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PLANT BIOTECHNOLOGY | ORIGINAL ARTICLE

Morphological, biochemical and molecular identification of the wild strain of *Agrobacterium tumefaciens* from crown gall infected mango tree

Mst Lailatul Ferdous, Md Najmul Hossain, Md Osman Ali, Md Shahidul Islam, Sabina Yasmin

Department of Biotechnology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

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Md Rashidul Islam
rasha740177@yahoo.com

**Corresponding Author*
Sabina Yasmin
sabina.yasmin@bau.edu.bd

ABSTRACT

Crown gall is one of the destructive diseases that is considered to be of great economic impact due to the significant losses in mango and other stone fruit tree nurseries. The plant pathogen that causes crown gall is *Agrobacterium tumefaciens*. The present study was conducted to isolate and identify *Agrobacterium tumefaciens* from crown gall samples. These samples were collected from BAU campus, Mymensingh. The bacterium was isolated and characterized by morphological, microscopic, biochemical and confirmed by molecular test. The morphological, microscopic and biochemical tests initially revealed that all the bacterial isolates are gram negative. Pathogenicity test: a carrot disc bioassay showed tumors in the plant because T-DNA of the bacteria was transferred and integrated into each of carrot disc. Antibiotic sensitivity tests revealed that the resistance to antibiotic. Moreover, DNA was isolated from infected tissue and after gel electrophoresis of PCR product, DNA band with expected 184bp was found and therefore, it was finally confirmed that the isolates were *A. tumefaciens*. Therefore, morphological, biochemical and PCR-based rapid detection techniques could effectively be used to detect the nature's best genetic engineer, *A. tumefaciens* causing crown gall disease.

Keywords: Disease, bacterium, confirmation, mango tree

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1 Introduction

Agrobacterium tumefaciens is a soil borne pathogen that has immense importance in plant genetic engineering. It causes crown gall tumors on a wide range of plants including most dicots, some monocots and some gymnosperms (Matthysse, 2006). For tumor formation, Ti plasmid of virulent *Agrobacterium* strains serves as a source for the transfer DNA (T-DNA), a DNA region that is imported into plant cells and integrated into the host genome (Tempe and Petit, 1982). The T-DNA contains genes that cause plant cells to overproduce hormones such as auxin and cytokinin which results in cell proliferation and the production of a gall (Finer et al., 2016). Due to its distinctive mode of infection, *Agrobacterium* is a natural genetic engineer of plant cells. It has great importance not only in plant genetic engineering but also in antitumor studies as well as in microbiological research purpose.

In Bangladesh, Mango (*Mangifera indica*) is a very popular fruit, also called 'The King of Fruits' due to its delicious taste, marvelous fragrance, and beautiful appearance. Ripened mango contains adequate quantity of carotene or vitamin A and minerals. Like other diseases, crown gall also affects mango plantations and decrease of vigour. However, numerous studies have been shown on isolation and characterization of the pathogenic wild strain of *A. tumefaciens* from infected leaves, stems and crown galls of different plants by using morphological, physiological, biochemical and molecular tests etc. (Ali et al., 2016;

Khan et al., 2016; Setti and Bencheikh, 2013; Islam et al., 2009; Tiwary et al., 2007). But there is little report on isolation and identification of *Agrobacterium* strains from diverse plants in Bangladesh.

Keeping in view the importance of *Agrobacterium* in plant biotechnology, the present study has been undertaken to isolate and characterize virulent *A. tumefaciens* strains from locally mango trees. Highly virulent strain could be used for construction of genetically engineered strains, *in vitro* antitumor studies of plant's extract and other biological purposes. So, the basic aim of the study was to isolate the *A. tumefaciens* from crown galls samples of infected mango trees using morphological, biochemical tests and Polymerase Chain Reaction (PCR).

