ISOLATION AND CHARACTERIZATION OF *AGROBACTERIUM TUMEFACIENS* FROM CROWN GALL AND SOIL SAMPLES

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Abstract

Agrobacterium tumefaciens is popularly known as nature's genetic engineer. It is a Gram-negative pathogenic bacterium which is generally found in soil. It causes tumour formation in plants due to its ability to perform inter-kingdom DNA transfer. Due to this characteristic of A. tumefaciens to act as a gene vector it is used as a gene jockeying tool. Its virulence is due to presence of Ti plasmid, T-DNA and vir regions of the plasmid. This paper focuses on the isolation and characterization of Agrobacterium tumefaciens isolated from two samples- crown gall present on Azadirachta indica (neem tree) and soil present around Phaseolus vulgaris (bean) plant. The tumour sample was surface sterilized using Tween-20, 70% ethanol and mercuric chloride solution while the soil sample was serially diluted. Isolation was done by streaking the samples onto MacConkey agar plates that is selective for Agrobacterium. On performing Gram staining it was determined that bacterium was Gram-negative. Antibiotic Sensitivity test performed using Kirby-Bauer method revealed that the isolate was sensitive to kanamycin (1.5 cm) and tetracycline (2.4 cm), visualized as zone of inhibition, while being resistant to cefuroxime and rifampin (0 cm). Biochemical tests revealed that the isolate was positive for motility test (highly motile), oxidase test (disc turns blue), catalase test (effervescence produced), citrate utilization test (Simmon's citrate agar turned blue) and H_2S production test (Kligler's iron agar turned black). Positive result was obtained for the pathogenicity test in carrot, which confirmed tumour forming ability of the isolate. Leaves of brinjal plant which were infected with the isolate were then tested for GUS activity and blue coloured spots were observed which are positive for GUS assay. From the obtained results, it was concluded that the organism isolated was indeed Agrobacterium tumefaciens. However, the strain can be confirmed only by using advanced techniques such as DNA sequencing and polymerase chain reaction (PCR).

Keywords: *Agrobacterium tumefaciens, crown gall, Ti plasmid, transformation, GUS assay.*

Introduction

Agrobacterium tumefaciens, known as *Rhizobium radiobacter*, is a Gram-negative rod-shaped bacterium found in soil. It causes crown gall disease, which is marked by tumour-like growths on mostly dicotyledonous plants. It damages the vascular system of the plant, making it difficult for transport of nutrients and water, ultimately killing the plant. The pathogen enters the host through any sites of injury, and then bacterial DNA gets integrated into the plant genome. These galls are caused due to unchecked cell division in the plant tissues caused by the pathogen. Smith and Townsend (1907) initially isolated *Agrobacterium*

tumefaciens from galls found on daisies. They confirmed the bacterium's pathogenicity by inoculating healthy plants with the isolated bacteria, resulting in creation of new galls. *A. tumefaciens* has significant impact on biotechnology, plant pathology, and genetic engineering.

1.1 Current Taxonomic Placement of *A. tumefaciens*

As a prokaryotic organism, *Agrobacterium tumefaciens* belongs to the domain Bacteria. Due to lack of complex organelles and membrane-bound nucleus. *A. tumefaciens* belongs to phylum *Pseudomonadota*. It belongs to the class *Alphaproteobacteria*, and has a symbiotic relationship with number of plants. *A. tumefaciens* is categorized under the order *Hyphomicrobiales*, down the taxonomic ladder and belongs to the *Rhizobiaceae* family.

1.2 Cellular Organization and Physiology of A. tumefaciens

Agrobacterium tumefaciens is a rod-shaped, strictly aerobic bacterium which is Gram-negative. The size of the bacteria ranges between 0.5–1.0 µm wide and 1.5–3.0 µm long. Between the inner and outer cytoplasmic membranes lies a thin layer of peptidoglycan. The outer membrane contains lipopolysaccharides (LPS), which acts confer pathogenicity to the bacteria. *A. tumefaciens* consists of peritrichous flagella. These whip-like appendages allow the bacteria to move through the soil, thus conferring motility to them. *A. tumefaciens* also contain pili, which are involved in conjugation and assist in the DNA transfer to host plants.

