-spore forming (0.6–1 μm by 1.5–3.0 μm in size) soil bacterium that occurs singly or in pairs. It has the ability to move via one to six peritrichous flagella. (Conn, 1942; Meyer *et al.,* 2000; Tzfira and Citovsky, 2000; Giri and Giri, 2007; Murugesan *et al.,* 2010). *A. rhizogenes* was first isolated by Riker *et al.* in 1930 (Brenner *et al.,* 2005) and is commonly found in the nodules of leguminous plants (Ron *et al.,* 2014). It is widely known for its ability to induce hairy root formation in various plant

species. It enters the plant roots via wounds or natural openings, which may result in the proliferation of secondary roots, leading to a ‘hairy root disease’.

1.1.2 Distribution and habitat

The host plant species of *A. rhizogenes* are distributed in moist to dry tropical and subtropical regions in South Africa such as Kwazulu-Natal, the Mpumalanga Escarpment and the Highveld. It is widely distributed in apple nurseries and orchards in the United States, and has also been found on roses in California and Texas (Uk *et al.*, 1965). Under natural conditions, the host plants of *A. rhizogenes* include apple, cucumber, tomato and melon with the microhabitat of these microorganisms being the rhizospheric zone of the above mention host plants. However, under laboratory conditions, more than 450 plant species are susceptible to infection by these bacteria (Veena & Taylor, 2007).

1.1.3 Nutrition and metabolism

A. rhizogenes is an aerobic bacterium that uses oxygen for its growth needs (Brenner *et al.*, 2005). The root-inducing plasmid (pRi) gives the bacterium the ability to induce the production and degradation of opine complex acids such as mannopine and agropinic acids as sole sources of nitrogen and carbon.

The conditions required for the successful culturing of *A. rhizogenes* under laboratory conditions are the following: a temperature between 25°C and 28°C, a pH level of 5–9 and biotin as a growth factor. It should be emphasized that this bacterium will not flourish at 35°C, in a medium with 2% NaCl, and do not generate 3-ketolactose. The colony morphology will exhibit characteristics of being convex, circular and smooth, with a colour ranging from non-pigmented to light beige (Brenner *et al.*, 2005).

1.1.4 Life cycle of *A. rhizogenes*

A. rhizogenes multiply through the process of binary fission, where a bacterial cell (called a parent cell) makes a copy of its DNA and grows larger by doubling its cellular content. The parent cell then splits, pushing apart the duplicated cellular material and forming two identical 'daughter' cells. Generally, bacteria reproduce either via binary fission or budding, however, on both cases, the DNA found in parent cells and the offspring is exactly the same.

A. rhizogenes relies on other means to introduce variation into its genetic material, such as gene transfer using the plasmid. The resulting genetic variation ensures that the bacterium can adapt and survive amid changing surrounding environment and ensure that its host is prepared to tolerate changes as well. Root plant cells would release compounds, which are sensed by the bacterium in the soil, and which triggers it to be attracted to that area. The bacterium then transfers DNA from the plasmid into the host cell and integrates the T-DNA into the plant cell gene structure. After integration, the plant undergoes root architectural changes and produces an abundance of opines, which are beneficial for the growth of *A. rhizogenes*.

1.1.5 Infection and colonization by *Agrobacterium rhizogenes*

a. Process of infection in leguminous plants

The mechanism by which *A. rhizogenes* infects wounded plant cells begins by the attraction of the bacterial cells to the wound site due to the production of phenolic compounds via chemotaxis. The genetic transformation can be divided into following steps: Agrobacterium sensing phenolic compounds released by plant roots, which triggers attachment of the bacteria to the root; Processing the T-DNA into Agrobacterium cells and T complex formation (mechanism similar to those of *A. tumefaciens*); transfer of T complexes (via the type IV protein secretion system) from the bacteria to the host plant genome. The *A. rhizogenes* T-DNA has two independent sequences, denoted TL (left) and TR (right) borders.

TL-DNA and TR-DNA are generally independently transferred and integrated into the host plant genome, however only the TL-DNA is essential (and hence sufficient) to induce hairy roots. Sequence analysis of TL-DNA revealed four open reading frames that are essential for hairy roots induction (rolA, B, C, and D). RolB gene appears to be the most important in hairy roots induction, since loss-of-function mutation at this locus renders the plasmid as avirulent. Once the bacteria reach the wound site, they infect the plant by integrating their pathogenic Ri plasmid into the plant genome which results in the development of hairy root disease.

b. Impact on plant growth and development

The plants infected by *A. rhizogenes* show hairy root formation which occurs as a result of protruding large numbers of small roots as fine hairs directly from the infection site (Chandra 2012). This plants exhibit plagiotropic root growth with short internodes, a high degree of lateral branching, wrinkled leaves, reduced apical dominance, reduced fertility, profusion of root hairs, abnormal flower production, advanced flowering, increased number of flowers, enhanced growth rates and changed secondary metabolite accumulation (Ackermann 1977; Tepfer 1983; Balandrin et al. 1985; Charlwood and Charlwood 1991; Pellegrineschi et al. 1994; Flores et al. 1999; Lee et al. 2001; Keil 2002; Ca-sanova et al. 2004; Veena and Taylor 2007)

1.1.6 Applications of *A. rhizogenes*

A. rhizogenes plays a crucial role in the growth and development of leguminous plants. These bacteria establish a symbiotic relationship with the roots of legumes, promoting various mechanisms that enhance plant productivity. *A. rhizogenes* aids in nitrogen fixation, phosphate solubilization, phytohormone production, and siderophore activity, all of which contribute to improved nutrient uptake and overall plant health. Additionally, the presence of

these beneficial rhizobacteria can lead to increased root and shoot growth, vigor, biomass production, and enhanced nodulation in legumes, ultimately resulting in higher crop yields. The multifunctional properties of *A. rhizogenes* make it a valuable asset for sustainable agriculture practices, especially in legume production.

It is commonly used to effect transformation, and has several features desirable for the production of secondary metabolites. For example, hairy root cultures can usually synthesize the same compounds as the roots of the intact plant (Muranaka *et al.,* 2010).

Agrobacterium rhizogenes strain K84 (formerly called *A. radiobacter*) is used worldwide as a commercial agent for the biocontrol of crown gall disease caused by tumorigenic *Agrobacterium* strains. *A. rhizogenes* is responsible for the development of hairy root disease in a wide range of dicotyledonous plants and characterized by a proliferation of excessively branching roots.

The plants transformed with *A. rhizogenes* are become increasingly popular for offering approaches to create cost-effective options in mass-producing desired plant metabolites and expressing foreign proteins. The data from numerous proof-of-concept studies including improved the nutritional quality, agronomical characteristics, production of plant-derived products encourages for the realization of scaling up *Agrobacterium* based practices. Studies on the molecular characteristics and taxonomy of *Agrobacterium* and *Rhizobium* are plentiful; however, studies on the isolation and characterization of Agrobacterium from root nodules of legumes, especially pulses, are limited. Considering all these factors, the present investigation was carried out with the isolation and characterization of *Agrobactierum* from the nodules of leguminous plant hosts such as *Arachis hypogaea*, *Vigna radiata* and *V. unguiculata*.

1.2 AIM AND OBJECTIVES

The aim of the present study was to isolate and characterize *Agrobacterium rhizogenes* obtained from root nodules of leguminous plants.

The objective of this study is as follows:

1) To isolate *Agrobacterium* from the root nodules of leguminous plants namely *Arachis hypogaea, Vigna radiata* and *V. unguiculata*.

2) To characterize and identify the isolated bacteria as *Agrobacterium rhizogenes* based on cultural and *Agrobacterium*-specific tests.

1.3 HYPOTHESES/ RESEARCH QUESTION

- *Agrobacterium rhizogenes* can be isolated from the root nodules of leguminous plants, and these isolates will exhibit diverse characteristics based on their origin.
- Different leguminous plant species will harbour different strains or species of *Agrobacterium rhizogenes* within their root nodules due to host specificity.

1.4 SCOPE

The study aims to isolate and characterize *Agrobacterium rhizogenes* from the root nodules of leguminous plants namely *Arachis hypogaea, Vigna radiata* and *V. unguiculata*. The scope includes:

- Collection of root nodules from leguminous plants
- Isolation of *A. rhizogenes strains* from roots an root nodules using selective media
- Characterization and identification of *A. rhizogenes* based on cultural and *Agrobacterium*-specific tests.

The study will focus on understanding the diversity of *A. rhizogenes* strains, their symbiotic relationship with leguminous plants and their potential for biotechnological and agricultural applications.

CHAPTER 2: LITERATURE REVIEW

Agrobacterium rhizogenes, now renamed *Rhizobium rhizogenes* (Young *et al.,* 2001) is a gram negative soil inhabiting bacteria, which produces a condition called 'hairy roots' as a result of the modified hormonal balance of the tissue that makes them vigorous and allows it to grow rapidly on artificial media (Abdul-Khaliq *et al.,* 2001).

A. rhizogenes infects plant roots, leading to the formation of hairy roots. These hairy roots exhibit increased branching and growth, contributing to enhanced nutrient uptake and overall plant vigor. The formation of these roots is due to the transfer of T-DNA (transfer DNA) from the bacterium's Ti plasmid into the plant genome. This genetic material contains genes responsible for the production of plant growth regulators, such as auxins and cytokinins, which promote root growth and branching (Veena *et al.,* 2003).

The discovery of *A. rhizogenes* as a plant pathogen capable of inducing hairy root formation was a significant milestone. It was first described by Muller in 1907, who observed the formation of hairy roots in plants infected with the bacterium. This discovery laid the foundation for subsequent research into the mechanisms underlying root transformation (Muller, 1907).

A. rhizogenes has been exploited in agriculture for its ability to induce hairy root formation in various plant species. This trait has been utilized in the development of transgenic plants with improved characteristics, such as enhanced resistance to biotic and abiotic stresses, increased yields, and improved nutrient uptake efficiency. Additionally, the hairy root culture system offers a rapid and efficient method for the production of valuable secondary metabolites, including pharmaceuticals, flavors, and fragrances (Capone *et al.,* 2019).

A. rhizogenes-mediated transformation has become a valuable tool in plant biotechnology for the introduction of desirable traits into crop plants. By harnessing the bacterium's natural genetic transfer capabilities, researchers can introduce genes encoding traits such as pest resistance, herbicide tolerance, and improved nutritional content into plant genomes. This technology has facilitated the development of genetically modified crops with enhanced agronomic traits, contributing to sustainable agriculture and food security (Lichtenthaler *et al.*, 2017).

