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Agrobacterium tumefaciens pathogenicity and characterization in rose crown gall disease

Zohaib Ahmad[1], Mohammed Abdulaziz Alfehaid[2], Sidra Gill[*3], Muhammad Atif[4], Abdul Manan[1], Zain-Ul-Abadin[5]

[1]*Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan*

[2]*Department of Biology, Imam Muhammad IBN Saud Islamic University, Saudi Arabia*

[3]*Department of Botany, Faculty of Chemical and Biological Sciences, The Islamia University of Bahawalpur, Pakistan*

[4]*Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan*

[5]*Ondokuz Mayis University, Turkey*

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Abstract

Agrobacterium is a bacterium that has a gigantic role in modern plant genetic engineering. As its beneficial role of the bacterium, it also causes crown gall diseases in many horticultural crops and causes losses all over the world. A crown gall-infected rose sample was collected and morphological, biochemical, and cultural characteristics using NA, YDC, YPDA and King's B mediums. Different tests were conducted to study the biochemical properties of the bacterium such as gram staining, KOH solubility test, Starch hydrolysis, Kovac's oxidase, Litmus milk, Hydrolysis of gelatin, Citrate utilization, Methyl red and Voges Proskauer test, fluorescent pigment, Urease and Catalase tests. A pathogenicity test was also performed on broad bean plants in the greenhouse to confirm the pathogen. All these test results exhibited that the crown gall disease in rose due to the attack of the *Agrobacterium tumefaciens*. These tests are essential to identify the pathogenic bacterium to develop the management strategies for the control of the pathogen. Several reports indicate that this soil-born pathogen is an emerging threat to several crops of horticulture as well as to ornamental plants around the globe.

* **Corresponding Author:** Sidra Gill ✉ sidragill68f@gmail.com

Introduction

Agrobacterium tumefaciens is a bacterium that lives in the soil. *A. tumefaciens* belongs to family Rhizobiaceae. These microscopic creatures are gram-negative, rod-shaped, motile, and actively growing without endospores (Davoodi and Hajivand, 2013). Its infectious strains cause crown gall disease all over the world and contaminate dicotyledonous plants of around 90 unique families and a couple of monocotyledonous plants (Cleene and Ley, 1976). *A. tumefaciens* contains an extrachromosomal DNA assigned as Ti (tumour inducing) plasmid (Zaenen *et al*., 1974). Ti-plasmid conveys two segments: *vir* and T-DNA locales required for the hereditary change (Tzfira *et al*., 2014). Ti-plasmid virulence (vir) genes and bacterial chromosomal virulence (chv) genes encode proteins that are part of the molecular machinery needed for T-DNA synthesis and transit into the host cell. (Gelvin, 2003; Tzfira and Citovsky, 2002; Zupan *et al*., 2000).

Any living cell, including various prokaryotes (Kelly and Kado, 2002), yeast (Piers, 1996), fungi (*Groot et al.,* 1998; Gouka *et al.,* 1999), and human cells (Kunik *et al*., 2001), can be altered by agrobacterium. Refreshed data of systems for T-DNA transfered to plant cells by *A. tumefaciens* is given, centred around the pretended by the various parts of the virulence framework (Riva *et al*., 1998). *A. tumefaciens* intervened change has generally been utilized for research in plant molecular science and hereditary development of harvests since 1983 (Park, 2006). Transformation is as of now utilized for hereditary control of over 120 types of at any rate 35 families, including the major monetary yields, vegetables, decorative, therapeutic plants, natural products, trees and field plants, utilizing Agrobacterium-interceded or direct transformation techniques (Birch, 1997).

Various types of research exhibited isolation of the infective wild strain of *Agrobacterium tumefaciens* from contaminated leaves, stems and crown galls of *Viciafaba* (Tiwary *et al.,* 2007), tobacco (Furuya *et al*., 2004), rose (Islam *et al*., 2010), apricot (Aysan and Sahin, 2003) and aster (Chenet *et al*., 1999). This demonstrates the massive probability of isolating different *Agrobacterium tumefaciens* strains from various plant types of our surroundings as well. Keeping in view the significance of Agrobacterium in plant biotechnology, the present examination is meant to separate and portray wild destructive strains of *A. tumefaciens* from privately contaminated hosts for multipurpose future uses, for example, change innovation or antitumour examinations.

Materials and methods

Samples of crown gall were collected from the heavily infected plant of rose (*Rosa* spp.) from Faisalabad. The collected sample was washed under the running water and let dry. After that, the sample was cut into small pieces and put in the Eppendorf tube containing sterilized distilled water. These pieces were crushed with the help of a sterilized needle in the Eppendorf tube. Making suspension of the sample. The next day after 24 hours streaked the suspension of the bacteria on two different media Nutrient Agar media (lab lamco powder1. 0g, yeast extarct2. 0g, peptone5. 0g, Sodium choloride5. 5g, Ager15.0inn 1liter distil water) and king's B media. Plates were incubated at 28 °C for 24 hours. The purified isolate was also cultured on two different media such as YPDA (yeast peptide dextrose carbonate agar) (Yeast extract 10g, peptone20g, dextores20g, agar20gin 1 litre distilled water) and YDC (yeast dextrose carbonate) media (dextores20g, yeast extract 10g, $CaCO_3$/lime powder 20g agar 20g in 1liter of water) (Schaad *et al*., 2001). The isolated Agrobacterium is stored in tubes or plates at 4°C.