The metabolism of A. tumefaciens mainly involves pathways such as the Entner-Dourdoff (ED), Phospatidylcholine (PC) and Tricarboxylic acid (TCA) cycle pathways for the consumption of carbohydrates. The Ti plasmid of *A. tumefaciens* encodes production of compounds called opines in plant tissues, which serve as carbon and nitrogen source to the pathogen. Based on the type of opine produced, Ti plasmids are classified into different types.

1.3 Tumour inducing (Ti) Plasmid

The Tumour inducing (Ti) plasmid, serves as a molecular vector to introduce certain DNA fragments, known as T-DNA, into the genomes of host plants leading to the formation of distinctive crown gall tumours. Ti plasmid usually disappears when Agrobacterium is cultivated at temperatures higher than 28° C. Investigating plant biotechnology and genetic engineering techniques requires an understanding of plasmid behaviour.

1.4 Ti Plasmid Classification Framework

Due to variations in the way their T-DNA is organized, the Ti plasmids can be generally divided into two types: octopine-type and nopaline-type plasmids. Both octopine and nopaline are nitrogen-rich substances (opines) that are essential in creating the tumour microenvironment. Octopine or nopaline are usually not found in healthy plants by nature; rather, they are produced in infected plants as a result of the genetic modification caused by *A. tumefaciens* during the development of crown gall tumours. The presence of these plasmids causes the synthesis of opines in the transformed plant cells.

1.5 Ti Plasmid Arrangement and Functional Layout

The replication mechanism of the Ti plasmid is still not well understood, but it has been observed thta on the RepC protein largely controls the initiation. RepC has two domains: a C-terminal domain and an N-terminal domain that interacts with DNA.

The *rep*C gene contains the *ori* sequence having 150 nucleotides. RepC binds to this *ori* site precisely in order to start replication and replicates on the plasmid that contains its gene.

1.6 Functional Architecture and Genetic Arrangement of the T-DNA Region in Plasmids

During the infection process by Agrobacterium species, T-DNA is transferred into plant cells. Three key genes- *iaa*M, *iaa*H, and *ipt*—are crucial for crown gall development. The *iaa*M and *iaa*H genes encode enzymes (*tryptophan-2-monooxygenase* and *indoleacetamide hydrolase*) that convert tryptophan into indole-3-acetic acid (IAA), forming part of the shooty tumour locus. Deleting these genes leads to the formation of shooty crown galls. The *ipt* gene encodes *isopentenyl transferase*, producing zeatin-type cytokinin isopentenyl adenine. T-DNA exhibits left (TL) and right (TR) borders. Mostly opine genes are localized near the TR border. In nopaline-type Ti plasmids, both TL and TR segments form one continuous DNA segment. The Ri plasmid of *A. rhizogenes* contains TL and TR sections. The *rol* locus in the TL comprises genes like *rol*A, *rol*B, and *rol*C, which enhance auxin sensitivity in infected plant cells. The right T-DNA region hosts genes involved in auxin and opine biosynthesis and releases TL and TR separately. Imported T-DNA genes possess eukaryotic regulatory sequences that restrict transcription in *A. tumefaciens*, permitting expression exclusively in plant cells.

1.7 The Virulence Region of Agrobacterium tumefaciens: Structure and Function

The virulence region found in the *A. tumefaciens* plasmid normally stays dormant under normal physiological settings, and it gets active when signals are detected from wounded areas of the plant (Gordon, 2014). Upon activation, the virulence region essential for the synthesis of virulence proteins which help transmit T-DNA into the cells of host plant. Eight operons, referred to as *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G, and *vir*H, together make up the virulence area of the Ti plasmid. VirA protein undergoes autophosphorylation and then phosphorylates the VirG protein, which induces the activation of the *vir* regulon. *A. tumefaciens* moves towards the plant wound site due to the induction, and can thus enter plant cells. Notably, the *vir*B operon, which codes for 11 VirB proteins is the largest operon in the virulence region. Two proteins, VirC1 and VirC2 that are encoded by the *vir*C operon have a major effect on the pathogenicity of the bacteria.

