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ISOLATION AND CHARACTERIZATION OF MICROBIAL COMMUNITIES FROM CICER ARIETINUM L. (CHICKPEAS) ROOT NODULES

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Abstract

The Agrobacterium of various leguminous plants show a number of variations, and they were identified and classified as Agrobacterium rhizogenes based on their antibiotic resistance, pathogenicity, microbiology, and presence of plasmid. The aim of present study was to isolation and characterization of microbial communities existing in the root nodules of chickpea plants, with a specific focus on the isolation of Agrobacterium species. The effective success of these objectives delivers appreciated insights into the diversity and potential functional characters of microbial communities associated with root nodules of chickpeas, specifically highlighting the existence of Agrobacterium as a significant component. The isolates from the root nodules were grown on LB agar media. Subsequently, the identification of Agrobacterium was carried out using various tests, including the antibiotic resistance test, gram-staining, microscopy, and pathogenicity test. As well as the plasmid DNA of the isolated bacterial strain was extracted and confirmed by the gel electrophoresis. Once the presence of Agrobacterium will be confirmed, knockouts can be generated by removing the tumor-causing genes from the Ti plasmid (tumor-inducing), resulting in the creation of Agrobacterium mutants. The mutants can be then subjected to sequencing analysis.

INTRODUCTION

Agrobacterium is a soil-born gram-negative phytopathogen that naturally infects plants at the site of the wound and causes crown gall disease by shifting their transferred DNA (T-DNA) into the host plant cells (Ali, 2016). Through the latest and most advanced molecular biology techniques scientists discovered various bacterial strains that are involved in tumorigenesis, during the past few years.

With the help of complete knowledge, of how Agrobacterium transfers their DNA into host plant cells. Agrobacterium has become the most popular and most useful tool for plant transformation nowadays. Any gene can be replaced with the tumor-causing gene in T-DNA to perform plant genetic transformation with Agrobacterium.

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In the scientific history, a peculiar disease affecting plants puzzled researchers. After a long time this disease became known as crown gall. In 1679 a biologist Malpighi first time described that, the phenomenon of abnormal growths on plants, called galls. He believed these curious formations appeared spontaneously, without any known cause. Fast forward to 1897, a Italian scientist Fridiano Carva, a diligent scientist in Napoli, Italy, isolated a bacterium from these tumors and named it Agrobacterium (Carva, 1897). It was the first step towards unraveling the mystery of crown gall disease (Kado, 2014).

Once it was confirmed that the crown gall-causing bacterium is Agrobacterium, the next question was the mechanism by which this bacterium causes tumors in plants. In 1917, a scientist used *Ricinus communis L.* as a host for the Agrobacterium to determine the mechanism of of tumor formation in plants. After a large number of experimental tests he stated that "Diluted ammonia induces intumescences, suggesting that ammonia released within the cell in small quantities by the enclosed bacteria likely contributes to excessive and abnormal cell proliferation in crown gall." Then it was thought that the Agrobacterium was penetrated into the host plant cells (Smith, 1917). Through mechanical inoculations, it was confirmed that almost 90 families of plant species are susceptible to the crown gall disease (Kado, 2010).

In 1942, scientist Armin C. Braun demonstrated crown gall formation derived from secondary crown gall, which means bacteria-free crown gall, by using serological and cultural methods. These findings introduced the idea of genetic transformation into host plant cells from infected plant cells. He also discovered that tumor cells grow well without phytohormones, as these plant hormones are required for the growth of normal plant cells (White P. R., 1942). After that, many scientists proposed that the induction of crown gall involved by DNA transformation from bacteria to host plant cells (Srivastava, 1969) (Srivastava B. I. S., 1970) (Stroun M., 1971). So, the question that arises after that is how bacterial DNA is transferred into the host plant cells? To answer these questions, Gelvin and Virts (Gelvin, 1985) conducted some experiments. They used infected *Petunia* protoplasts with Agrobacterium and found bacterial DNA in the host plant cells within 2 to 6 hours, but the DNA started degrading rapidly. Later in 1986, it was confirmed that the bacterial cellulose fibrils played an important role in attachment (Matthysee, 1986). In 2000, scientists confirmed that plant genes and Agrobacterium are involved in T-DNA transformation. The large extrachromosomal plasmid DNA, also known as Ti (tumor-inducing plasmid), contains some virulence genes responsible for tumor formation (Gelvin, 2000).