The study isolated 57 colonies of *Agrobacterium* species from the root nodules of five different leguminous plants using yeast extract mannitol agar (YEMA). The isolated bacteria were identified as *A. rhizogenes* based on morphological, biochemical, cultural, and pathogenicity tests. Biochemical constituents like exopolysaccharide, glycogen, total protein, free amino acids, and total lipids were characterized, showing variations among the agrobacterial isolates from different plant species. The bacteria were characterized by specific tests, and pathogenicity tests confirmed their species. The findings revealed variations in biochemical constituents among the agrobacterial isolates from different host plants (Murugesan *et al.*2010).

Murugesan *et al.* (2011) isolated *Agrobacterium* strains from five different leguminous plants viz., *Pisum sativum, Sesbania rostrata, Vigna mungo, V. radita* and *V. ungiculata*. The isolated bacteria were characterized and identified as *Agrobacterium rhizogenes* based on their morphological, biochemical, cultural and pathogenicity tests. Antibiogram and protein profile of isolates were also studied by disc diffusion and SDS-PAGE methods respectively.

Nguyen *et al.* (2017) isolated total 274 colonies of *Agrobacterium* species on selective MG Te medium from 235 samples of rhizophere soils. Only thirteen colonies belonged to *A. rhizogenes* being able to efficiently induce the hairy root formation of *Catharanthus*

roseus in vivo based on the morphological, biochemical, and pathogenicity tests. These thirteen colonies were identified as *A. rhizogenes* based on 16S rRNA sequence and possessing rolABC genes. This has provided a valuable transgenic tools for future research on this plant trait.

Strobe *et al.* (1985) treated bare root stock almond trees with *A. rhizogenes* 232 shown a larger root number and root mass after 90 d than those treated with autoclaved or filter-sterilized bacterial suspensions, or with medium alone. Treatment with *A. rhizogenes* 232 also led to significant increases in leaf number, stem diameter and shoot elongation during the first growing season after treatment.

The altered phenotypes obtained from *A. rhizogenes*-mediated transformation such as increased flowering, shortened stature, and increased secondary products are of interest for horticultural purposes (Christey, 2005). Hairy roots were used to produce artificial seeds. (Uozumi *et al.*, 1997) harvested a large quantity of root fragments with apical meristems or lateral roots from a whole root culture. Hairy roots were treated with various concentrations of auxin, then randomly-picked root fragments were encapsulated and transferred to Murashige and F. Skoog liquid medium in the light.

Zeng *et al.* (2017) developed a technique for efficient transformation of hairy roots of *Arachis hypogaea* L. using *A. rhizogenes* K599, and have validated this approach for the investigation of gene function. As a model transgene, AhAREB1, a drought-resistance gene from peanut, was fused to green fluorescent protein, and four parameters that might influence the transformation efficiency were tested. The optimal procedure involved the use of petioles with four expanded leaves as explants, infection by K599 at optical density (OD_{600}) of 0.6 for 15 min and co-cultivation for 2 d, giving transformation efficiencies of up to 91%. Hairy roots from transgenic peanut plants overexpressing

AhAREB1 were unaffected by treatment with polyethylene glycol (PEG), demonstrating increased drought tolerance, whereas control roots showed clear signs of plasmolysis. Transgenic roots accumulated less superoxide anion (O_2 −) than control roots under drought conditions.

The importance of endangered *B. aristata* lies in its medicinal applications as some of its metabolites like benzylisoquinoline (berberin), a natural alkaloid, have shown anticancer activity as well as aiding the treatment of diabetes and malarial fever. Brijwal *et al.* (2015) develop hairy root induction protocol using two different strains of *Agrobacterium rhizogenes*, MTCC 532 and 2364 , which would increase the yield of valuable berberin thereby, reducing the pressure on the endangered plant in its natural population and consequently preventing the overharvesting of *B. aristata* in the native environment. The strain 532 was more effective than strain 2364 in hairy root induction.

Sathasivam *et al.* (2022) examined the transformation efficiency of various *A. rhizogenes* strains (ATCC 13333, ATCC 15834, A4, R1000, R1200, and R1601) for transgenic hairy root (HR) induction in *Ocimum basilicum*. A total of 55 metabolites were identified using high performance liquid chromatography (HPLC) and gas chromatography–time-of-flight mass spectrometry (GC-TOFMS). *Agrobacterium* strains R1601 is one of the best and most promising strains for inducing mass HR production and enhanced levels of secondary metabolites in *O. basilicum*.

CHAPTER 3: METHODOLOGY

3.1 SAMPLE COLLECTION SITE

For the present study, legumes such as *Arachis hypogaea*, *Vigna radiata* and *V. unguiculata* were collected along with root nodules from the field which is located at Shingane Vagurem, Sattari, North Goa. The location of site (15.4994681°N, 74.0846971°E) was recorded by Google map.

3.2 ISOLATION OF *AGROBACTERIUM*

3.2.1 Preparation of root nodules extract

The leguminous plants were carefully uprooted from the soil, and the root system was washed with running water to remove soil particles. Firm, undamaged root nodules were selected visually and excised from the roots. The nodules were kept immersed in a 0.1% acidified mercuric chloride solution for 5 minutes and washed repeatedly with sterile distilled water. Then they were immersed in 70% ethyl alcohol for 30 seconds. This treatment was followed by repeated washing with sterile distilled water. These sterilized root nodules were crushed simply with a pestle and mortar and extracted with sterile distilled water.

Agrobacterium isolates were isolated by using serial dilution and pour plate techniques. The root nodule extract was serially diluted up to 10^{-7} with sterile distilled water, and 1 ml of the diluted sample was inoculated into sterile Petri plates and poured with the sterilized yeast extract mannitol agar (YEMA) medium. The plates were incubated at 28°C for 2 to 3 days. This medium allowed both *Agrobacterium* and *Rhizobium* to grow and develop into colonies.

3.2.2 Preparation of root extract

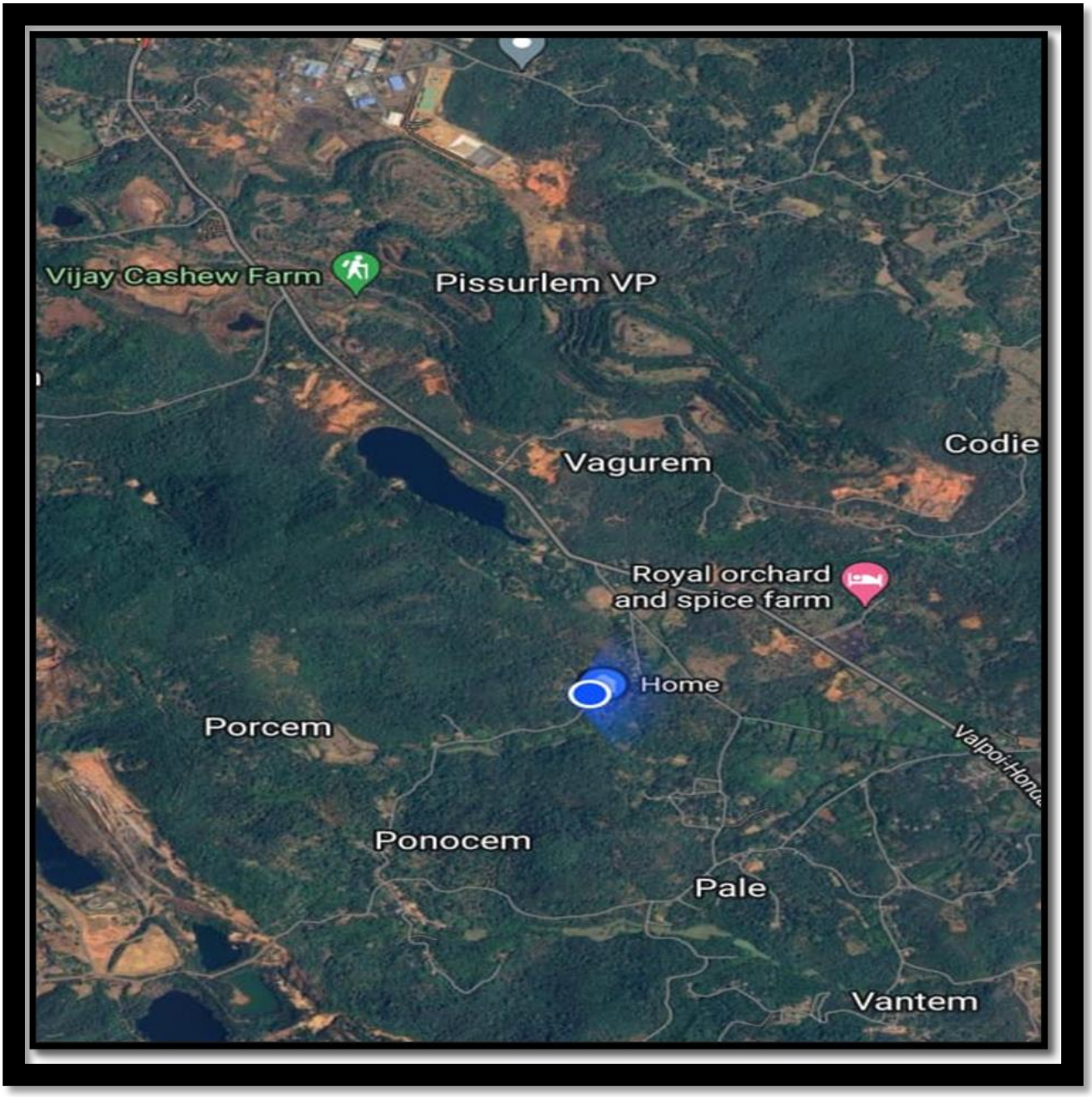


Plate 3.1: Sample collection site (15.4994681°N, 74.0846971°E)

To isolate *Agrobacterium rhizogenes*, the roots were gently shaken by immersing them in the sterile distilled water to remove the loosely bound soil clumps, leaving behind the soil firmly adhering to the roots. This was further used for the serial dilution. The roots were cut into small pieces using sterile blade and placed into a sterile vial containing 4ml of sterile distilled water to prepare a root extract. This extract was then serially diluted up to 10^{-4} with sterile distilled water, and 1ml of the diluted sample was spread plated on the Yeast Extract Mannitol Agar (YEMA) medium. The plates were incubated at 28°C for 2 to 3 days.