1.8 Pathways of T-DNA Incorporation into Plant Genetic Material

The virulence region, is responsible for executing the transfer of T-DNA into plant cells. Two molecules, acetosyringone and alpha - hydroxyacetosyringone, cause the activation of this region. These compounds are produced when plant tissues sustain injury. The VirA protein, influenced by the signals released, gets activated and binds to it. The VirG protein requires phosphorylation by VirA to perform binding. Once phosphorylated, VirG activates all 8 operons in the virulence region, which activates transcription. The VirD1 protein having

topoisomerase activity, enables it to attach to the T-DNA's right border sequence. The *vir*D gene encodes the VirD2 protein, which cleaves the T-DNA's right border 5'-end to keep the two molecules together during transfer. Further, during DNA synthesis, the 3'-end created during this cleavage acts as a primer, and single-stranded (ss) T-DNA are created.

T-DNA is transmitted between the bacterial and plant cells via conjugative pilus created by VirB and VirD4 proteins. VirB11, which demonstrates ATPase activity to generate the required energy for T-DNA translocation into host cells, facilitates this energy-dependent transfer. T-DNA enters the plant cell and nucleus through nuclear pore complexes (NPCs), which control the selective movement of molecules. The single-stranded T-DNA is changed into double-stranded DNA in the nucleus. The plant genome then randomly incorporates this double-stranded T-DNA into its genome.

1.9 The Integral Role of A. tumefaciens in Advanced Plant Genetic Engineering

Plant genetic engineering progressed significantly following the introduction of A. tumefaciens as a gene vector. *Agrobacterium*-mediated transformation (AMT), is a process in which genetically modified *Agrobacterium* cells are added to plant cells to promote the incorporation of particular DNA segments and desired gene replicates as target DNA segments, which are incorporated into the plant genomic DNA.

Modifications are done to *A. tumefaciens* to create disarmed strains by removing oncogenes from the plasmids and replacing them with the desired gene. In disarming, it must be noted that the ability of *Agrobacterium* to aid in the incorporation of its DNA into plant cells must be preserved, but the pathogenicity of the bacterium has to be eliminated. The gene of interest may encode for stress tolerance, pest resistance, disease resilience, and other beneficial characteristics. It is also possible to introduce genes into plant cells to improve the harvestable fruits' taste, appearance, and shelf life. These developments in *Agrobacterium*-mediated transformation could support sustainable farming methods by boosting crop yields and reduced reliance on chemical pesticides.

1.10 GUS Reporter Gene System

The innate ability of the Ti plasmid of *A. tumefaciens* to transfer DNA fragments, into the host plant's genome can be exploited to carry out the insertion of foreign genes into plant cells by integrating these genes in the T-DNA region. The verification of successful integration and expression of the target gene after transfer is essential to know whether transformation had occured. Reporter genes such as β-glucuronidase (GUS), Luciferase (Luc) and green fluorescent protein (GFP) are commonly used to qualitatively and quantitatively assay the regulation of gene expression in cells. These reporter genes are linked to gene sequences whose expression has to be detected and studied. The GUS reporter gene system is commonly used in plants to check for the patterns of expression of different genes. The activity of any gene of interest can be studied by fusing that gene with the GUS gene and introducing it into host cells. Then a substrate such as X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) is added, which reacts with GUS and produces blue colour in

the plant tissues. Thus, the successful integration of any foreign gene in plant tissues can be detected using this assay.

Materials and Methods

2.1 Sample Collection

Samples of crown gall were collected from *Azadirachta indica* while the soil sample was collected from soil of *Phaseolus vulgaris*.

2.2 Isolation of Bacteria from the Samples

The collected crown gall sample was first cut into pieces and then surface sterilized. The pieces were first rinsed thoroughly with tap water in a beaker thrice or for 30 minutes. One drop of detergent was added to the beaker and washed with tap water thrice or till no more frothing was seen. The pieces were then washed with distilled water three times under a laminar air flow (LAF) cabinet to prevent contamination. The pieces were then submerged in 70% alcohol for 30 seconds following which they were thoroughly washed with thrice distilled water to remove any excess alcohol present. The crown gall pieces were then immersed in 1% mercuric chloride ($HgCl_2$) for 1 minute. This was followed by washing the pieces thrice using distilled water. The pieces were then submerged in distilled water and the beaker was secured with a muslin cloth. It was then incubated at 30°C in the incubator overnight so that the bacterial cells would get suspended in the distilled water.