2 Materials and Methods

2.1 Crown galls collection

Three crown gall samples were collected from the different mango trees of Bangladesh Agricultural University Area, Mymensingh, Bangladesh. These samples were taken into laboratory and used for *Agrobacterium* isolation (Fig. 1).

2.2 Preparation of crown gall samples

The crown gall samples were labeled and rinsed with tap water to remove the hazardous materials and other soil particles from the samples. The gall samples were dipped in the solution of 10% commercially available bleach for 5-8 min (Ali et al., 2016). Subsequently, the galls were washed with sterilized distilled water to remove the traces of the bleach solution. Then the crown gall samples were kept in sterilized distilled water for 6-8 days to make it soft for sample collection. Then galls were chopped into small pieces and were kept in sterilized distilled water for 3 days.

2.3 Culture of bacteria

Loopful of tissue suspensions were streaked on yeast mannitol agar (YMA) medium. Plates were incubated at 28 °C for 3-5 days. In the sub culturing, a single colony was picked from each plate with the help of loop and inoculated into fresh media by a streaking plate method. The process was repeated continuously. For incubation, the petri plates were kept in the incubator for 2-3 days at 28 °C (Ali et al., 2016). The pure culture was used for different tests for the identification of *A. tumefaciens*.

2.4 Characterizations of *A. tumefaciens*

The bacterial species could be identified by observing from certain morphological, biochemical and physiological characteristics. The morphological characteristics such as texture, color of many bacterial colonies were taken into consideration. Different biochemical tests were performed to confirm the presence of *A. tumefaciens* in galls. The biochemical tests such as potassium hydroxide test, catalase production test were conducted. Microscopic test was performed. Pathogenicity tests (carrot-disc bioassay) as well as antibiotic sensitivity test were carried out for the identification of *A. tumefaciens*.

2.4.1 Morphological characterization

The color and texture were observed to ensure whether the isolates were gram negative or positive.

2.4.2 Microscopic characterization

In microscopic characterization, gram staining was performed and after staining, the color was observed.

2.4.3 Biochemical characterization

Potassium hydroxide test A single drop of KOH (3%) was put on a slide and overnight culture were mixed on the slide. The slides were rotated for 10-15 sec. Subsequently, the solution on the slide was picked up by tooth pick and a sticky thread like paste was observed (Ali et al., 2016).

Catalase production test A slide was swabbed with 95% ethanol. Fresh isolate was transferred to a clean slide using sterilized toothpick and thoroughly mixed with a small drop of sterile distilled water. Then, a drop of 3% hydrogen peroxide (H_2O_2) was added on the smear and the bubble was formed (Reiner, 2010).

2.4.4 Pathogenicity test

Carrot-disk bioassay was performed as pathogenicity test. In this test, carrot discs were placed and poured overnight bacterial culture on it; the plates were incubated at 28 °C for 20 days. The formation of young galls on the carrot was observed (Ali et al., 2016).

2.4.5 Antibiotic sensitivity tests of isolates

Antibiotic sensitivity tests were performed according to the Bauer et al. (1966) method. The antibiotics discs of kanamycin and rifampicin, and overnight bacterial culture were used. Bacterial isolates were spread on YMA media and placed two antibiotic discs. The size of inhibition zone was observed after incubation at 28 °C for 24 hrs.

2.5 Molecular identification

Molecular identification was done at the molecular level without any effect of environment or development or physiological state of the organism.

Figure 1. Crown galls formation in mango trees. (a) Crown gall infected mango tree (b) Crown gall sample