After incubation, the bacterial colonies were purified by the streak plate technique on *Agrobacterium* mannitol medium [Tryptone 5 g/L; mannitol 5 g/L; yeast extract 2.5 g/L; L-glutamic acid 1 g/L; KH_2PO_4. 25 g/L; NaCl 0.1 g/L; $MgSO_4$.$7H_2O$ 0.1 g/L; biotin 10 µl (0.1 mg/ml stock); pH 7.0; agar 15 g/L]. Pure agrobacterial cultures were maintained in both Yeast Extract Mannitol Agar and *Agrobacterium* mannitol medium.

3.3 Gram Staining Test

The pure cultures of bacterial strains were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar air flow hood. The slides were firstly washed with ethanol and colonies were marked on the slides with the help of inoculating needle. The slide was air-dried and heat fixed. Then, with the help of a dropper, crystal violet dye was added to the slide for 30 seconds. Rinse the slide with distilled water to remove the access dye. Then gram iodine was added to the slide for one minute. Then 95% ethanol was applied and rinsed the slide with distilled water. Then, safranine was applied and again rinsed the slide with distilled water and blot dried. After this red colour will show which confirms that the bacteria are gram-negative bacteria (Ali et al. 2016).

3.4 CHARACTERIZATION OF *AGROBACTERIUM* ISOLATES

The *Agrobacterium* isolates were characterized based on cultural and physiological characteristics such as the Congo red test, growth in glucose peptone agar and Lactose Agar with Benedict's reagent. *Agrobacterium*-specific tests such as growth on potato dextrose agar (PDA), ketolactose test and citrate utilization test were also carried out.

3.4.1 Cultural Tests

A. Congo red test

The isolates were spot inoculated on Congo Red Yeast Extract Mannitol Agar medium. This medium was prepared by addition of 0.025g/L of Congo red in YEMA medium. The plates were incubated at 28°C for 2 to 3 days. In general, Agrobacteria take up the dye strongly whereas Rhizobia absorb the dye weakly and produce white colonies.

B. Glucose Peptone Agar

This test was performed to check the ability of the isolates to utilize glucose as the sole carbon source. Glucose peptone agar medium contains glucose 40 g/L, peptone 20 g/L, agar15 g/L pH 7.0 to differentiate rhizobia from Agrobacteria. *Agrobacterium* shows massive growth on this media.

C. Lactose Agar with Benedict's reagent

The isolates were spot inoculated on Lactose Agar medium (1 g/L yeast extract; 10g/L lactose 15g/L agar). The plates were incubated at 28°C for 2 to 3 days. The plates were flooded with Benedict's reagent and kept at room temperature. Wait for 1-2 hours or until a bright, yellow product is observed in the agar. The yellow colour confirms the presence of *Agrobacterium* sp.

3.4.2 *Agrobacterium*-specific Tests

A. Potato Dextrose Agar (PDA)

The bacterial isolates were inoculated on PDA medium. The plates were incubated at 28°C for 2 to 3 days. On PDA medium, the *Agrobacterium* will show well pronounced growth.

B. Ketolactose test

Ketolactose agar medium (Lactose 10 g/L, KH2PO4 0. 5 g/L, MgSO4. 7H2O 0.2 g/L, NaCl 0. 1 g/L, Yeast extract 1 g/L, Agar 15 g/L, pH adjusted to 6.8) was poured into the sterilized Petri dishes and allowed to solidify. The bacterial isolates were streaked on the Ketolactose agar medium and incubated for 2-3 days. The plates were flooded with Benedict's reagent and kept at room temperature for 1-2 hours. The *Agrobacterium* produced yellow ring of precipitate of Copper (II) oxide (CuO_2) around the colonies of the bacterium when plates were flooded with Benedict's reagent.

C. Citrate utilization test

The citrate utilization test is used to determine the ability of an organism to utilize citrate as its sole carbon source. In the test, the medium contains citrate as the only carbon source, along with a pH indicator such as Bromothymol Blue. If the bacterium can utilize citrate, it produces alkaline by-products, causing the pH of the medium to rise, which changes the colour of the indicator from green to blue. Growth of *Agrobacterium rhizogenes* on the citrate utilization medium and a change in colour of the medium from green to blue indicates that the organism can utilize citrate as a carbon source.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Arachis hypogaea*

A. GRAM STAINING TEST

Table 4.1. Gram staining results of bacterial colonies isolated from the root nodules of *Arachis hypogaea*

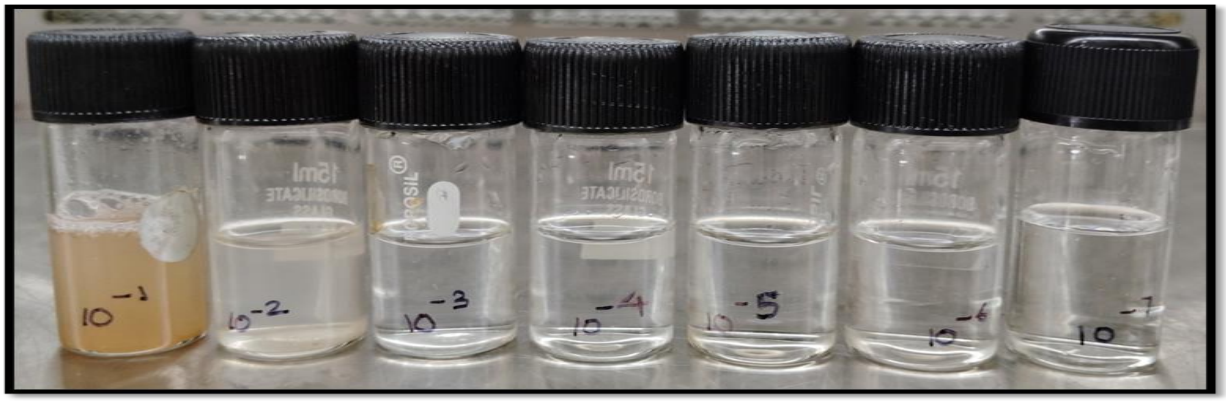
Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	1	3	4
10^{-2}	1	0	1
10^{-3}	0	0	0
10^{-4}	0	0	0
10^{-5}	0	0	0
10^{-6}	0	0	0
10^{-7}	0	0	0

Table 4.2. Gram staining results of bacterial colonies isolated from the roots of *Arachis hypogaea*

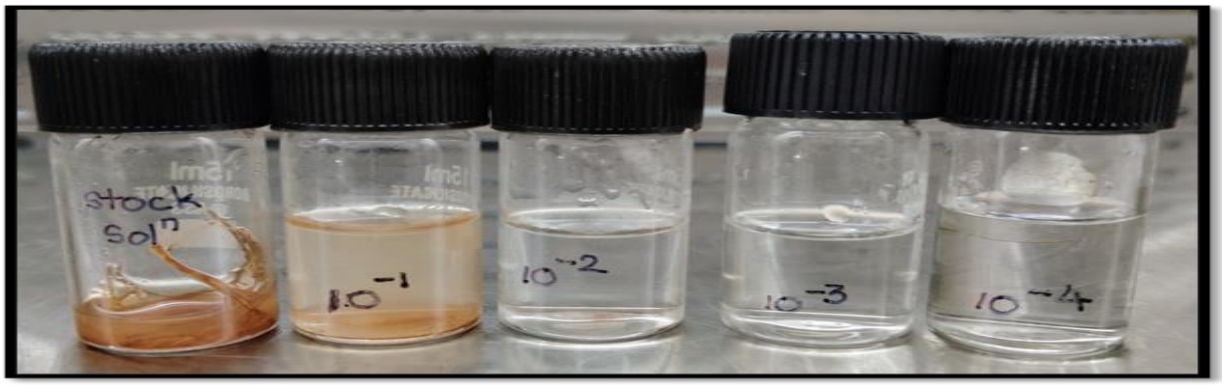
Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	Over growth		
10^{-2}	4	0	4
10^{-3}	3	0	3
10^{-4}	0	3	3

A. Plant of *Arachis hypogaea*

B. Root nodules of *Arachis hypogaea*



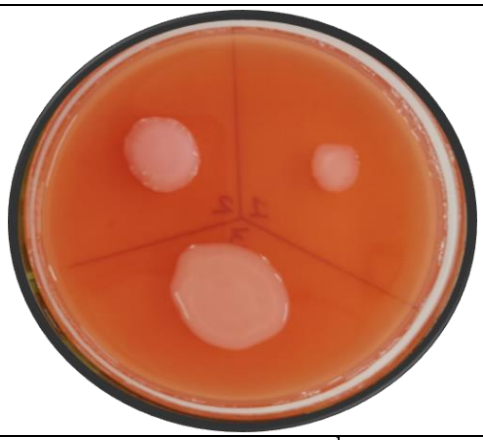
C. Serial dilution of root nodules extract



D. Serial dilution of root extract

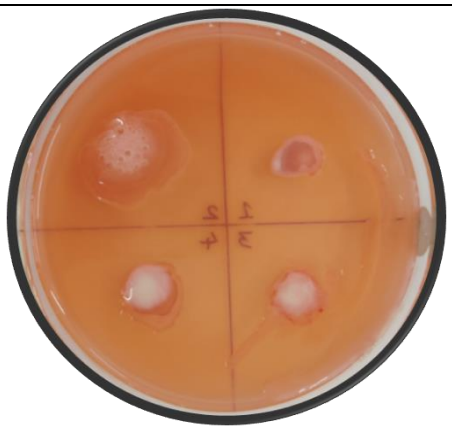
Plate 4.1: Isolation of *Agrobacterium rhizogenes* from the root nodules of *Arachis hypogae*

Root nodules



Dilution 10^{-1}

Roots



Dilution 10^{-4}

Plate 4.2: Growth of bacterial isolates on CRYEMA medium

B. CULTURAL TESTS

Table 4.3. Growth of bacterial isolates (Root nodules) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-1}	1	Don't absorb Congo Red
	2	Don't absorb Congo Red
	3	Don't absorb Congo Red

Table 4.4. Growth of bacterial isolates (Roots) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-4}	1	Absorb Congo red
	2	Absorb Congo red
	3	Absorb Congo red
	4	Absorb Congo red