Agrobacterium and rhizobium are closely related and both have a unique capacity for root proliferation. These bacterial species are very important in the agricultural industry and are important tools to understand the mechanisms of pathogenesis, plant morphogenesis, and plant-microbe interactions (Balows, 1992). There are some specific features of pathogenic strains of Agrobacterium. They contain at least one large plasmid which contains some virulent genes. The virulence of the plasmid DNA can be determined by specific regions of plasmid DNA, including the virulence genes and transferred DNA (T-DNA) (S. Murugesan, 2011).

This research will contribute to our understanding of the root-associated microbial communities in *Cicer arietinum* and their potential contributions to plant health and productivity. Our spatial focus was on the isolation and identification of some local strains of Agrobacterium from chickpea root nodules. After the isolation of bacteria, various experimental tests were done for the identification of isolated bacteria including, antibiotic resistance tests, pathogenicity tests, gram staining and microscopy, plasmid DNA extraction and gel electrophoresis.

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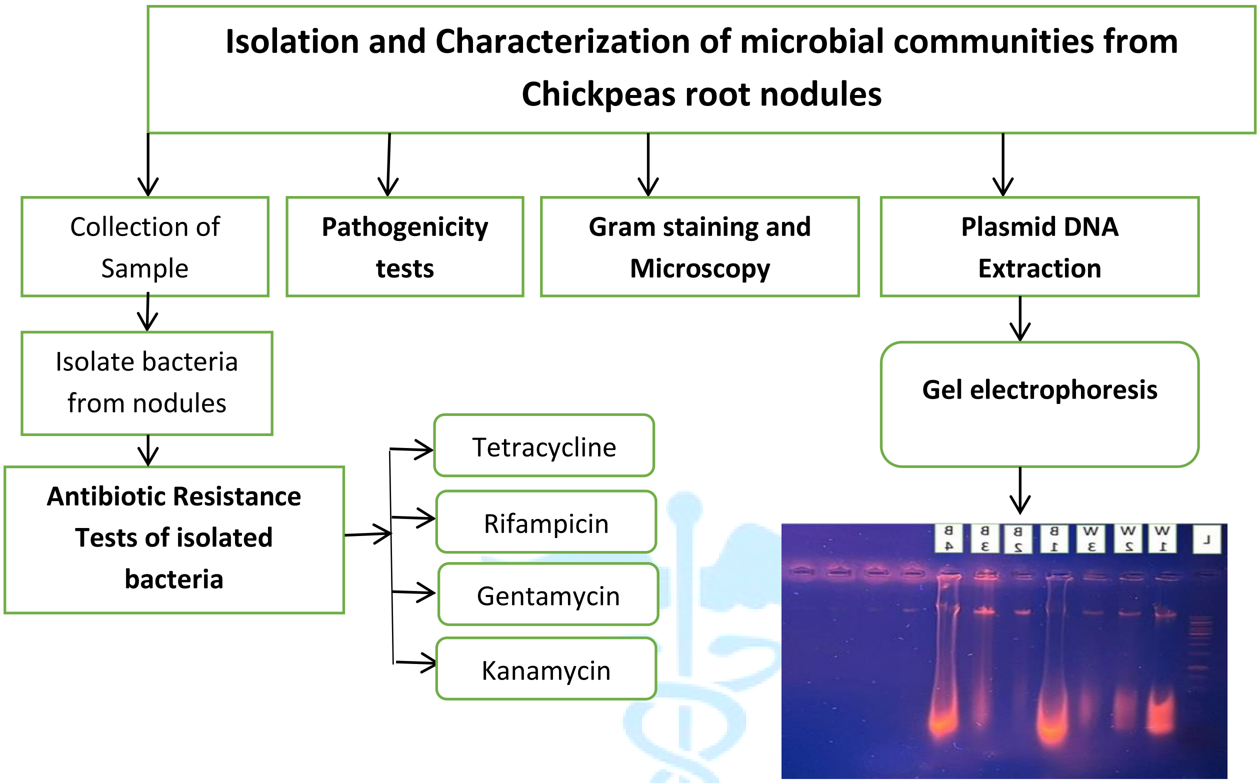