On the other hand, the soil sample was serially diluted. Firstly, 1g of the soil was sample weighed and then added to 100 mL sterile distilled water to prepare the stock solution. From the stock solution 0.5 mL was pipetted into 1st tube which contains 4.5 mL distilled water or saline. Then 0.5 mL from the 1st tube was pipetted into the 2nd tube also containing 4.5 mL of sterile distilled water. This was then repeated up to 10 test tubes and 0.5 mL was discarded from the last test tube. With each increasing dilution the number of bacterial organisms decrease.

2.3 Streaking on Selective Media

From the incubated sample of crown gall pieces, 0.1 mL was streaked onto MacConkey Agar that is selective for *Agrobacterium tumefaciens*. In the case of the soil sample, 0.1 mL of dilutions from 10^{-6} to 10^{-9} were streaked onto MacConkey Agar plates. As microorganisms exist in a mixed population the organism of interest can be isolated by streaking. A loopful of inoculum was taken and streaked in a way that the first quadrant has the densest concentration of the inoculum while the number of organisms decreased gradually with the second quadrant, third quadrant and fourth quadrant.

2.4 Gram Staining

In order to determine the Gram nature of the isolate, Gram staining was performed. The isolate was heat-fixed onto a clean glass slide. Crystal violet was then added to the slide and was incubated for 1 minute. After which it was washed and then the mordant, Gram's iodine was added to the slide. It was washed off after 1 minute. Following this alcohol was added

and washed off after 20-30 seconds. Finally, counterstain safranin was added and washed off after 2 minutes. The slide was air-dried and observed under a compound microscope.

2.5 Antibiotic Sensitivity Test using Kirby-Bauer Disc Diffusion Method

To measure the susceptibility of bacteria against various antibiotics, antibiotic sensitivity test was performed. Using a pipette 2-3 drops of the active culture was inoculated and spread onto the surface of Luria Bertani (LB) agar plates. Using sterile forceps two of the four antibiotic discs, namely Tetracycline (30µg), Rifampin (5µg), Cefuroxime (30µg) and Kanamycin (30µg) were placed equidistantly in the centre of the plate. This was repeated for the other plates as well. The plates were sealed with parafilm and wrapped in aluminium foil to prevent light exposure and placed in the incubator overnight at 30°C.

2.6 Pathogenicity Test

In order to check the tumour forming capacity of the isolate pathogenicity test was performed. One potato and one carrot were peeled and sliced into thin discs which were then surface sterilized. Following this, the discs were blotted dry and then placed aseptically on water agar using sterile forceps. From the culture of isolate, 100µl was loaded evenly onto carrot disc while 50µl was loaded onto potato disc. The plates were sealed and incubated for 5-6 weeks at 30°C.

2.7 Motility Test

To determine whether the isolate was motile or not, motility test was performed using hanging drop method. On all four edges of a cover slip, petroleum jelly was applied and then 2-3 drops of bacterial suspension of the isolate was added in the centre of the cover slip. A cavity slide was inverted and placed on the cover slip such that the cavity covers the drop. The slide wass inverted again and observed under 40X.

2.8 Catalase Test

A drop of hydrogen peroxide was added to glass slide and a loopful of culture was added to the drop aseptically. After few seconds the slide was observed for effervescence.

2.9 Citrate Utilisation Test

Using an inoculation loop, a loopful of culture was inoculated onto Simmon's Citrate Agar, which was prepared by dissolving 2.428 grams of Simmon's Citrate Agar in 100 mL distilled water and autoclaving it at 121°C at 15 lbs pressure for 15 minutes. This was followed by streaking the culture on the agar slant. The slants were then incubated for 48 hours at 37°C in the incubator. After incubation agar slants were observed for colour change.

2.10 H_2S Production Test

A loopful of the bacterial culture was streaked onto the surface of Kligler's Iron Agar that was prepared by dissolving 5.725 grams in 100 mL distilled water and autoclaving at the same conditions mentioned above. Once streaking was done, a Whatman paper strip impregnated with lead acetate impregnated was aseptically placed on the surface of the slant in order to submerge it. The agar tubes were incubated for 48 hours at 37°C in the incubator. After incubation, the agar slants were observed for colour change.

2.11 Oxidase Test

An oxidase disc was aseptically placed on a glass slide and a loopful of culture was streaked onto the disc while the disc was held in place using sterile forceps. The disc was observed for colour change after 5-10 minutes of incubation.

