

Isolation and identification of *Agrobacterium tumefaciens* from the galls of peach tree

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Key Message *Agrobacterium tumefaciens* attacks on peach plant and it causes crown gall disease. The present study was conducted to isolate and identify *Agrobacterium tumefaciens* from crown gall samples of peach plants.

ABSTRACT Peach (*Prunus perisca*) is a very important fruit and it is attractive all over the world due to its delicious aroma and flavor. Peach is susceptible to various types of pathogens that result in decline of its fruit production. An important pathogen, *Agrobacterium tumefaciens* attacks on peach plant and it causes crown gall disease. The present study was conducted to identify the peach plants infected with *Agrobacterium tumefaciens* and isolate as well as identify the bacterium from the crown gall samples. These samples were collected from different locations including district Swat and Shangla. The bacterium was isolated from the samples using MacConkey selection media that is used specifically for identification of *Agrobacterium tumefaciens*. Two different biochemical tests i.e. Gram staining and KOH test were performed to confirm that the isolated bacterium is gram negative. The biochemical tests reveal that all the bacterial isolates are gram negative. Furthermore, two pathogenicity tests i.e. potato disc and carrot disc bioassay were conducted that confirmed the isolates causing tumors in the infected plant tissues. Antibiotic sensitivity tests reveal that the bacterial isolates are resistant to rifampicin antibiotic. All the morphological as well as the biochemical features of the bacterial isolates suggested that the samples isolated from crown galls were *Agrobacterium tumefaciens*. Our study provides the basis for further molecular characterization of the pathogens and to devise strategies for reducing the risk of bacterial infection and to enhance the yield of fruits.

Keywords: *Agrobacterium tumefaciens*, Antibiotics, Disc bioassay, Gram staining, KOH test

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INTRODUCTION

Peach (*Prunus perisca*) belongs to Rosaceae family (Parveen et al., 2016). The production of peach ranked second among different fruits in Pakistan. Peach is a very important fruit because of its flavor, attractive colour, dietary value and medicinal worth. This fruit is enriched with carotenoids, ascorbic acid and phenolic compounds that act as antioxidants (Tomas-Barberan et al., 2001; Byrne, 2002). The peach was first grown in Asia, cultivated in Europe and later on it was introduced into Persia. In Pakistan, cultivation of peach occurs at about 15200 hectares and its annual production is 52600 tonnes (Agriculture Statistics of Pakistan, 2010-11). In Pakistan, the cultivation of peach occurs in different cities i.e. Peshawar, Swat valley, Quetta as well as many parts of Kohistan hills. More than 100 different families of dicotyledonous plants are infected by the gram negative bacteria called *Agrobacterium tumefaciens* (Lacroix & Citovsky, 2016). The bacterium own Ti-plasmid (Tumor inducing plasmid) that result in the formation of tumor at wound site in dicotyledonous peach tree (Roy, 2015). The size of Ti-plasmid is about 200 kb that carry 27 genes. The T-DNA region of the Ti-plasmid is excised, transformed into the host plant tissue and integrated into the genome of the plant (Kwon, 2016). The transformation of T-DNA and the subsequent formation of tumor require hormonal and virulence genes present on the Ti-plasmid (Kwon, 2016). The Ti-plasmid have region called transferred DNA

with *tmr* and *tms* genes and these genes are responsible for the over production of plant hormones such as cytokinins and auxins, respectively (Nester, 2015).

There are mainly two bacterial species namely *Agrobacterium vitis* and *Agrobacterium rubi* that are involved in the formation of crown gall disease. This disease produces tumor in plants and it is a very hazardous for the peach plants. The habitat of these pathogenic species is the soil (Roy, 2015). In dicotyledonous plants, the crown gall is caused by the *Agrobacterium* species that are polyphagous bacteria and are known as tumorigenic species of *Agrobacterium*. A huge loss occurs in walnut, pome, stone fruits and grapevine due to the crown gall disease. The cultivation of different host plants shows different levels of susceptibility. Some members like Arales and Liliales of monocots show susceptibility to this disease. *Agrobacterium* namely *rhizogenes* biovar 2 and *Agrobacterium* biovar 1 show a wider host range than that of *Agrobacterium vitis*, *Agrobacterium rubi* and *Agrobacterium larrymoorei*. Some strains show a wider host range, while others show to only one plant. The supervirulent strain shows a wide host range (Pulawska, 2010).