Biochemical analysis

Using the bacterial isolates for a variety of biochemical tests, including Gram Staining, KOH, H_2O_2, Starch hydrolysis, Simmons citrate test, Urease test, Methyl red test, Vogues Proskauer's test, Kovac's oxidase test, Litmus milk test, Hydrolysis of gelatin, and Pathogenicity test, the biochemical characteristics of the bacterium were investigated..

Gram staining

Crystal violet, Lugol iodine, Acetone, and a counterstain of Safranin were used to make Gram reagent. Isolates were created by taking pure culture with a sterilized needle and smear it on a clear slide with distilled water.

A drop of crystal violet was applied, rubbed on, and left for 30 seconds before being washed with sterile water. Following the washing with a DAW, a drop of Lugol's iodine was added, and the item was then permanently cleaned with pure acetone. At last, a safranin drop was added, allowed to sit for 30 seconds, and then rinsed with water. After using blotter paper to dry the mounts, a drop of Canada balsam was applied to the stained area, and the entire thing was examined at a magnification of 100X (American Society for Microbiology, 1957).

KOH test

A 24-hour-old culture was combined with a sterile needle, agitated for a short while, and then a drop of 3% potassium hydroxide was poured on the glass slide. The culture was then checked for thin threads. If the loop is visible when the needle is elevated, the bacteria are gram-negative (Ryu, 1940).

Catalase test

After the culture had been growing for 24 hours, it was put on the spotless glass, combined with a drop of 3% hydrogen peroxide, and left to react for a few minutes before being checked for bubble formation (Lelliott and Stead, 1987).

Urease test

It was prepared with 5g sodium chloride, 1g peptone, 2g potassium dihydrogen phosphate, agar-agar 20g and 1000ml distil water adjusted pH of 6.8. After that autoclave it at 121°C at 15 psi for 20 minutes and cools at 50°C. Then we added in it 1g glucose, phenol red (0.2 percent of the solution) 6 ml (molten base and steam for 1 hour, cool it at 50°C); urea, 20 percent aqueous solution 100ml. Mixed well pour it into test tubes and allow it to solidify in a slanting position. After that inoculate the tubes with the test bacterium (Agrobacterium) and incubate it for 24 to 48 hours at 37°C (Stuart *et al.,* 1945).

Litmus milk test

Sterile tubes of litmus milk medium were autoclaved at 7psi for 20 minutes. Label each tube and inoculate with test bacteria, i.e. *Agrobacterium* with the help of an inoculation loop and keep one tube uninoculated for control. Incubate the tubes at 28°C for 24 to 48 hours. Refrigerate the uninoculated control tube of litmus milk. After that, the tubes were observed for colour change (Dye, 1968).

Hydrolysis of gelatin

Autoclaved gelatin agar medium allowed to cool at 40 to 45°C and poured into sterile plates or test tubes. Allow the medium to solidify and then the bacterium was inoculated into the tubes by stab inoculation. One uninoculated tube was used as a control. Incubated all the tubes for 28°C for 4 to 7 days. After incubation tubes were placed in the refrigerator at 4°C for 15 minutes. Flooded the tubes with mercuric chloride solution and allowed the tubes to stand for 5 to 10 minutes (Schaad *et al.*, 2001).

Hydrolysis test of starch

After making the starch agar medium (using potato starch), pouring it into the Petri plate, and letting it set and streak after streaking, the test bacteria was inverted and kept at 28°C for 96 hours. Following the incubation plates, iodine solution was applied to the surface using a dropper and left for 30 seconds. Keep an eye on how the medium's color changes along the growth line (GJ, 1983).

Simmons citrate test

1g ammonium dihydrogen phosphate, 1g dipotassium phosphate, 5g sodium chloride, 0.2g magnesium sulphate, and 15g agar were used to make Simmons citrate agar. Except for the phosphate, which was dissolved separately in 100ml of water, bromothymol blue 0.8g was dissolved in 1000ml of distil water. Mix them to make 1000ml, retain the pH at 6.9, and autoclave it. After that, spoon it into plates and place them in the freezer to firm. Incubate the bacterium at 280 degrees Celsius for 28 hours. Examine the coloring and development of the culture (Simmons, 1926).

Methyl red and Voges Proskauer test

These ingredients were used to make MR-VP broth. Peptone (7g), dextrose (5g), potassium phosphate (5g), and 1000ml distil water. Fill each test tube with 5ml broth and autoclave. Incubate the test tubes inoculated with test bacteria at 280°C for 48 hours, then divide the tubes into two sets.