2.12 GUS Assay

The leaves of *Solanum melongena* (brinjal) were taken as explants. They were surface sterilized and then cut into small pieces of size 1 cm^2 in a sterile petri plate. Then 10-15 mL of the active bacterial culture was taken and added to brinjal leaves. The leaves were meticulously mixed with the culture by gentle swirling for about 30 minutes. The bacterial suspension was then decanted and leaves were blotted dry. They were air-dried for 10-15 minutes and then transferred to MR medium. The plates were then sealed and co-cultured with the isolate at 28°C for 3 days. The brinjal leaf pieces were taken and transferred to new sterile plates. The leaves were pinched to induce injury. 50µL of Acetosyringone solution was sprayed onto the leaves and incubated for 15 minutes at room temperature. After incubation the leaves were sprayed with GUS Detection Solution at the site of injury and incubated in dark for 15-30 minutes to view for the appearance of blue spots.

Results

3.1 Sample Collection

Figure 1: Plants from which Sample was Obtained Crown Gall Sample (left) and Soil Sample (Right).

3.2 Sterilization of Collected Sample

Figure 2: Surface Sterilized Tumour Sample

3.3 Streaking of Bacteria Isolate on MacConkey Agar Plates

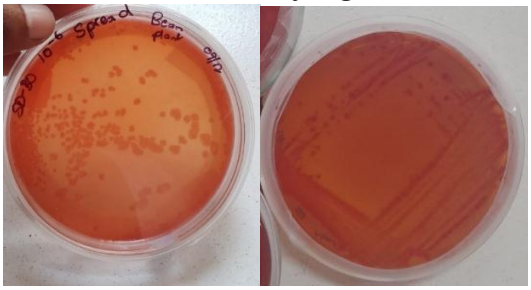


Figure 3: Pink Colour Colonies were Developed on MacConkey Spread Plate

3.4 Gram Staining

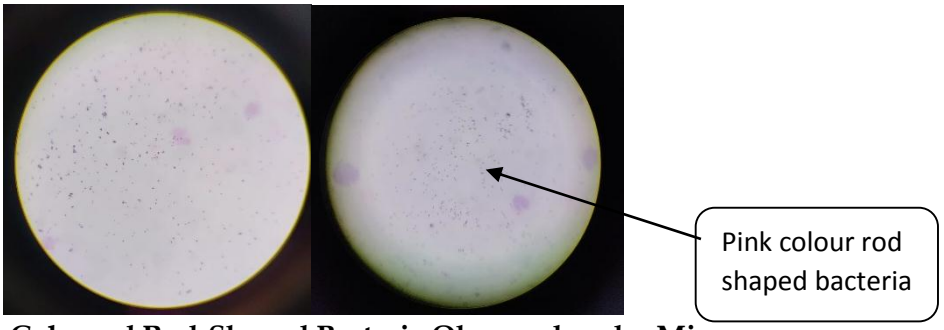


Figure 4: Pink Coloured Rod-Shaped Bacteria Observed under Microscope

3.5 Antibiotic Sensitivity

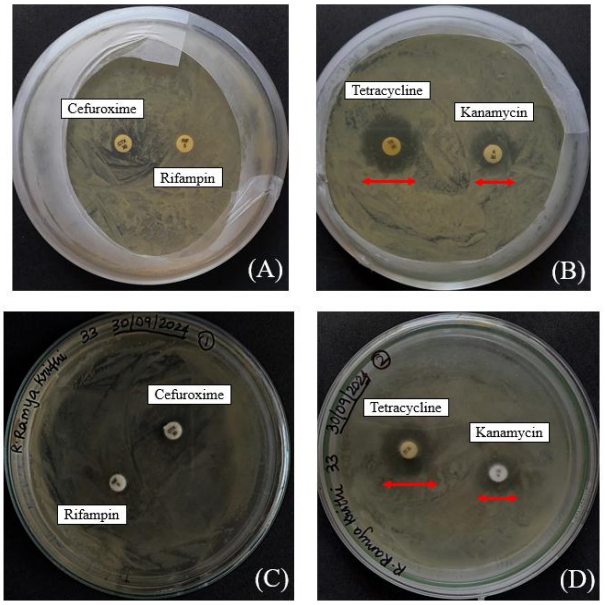


Figure 5: Kirby-Bauer method to test sensitivity of isolate to antibiotics Cefotoxime, Rifampin, Tetracycline and Kanamycin. (A) Plate 1- bottom side; (B) Plate 2- bottom side; (C) Plate 1- top view; (D) Plate 2- top view. (Red arrows depict the zones of inhibition)