Agrobacterium tumefaciens has been isolated from different plant tissues including stem, leaf and crown gall samples of aster (Chen et al., 1999), from galls of apricot (Aysan et al., 2003), galls of rose (Aysan and Sahin, 2003), root nodules of *Vicia faba* (Tiwarly et al., 2007) and tobacco (Furuya et al., 2004). Different selection media have been utilized for isolation of *A. tumefaciens* from plant samples including MacConkey media and yeast extract mannitol agar (YEMA) (Aysan et al., 2003; Aysan & Sahin, 2003). Crown galls have been isolated from different plant species and they belong to different dicotyledonous plants i.e. *Tectona grandis*, *Artocarpus heterophyllus*, *Anthcephalus codomba*, *Terminalis arjuna*, *Rosa chinensis* and *Solanum lycopersicum* (Sarker et al., 2011).

The characterization and identification of *Agrobacterium tumefaciens* is usually conducted by morphological, biochemical, pathogenicity, antibiotic sensitivity and molecular methods. The morphological methods include the observation based on size, shape, colony surface, colour, opaqueness, elevation, consistency and margin type. The biochemical method for bacterial identification is also method of choice for identification and confirmation of bacterial samples. The biochemical is usually conducted according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). In antibiotic sensitivity tests, the isolates are treated with antibiotics according to the Bauer Kirby method (Bauer et al., 1966) and confirm the bacterium by the formation of the inhibition zones. It shows resistance to some antibiotics, while it exhibits susceptibility to several other antibiotics. *A. tumefaciens* was also confirmed performing pathogenicity tests that were done on potatoes (*Solanum tuberosum*) (Hussain et al., 2007) and carrot (*Daucus carota*) (Aysan et al., 2003).

The crown gall disease limits the economic importance of dicotyledonous plants e.g. apple, rose, pear, almond and cherry (Pionnat et al., 1999). The tumor found in crown portion causes disruption of the vascular system as well as inferior development (Moore et al., 2001). The objectives of our study were isolation of *Agrobacterium* from crown galls samples of peach trees. We were also interested in cultural and biochemical characterization of the isolates.

The basic aim of this study was to isolate the virulent *Agrobacterium tumefaciens* from peach trees. We were also interested to confirm this bacterium by different morphological, biochemical, and pathogenicity and antibiotics sensitivity tests.

MATERIALS AND METHODS

Crown galls collection

Crown gall samples were isolated from the dicotyledonous plant i.e. peach (*Prunus perisca*). The samples were collected from the different areas of the district Swat and Shangla. These samples were taken into laboratory with great care to avoid contamination and used for sample isolation. The experimental period was during March to July, 2016.

Preparation of crown gall samples

The crown gall samples were labeled in the laboratory and rinsed with tap water to remove the hazardous materials and other soil particles from the samples. A solution of 10% commercially available bleach was prepared. The gall samples were immersed in the solution for 3-5 min but it depends on the galls nature. Subsequently, the galls were washed with sterilized distilled water to remove the traces of the bleach solution. The crown gall samples were kept in sterilized distilled water for 5-6 days to make it soft for sample collection. Then galls were chopped into small pieces and were kept in sterilized distilled water for 2-3 days.

MacConkey media preparation

The MacConkey media is composed of the following ingredients: 1.5 g bile salts, 17 g pancreatic digest of gelatin, 10 g lactose monohydrate, 3 g peptones (casein and meat), 13.5 g agar, 0.001 g crystal violet, 5 g NaCl, 0.03 g neutral red and pH 7.1 (Bopp et al., 1999). MacConkey media (49 g) was weighed according to the manufacturer instructions and dissolved in 1 liter distilled water. Then the solution was completely mixed and autoclaved for 15 min at 121 °C. The media was allowed to cool and then poured into petri plates under sterilized condition. The media in the petri plates were allowed to solidify and then sealed. The plates were kept for 24 h in the incubator at room temperature.

