Identification of Virulent *Agrobacterium tumefaciens* Strains from some Dicotyledonous Plants in Bangladesh

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Summary

Wild type virulent *Agrobacterium tumefaciens* strains viz. AtTp0120, AtTe0121, AtMo0122 and AtMi0123 (accession number was given according to their respective host plants) were identified from four dicot plants viz. *Tagetes patula*, *Tagetes erecta*, *Moringa oleifera* and *Mangifera indica*, respectively, of Rajshahi University campus, Rajshahi, Bangladesh. Isolated strains were confirmed as *A. tumefaciens* on the basis of their morphological, physiological and biochemical features, antibiotic sensitivity, phytopathogenicity tests, and agarose gel analysis of plasmid DNA in comparing with type strian of *A. tumefaciens* (ATCC23308^{T}). Supported by statistical analysis of the number of induced tumors in potato disc strain AtTp0120 from *Tagetes patula* was identified as more virulent than the other isolates.

Key words

Agrobacterium tumefaciens, wild type, virulence, phytopathogenicity, antibiotic sensitivity and plasmid DNA

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Introduction

Agrobacterium tumefaciens is a soil borne bacterium; *A. tumefaciens* is a member of family-Rhizobiaceae. These are Gram-negative, rod-shaped and motile bacteria that grow aerobically without forming endospores (Collins, 2001). Its virulent strains cause crown gall disease throughout the world and infect dicotyledonous plant of about 90 different families and a few monocotyledonous plants (Cleene and Ley, 1976). *A. tumefaciens* is containing an extra-chromosomal DNA designated as Ti (tumor inducing) plasmid (Zaenen *et al*., 1974). Ti-plasmid carries two components: vir and T-DNA regions needed for genetic transformation (Tzfira *et al*., 2004). The molecular machinery needed for T-DNA generation and transport into the host cell comprises proteins that are encoded by the bacterial chromosomal virulence (chv) genes and encoded by the Ti-plasmid virulence (vir) genes (Gelvin, 2003; Tzfira and Citovsky, 2002; Zupan *et al*., 2000).

Agrobacterium can transform virtually any living cell, from other prokaryotes (Kelly and Kado, 2002) to yeast (Piers *et al*. 1996) and fungi (Groot *et al*., 1998; Gouka *et al*., 1999) to human cells (Kunik *et al*., 2001). Updated information of mechanisms for T-DNA transfer to plant cells by *A. tumefaciens* is provided, focused on the role played by the different components of the virulence system (Riva *et al*., 1998). *A. tumefaciens*-mediated transformation has widely been used for research in plant molecular biology and for genetic improvement of crops since 1983 (Park, 2006). Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal plants, fruit, trees and pasture plants, using *Agrobacterium*-mediated or direct transformation methods (Birch, 1997). This is part of our research looking for virulent strains of *A. tumefaciens* (Islam *et al*., 2010).

Materials and methods

Plant samples

Crown gall tissues were collected from four different dicot plants: *Tagets patula*, *Tagetes erecta*, *Moringa oleifera* and *Mangifera indica* available in Rajshahi University Campus, Rajshahi, Bangladesh. Samples were immediately transferred to the laboratory. Special care was taken to avoid contamination. The experimental period was from February to December, 2009.

Gall extraction

Samples were rinsed with tap water to remove soil and hazardous materials. Galls were sterilized with ten percent (10%) commercial bleach (Savlon, ACI limited, Bangladesh) for 1.5-3.0 min according to the nature of galls. After washing three more times with sterilized distilled water (SDW), galls were finely chopped and immersed in SDW and incubated overnight at room temperature (27-30^{0}C).

Isolation of bacteria

Overnight incubated crown gall extracts were streaked on to two different media i.e., MacConkey agar (Bopp *et al*., 1999) and Clark's selective medium designated as NASA (Serfontein and Staphorst, 1994). Plates were incubated at 28-30^{0}C for 18 to 24 h and examined for growth and color development. Bacterial colonies were selected based on colonies form, elevation, surface, color etc. Four individual colonies were transferred onto the same medium (NASA) and purified on MGY agar media (Putnam, 2006) for further purification. Purified isolates were cultured on Luria-Bertani (LB) medium described by Miller (1987) and preserved in glycerol (25%) stock for further experimentation.