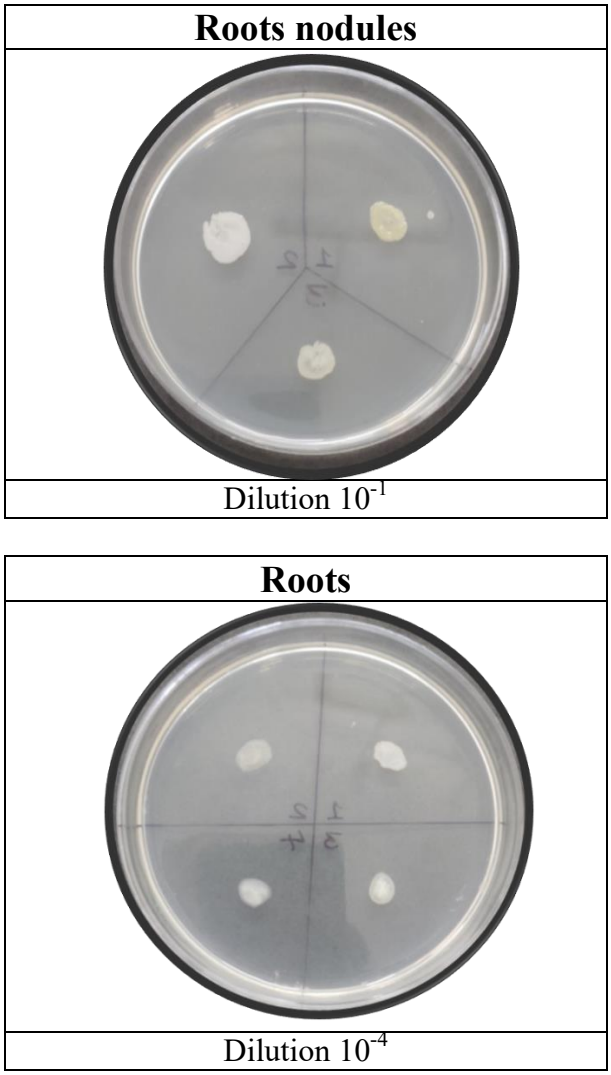


Plate 4.3: Growth of bacterial isolates on Glucose Peptone Agar medium

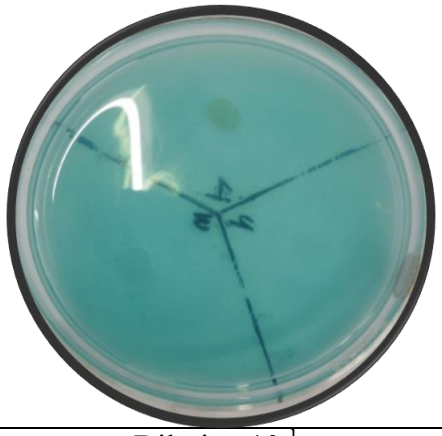
Table 4.5. Growth of bacterial isolates (Root nodules) on Glucose Peptone Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	Moderate growth
	2	Moderate growth
	3	Moderate growth

Table 4.6. Growth of bacterial isolates (Roots) on Glucose Peptone Agar medium

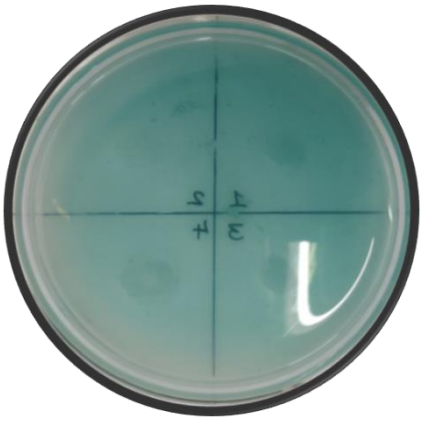
Dilutions	Colony no.	Observations
10^{-4}	1	Moderate growth
	2	Moderate growth
	3	Moderate growth
	4	Moderate growth

Root nodules



Dilution 10^{-1}

Roots



Dilution 10^{-4}

Plate 4.4: Growth of bacterial isolates on Lactose Agar medium with Benedict's reagent

Table 4.7. Growth of bacterial isolates (Root nodules) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration

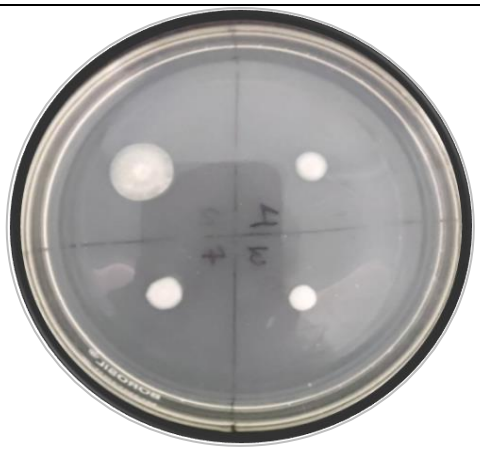
Table 4.8. Growth of bacterial isolates (Roots) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-4}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
	4	No yellow coloration

Root nodules

Dilution 10^{-1}

Roots



Dilution 10^{-4}

Plate 4.5: Growth of bacterial isolates on Potato Dextrose Agar medium

C. *AGROBACTERIUM-SPECIFIC TESTS*

Table 4.9. Growth of bacterial isolates (Root nodules) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	High growth
	2	Moderate growth
	3	Moderate growth

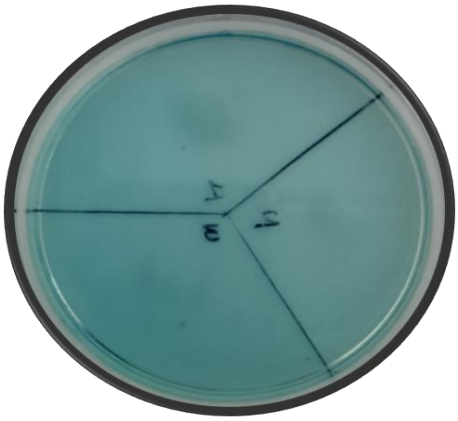
Table 4.10. Growth of bacterial isolates (Roots) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-4}	1	Moderate growth
	2	Moderate growth
	3	Moderate growth

Root nodules

Dilution 10^{-1}

Roots



Dilution 10^{-4}

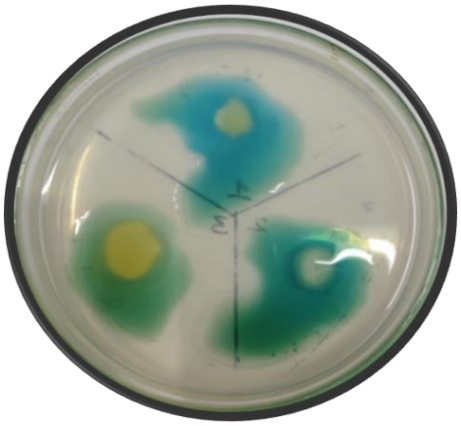
Plate 4.6: Production of ketolactose by bacterial isolates

Table 4.11. Production of ketolactose by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration

Table 4.12. Production of ketolactose by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-4}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration

Root nodules

Dilution 10^{-1}

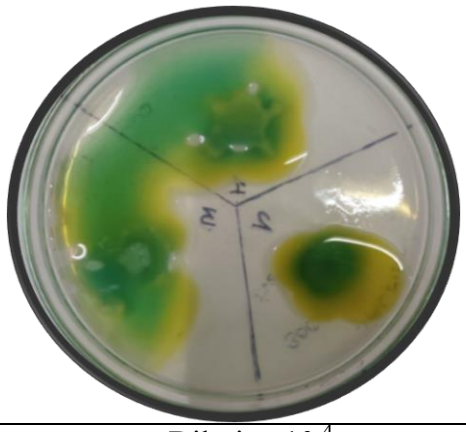
Roots

Dilution 10^{-4}

Plate 4.7: Citrate utilization activity by bacterial isolates

Table 4.13.Citrate utilization activity by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	Blue
	2	Green
	3	Green

Table 4.14. Citrate utilization activity by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-4}	1	Greenish yellow
	2	Greenish yellow
	3	Greenish yellow

4.1.2 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Vigna radiata*

A. GRAM STAINING TEST

Table 4.15.Gram staining results of bacterial colonies isolated from the root nodules of *Vigna radiata*

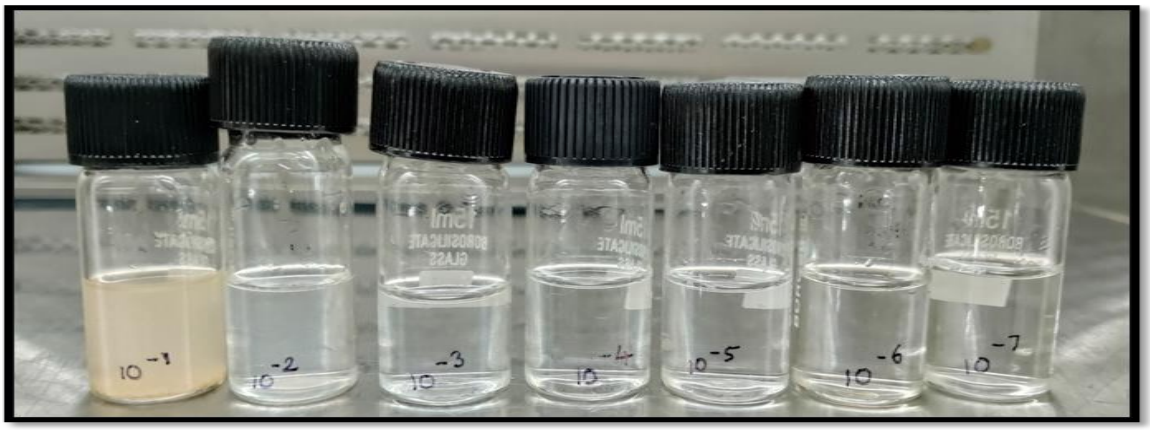
Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	5	1	6
10^{-2}	4	0	4
10^{-3}	4	0	4
10^{-4}	3	0	3
10^{-5}	2	0	2
10^{-6}	2	0	2
10^{-7}	0	1	1

Table 4.16. Gram staining results of bacterial colonies isolated from the roots of *Vigna radiata*

Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	Over growth		
10^{-2}	Over growth		
10^{-3}	1	3	4
10^{-4}	0	1	1