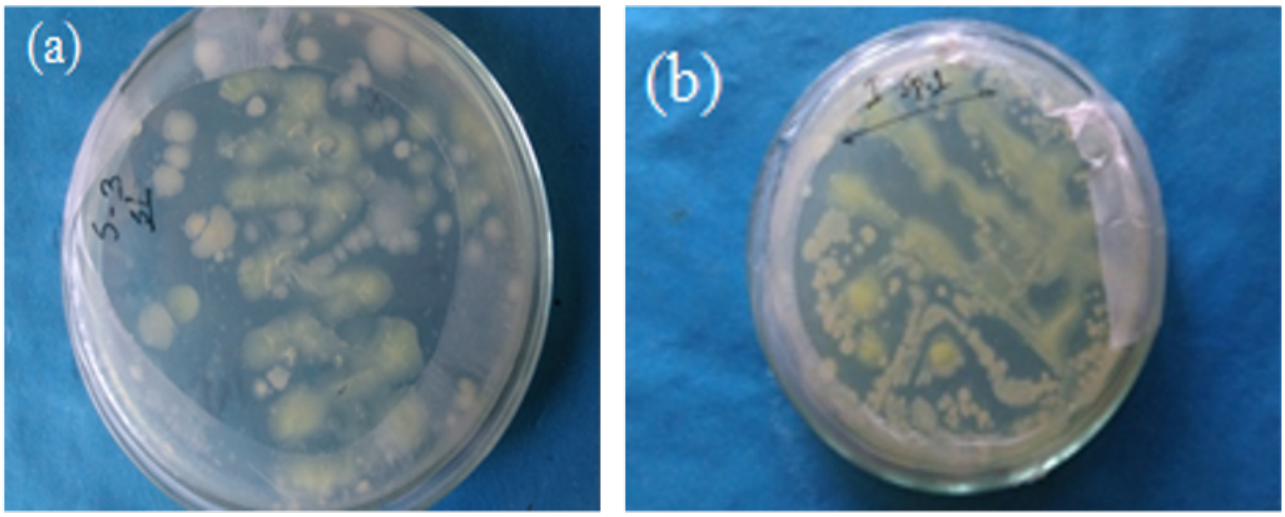

Figure 2. *Agrobacterium tumefaciens* isolated from crown galls on YMA media. (a) Streaking method (b) Spreading method

2.5.1 DNA Extraction of the strain

Bacterial cell were grown overnight in LB broth at 28 °C to extract genomic DNA from the pure culture. DNA was extracted using the protocol of AddPrep Genomic DNA Extraction Kit (Addbio, korea). Extracted DNA was confirmed by 0.8% agarose gel electrophoresis and quantified using a Nanodrop.

2.5.2 PCR amplification and agarose gel electrophoresis

The extracted genomic DNA was added into ready-made PCR reaction mixture. The volume of reaction mix was 20 µLcontaining 10 µL AddTaq Master mix (Addbio, Korea), 0.5 µL Universal Forward Primer (UF) 5′-GTA AGA AGC GAA CGC AGG GAA CT-3′ and 0.5 µL Biovar-1 Reverse Primer (B1R) 5′-GAC AAT GAC TGT TCT ACG CGT AA-3′3′ (Puławska et al., 2006), 8 µL DNA template (conc. 25ng/μl) and 1 µL nuclease-free water. PCR was done using thermal Cycle with an initial denaturation step at 95°C for 5 min, followed by: 30 cycles of 95°C for 30s, 60°C for 30 s, 72°C for 30 s, and final extension at 72 °C for 5 min. The PCR products were run on 2% agarose gel. Gel was stained with safe gel stain dye (Addbio, Korea), visualized and photographed using a gel documentation system.

3 Results

3.1 Characteristics of isolates

3.1.1 Morphological characteristics

The morphological characteristics like color and texture of the bacterial colonies were observed on YMA media Fig. 2. The color of the bacteria was gray to bright ashen and the texture showed that colonies were smooth, circular, micoud, translucent and shiny appearance.

3.1.2 Microscopic characteristics

Three cultures were tested with gram staining to confirm the identity of the bacterium whether as gram negative or positive. Three bacterial isolates produced pink color after staining. It is presumed that all bacterial cultures were gram negative (Fig. 3).