Characterization of *A. tumefaciens*

Biochemical test

Bicochermical test of isolates was done according to Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994; Moore *et al*., 1988; Sawada and Ieki, 1992). The following tests were carried out: (i) Gram stain and motility at room temperature; (ii) catalase and oxidase production; (ii) utilization of lactose and mannitol; (iv) production of 3-ketolactose; (v) salt tolerance (2%); (vi) H_2S production; (vii) utilization of L-tyrosine; (viii) citrate utilization; (ix) growth on Mac Conkey, NASA and Luria-Bertain agar; (x) growth and pigmentation in ferric ammonium citrate.

Antibiotic sensitivity test

The antibiotic sensitivity of selected isolates was determined according to the method of Bauer-Kirby (Bauer *et al*., 1966). The following antibiotics i.e., Kanamycin (30 µg mL^{-1}), Cefuroxime (30µg mL^{-1}), Tetracycline (30 µg mL^{-1}) and Rifampicin (10 µm mL^{-1}) were used. Whatman No. 1 filter paper discs (6 mm in diameter) were impregnated with 10 µL of antibiotics solution with particular concentration followed by air-drying and then placed on seeded Luria-Bertani (LB) agar plates. Twenty microliter standard bacterial cultures (10^8 cfu mL^{-1}) were used for preparing seeded agar plates. The petri plates were incubated at 30°C for 24 h. Antibiotic susceptibility was determined by measuring the size of inhibition zone.

Phytopathogenicity test

Phytopathogenicity tests were done using both carrot (Chen *et al*., 1999; Aysan *et al*., 2003) and potato (Hussain *et al*., 2007) disc bioassays. Type strain of *A. tumefaciens* named ATCC23308^T was used as control in both cases.

Carrot disc bioassay

Carrot (*Daucas carota* L.) was collected from local market in Rajshahi city, Bangladesh. Carrots were sterilized with commercial bleach (Savlon, ACI limited, Bangladesh) followed by washing with SDW for three times and sliced. Each disc was overlaid with 100 µL of bacterial suspension (10^8 cfu mL^{-1}). Petri plates were sealed by parafilm and incubated in growth chamber (controlled environment, 25-30°C). Discs were checked (after 21 days) for young galls (tumors) developing from meristematic tissue around central vascular system of carrot.

Potato disc bioassay

Red skin potato (*Solanum tuberosum* L.) was also collected from local market. Potatoes were sterilized with commercial bleach (savlon) and 0.1% $HgCl_2$. Sliced potato discs (5×8 mm) were placed on water agar plates (1.5g/100mL). Each disc was overlaid with 50 µL of bacterial suspension (10^8 cfu mL^{-1}). Petri plates were sealed by parafilm and incubated at room temperature (25-30°C). After 21 days, discs were stained by Lugol's iodine solution (10% KI and 5% I_2) for 30 min and tumors were observed under stereo microscope, where the tumor cells lack starch (Hussain *et al*., 2007).

Table 1. Phenotypic characteristics of the selected strains of *A. tumefaciens*

Biochemical tests	Selected strains				Type strain of *A. tumefaciens*
	AtTp0120	AtTe0121	AtMo0122	AtMi0123	ATCC23308^T
Gram stain	–	–	–	–	–
Motility test	+	+	+	+	+
Catalane	+	+	+	+	+
Oxidase	+	+	+	+	+
Utilization of carbohydrates					
Lactose	+	+	+	+	+
Mannitol	+	+	+	+	+
3-keto lactose production	+	+	+	+	+
Salt tolerance	+	+	+	+	+
H_2S production	+	+	+	+	+
L-tyrosine utilization	–	–	–	–	–
Antibiotic sensitivity test					
Kanamycin	S	S	S	S	S
Cefuroxime	S	S	S	S	S
Tetracycline	R	R	R	R	R
Rifampicin	R	R	R	R	R
Phytopathogeneisity test	+	+	+	+	+