Crown gall extracts culturing on MacConkey media

Galls extracts were taken out from the sterilized water. The galls were cut into small pieces through a sterilized blade. Small pieces of samples were picked by a loop and inoculated into the MacConkey media by a Streak plate method. Two replicates were produced from each sample. Then petri plates were kept in the incubator at 28 °C for 2-3 days.

Sub culturing on MacConkey media

The initial bacterial culture was sub cultured on MacConkey media to produce pure culture of *Agrobacterium*. In the sub culturing, a single colony was picked from each plate with the help of loop and inoculated into MacConkey media by a Streak plate method. The process was repeated continuously and from each plate, one replicate was formed. After sub culturing, the petri plates were kept in the incubator for two to three days at 28 °C. The pure culture appeared on MacConkey media that was used for different tests for the confirmation of *Agrobacterium tumefaciens*.

Identification of *Agrobacterium tumefaciens*

The bacterial species can be identified from certain biochemical and physiological characteristics by observing its physiological nature and morphological characteristics (Adenemo & Onilude, 2014). During this research study, the morphological characteristics such as texture, colour and shape of many bacterial colonies were taken into consideration. Different biochemical tests were performed to confirm the presence of *Agrobacterium tumefaciens* in galls. The biochemical tests such as potassium hydroxide test, gram staining, pathogenicity tests (carrot-disc bioassay and potato-disc bioassay) as well as antibiotic sensitivity test were carried out for the identification of *Agrobacterium tumefaciens*.

Potassium hydroxide test

In this test, 3% KOH solution was prepared. A single drop of KOH was put on a slide. Bacterial colony was picked from bacterial pure culture and mixed with KOH on the slides. The slides were rotated for 10-15 sec. Subsequently, the solution on the slide was picked up by tooth pick that revealed a sticky thread like paste. The formation of thread like paste confirmed that the isolated bacterial culture was gram negative.

Gram staining

The slide was washed with 95% ethanol. A drop of distilled water was placed on a slide. Then bacterial colony was picked from the bacterial culture, placed on the slide and properly mixed. The slides were air dried. Crystal violet dye was applied to the slide with the help of dropper for 30 sec. The slides were rinsed with sterile water to remove the excess dye. Gram Iodine was applied to the slide for 1 min and washed. Then 95% ethanol was applied and rinsed the slide again with the distilled water. The slides were further treated with Safranin called counter stain for 1 min and rinsed. After gram staining, our results showed red colour confirming the presence of gram negative bacteria.

Pathogenicity test

Carrot-disk bioassay and potato-disk bioassay were performed as pathogenicity test.

Carrot and potato-disc bioassays

In this test, carrot (*Daucus carota*) discs were used (Aysan et al., 2003). Carrot was cut into small disc and properly washed with 95% of Bleach solution for 3 min and then washed with double distilled water. Sterilized filter paper was kept on each petri-dish where the carrot disc was placed. A single colony of bacterial culture was picked up from petri plates and poured into each disc present in the petri dish. Subsequently, these plates were kept in incubator for 20 days to observe young galls formation on the carrot disc. The same method of carrot disc bioassay was repeated for potato disc (Hussain et al., 2007).

Antibiotic sensitivity tests of isolates

The antibiotic sensitivity tests were performed on the isolates according to the Bauer Kirby method (Bauer et al., 1966). The antibiotics namely cefotaxime, kanamycin, rifampicin and tetracycline were used. A standard bacterial culture (20 µl) was used in antibiotic sensitivity tests. A solution of 10 µl antibiotics was prepared and a filter paper was soaked in it and placed on the designated isolates. The plates were kept at 30 °C for 24 h in an incubator. The measurement of the size of inhibition zone shows vulnerability to antibiotics.

RESULTS

Collection of crown gall sample

The fresh crown gall samples were identified and collected from peach trees. The crown gall samples have been shown in fig.1.

Isolation of *Agrobacterium tumefaciens*

After inoculating crown gall samples on MacConkey media, the colonies of bacterial culture were noticed 2-3 days after inoculation at 28 °C. The initial bacterial culture plates have been shown in fig. 2. After sub-culturing of bacterial plates, the pure culture isolation was obtained. The inoculated plates showed the presence of *Agrobacterium tumefaciens*.