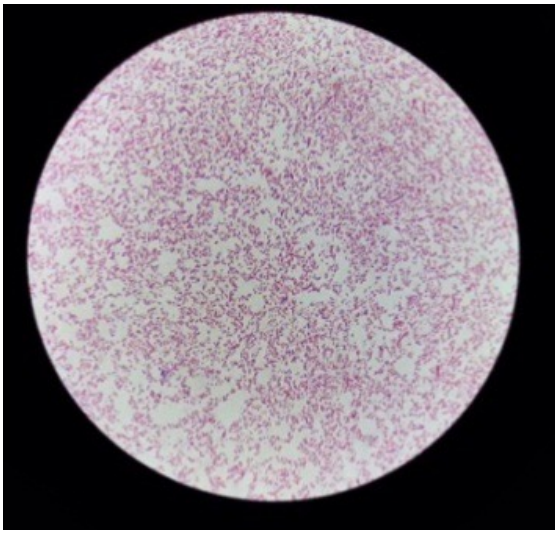

Figure 3. Microscopic test for gram negative confirmation

3.2 Biochemical tests

3.2.1 Potassium hydroxide test

The potassium hydroxide test was conducted in order to confirm whether three bacterial isolates were either gram negative or positive. The thread appeared like paste, sticky when solution was picked up by tooth pick. It was assumed that the culture of bacteria was gram negative (Fig. 4).

3.2.2 Catalase production test

The catalase production test was performed in order to confirm three bacterial isolates were gram positive or negative. After the test, three isolates were produced bubble. It can be allowed that all cultures were gram negative (Fig. 5).

3.3 Pathogenicity tests

In this test, the discs of carrot were infected with the bacterial culture in lab conditions. The results revealed that galls formation was initiated in all the petri dishes inoculated with bacterial isolates (Fig. 6).

3.4 Antibiotic sensitivity tests

The bacteria showed resistance or became vulnerable to these antibiotics. Three bacterial isolates were found resistant to rifampicin, and no zone of inhibition was formed around the antibiotic disc on petri dish by *A. tumefaciens*. The other antibiotic i.e. kanamycin formed clear zone of inhibition around the disc (Fig. 7).

3.5 The identification of *A. tumefaciens*

Two replications of each isolate were used for PCR. UF and BIR primers were used for amplification of 23S rRNA gene sequence of isolates and visualizing 184bp 23sRNA for the identification and confirmation of *A. tumefaciens* (Fig. 8).

4 Discussion

Crown gall disease is caused by *A. tumefaciens* which are gram negative soil bacteria (Nester, 2015). *A. tumefaciens* infects dicotyledonous plants; both herbaceous and woody plants. 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 culture on specific selection medium (Rhouma et al., 2006). In this study, the isolated bacteria from crown gall samples were initially cultured on YMA media and identified as *A. tumefaciens* by different morphological, biochemical, microscopic, pathogenicity and antibiotics sensitivity tests. In addition, bacteria were also confirmed at molecular level by PCR.

In morphological study, all bacterial isolates were gray color when cultured on YMA selection media. YMA media has been previously used as a selective media for the isolation of *A. tumefaciens* from crown gall samples. The culture colonies of *A. tumefaciens* showed gray white on YMA selection media and showed resemblance with the morphological features of *A. tumefaciens* (Song et al., 2004). During microscopic test, three isolates of bacteria showed pink color which was similar to the findings of Ali et al. (2016). It confirmed that three isolates were the gram negative nature of *A. tumefaciens*. Different biochemical tests were conducted including KOH test, catalase production test, carrot disc assay as pathogenicity test and antibiotic sensitivity test (Figs. 4 to 7). 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 approaches were used in the previous research studies for the identification of *A. tumefaciens* from crown gall samples of different plant species (Chen, 1999). In potassium hydroxide test, the thread was appeared like paste, mucoid, sticky which showed similarity with potassium hydroxide test of Ali et al. (2016). In catalase production test, three isolates were produced bubble which showed resemblance with