A. Plant of *Vigna radiata*

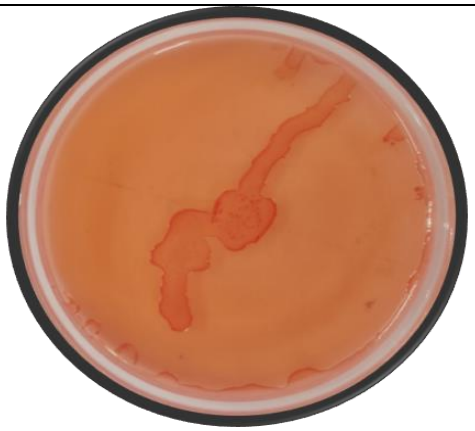
B. Root nodules of *Vigna radiata*



C. Serial dilution of root nodules extract

D. Serial dilution of root extract

Plate 4.8: Isolation of *Agrobacterium rhizogenes* from the root nodules of *Vigna radiata*

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

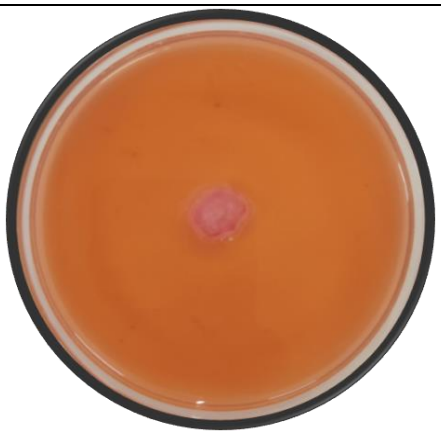
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.9: Growth of bacterial isolates on CRYEMA medium

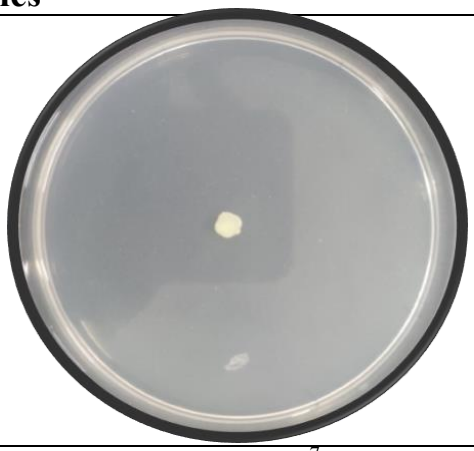
B. CULTURAL TESTS

Table 4.17.: Growth of bacterial isolates (Root nodules) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-1}	1	Absorb Congo red
10^{-7}	1	Absorb Congo red

Table 4.18. Growth of bacterial isolates (Roots) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-3}	1	Absorb Congo red
	2	Absorb Congo red
	3	Absorb Congo red
10^{-4}	1	Absorb Congo red

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

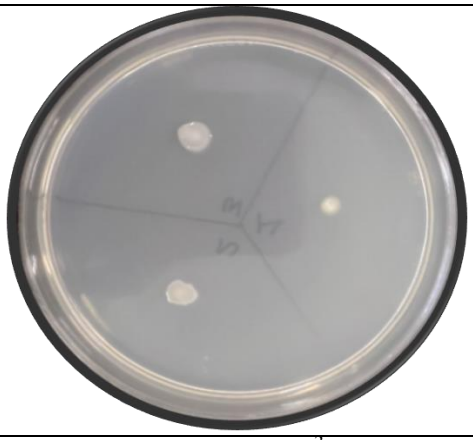
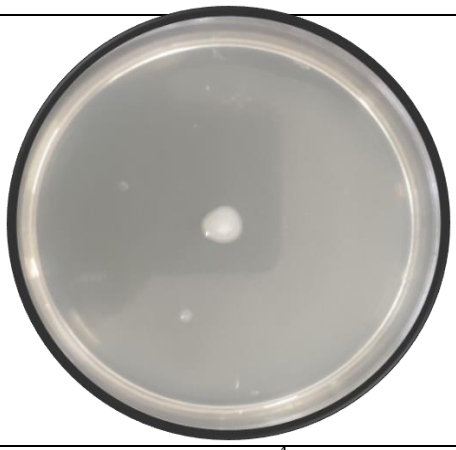
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.10: Growth of bacterial isolates on Glucose Peptone Agar medium

Table 4.19. Growth of bacterial isolates (Root nodules) on Glucose Peptone Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	Less growth
10^{-7}	1	Less growth

Table 4.20.Growth of bacterial isolates (Roots) on Glucose Peptone Agar medium

Dilutions	Colony no.	Observations
10^{-3}	1	Less growth
	2	Moderate growth
	3	Moderate growth
10^{-4}	1	Moderate growth

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

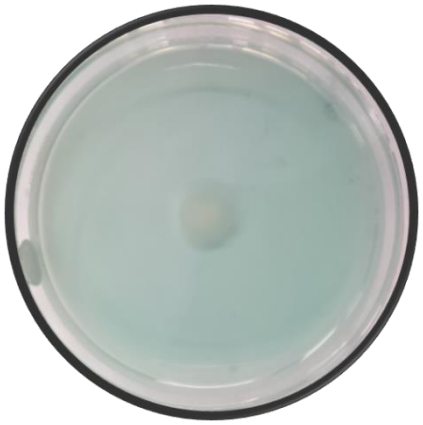
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

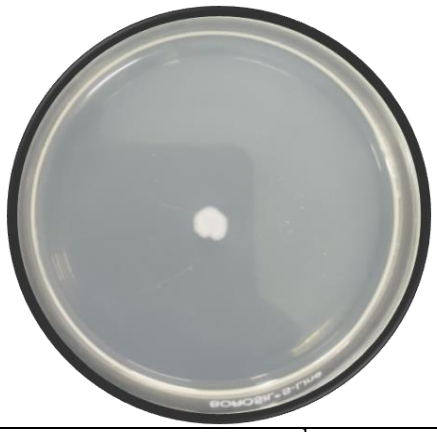
Plate 4.11: Growth of bacterial isolates on Lactose Agar medium with Benedict's reagent

Table 4.21. Growth of bacterial isolates (Root nodules) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
10^{-7}	1	No yellow coloration

Table 4.22. Growth of bacterial isolates (Roots) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-3}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
10^{-4}	1	No yellow coloration

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

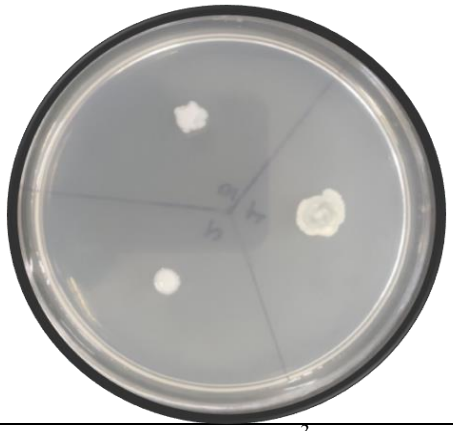
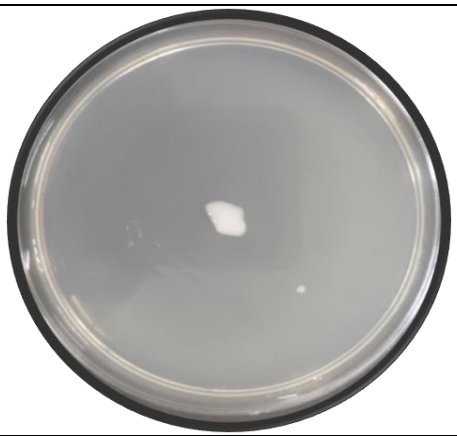
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.12: Growth of bacterial isolates on Potato Dextrose Agar medium

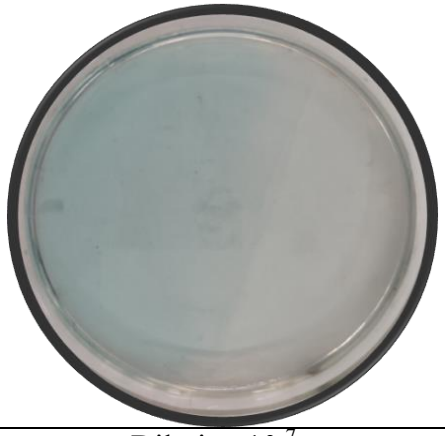
C. *AGROBACTERIUM*-SPECIFIC TESTS

Table 4.23. Growth of bacterial isolates (Root nodules) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	Less growth
10^{-7}	1	Moderate growth

Table 4.24. Growth of bacterial isolates (Root) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-3}	1	High growth
	2	Moderate growth
	3	Moderate growth
10^{-4}	1	Moderate growth

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

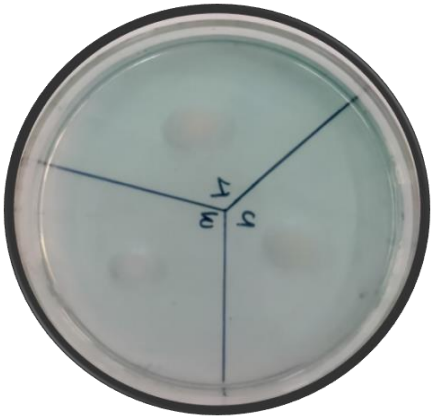
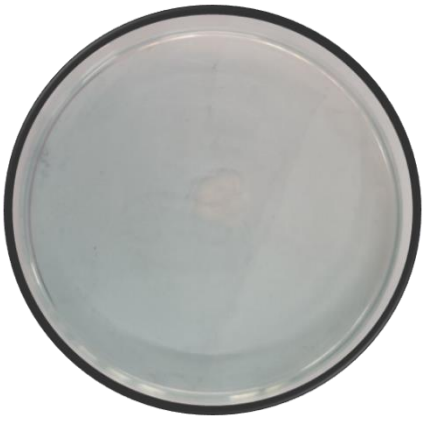
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.13: Production of ketolactose by bacterial isolates

Table 4.25. Production of ketolactose by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
10^{-7}	1	No yellow coloration

Table 4.26. Production of ketolactose by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-3}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
10^{-4}	1	No yellow coloration