Fig. 1 Schematic representation of the experimental design for isolating and characterizing microbial communities from chickpea root nodules, including sampling, isolation, and identification tests.

1. Material and Methods:

2.1 Collection of samples:

The samples were taken from the Center of Advanced Studies (CAS) department, University of Agriculture, Faisalabad. The samples of plants along with roots were collected from the field after the sowing of 50 days and rinsed carefully with water to remove soil and large and pink nodules were selected for the isolation of Agrobacterium, after the collection of healthy and unharmed plant samples of chickpeas (*Cicer arietinum*) from the different fields. The plants were uprooted and shaken to remove the loosely attached soil, and then the nodules were washed with water to remove the adhered soil.

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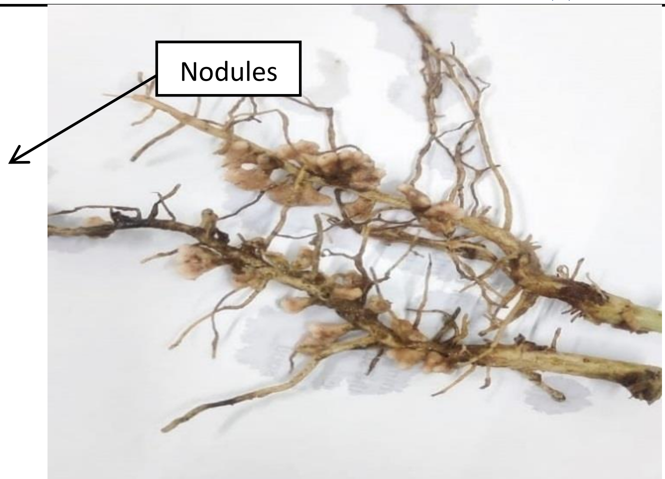

Fig. 2.1. Roots of chickpea plants showing nodules. Healthy roots with visible nodules used for bacterial isolation

2.2 Isolation of bacterial strains from root nodules:
After the collection of samples, the nodules were separated carefully. The large size of nodules was selected to isolate the bacteria. The nodules were washed with 90% alcohol for 5-10 seconds. Then nodules were submerged in 0.1% mercuric chloride solution for 5 minutes. Then rinsed with sterilized distilled water thoroughly six times to eliminate the chemicals. Then the nodules were crushed aseptically using an autoclaved pestle and mortar and the extract from the root nodule was streaked on the LB (Luria-Bertani) agar plates and incubated these plates at 28^{o}C for 3-4 days. The growth of rhizobium was shown on plates, then the Agrobacterium was isolated by using selective media. The bacteria were grown on the plates and after incubation developed into colonies.