In one set of tubes, put 5 drops of methyl red indicator. Take note of the color shift. Set2 tubes should have 12 drops of V-P reagent 1 and 2-3 drops of V-P reagent 2. To expose the media to oxygen, shake the tubes for 30 seconds with the caps off. The reaction takes between 15 and 30 minutes to complete. Take note of the color shift (American Society for Microbiology, 1957).

Kovacs oxidase test

For the test, 1% Kovac reagent was poured into the center of Whatman filter paper No.1 and fresh bacterial culture was employed. The bacterium was gently rubbed on filter paper with a platinum needle. If the purple color appears within 30 to 60 seconds, the result is good; otherwise, the negative color appears after 60 seconds or does not appear at all (Haider *et al*., 2020).

Pathogenicity test

The pathogenicity test served as the foundation for confirming the pathogen. The pathogenicity of isolated Agrobacterium tumefacien was tested in 2-week-old broad bean plants (Viciafaba) injected by wounding stems in two areas with a surgical tool knife recently inserted into a significant bacterial growth on Nutrient Agar. The injuries were covered with parafilm tape after inoculation, and the plants were housed in a glasshouse at 20°C (Spiers, 1979).

Results and discussion

The main purpose of the current research was the collection of the highly virulent strain of *A. tumefaciens*, isolation, storage, and confirmation of their characteristic using morphological, physiological, biochemical characterization and Pathogenicity test (tumour forming ability). The bacterium *Agrobacterium* is frequently located in the rhizosphere, which is the area around root surfaces. It can effectively be detached for recognizable proof from nerve tissue, soil or water (Davoodi and Hajivand, 2013). For this purpose, the sample was collected from rose (*Rosa*) horticultural nurseries of Faisalabad. The initial bacterium was isolated on two different mediums such as on nutrient agar and YPDA. *Agrobacterium* has been generally distinguished as gram-negative microorganisms that don't create fluorescent shade on King's B medium when plates were taken under UV light (King *et al.,* 1954). Similarly, the bacterium was streaked on the YDC media (Schaad *et al*., 2001). The isolate was found to be a gram-negative, dispersed motile rod under the oil lens when the gram-staining test was done. When we performed the catalase test it was found to be catalase-positive showing them as aerobic microorganisms and forming the bubbles. The isolate was motile, the gram-negative rod, and positive to Kovac's oxidase test and formed a pink or purple colour. In the case of the KOH test, the bacterium was positive forming the loop when mixed with the drop of the potassium hydroxide. The bacterium was urease positive when a urease test was done on it. When the bacterium was incubated possessed urease produced ammonia that raised the pH of the medium. As pH of medium-high the phenol red changes from a yellow colour to a red or deep pink colour. In (Simmons, 1962) citrate test bacterium utilized citrate showing a change of colour from green to blue. This change of colour was due to the presence of the enzyme citrate produced by a bacterium that breaks down the citrate to oxaloacetic acid and acetic acid. They were negative in the methyl red and Voges Proskauer test showing no reaction. This test was used to differentiate between two major types of bacterium that produced a large amount of acid and that produced neutral acetone as a product. When the starch hydrolysis test was performed bacteria were unable to form a clear zone when the iodine solution was Sameer on the growth of the bacterium in the plate and indicated that it was starch hydrolysis negative. In the case of hydrolysis of the gelatin test, the bacterium was negative because, after the incubation of the plates, the gelatin medium remained solid and exhibited a negative reaction. When a litmus milk test was also performed its milk was the best source of proteins, vitamins, minerals and water acts an as excellent source of growth for bacterium while litmus was used as a pH indicator. It was incorporated into the medium for the detection of the production of alkali or acidic and oxidation and reduction activities. Several changes occur in milk; it depends upon which milk is utilized by the bacterium.

Table 1. Biochemical characteristic *Agrobacterium tumefaciens*

Test	Result
KOH Test	+
H_2O_2 Test	+
Gram Staining	_
Oxidase Test	+
Urease Test	+
Methyl Red Test	_
Voges Proskauer Test	_
Citrate Utilization	+
Starch Hydrolysis Test	_
Litmus Milk Test	ALK
Hydrolysis of Gelatin Test	_
Growth on Nutrient Agar (± 28.4°C)	+
Growth on YDC	+
Growth on YPDA	+
Growth on KING'S B. Media Grows on it. But no fluorescence is produced.	_

It may depend on the type of enzyme that the bacterium produced when grown litmus milk medium. In that case, the bacterium showed an alkaline reaction on the litmus paper. For the final confirmation of the pathogen, the pathogenicity test was performed on the broad bean plants and kept in the glasshouse for more than two months (Table 1). Pathogen expressed the symptoms by producing the galls on the plants. Galls development was recorded at the plant after 2 months at the initial stage the size of the galls was very small with time the size of the galls increased. The identification of the pathogen is essential and plays a significant role in the proper management of the pathogen. Further work is also needed to investigate the pathogen hosts. Many research reports indicate that the bacterium is a threat to several horticulture crops all over the world because the bacterium has a wide range of hosts. Similar work was also done by several investigators such as (Khan *et al.,* 2016; Rouhrazi and Rahimian, 2014; Davoodi and Hajivand, 2013; Sarker *et al.*, 2011).

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