Plate	Antibiotic discs	Concentration of the antibiotic (µg/mL)	Diameter of zone of inhibition (cm)
Plate 1	Cefotoxime (CTX)	30	0.0
	Rifampin (RIF)	5	0.0
Plate 2	Tetracycline (TE)	30	2.4
	Kanamycin (K)	30	1.5

3.6 Pathogenicity Test on Carrot and Potato

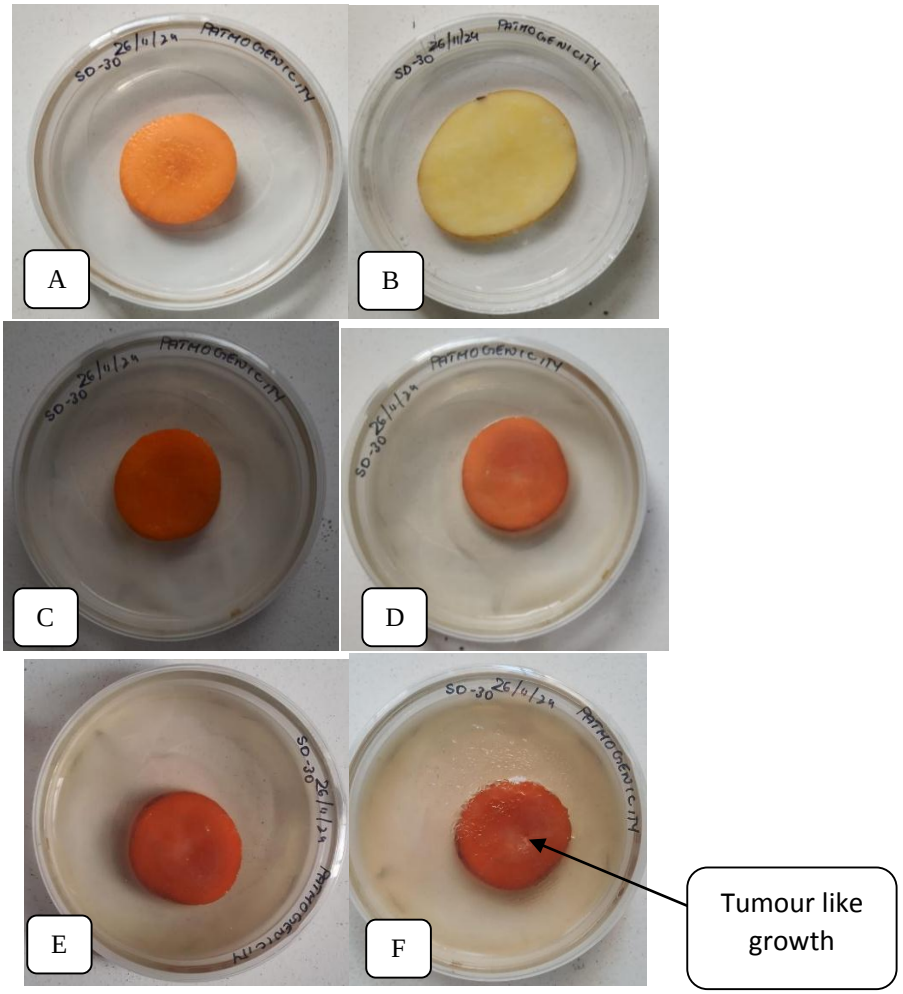
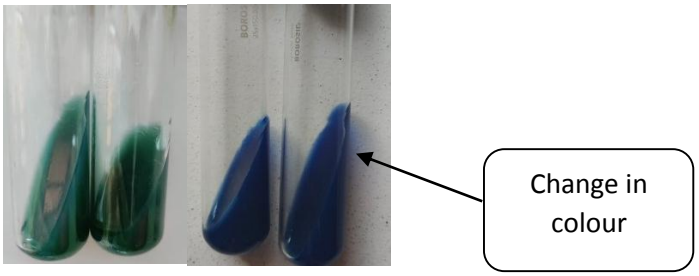


Figure 6: Formation of Tumours Due to Agrobacterium Tumefaciens. Figures (A), (B) Before Incubation; Figure (C) 1st Week of Incubation; Figure (D) 2nd Week of Incubation; Figure (E) 3rd Week of Incubation; Figure (F) After 4-5weeks of Incubation.