Root nodules	
	
Dilution 10^{-1}	Dilution 10^{-7}

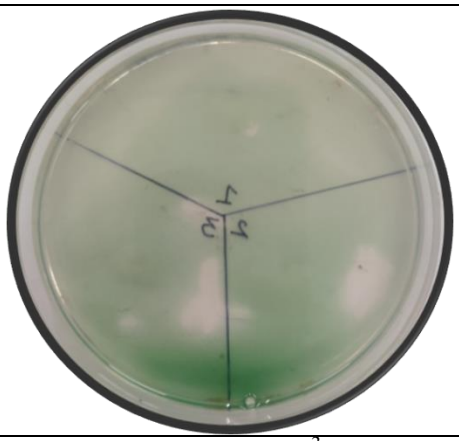
Roots	
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.14: Citrate utilization activity by bacterial isolates

Table 4.27. Citrate utilization activity by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	Green
10^{-7}	1	Green

Table 4.28. Citrate utilization activity by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-3}	1	Green
	2	Green
	3	Green
10^{-4}	1	Green

4.1.3 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Vigna unguiculata*

A. GRAM STAINING TEST

Table 4.29. Gram staining results of bacterial colonies isolated from the root nodules of *Vigna unguiculata*

Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	1	4	5
10^{-2}	1	0	1
10^{-3}	0	0	0
10^{-4}	0	0	0
10^{-5}	0	0	0
10^{-6}	0	0	0
10^{-7}	0	0	0

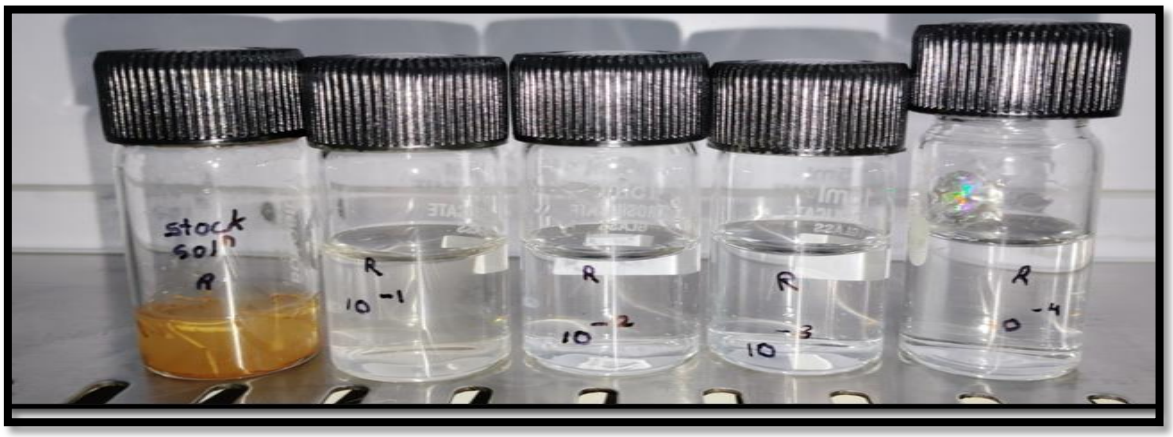
Table 4.30. Gram staining results of bacterial colonies isolated from the roots of *Vigna unguiculata*

Dilutions	No. of Gram positive bacterial colonies	No. of Gram negative bacterial colonies	Total no. of bacterial colonies
10^{-1}	3	2	5
10^{-2}	1	3	4
10^{-3}	1	2	3
10^{-4}	0	1	1

A. Plant of *Vigna unguiculata*

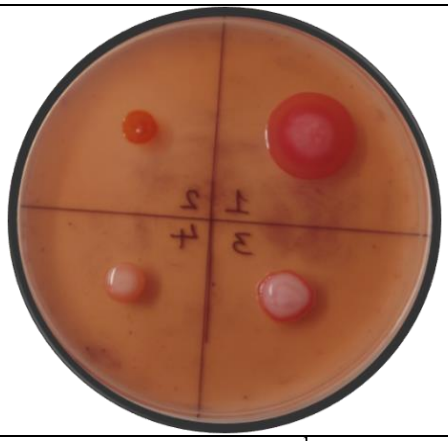
B. Root nodules of *Vigna unguiculata*

C. Serial dilution of root nodules extract



D. Serial dilution of root extract

Plate 4.15: Isolation of *Agrobacterium rhizogenes* from the root nodules of *Vigna unguiculata*

Root nodules

Dilution 10^{-1}

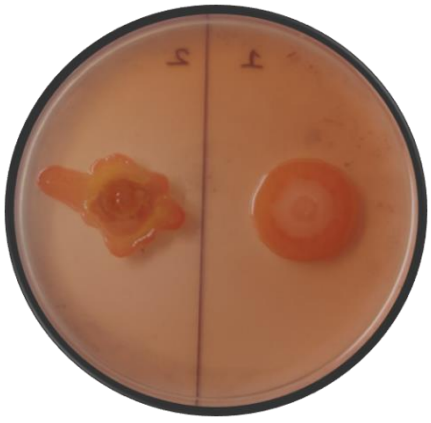
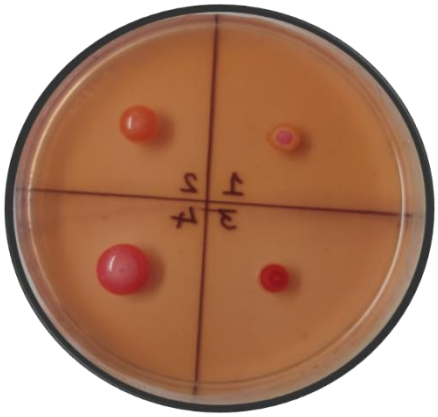
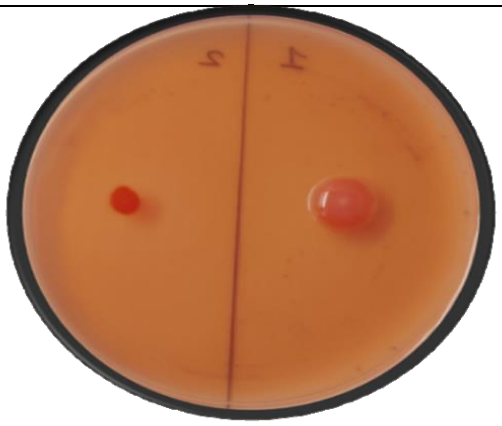
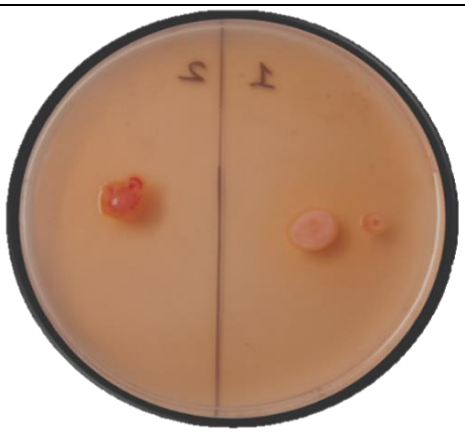
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.16: Growth of bacterial isolates on CRYEMA medium

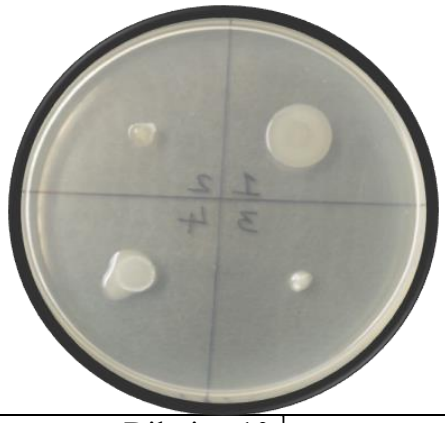
B. CULTURAL TESTS

Table 4.31. Growth of bacterial isolates (Root nodules) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-1}	1	Absorb Congo red
	2	Absorb Congo red
	3	Absorb Congo red
	4	Don't absorb Congo red

Table 4.32. Growth of bacterial isolates (Roots) on CRYEMA medium

Dilutions	Colony no.	Observations
10^{-1}	1	Don't absorb Congo red
	2	Don't absorb Congo red
10^{-2}	1	Don't absorb Congo red
	2	Absorb Congo red
	3	Absorb Congo red
	4	Absorb Congo red
10^{-3}	1	Absorb Congo red
	2	Absorb Congo red
10^{-4}	1	Don't absorb Congo red
	2	Don't absorb Congo red

Root nodules

Dilution 10^{-1}

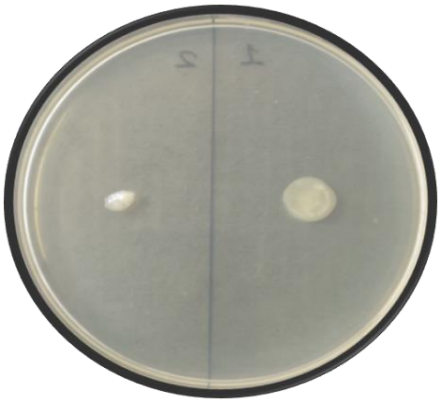
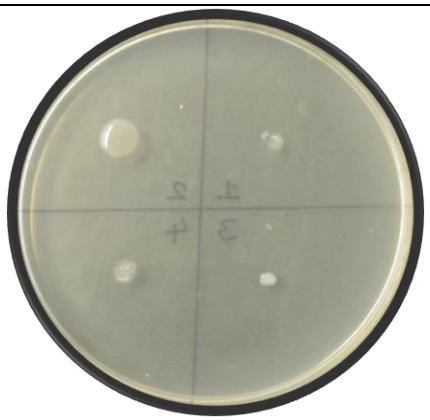
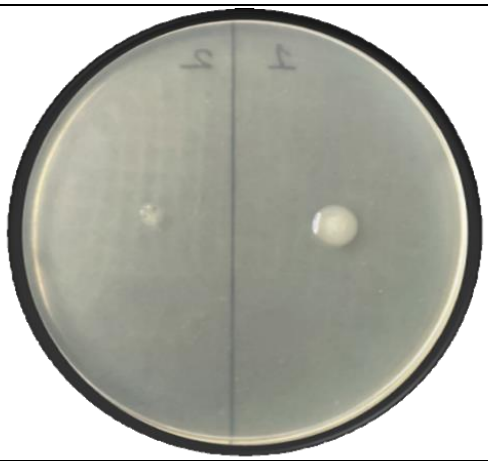
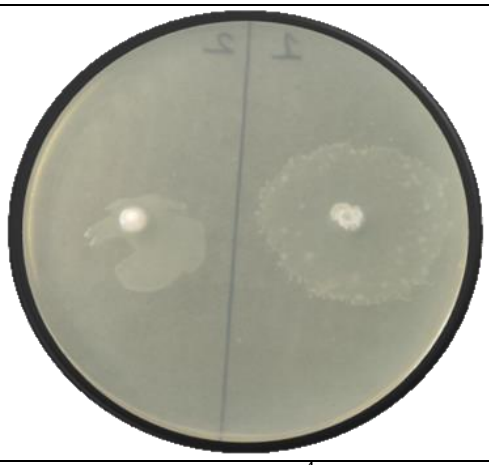
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