Identification of bacterium by morphological characteristics of the colonies

The morphological characteristics like shape, color and texture of the bacterial colonies were observed on MacConkey media. The shape of the bacterium was convex, colour of the bacteria was pink to brick red and the texture showed that colonies were smooth, circular, micoud, translucent and shiny appearance. All the observed characteristics were similar to that of *Agrobacterium tumefaciens* culture.

Table 1 KOH tests of five isolates of bacterium

Biochemical test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Gram staining	-ve	-ve	-ve	-ve	-ve

-ve = Gram negative

Table 2 Gram staining of five isolates of bacterium

Biochemical test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Gram staining	-ve	-ve	-ve	-ve	-ve

-ve = Gram negative

Table 3 Carrot and Potato disc bioassay of five isolates of bacterium

Pathogenicity test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Carrot-disc assay	+ve	+ve	+ve	+ve	+ve
Potato-disc assay	+ve	+ve	+ve	+ve	+ve

+ve = Tumor formation in the discs



Fig. 1 Crown galls formation in different peach trees

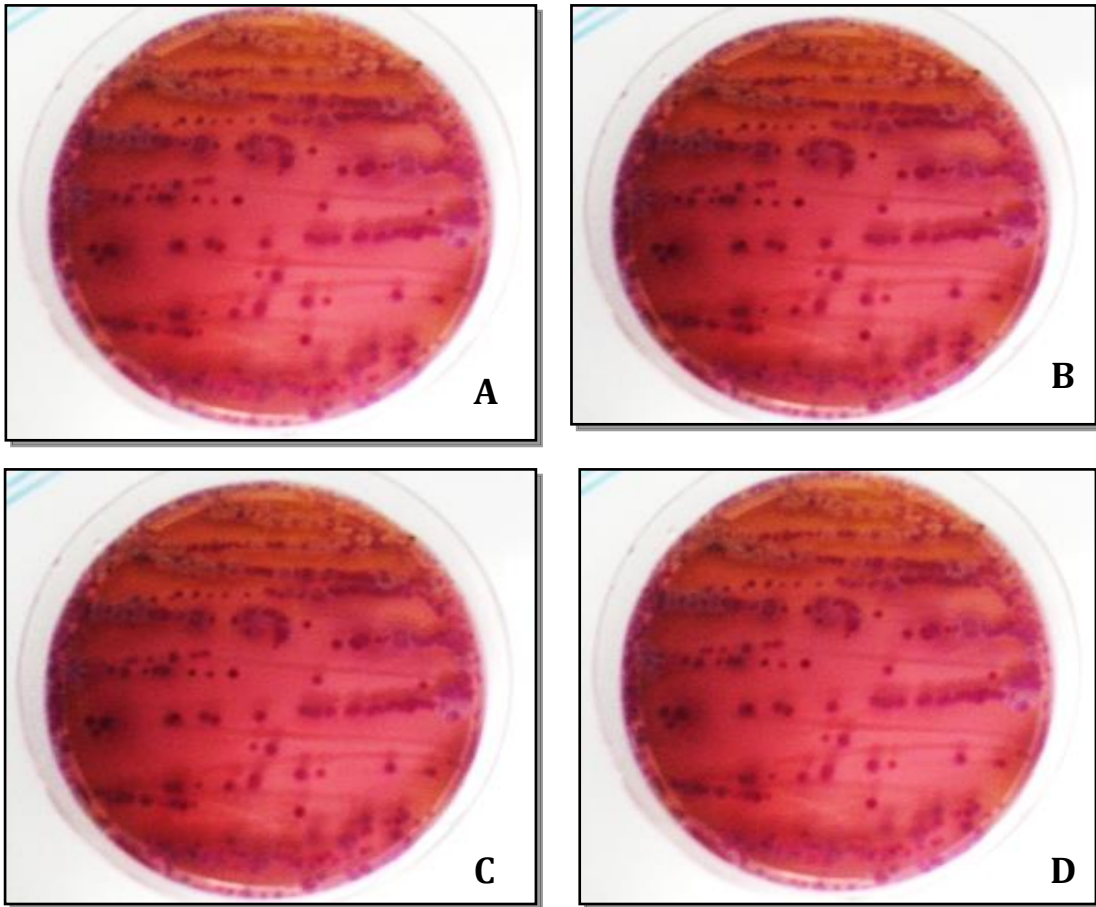


Fig. 2 *Agrobacterium tumefaciens* isolated from crown galls on MacConkey media

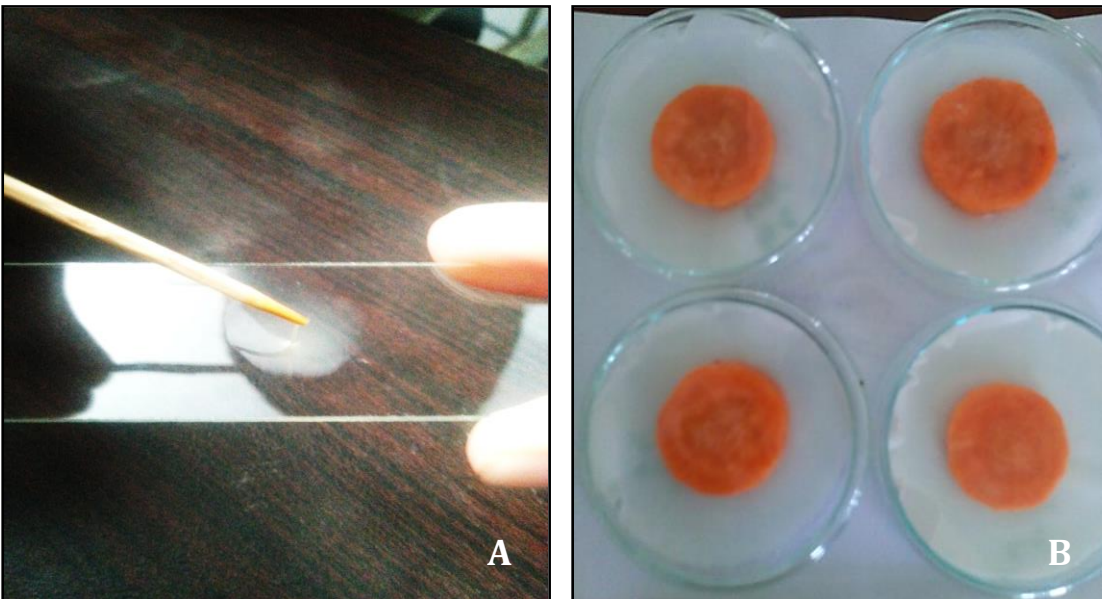


Fig. 3 (A) KOH test of gram negative (B) Pathogenicity test of carrot disc assay

Biochemical tests for identification of bacterial isolates

Two different biochemical tests were conducted to confirm the identity of bacterium as gram negative.