3.7 Catalase Test

Figure 7: Effervescence Produced after Addition of Hydrogen Peroxide to Culture.

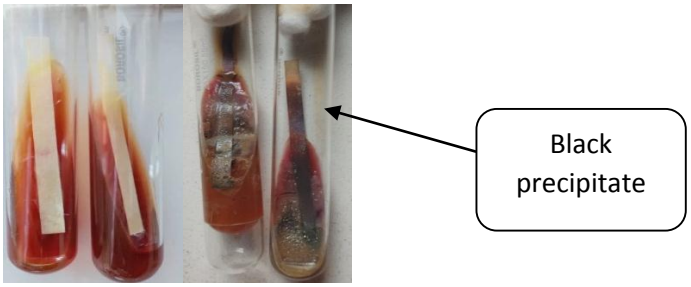
3.9 Citrate Utilization Test



A) Before B) After

Figure 9: Change in Colour of Simmon's Citrate agar from Green to blue 24 Hours after Incubation with Bacterial Culture.

3.10 H2S Production Test



A) Before B) After

Figure 10: Black Precipitate on Edge of Lead Acetate Observed after 48 Hours of incubation of culture on Kligler's iron agar.

3.8 Oxidase Test

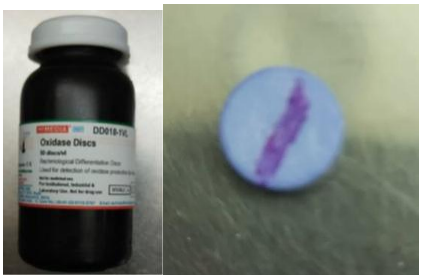


Figure 8: Purple Colour Observed 5 Minutes after Culture was Streaked on Oxidase Disc

3.11. GUS Assay

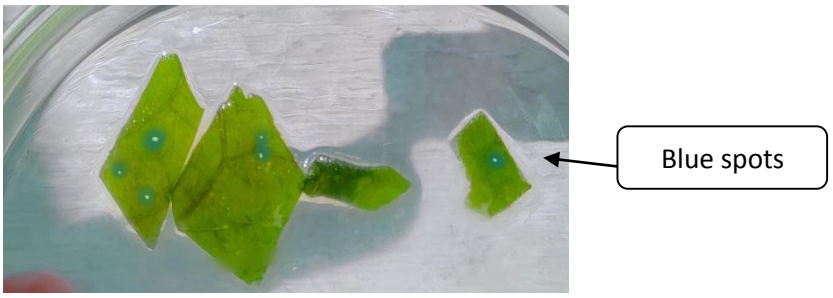


Figure 11: Blue Colour Observed at Wounded Sites of *Solanum Melongena* (Brinjal) Leaves

Disscussion

The objective of this project was to isolate and characterise *Agrobacterium tumefaciens* obtained from crown gall and soil samples. Initially, bacteria were isolated from the crown gall present on *Azadirachta indica* (neem tree) and from soil present around *Phaseolus vulgaris* (bean) plant. The crown gall sample was surface sterilized and then fragmented, whereas the soil sample was serially diluted. When the bacterial isolate was streaked on MacConkey agar, pink coloured colonies growing luxuriantly on the agar were observed. MacConkey agar is both a differential and selective media, which is used for differentiating Gram-negative bacteria based on their lactose fermenting ability. Therefore, it can be inferred that the isolate was a lactose-fermenting bacteria. Gram staining of the isolate revealed pink rod-shaped bacteria under compound light microscope (40x magnification), implying that the bacteria was Gram-negative as it was unable to retain crystal violet in their cell wall on addition of alcohol (decolourizing agent), and ended up retaining safranin. Kirby-Bauer disc diffusion test for finding out the sensitivity of the isolate to different antibiotics revealed that the isolate was sensitive to tetracycline and kanamycin, as zone of inhibition was formed around the 2 discs, measuring 2.4 cm and 1.5 cm respectively. The isolate was observed to be resistant to rifampin and cefuroxime due to the lack of zone of inhibition. Pathogenicity test was performed on potato and carrot slices to test the ability of *Agrobacterium tumefaciens* to cause tumour in the plants. Tumour like growth was detected on carrot slices, which indicated that the isolate was virulent and had tumorigenic ability. When hanging drop method was carried out to check for the motility of the isolate, bacteria were observed to be highly motile when viewed under the microscope.