Table No. 2.1. Recipe for the preparation of LB media

Ingredients	**Quantity**
Tryptone	1 g/L
NaCl	1g/L
Yeast Extract	0.5g/L
Agar	1.5g/L

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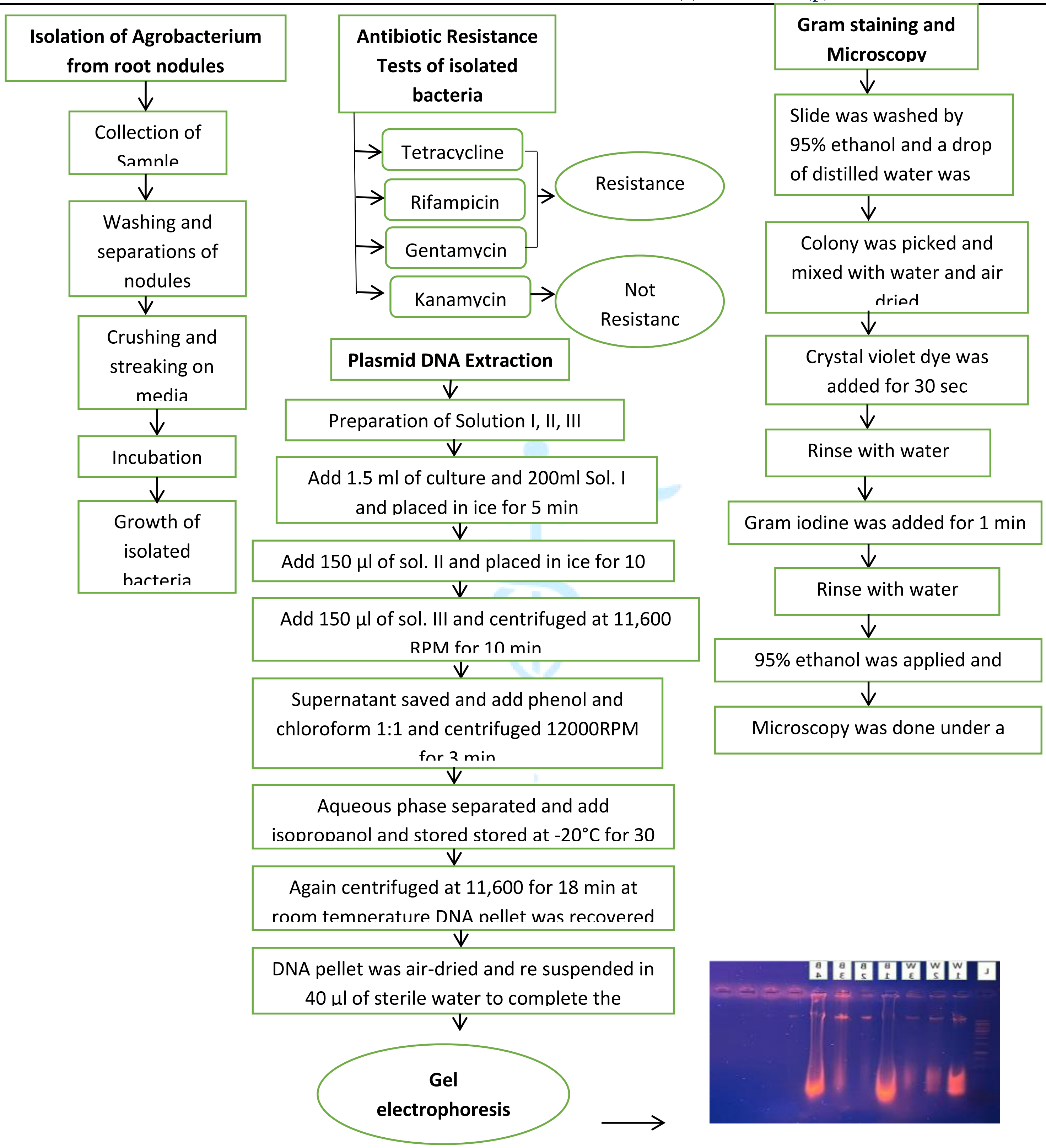

Fig. 2.2 Technical roadmap of the study. Flowchart of the experimental workflow for isolation and characterization of Agrobacterium.