Potassium hydroxide test

The KOH test was conducted in order to confirm that the bacterium was gram negative or positive. The thread like slime appearance of the smear of bacterial isolates after KOH test revealed that the isolated bacterial culture was gram negative (Table 1; Fig. 3A). The gram negative result further confirmed that the bacterial isolates were *Agrobacterium tumefaciens*.

Gram staining test

The isolated bacterial culture was further tested with gram staining to confirm the identity of the bacterium as gram negative. The color produced by the bacterial isolates after gram staining was red that further revealed similarity with *Agrobacterium tumefaciens* (Table 2).

Pathogenicity tests

Pathogenicity test with two different dicotyledonous plant samples i.e. carrot and potato were conducted to confirm the pathogenicity of *Agrobacterium tumefaciens* causing crown gall under *in vitro* conditions.

Carrot-disc and potato-disc bioassays

In both bioassay tests, the discs of both plants species were infected with the bacterial culture in lab conditions. The test was conducted to confirm whether the bacterium inoculated in the discs initiated the formation of galls. The results revealed that galls formation was initiated in all the petri dishes inoculated with bacterial isolates (Table 3). Hence, it was confirmed that the bacteria isolated were *Agrobacterium tumefaciens*. The discs with crown galls produced by bacterial isolates have been shown in fig. 3B.

