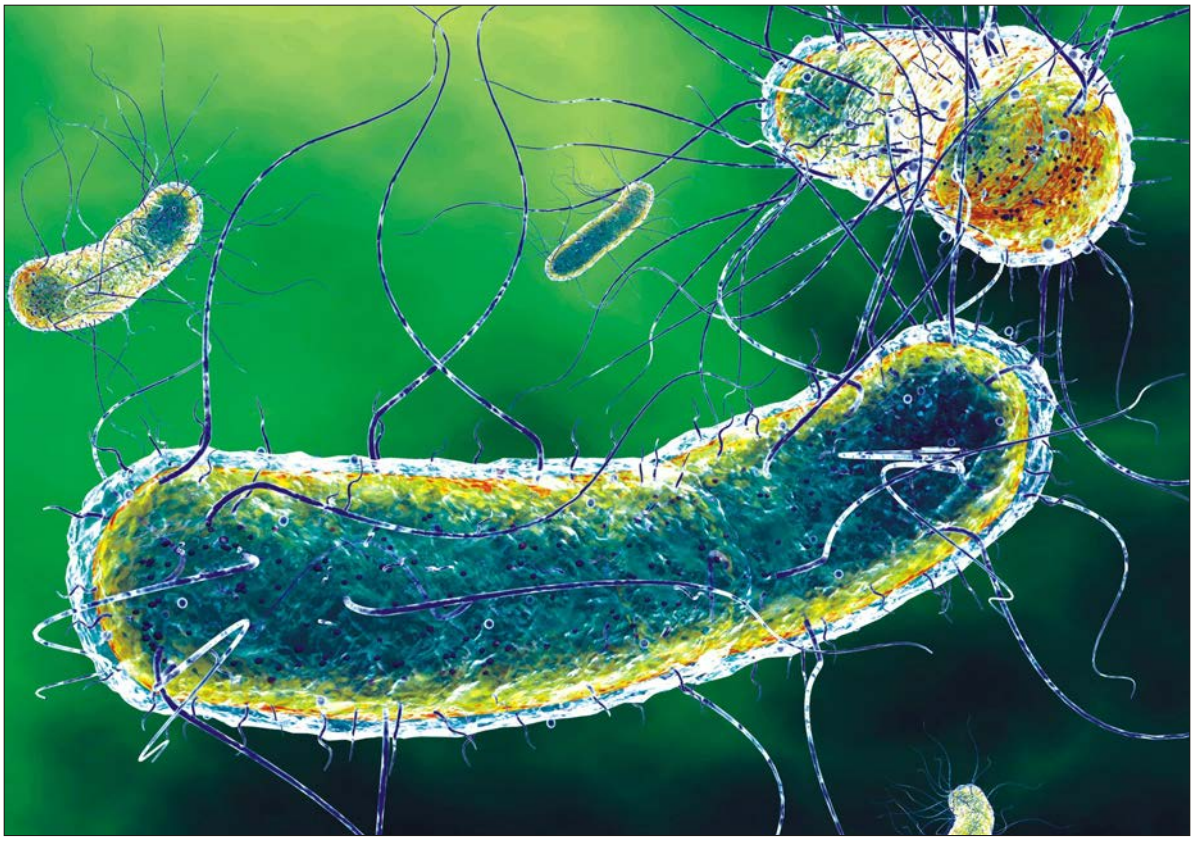

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Isolation of *Agrobacterium tumefaciens* Strains from Crown Gall Sample of Dicot Plants in Bangladesh

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Abstract: *Agrobacterium tumefaciens* strains were identified isolated from six different dicot plants viz. *Artocarpus heterophyllus*, *Tectona grandis*, *Terminalia arjuna*, *Anthocephalus codomba*, *Solanum lycopersicum* and *Rosa chinensis* of Rajshahi University campus, Rajshahi, Bangladesh. The isolated strains were confirmed as *Agrobacterium* using different biochemical, antibiotic resistance and pathogenicity (tumor forming ability) tests in comparing with standard *Agrobacterium tumefaciens* (ATCC23308^T). Accession No. of the strains was given according to their respective host plants. Statistical analysis shows that strains AtSl0105 and AtAc0114 were found more virulent than the other isolates in aspect of their tumor forming ability on potato discs.