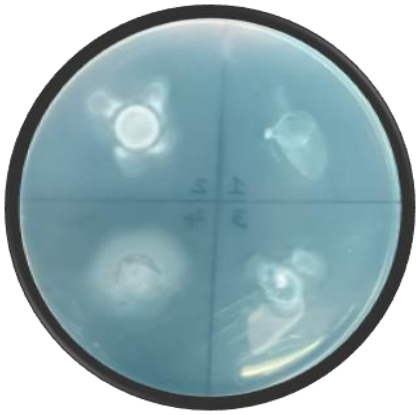
Plate 4.17: Growth of bacterial isolates on Glucose Peptone Agar medium

Table 4.33. Growth of bacterial isolates (Root nodules) on Glucose Peptone Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	High growth
	2	Less growth
	3	Less growth
	4	Moderate growth

Table 4.34. Growth of bacterial isolates (Roots) on Glucose Peptone Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	Less growth
	2	Moderate growth
10^{-2}	1	Less growth
	2	Moderate growth
	3	Less growth
	4	Less growth
10^{-3}	1	Moderate growth
	2	Less growth
10^{-4}	1	Less growth
	2	Less growth

Root nodules

Dilution 10^{-4}

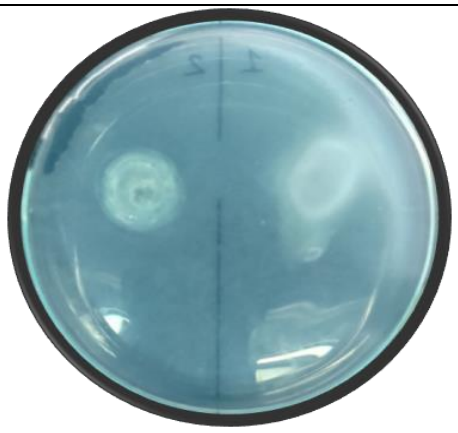
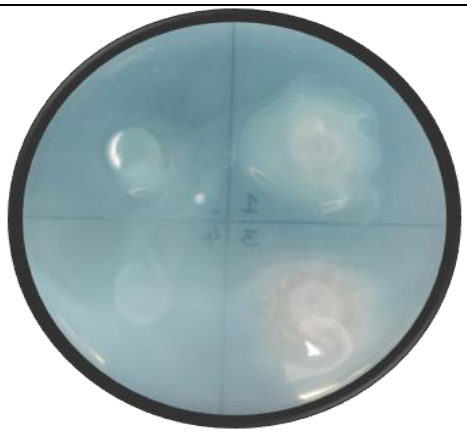
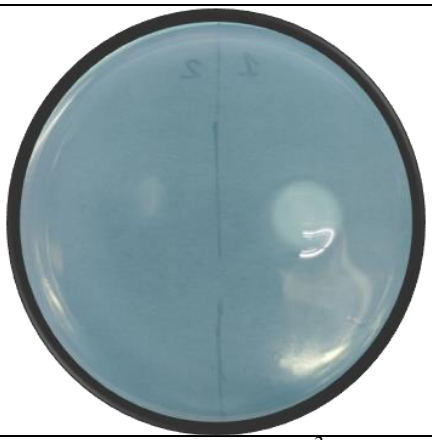
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

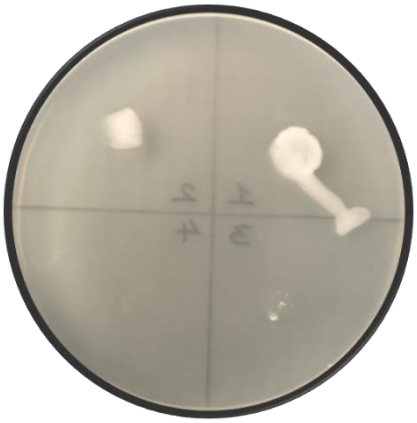
Plate 4.18: Growth of bacterial isolates on Lactose Agar medium with Benedict's reagent

Table 4.35. Growth of bacterial isolates (Root nodules) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
	4	No yellow coloration

Table 4.36. Growth of bacterial isolates (Roots) on Lactose Agar medium with Benedict's reagent

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
10^{-2}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
	4	No yellow coloration
10^{-3}	1	No yellow coloration
	2	No yellow coloration
10^{-4}	1	No yellow coloration
	2	No yellow coloration

Root nodules

Dilution 10^{-1}

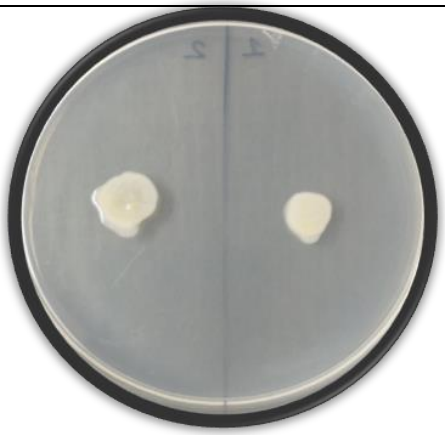
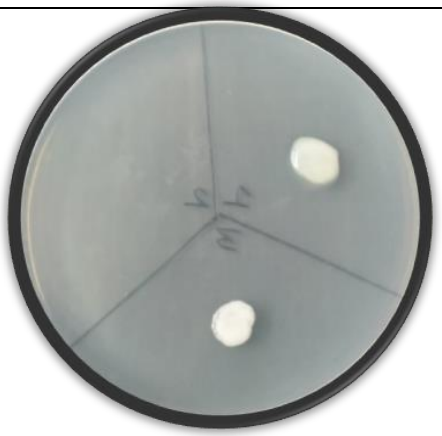
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.19: Growth of bacterial isolates on Potato Dextrose Agar medium

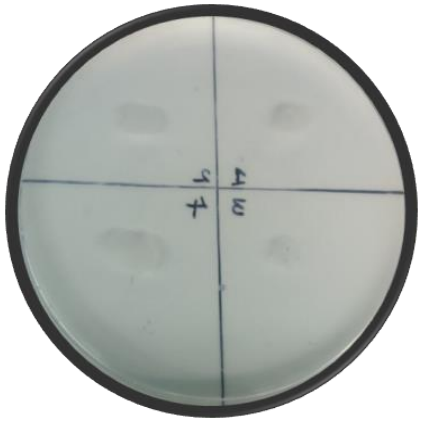
C. *AGROBACTERIUM* -SPECIFIC TESTS

Table 4.37. Growth of bacterial isolates (Root nodules) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	High growth
	2	Moderate growth
	3	No growth
	4	No growth

Table 4.38. Growth of bacterial isolates (Roots) on Potato Dextrose Agar medium

Dilutions	Colony no.	Observations
10^{-1}	1	Moderate growth
	2	Moderate growth
10^{-2}	1	Moderate growth
	2	No growth
	3	Moderate growth
10^{-3}	1	Moderate growth
	2	No growth
10^{-4}	1	Moderate growth

Root nodules

Dilution 10^{-1}

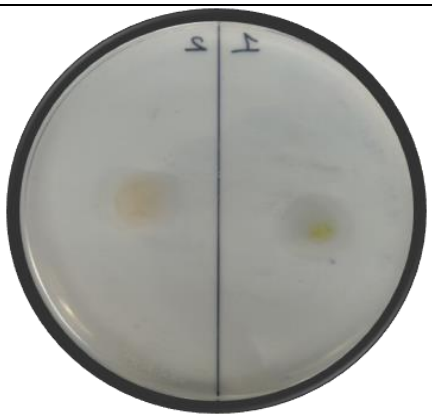
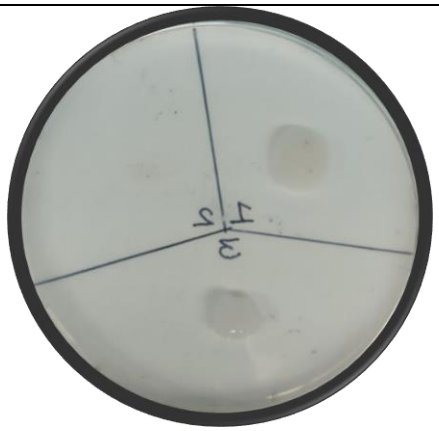
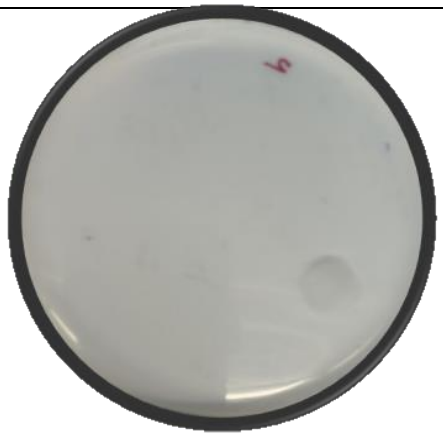
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

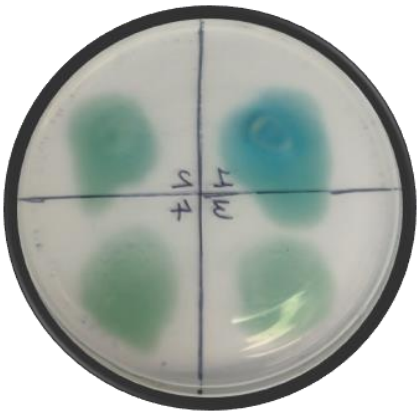
Plate 4.20: Production of ketolactose by bacterial isolates

Table 4.39. Production of ketolactose by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
	4	No yellow coloration

Table 4.40. Production of ketolactose by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-1}	1	No yellow coloration
	2	No yellow coloration
10^{-2}	1	No yellow coloration
	2	No yellow coloration
	3	No yellow coloration
10^{-3}	1	No yellow coloration
	2	No yellow coloration
10^{-4}	1	No yellow coloration