Antibiotic sensitivity tests of isolates

The antibiotic sensitivity test was performed on the isolates whether the bacteria showed resistance or became vulnerable to these antibiotics. The bacterial isolates were found resistant to tetracycline and ampicillin and no zone of inhibition was formed around the respective antibiotic disc on petri dish by *Agrobacterium tumefaciens*. The two other antibiotics i.e. cefotaxime and kanamycin formed clear zone of inhibition around the discs and these zones showed that bacteria were vulnerable to these antibiotics.

DISCUSSION

In present study, we identified peach trees with crown gall disease most commonly caused by *Agrobacterium tumefaciens* and then we isolated the gall samples. The isolated bacteria from crown gall samples were confirmed as *A. tumefaciens* by different morphological, biochemical, and pathogenicity and antibiotics sensitivity tests.

Crown gall disease is caused by *A. tumefaciens* that is gram negative soil bacteria (Nester, 2015). *Agrobacterium tumefaciens* infects dicotyledonous plants; both herbaceous and woody plants (Rhouma et al., 2006). In dicots, *A. tumefaciens* is mostly present on the stem of the plants resulting in crown gall formation. Different methods have been used to isolate the bacterium from the galls including sample collection and culturing it on specific selection medium (Collins, 2001). In our study, we isolated the bacteria using MacConkey selection media. MacConkey media has been previously used as a selective media for the isolation of *Agrobacterium tumefaciens* from crown gall samples (Bopp et al., 1999). The culture colonies of *A. tumefaciens* showed brick red colour on MacConkey selection media (Bergey's Manual of Determinative Bacteriology) (Holt et al., 1994). The bacterial isolates in our research study also revealed brick red color

when cultured on MacConkey selection media that showed resemblance with the morphological features of *A. tumefaciens*.

We conducted different biochemical and pathogenicity tests including KOH test, gram staining, potato disc assay and carrot disc assay. All these tests revealed that the isolated bacterium was gram negative and had the ability to cause tumor in plant disc sample under *in vitro* conditions. The biochemical and pathological approaches have been authenticated in the previous research studies for the identification of *A. tumefaciens* from crown gall samples of different plant species (Chen et al., 1999).

We were interested to assess the sensitivity of *Agrobacterium tumefaciens*. Therefore, we conducted antibiotic sensitivity tests to appraise the sensitivity of this bacterium to various antibiotics i.e. tetracycline, rifampicin, cefotaxime and kanamycin using Bauer Kirby method. The bacterium exhibited natural resistance to tetracycline and rifampicin, while it was susceptible to cefotaxime and kanamycin. A similar type of study was conducted by Bauer et al. (1966) in which the bacterial samples showed resistance to tetracycline and rifampicin forming inhibition zones and were found susceptible to cefotaxime and kanamycin. The molecular identification of this bacterium by PCR is also the most efficient method of its identification (Koivunen et al., 2004; Rhouma et al., 2006). During this study we also confirmed the gram negative nature of *Agrobacterium tumefaciens* by different biochemical and pathogenicity tests that have also been reported by Chen et al. (1999).

CONCLUSION AND RECOMMENDATIONS

In the present study, we have identified crown gall formation in different fruit trees of peach. The bacterial pathogens causing crown gall disease was confirmed as *Agrobacterium tumefaciens* by different biochemical tests, pathogenicity tests and antibiotic sensitivity tests. The identification of the bacterium can help for future research to devise strategies for the control of this pathogen. Further study of this bacterium can also help to utilize it for genetic engineering purpose in plants. The following recommendations were made in the light of this project: The research should be broadening to other fruit trees like cherry, apricot etc. These bacterial isolates should be further confirmed by PCR.

Author Contribution Statement Nizar Ali and Akbar Zada carried out the experiment. Murad Khan helped during the experiment. Zahid Hussain participated in the design, arrangements and coordination of the study and helped to draft the manuscript. All the authors read and approved the final manuscript.

Conflict of Interest All the authors have approved the final version of the manuscript being submitted. The article is the authors' original work. Our submitted manuscript is not under consideration for publication in another journal. The authors declare that they have no conflict of interest.

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