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.3 Identification and Characterization:

Some tests were performed for the identification and characterization of Agrobacterium from the isolates of nodules. Such as antibiotic resistance tests, gram staining and microscopy, pathogenicity tests, and polymerase chain reaction (PCR).

2.3.1 Antibiotic Resistance Test:

The Luria Broth (LB) media was prepared and the colonies were grown on the LB agar media containing the antibiotics such as tetracycline. The solutions of antibiotics were formed and tested the colonies on different antibiotics including tetracycline, rifampicin, kanamycin, and gentamicin to confirm our bacteria. Tetracycline is the best antibiotic for the selection of Agrobacterium cells.

Table No. 2.2. Recipe of 10 ml Antibiotic solutions preparation

Ingredients	Quantity mg	Distilled Water ml
Kanamycin	0.5 g	10 ml
Gentamycin	100 mg	10 ml
Rifampicin	0.1 g	Methanol 5ml

2.4 Preparation of Primary Culture:

The primary culture was prepared for the making of dilutions of the bacteria for the gram staining and microscopy and for the isolation of the plasmid DNA of the bacteria. For the preparation of primary culture test tubes were autoclaved. LB broth was also prepared and poured the LB broth media into the test tubes the colony was picked up by the autoclaved toothpick from the cultured plates and inoculated into the test tubes which contains LB broth. Then the test tubes were placed on a shaking incubator for 2 days at 28 °C at 120 RPM.

2.5 Gram-Staining and Microscopy:

Gram staining was done to check whether the bacteria are gram-positive or gram negative. The slide was washed with 95% ethanol then one drop of distilled water was added to the slide and a bacterial colony was picked and mixed with water. And the slide air-dried. Then, with the help of a dropper, crystal violet dye was added to the slide for 30 seconds. The slide was rinsed 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 plate with distilled water. Then, safranine was applied and again rinsed the slide with distilled water. After this red color was shown which confirmed that the bacteria are gram negative.

2.5.1 Microscopy:

After the gram-staining, microscopy was done to confirm the isolated bacteria. The slide was dried completely and microscopy was done under a 100X lens. Here it was confirmed that the isolated bacteria are gram-negative.

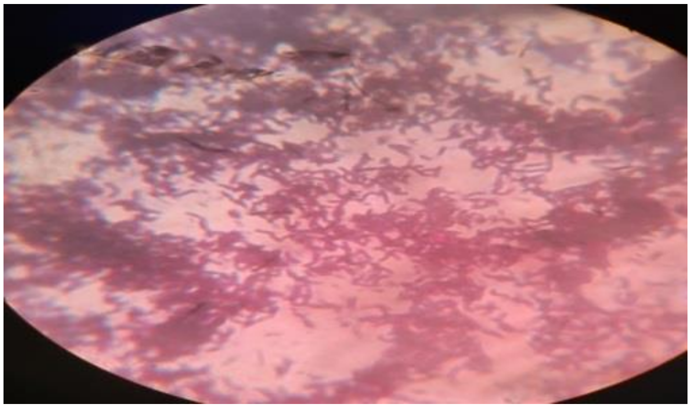

Fig. 2.4 Microscopy of isolated bacteria. Gram-stained cells observed under 100X magnification, confirming Gram-negative morphology.

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2.6 Pathogenicity test:
Chickpeas were grown in the growth room of the lab. After 15 to 20 days of sowing, the bacteria were inoculated into the chickpea. To check the pathogenicity of isolated Agrobacterium.