Root nodules

Dilution 10^{-1}

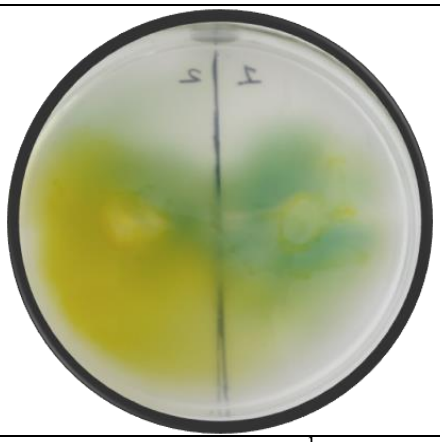
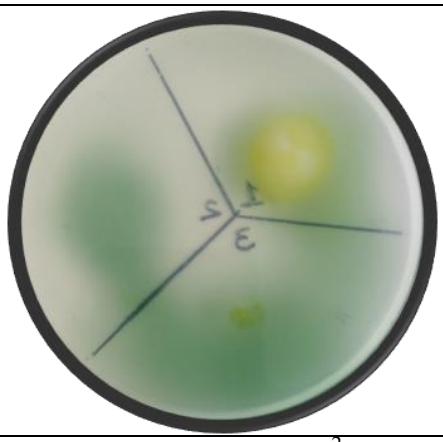
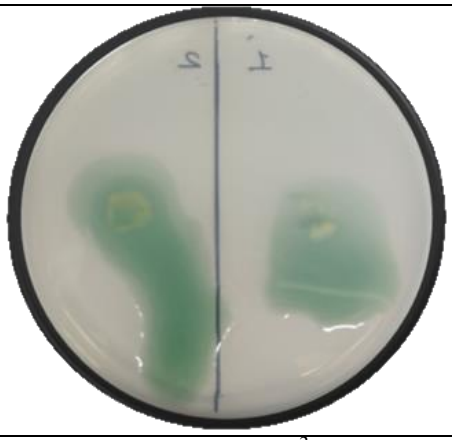
Roots	
	
Dilution 10^{-1}	Dilution 10^{-2}
	
Dilution 10^{-3}	Dilution 10^{-4}

Plate 4.14: Citrate utilization activity by bacterial isolates

Table 4.41. Citrate utilization activity by bacterial isolates (Roots nodules)

Dilutions	Colony no.	Observations
10^{-1}	1	Blue
	2	Green
	3	Green
	4	Green

Table 4.42. Citrate utilization activity by bacterial isolates (Roots)

Dilutions	Colony no.	Observations
10^{-1}	1	Green
	2	Yellow
10^{-2}	1	Yellow
	2	Green
	3	Green
10^{-3}	1	Green
	2	Green
10^{-4}	1	Green

4.2 DISCUSSION

Agrobacterium rhizogenes is a soil bacterium known for its ability to cause hairy root disease in plants. It infects numerous plant species, integrating a segment of its DNA, known as the Ri plasmid, into the host plant's genome. This genetic transfer leads to the formation of hairy roots at the infection site, characterized by rapid growth and increased production of secondary metabolites. These hairy roots are valuable for biotechnological applications, particularly in the production of pharmaceuticals, phytochemicals, and genetic engineering studies. Additionally, *A. rhizogenes* is studied for its potential role in enhancing plant growth, stress tolerance, and soil remediation.

Gram staining is a fundamental technique used to differentiate bacteria based on their cell wall composition. The results of the Gram staining test revealed a notable presence of Gram-negative bacterial colonies, which is consistent with the characteristics of *A. rhizogenes*. In the root nodules, a significant proportion of colonies were Gram-negative, indicating the presence of *A. rhizogenes*. However, in the roots, the results varied, with some dilutions showing Gram-positive colonies, possibly indicating the presence of other bacterial species. In the present study, total 24 gram negative bacterial colonies were isolated from both root nodules and roots of *Arachis hypogaea, Vigna radita* and *V. ungiculata.*

4.2.1 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Arachis hypogaea*

The isolation and characterization of *Agrobacterium rhizogenes* from the root nodules and roots of *Arachis hypogaea* (commonly known as groundnut or peanut) provide valuable insights into the interactions between this bacterium and leguminous plants. The results obtained from various tests shed light on the characteristics and behavior of *A. rhizogenes* in the context of root nodulation.

- **Cultural Tests**

A. Congo red test

CRYEMA is designed to differentiate between cellulose-producing and non-cellulose-producing bacteria. *A. rhizogenes* absorb Congo red due. The bacterial colonies from the root nodules did not absorb Congo red. However, in the roots, there was some variability, with colonies showing both absorbing characteristics.

B. Glucose Peptone Agar test

The growth observed on Glucose Peptone Agar medium was moderate, indicating the ability of A. rhizogenes to utilize glucose and peptone as carbon and nitrogen sources, respectively. This further supports the presence of *A. rhizogenes* in both root nodules and roots.

C. Lactose Agar Medium with Benedict's reagent

The lack of yellow coloration indicates the inability of the bacterial isolates to ferment lactose. This is consistent with *A. rhizogenes*, which typically does not ferment lactose.

- ***Agrobacterium*-Specific Tests**

A. Potato Dextrose Agar test

The high to moderate growth observed on PDA medium suggests that *A. rhizogenes* can utilize potato dextrose as a carbon source. The results from both root nodules and roots confirm the presence of *A. rhizogenes*.

B. Ketolactose test

Ketolactose production is a characteristic feature of *A. rhizogenes*. The lack of yellow coloration in both root nodules and roots indicates the absence of ketolactose production, which is consistent with *A. rhizogenes*.

C. Citrate Utilization Test

Citrate utilization activity provides further evidence of the metabolic capabilities of *A. rhizogenes*. The bacterial colonies show blue and greenish-yellow coloration. The presence of colonies showing blue coloration indicates citrate utilization which may be the identity of *A. rhizogenes*.

4.2.2 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Vigna radiata*

The isolation and characterization of *Agrobacterium rhizogenes* from the root nodules and roots of *Vigna radiata* (commonly known as mung bean) provide valuable insights into the interactions between this bacterium and leguminous plants. The results obtained from various tests are as follows:

- **Cultural Tests**

A. Congo red test

The ability of colonies to absorb Congo red indicates cellulose production. The bacterial colonies isolated from both root nodules and roots don't absorb Congo red, which may include *A. rhizogenes* among other species.

B. Glucose Peptone Agar test

The growth observed varied, with some dilutions showing moderate growth and others showing less growth. This suggests that *A. rhizogenes* may have variable growth patterns depending on the nutrient availability in the medium and the dilution of the inoculum.

C. Lactose Agar Medium with Benedict's Reagent

The absence of yellow coloration indicates that the bacterial isolates do not ferment lactose. This is consistent with the typical characteristics of *A. rhizogenes*.

- **Agrobacterium-Specific Tests**

A. Potato Dextrose Agar test

The growth observed on this medium varied, with some dilutions showing high or moderate growth, while others showed less growth. This indicates that *A. rhizogenes* may utilize potato dextrose as a carbon source under certain conditions.

B. Ketolactose test

The absence of yellow coloration indicates that ketolactose was not produced by the bacterial isolates. This is consistent with *A. rhizogenes*, which typically does not produce ketolactose.

C. Citrate Utilization test

The bacterial colonies isolated from both root nodules and roots show green coloration .This indicates the absence of *A. rhizogenes* in the both root nodules and roots of *Vigna radiata*.

4.2.3 Isolation of *Agrobacterium rhizogenes* from the root nodules and roots of *Vigna unguiculata*

The isolation and characterization of Agrobacterium rhizogenes from the root nodules and roots of *Vigna unguiculata* (cowpea) provide insights into the diversity of bacterial interactions with leguminous plants. The results obtained from various tests are as follows:

- **Cultural Tests**

A. Congo red test

The ability of colonies to absorb or not absorb Congo red indicates cellulose production. The results indicate variability in cellulose production among the isolates, which may suggest differences in metabolic activity and strain diversity.

B. Glucose Peptone Agar test

The growth observed varied, with some dilutions showing high or moderate growth and others showing less growth. This variability may reflect differences in the nutrient utilization capabilities of the bacterial isolates.

C. Lactose Agar Medium with Benedict's Reagent

The absence of yellow coloration indicates the inability to ferment lactose, which is consistent with the characteristics of *A. rhizogenes*.

- **Agrobacterium-Specific Tests**

A. Potato Dextrose Agar test

The growth observed varied among dilutions, indicating different metabolic capabilities and responses to the medium.

B. Ketolactose test

The absence of yellow coloration indicates that ketolactose was not produced, which aligns with the typical characteristics of *A. rhizogenes.*

C. Citrate Utilization test

The observed colours indicate varying levels of citrate utilization among the isolates. This variability may reflect differences in metabolic activity and strain diversity. A colony show blue coloration due to citrate utilization activity. This indicates the presence of *A. rhizogenes* in the root nodules of *Vigna unguiculata.*

CHAPTER 5: CONCLUSION

In the present study, total 24 gram negative bacterial colonies were isolated from both root nodules and roots of *Arachis hypogaea, Vigna radita* and *V. ungiculata.* Out of which only one agrobacterial colony was isolated from the root nodules (Dilution10^{-1}; Colony no. 1) of *Vigna ungiculata.* In YEMA medium, the agrobacteria absorbed Congo red. This colony showed well pronounced growth in glucose peptone agar. Yellow colouration was not found in lactose agar with Benedict's reagent. These studies confirmed that *Agrobacterium* species and not *Rhizobium* was isolated from the root nodules of *V. ungiculata.*

The confirmation of *Agrobacterium* was made by the specific tests such as growth on Potato Dextrose Agar medium, ketolactose test and citrate utilization test. On Potato Dextrose Agar medium, the isolates showed well pronounced growth. In ketolactose test, no yellow coloration was found around the colony of the bacterium when plates were flooded with Benedict's reagent. In the citrate utilization test using bromothymol blue solution, a change in colour of the solution from green to blue was observed. This indicated the utilization of citrate as a carbon source.

Based on cultural (Congo red Test, Growth on Glucose Peptone Agar and Lactose Agar Test) and *Agrobacterium* specific tests (Growth on Potato Dextrose Agar, Ketolactose Test and Citrate utilization Test) the isolated organism from the root nodules of *Vigna unguiculata* (Dilution 10^{-1}; Colony no.1) appears to be *Agrobacterium rhizogenes*.

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