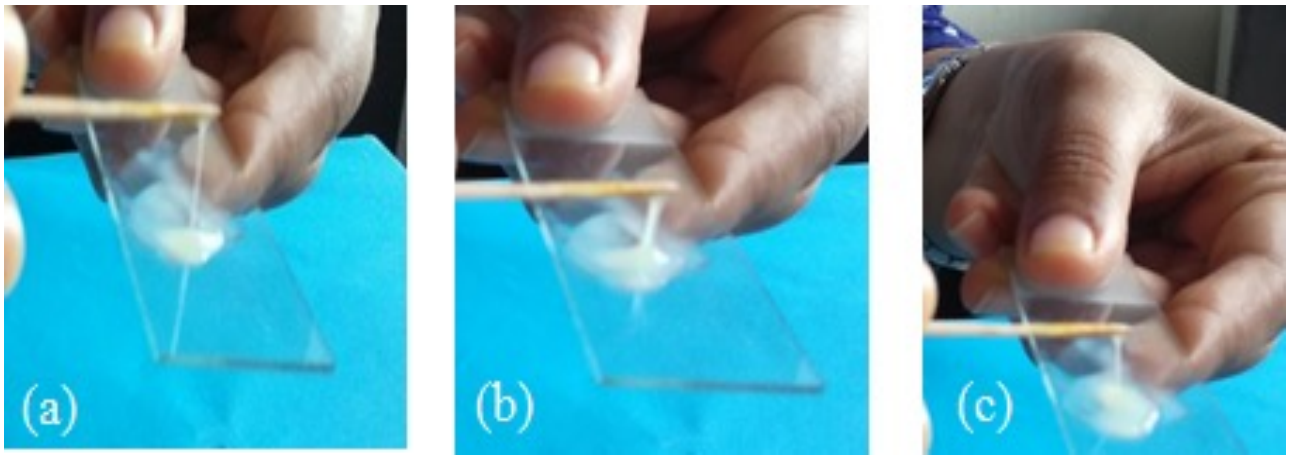

Figure 4. KOH test of gram identification. (a) Sample I, (b) Sample II, and (c) Sample III

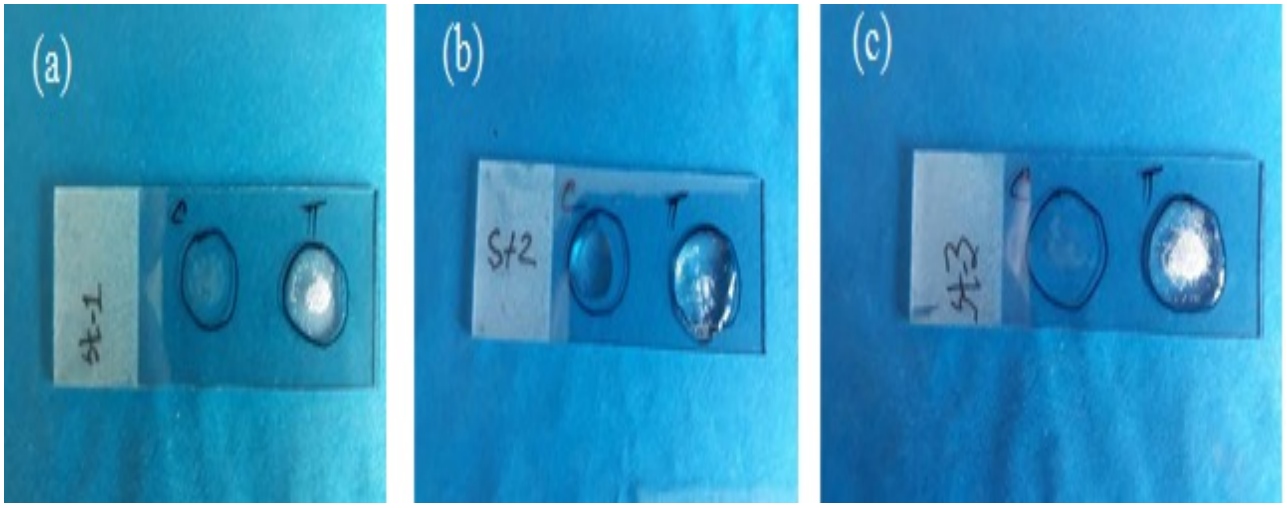

Figure 5. Catalase production test (a) Sample I, (b) Sample II, and (c) Sample III