2.7 Extraction of Plasmid DNA:
Plasmid DNA was extracted from the primary culture. Three solutions were prepared including, Solution I, solution II, and solution III to extract plasmid DNA. Cells were collected by centrifugation at 11,600 x 8 for I min after 14-16 hours of incubation at 28 degrees Celsius. Using 1.5 ml of culture as a starting point, 200 ml of solution I was added to the pelleted cells to resuspend them. After they had been vortexed, the tubes were placed on ice for 5 minutes to allow them to cool down. 150 ul of solution II was administered to the cells, which were then placed for 10 min on ice to denature the contents of their cytoplasm.
In the next step, 150 µl of solution III was added, carefully vortexed, and allowed to incubate on ice for 10 minutes. Then it was centrifuged at 11,600 RPM for 10 min, the supernatant was shifted into fresh Eppendorf tubes for further analysis. In the reaction vessel, a comparable volume of phenol and chloroform 1 ratio 1 was poured and well stirred until the phenol was completely dissolved. This was centrifuged at 12000RPM for 3 min to separate the phenolic and aqueous phases. The top aqueous phase was transferred to a fresh Eppendorf tube for further analysis The two phases were divided once again by centrifugation at 11,600 rpm for 2-3 minutes at 4 degrees Celsius, followed by the addition of an equivalent amount of chloroform and mixing the two phases. The use of chloroform at this point improves the quality of the DNA in the upper phase of the reaction. The aqueous layer at the top of the tube was moved to a new tube in the following step. A chilling dose of isopropanol (2.5 volumes) was added to the upper aqueous phase after thorough mixing, and the mixture was then stored at -20°C for 30 min. DNA precipitation would be aided as a result of this. After centrifuging at 11,600 for 18 min at room temperature, the DNA pellet was recovered. The supernatant was carefully discarded, and the DNA pellet was air-dried and re suspended in 40 µl of sterile water to complete the reaction.

Table No. 2.3. Composition of solution for Miniprep

Ingredients	Quantity (per Liter)
Sol I	
RNase	1.0 uL
d_3H_2O	92 mL
0.5 M EDTA	2.0 mL
2M Glucose	2.5 mL
1M Tris	2.5 mL
Sol II	
d_3H_2O	8.5 mL
10% SDS	1.0 mL
5M NaOH	0.5 mL
Sol III	
5 M KOH	28.5 mL
d_3H_2O	60 mL
96% Acetic Acid	11 mL

2.7.1Gel Electrophoresis:
An agarose gel with a concentration of 0.8 percent was run to examine the quantity and size of plasmid DNA based on charge and mass ratio. TBE buffer with a concentration of 0.5X was used for the manufacture of agarose gel, and the gel tank was

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filled. TBE Buffer with a concentration of twenty times the stock solution was produced. It was withdrawn from the graduated cylinder containing 150 ml of TBE buffer (0.5X), and it was put into a graduated flask containing 250 ml. To make a gel with a concentration of 0.8 percent, 1.2 grams of agarose were added, and the mixture was cooked for two minutes in the oven. After the mixture had cooled, 0.5 microliters of ethidium bromide were added before the flask was gently shaken. A casting tray was taken, and the gel was put into it. The wells in the gel were created with a comb. After the gel has been solidified, add three microliters of bromophenol blue dye to the sample, and mix it well. We started by loading the wells with the sample. Additionally, a 1 KB ladder was inserted into a separate well. The apparatus was left at 85 volts for a period of half an hour. At the very end, we removed the flowing gel, positioned it over the UV transilluminator, and examined the bands on the samples.

Tabe No. 2.4. Composition of 20X TBE Buffer

Ingredients	**Tris Quantity (g\I)**
Tris Base	216
Boric Acid	110
EDTA	14.85

Table No. 2.5. Composition of 0.5X TBE Buffer

Ingredients	**Quantity**
20X TBE	25ml
Water	1L

Table No. 2.6. Buffers for agarose gel electrophoresis

50X Tris acetate EDTA buffer (TAE)		
Tris base	121.0 g	A small amount of tris base was diluted, and the remaining chemicals were added in the proper proportions. Finally, using distilled water, then the total volume increased to 500 mL.
Glacial acetic acid	28.55 ml	
0.5 M EDTA (pH 8.0)	100 ml	