[*Note*: +: Positive, –: Negative, R: Resistant, S: Susceptible]

Agarose gel analysis of closed circular plasmid DNA

Plasmid DNA isolation has been carried out according to the alkaline lysis method of Birnboim and Doly (1979). Agarose gel preparation and electrophoresis have been performed according to Chawla (2004).

Results

Isolation of *A. tumefaciens*

Four bacterial colonies were observed and screened, isolated from four crown gall samples on the basis of their color development on selective medium. After 24 h of incubation, the bacterial colonies were visible on MacConkey agar plate, and after 28 h colonies were turned into pink to brick-red color. Colonies cultured on NASA medium turned brick-red color after two days of incubation. From these initial results, isoloated bacteria were tentatively identified as *A. tumefaciens* strains.

Charaterization of *A. tumefaciens*

Biochemical test

Biochemical features of the selected isolates are presented in Table 1 and Figure 1. Gram reaction indicates that selected isolates were Gram negative. Isolates were also negative for L-tyrosine utilization, and positive for motility, catalase, oxidase, lactose, mannitol, 3-keto lactose production and H_2S production. Similar reactions were also observed for type strain.

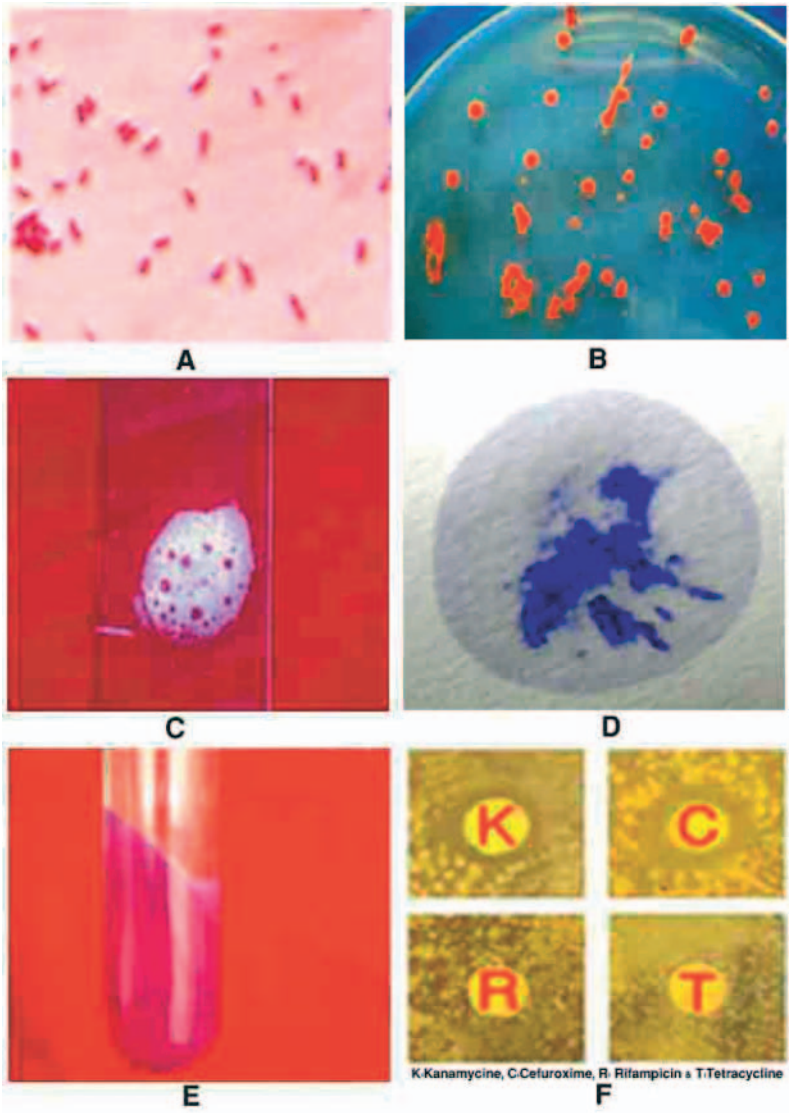

Figure 1. Various type of morphological, physiological and biochemical tests: A. Gram staining, B. Young bacterial colonies on NASA medium, C. Catalase test, D. Oxydase test, E. Urease test and F. Antibiotic sensitivity test using Kanamycine (K), Cefuroxime (C), Rifampicin (R) and Tetracycline (T).

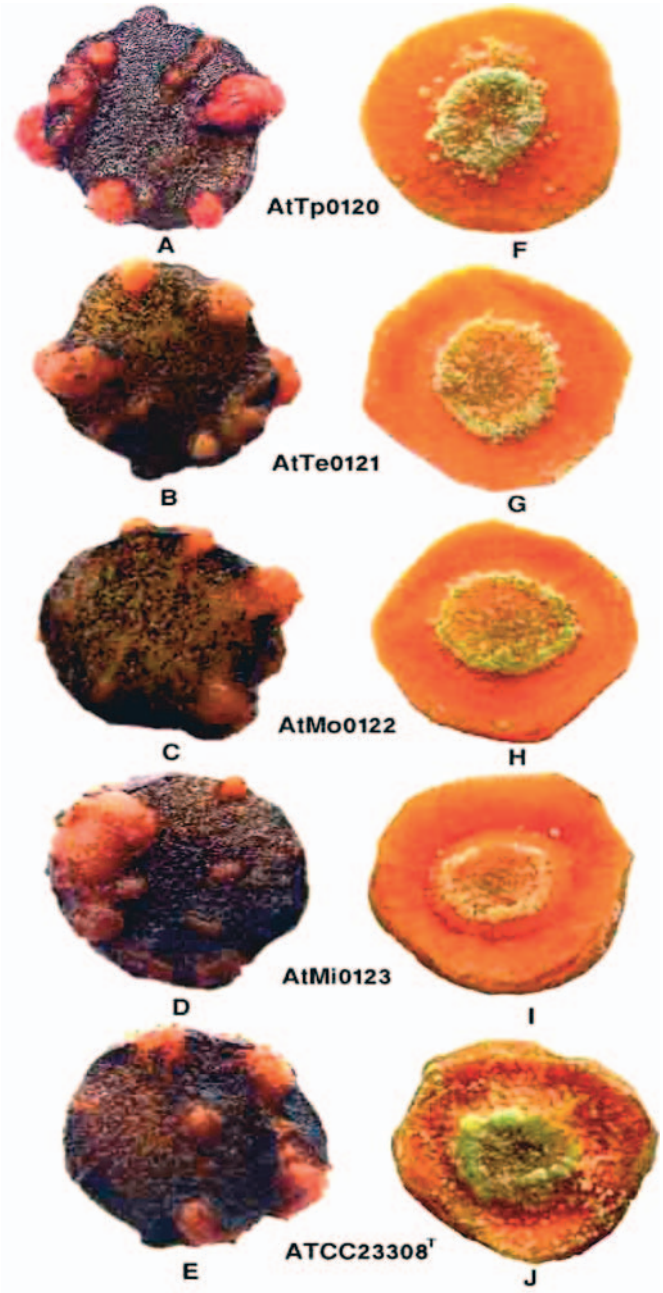

Figure 2. Showing the positive phytopathogenicity of selected *A. tumefaciens* strians (including with type strain of *A. tumefaciens*) on potato (A-E) and carrot (F-J) discs.

Antibiotic sensitivity test

Results showed (Table 1, Figure 1) that isolates were susceptible to Kanamycin and Cefuroxime (showing zone of inhibition) and resistant against Rifampicin and Tetracycline (showing no zone of inhibition).