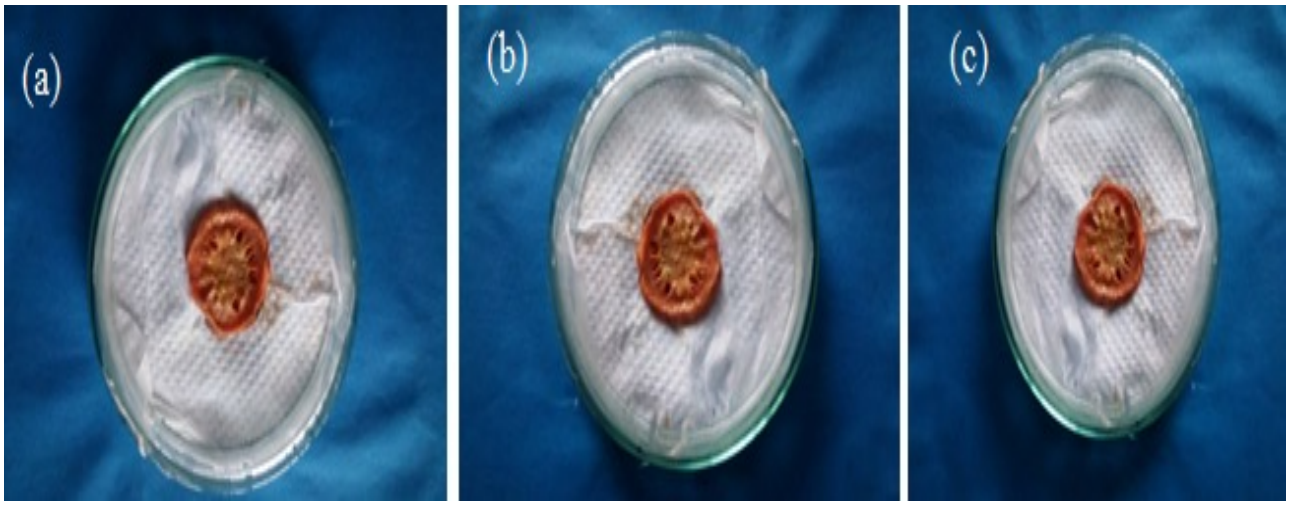

Figure 6. Pathogenicity test of carrot disc assay by using overnight culture bacteria. (a) Sample I, (b) Sample II, and (c) Sample III