Table No. 2.7. Ethidium Bromide Solution

Ethidium Bromide Solution		
Ethidium Bromide (5 mg/L)	0.5 g	With the help of a magnetic stirrer, mix the ingredients until the volume is 100 mL. Store in the refrigerator or cover the container in aluminum foil.
Water	45 ml`	

Table No. 2.8. Prepration of 1 % Agrose gel

Ingredients	**Quantity g/ml**
Agarose	2 g

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TBE Buffer	200 ml
Pyrex Bottle	500 ml

2. **Results and Discussion:**

In the present study, we did some experiments to identify the isolated bacteria strains from the root nodules of chickpeas by different tests like biochemical tests, pathogenicity tests, antibiotic sensitivity tests, gram-staining, and finally the PCR will be done to confirm the isolated bacterial strains from the root nodules of chickpeas.

The appearance and the color of the colonies were milky white and brown. The colonies we obtained from the root nodules of chickpeas were of two types. One was milky white and the second was brown. Both were antibiotic-resistant (tetracycline, rifampicin, and gentamicin) and both were gram-negative.

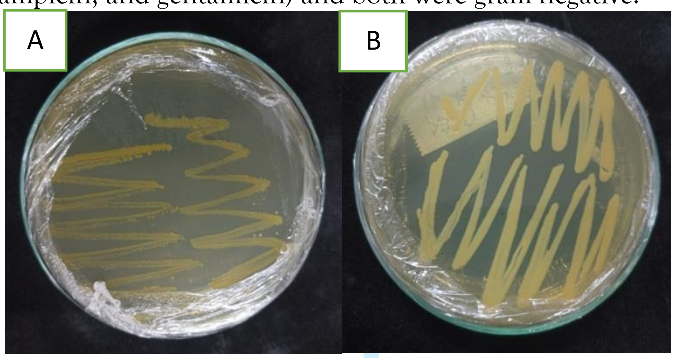

Fig. 3.1. Colony morphology. (A) Brown colonies of Agrobacterium. (B) White colonies isolated from chickpea nodules

We did antibiotic resistances test, the isolated bacterial strains were shown resistance to antibiotics including tetracycline, rifampicin, and gentamicin and the both strains were not resistant to kanamycin antibiotic.

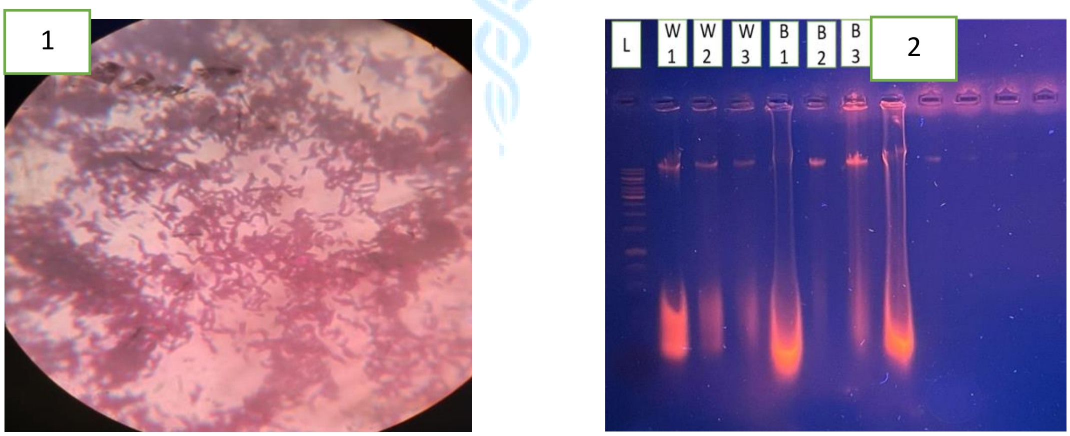

Fig. 3.2. Microscopy and plasmid DNA electrophoresis. (1) Microscopic view of Gram-negative isolates. (2) Gel electrophoresis: Lane L = DNA ladder; lanes W1–W3 = plasmid DNA from white colonies; lanes B1–B4 = plasmid DNA from brown colonies.