These tests indicated that the isolate could be *Agrobacterium tumefaciens*. To confirm the identity of the isolate, biochemical tests such as catalase test, citrate utilization test, H_2S production test and oxidase test were performed. On addition of hydrogen peroxide to the culture, effervescence was observed, which indicated that catalase enzyme was present in the isolate, which reacted with the hydrogen peroxide to produce oxygen (bubbles) and water. Citrate utilization test was performed to examine whether citrate can be utilized by the bacteria as a carbon source. Simmon's citrate agar changed colour from green to blue after being incubated with the culture. As the isolate contained citrate permease enzyme,

citrate could be utilized as carbon source leading to production of bicarbonates and carbonates, which increased the pH of the agar, which in turn resulted in bromothymol blue indicator changing to blue colour. Hydrogen sulphide (H_2S) production test was performed to determine the ability of the isolate to produce H_2S gas. The formation of black precipitate was observed on Kligler's iron agar after incubation, which showed that the isolate was a H_2S producer. The H_2S gas produced reacted with the ferrous sulphate in the agar, to form black-coloured ferrous sulphide. When oxidase test was performed, it was observed that there was a change in the colour of oxidase disc from colourless to purple at the region where culture was streaked. This indicated that the isolate was positive for oxidase test, and therefore contained the enzyme cytochrome C. The biochemical tests confirmed that the isolate was *Agrobacterium tumefaciens* as all the tests yielded positive results. Finally, GUS (β-Glucuronidase) assay was performed using *Solanum melongena* (brinjal) leaves. Blue spots were detected on the wounds of the leaves, which indicated that the β-glucuronidase (GUS) enzyme reacted with the substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). Thus, the blue colour seen confirmed that the strain of *Agrobacterium* tumefaciens isolated was capable of transforming plant cells, and could therefore be used for *in planta* transformation experiments.

Therefore, it could be concluded from all the microbiological and biochemical tests that the isolate obtained was *Agrobacterium tumefaciens*, which had pathogenicity and transformative ability. However, advanced molecular techniques such as DNA sequencing and polymerase chain reaction (PCR) would have to be performed in order to investigate the strain of the isolate.

Conclusion

This paper intended to isolate and characterize *Agrobacterium tumefaciens* from samples collected from crown gall present on *Azadirachta indica* tree, as well as from soil present around *Phaseolus vulgaris* plant. Bacterial isolation was done by streaking the sample onto MacConkey agar, which is selective for *Agrobacterium tumefaciens*. Pink colonies grew opulently on the agar, and single, well-isolated colonies obtained by streaking were used to prepare pure culture. Microbiological tests were performed to understand the nature and the morphology of the isolate. The bacterium was identified to be Gram-negative and was rod-shaped by Gram staining. Antibiotic sensitivity test by Kirby-Bauer disc diffusion method revealed that the organism was sensitive to tetracycline and kanamycin (due to the formation of zone of inhibition), and resistant to rifampin and cefuroxime.

Pathogenicity test resulted in the formation of small tumours, which were observed only on the carrot discs, but not on potato disc. The formation of tumours indicates that the isolate is pathogenic and can cause formation of tumours in plants. The isolate was positive for the biochemical tests performed, which included catalase test, citrate utilization test, hydrogen sulphide production test and oxidase test, which all point toward the identity of the isolate being *Agrobacterium tumefaciens*. Thus, the microbiological and biochemical tests confirmed that the isolate was *Agrobacterium tumefaciens*. The appearance of blue coloured spots on the brinjal leaves in GUS assay is indicative of the isolate having the ability to transform plant

cells. Therefore, the isolate could be used in experiments involving transformation of plant cells. However, to find out the strain of the bacterium, tests like DNA sequencing and PCR would be required. The results of this study aided in understanding the structure and properties of *Agrobacterium tumefaciens*, and also encourage further research on *Agrobacterium tumefaciens* and its role as a natural gene vector in plants.

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