Phytopathogenicity test

All four isolates and type strain were positive in phytopathogenicity test (Table 1) and produced young galls (tumor) on carrot and potato discs (Figure 2). The most virulent strain has been identified by comparison of mean number of produced gall on potato discs (replication number was three and 10 potato discs used for each replication) and results were confirmed by ANOVA. Statistical analysis proved that isolates are significantly different ($p<0.001$, df=4) in gall forming ability on potato disc.

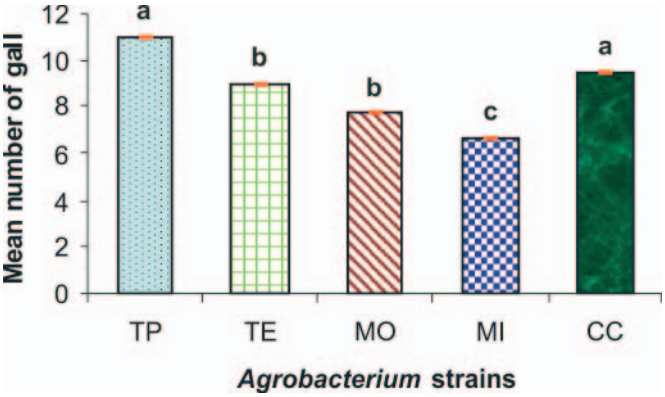

Figure 3. Virulence comparison among four *A. tumefaciens* strains. *Note*: TP: AtTp0120, TE: AtTe0121, MO: AtMo0122, MI: AtMi0123, CC: ATCC23308^T and mean number (three replicates) of gall is 11±0.58, 9±0.58, 7.67±0.88, 6.67±0.33, 9.5±0.33, respectively.

Agarose gel analysis of closed circular plasmid DNA

Isolated closed circular plasmid DNA of four *A. tumefaciens* strains (viz. AtTp0120, AtTe0121, AtMo0122 and AtMi0123) including with type strain of *A. tumefaciens* (ATCC23308^T) and GeneRuler^{TM} 1kb DNA Ladder was used for agarose gel analysis. It was observed that the plasmid DNA of these strains has been fall within the size range approximately 200-250 kb in comparison with 1kb DNA Ladder (Figure 4).

Discussion

Aim of this study was isolation of wild type highly virulent *A. tumefaciens* strains from different natural host dicot plants and confirmation of their characteristics using different morphological, physiological, biochemical, antibiotic sensitivity, and phytopathogenicity (tumor forming ability on carrot and potato discs) tests, and molecular analysis (agarose gel analysis of plasmid DNA). *A. tumefaciens* can generally be found on and around root surfaces known as the rhizosphere. It can effectively be isolated for identification from gall tissue, soil or water (Collins, 2001). In this study galls were collected from different dicot plants species: *Tagets patula*, *Tagetes erecta*, *Moringa oleifera* and *Mangifera indica* found in different places of Rajshahi University campus. On the basis of color development, desecrate four colonies were isolated from selective media (NASA). Isolates grew as pink to brick-red colonies on MacConkey agar and putative brick red colonies on NASA medium, tentatively identified as Gram negative *A. tumefaciens* strains. Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994) indicates that Gram negative bacteria generally grow as pink to brick-red colonies on MacConkey agar which was similar to our colonies. also Our results with *A. tumefaciens* strains were supported by work of Chen *et al.* (1999) because they also cultured crown gall