To solidify the bacterial identity, gram-staining was employed on the isolated bacteria to determine whether they were gram-positive or gram-negative. The resultant coloration exhibited by the bacterial culture after staining was distinctly red, thereby providing conclusive evidence that the isolated bacteria indeed belonged to the Agrobacterium genus.

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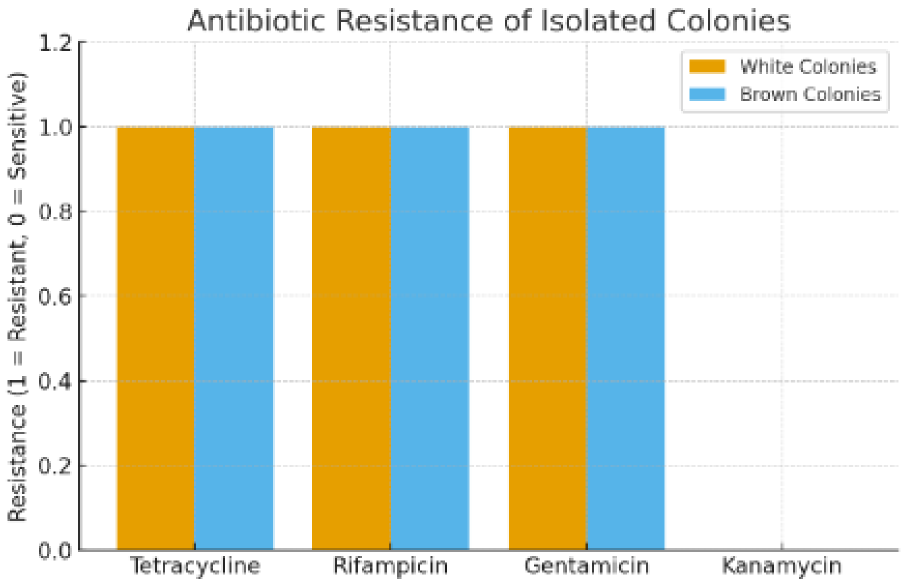

Fig. 3.3. Antibiotic resistance profile of isolated colonies.

Bar chart showing resistance patterns of white and brown colonies isolated from chickpea root nodules. Both strains were resistant to tetracycline, rifampicin, and gentamicin, while both were sensitive to kanamycin. Resistance was scored as 1 (Resistant) or 0 (Sensitive).

Characteristic	White Colonies	Brown Colonies
Colony Morphology	Milky white	Brown
Gram Staining	Gram-negative	Gram-negative
Plasmid DNA	Present	Present
Tetracycline	Resistant	Resistant
Rifampicin	Resistant	Resistant
Gentamicin	Resistant	Resistant
Kanamycin	Sensitive	Sensitive

Fig. 3.4 Summary of phenotypic and molecular characteristics of isolated colonies.

Comparison of white and brown colonies obtained from chickpea root nodules. Both strains were Gram-negative, carried plasmid DNA, and showed resistance to tetracycline, rifampicin, and gentamicin but were sensitive to kanamycin.

Discussion:

The study confirmed the presence of Agrobacterium in chickpea root nodules. The isolates showed resistance to multiple antibiotics, Gram-negative staining, and plasmid DNA consistent with Agrobacterium rhizogenes. These findings align with previous reports highlighting the role of plasmid-borne virulence genes in pathogenicity (Murugesan, 2011).

Future work will include PCR-based confirmation and sequencing to further characterize these isolates. Understanding their genetic features may provide insights into plant–microbe interactions and potential applications in biotechnology.

Declaration of interest:

The authors declare no conflict of interest.

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