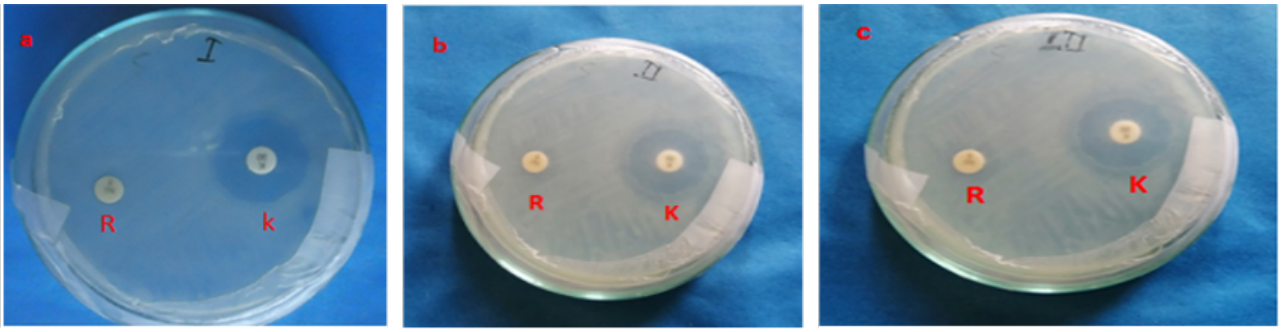

Figure 7. Antibiotic sensitivity test of bacteria by using antibiotic discs. (a) Overnight culture of Sample I, (b) Overnight culture of Sample II, and (c) Overnight culture of Sample III; R= rifampicin antibiotic disc and K = kanamycin antibiotic disc

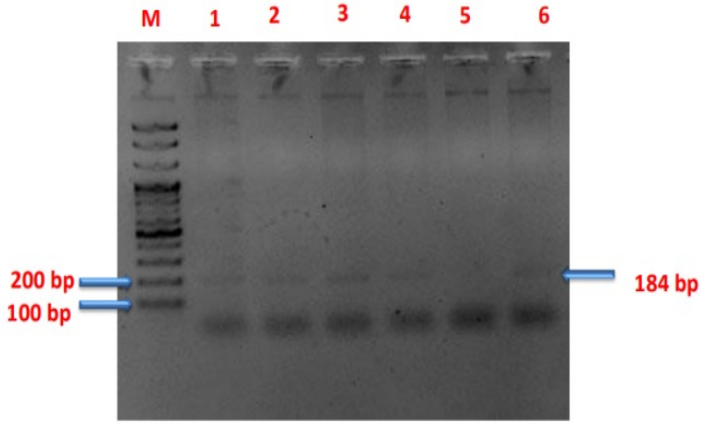

Figure 8. Confirmation of presence of DNA by agarose gel (2%) electrophoresis after DNA extraction using Addprep genomic DNA extraction kit

catalase production test of *A. tumefaciens* (Chen, 1999). Carrot disc bioassay was performed as pathogenicity test. Young galls were formed on carrot disc. Ti-plasmid of isolates was transferred and integrated into dicotyledonous carrot disc. A similar type of study was conducted by Aysan and Sahin (2003) in which, galls were formed on carrot disc. It was interesting to assess the sensitivity or zone of inhibition of *Agrobacterium tumefaciens*. Therefore, antibiotic sensitivity test was conducted to appraise the sensitivity of this bacterium to various antibiotics i.e. rifampicin and kanamycin using Bauer Kirby method. The bacterium exhibited natural resistance to rifampicin, while it was susceptible to kanamycin. A similar type of study was conducted by Bauer et al. (1966) in which the bacterial samples showed resistance to rifampicin forming no inhibition zones and were found susceptible to kanamycin.

The molecular identification of bacterium by PCR is the most efficient method for its identification (Koivunen et al., 2004; Rhouma et al., 2006). Genomic DNA of three isolates was extracted and two replications of each isolate were used. UF and B1R primers were used for amplification of 23S rRNA gene sequence of isolates for the identification of *A. tumefaciens*. Extracted genomic DNA sample was used in PCR for the amplification of 23S rRNA gene sequence. The expected band of 184 bp confirmed whether isolates were *A. tumefaciens* or not. In our study, each of the six genomic DNA samples gave 184 bp bands of all isolates (Fig. 8). Similar study was done by Puławska et al. (2006) where all PCR reactions were performed using the combination of UF and B1R primers.

5 Conclusion

Mango is susceptible to various types of pathogens that result in decline of its fruit production. *A. tumefaciens* attacks on mango plant and it causes crown gall disease. We have isolated and identified crown gall formation in different fruit trees of mango.The bacterial pathogens causing crown gall disease was identified as *A. tumefaciens* by different morphological test, biochemical test, pathogenicity test, antibiotic

sensitivity test and confirmed by molecular test. The crown gall caused by *A. tumefaciens* is becoming a great threat to nursery and fruit production. The identification of this 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.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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