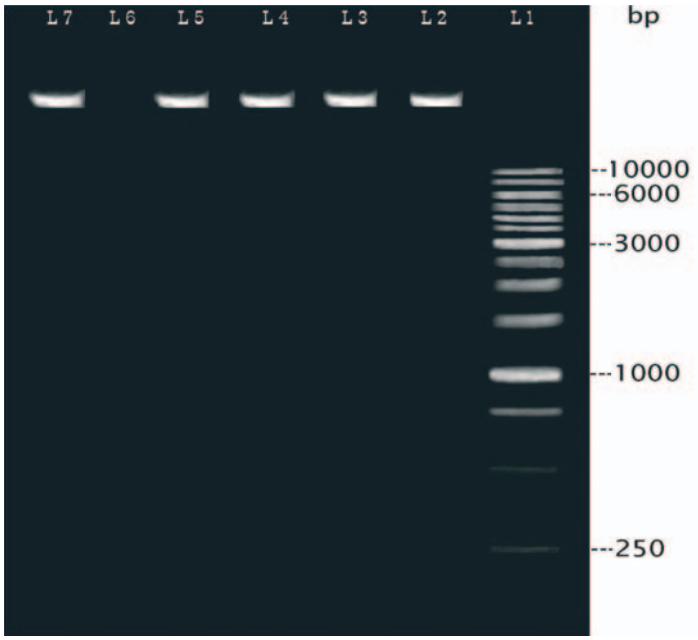

Figure 4. Agarose gel analysis of plasmid DNA of four *A. tumefaciens* strains (viz. AtTp0120, AtTe0121, AtMo0122 and AtMi0123) including with type strain of *A. tumefaciens* and compare at different base pair (bp) with Marker DNA (GeneRuler^{TM} 1kb DNA Ladder). *Note*: L1: Marker DNA, L2: AtTp0120, L3: AtTe0121, L4: AtMo0122, L5: AtMi0123, L6: Only buffer (negative control), L7: ATCC23308^{T}.

extracts. For further confirmation biochemical tests were performed according to Moore *et al*. (1988). Several workers (Chen *et al*., 1999; Koivunen *et al*., 2004) used series of biochemical tests (Moore *et al*., 1988) and their obtained results are in good agreement with ours.

Antibiotic resistance test showed that all the isolates were resistant to Rifampicin and Tetracycline, and susceptible to Kanamycin and Cefuroxime which was supported by Koivunen *et al*. (2004) and Karthy *et al*. (2009). It is another parameter to confirm *A. tumefaciens* strains.

Tumor forming ability of four selected isolates finally confirmed them as virulent *A. tumefacines* strains. Significant difference among the isolates was observed in tumor forming ability on potato discs. Among the locally isolated indigenous *A. tumefaciens* strains (viz. AtTp0120, AtTe0121, AtMo0122 and AtMi0123), AtTp0120 exhibited the highest virulence according to the mean number (replication number was three and 10 discs for each replication) of galls. The difference may be attributed to the nature of host plant, internal physiology of *A. tumefaciens* and also environmental condition. Chen *et al*. (1999) isolated *A. tumefaciens* strains from different types of gall tissue of aster and presented diverse virulence.

This is the first report of virulent *A. tumefaciens* isolated from *Tagets patula*, *Tagetes erecta*, *Moringa oleifera* and *Mangifera indica* (Turegon described crown gall of mango in 1982) in Bangladesh and also the world, whereas Chen *et al*. (1999), Aysan and Sahin (2003), Aysan *et al*. (2003) proved only tumor forming ability of *S. lycopersicum*, Aysan and Sahin (2003) reported crown gall disease of *Rosa* sp., and Islam *et al*. (2010) have been isolated *A. tumefaciens* strains from six dicot plants: *A. heterophyllus, T. grandis, T. arjuna, A. codomba, S. lycopersicum and R. chinensis*.

Conclusion

On the basis of *in vitro* phytopathogenicity, different biochemical and antibiotic sensitivity tests, agarose gel analysis of plasmid DNA, four isolates with the accession No. AtTp0120, AtTe0121, AtMo0122 and AtMi0123 were identified as wild type virulent *A. tumefaciens* strains. First two strains are newly reported regarding host plants (*Tagets patula* and *Tagetes erecta*) while the other two strains are reported for the first time in Bangladesh. Highly virulent strain could be used for construction of genetically engineered strains, *in vitro* antitumor studies of plant's extract and other biological purposes.

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