Key words: Virulent, *Agrobacterium tumefaciens*, pathogenicity, antibiotic resistance

INTRODUCTION

Agrobacterium tumefaciens is a Gram negative soil bacterium has worldwide distribution (Furuya *et al*., 2004). It causes crown gall tumors on a wide range of plants including most dicots, some monocots and some gymnosperms (Matthysse, 2006). The crown gall tumour-inducing capability of *A. tumefaciens* requires the presence of a large plasmid, designated the Ti plasmid (Onyesom, 2006). During the course of infection, the bacterium transfers a specific portion (T-DNA) of Ti plasmid to a host cell, which then integrates itself into the host genome (Winans, 1992). After its integration into the plant genome, the T-DNA genes encode enzymes responsible for the uncontrolled synthesis of the plant hormones auxin and cytokinin which account for the appearance of abnormal tissue proliferation and gall formation on the crown, roots and in some cases on stems (Rhouma *et al*., 2006). The DNA transmission capabilities of *Agrobacterium* have been discovered by Schell and Van Montagu (1977) and development of methods to alter *Agrobacterium* into an efficient delivery system for gene engineering in plants and makes it of great concern to the agriculture (Moore *et al*., 1997). Under laboratory conditions the T-DNA has also been transferred to human cells, demonstrating the diversity of insertion application (Kunik *et al*., 2001). The mechanism by which *Agrobacterium* inserts materials into the host cell by a type IV secretion system, is very similar to mechanisms used by animal pathogens to insert materials (usually proteins) into human cells also by type IV secretion

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(Lai and Kado, 2000). This makes *Agrobacterium* an important topic of medical research as well. Besides, it plays a vital role in aspect of antitumor studies (Hussain *et al*., 2007; Ibrahim *et al*., 2005).

Several studies have been shown *Agrobacterium tumefaciens* can be effectively isolated from leaf, stem and crown gall samples of aster (Chen *et al*., 1999), from crown gall of rose (Aysan and Sahin, 2003), apricot (Aysan *et al*., 2003), tobacco (Furuya *et al*., 2004) and root nodules of *Vicia faba* (Tiwary *et al*., 2007). All over the World huge amount of plant species are remain to isolate diverse *Agrobacterium tumefaciens* strains. Bangladesh has a rich heritage of diverse plant species among the countries of the world that could be a potential source of virulent *Agrobacterium tumefaciens* strains. With the best our knowledge, there is no report on isolation of *Agrobacterium tumifaciens* strains from natural habitat in Bangladesh before this. *Agrobacterium tumefaciens* has immence importance for plant genetic engineering as well as antitumor studies and other microbiological research purposes. The present study was undertaken to isolate and characterize virulent *Agrobacterium tumefaciens* strains from locally available host dicot plants in aspect of multipurpose uses.

MATERIALS AND METHODS

Collection of Crown Gall Tissues

Crown gall tissues were collected from six different dicot plants viz. *Artocarpus heterophyllus* Lam., *Tectona grandis* L., *Terminalia arjuna* L., *Anthocephalus codomba* Roxb., *Solanum lycopersicum* L. and *Rosa chinensis* Jacq available in Rajshahi University Campus, Rajshahi, Bangladesh. After collection in a sterilize container, samples were immediately transferred to the laboratory for necessary action and special care was taken to avoid contamination. The experimental period was during February to December, 2008.

Crown Gall Extract Preparation

After collection and labeling, the samples were rinsed with tap water for removing soil and hazardous materials. Galls were sterilized with ten percent (10%) household bleach (Savlon, ACI limited, Bangladesh) for 1.5-3.0 min according to the nature of the galls. After washing three more times with Double-Distilled Water (DDW), galls were finely chopped and immersed in DDW and incubated overnight at room temperature (27-30°C).

Culture of Crown Gall Extract on Selective Media

Overnight incubated crown gall extracts were streaked on to two different selective 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°C for 18 to 24 h and examined for growth, fermentation and color development.

Selection and Culture of Desired Colonies

Bacterial colonies were selected based on colonies form, elevation, surface, color etc. and eight individual colonies were transferred onto the same medium. Finally selected individual colonies were transferred to the MGY agar media (Putnam, 2006) for further purification. The purified eight colonies were cultured on Luria-Bertani (LB) medium described by Miller (1987) and preserved in glyceral (25%) stock for further experimentation.

Characterization of *A. tumifaciens*

Biochemical Test

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

Test of Antibiotics Resistance on Isolates

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}), Cefotaxime (30 μg mL^{-1}), Tetracycline (30 μg mL^{-1}) and Rifampicin (10 μg 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-dried 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 dishes were incubated at 30°C for 24 h. Antibiotic susceptibility was determined by measuring the size of the inhibition zone.

Pathogenicity Test

Pathogenicity tests were done using both carrot disk (Chen *et al*., 1999; Aysan *et al*., 2003) and potato disc bioassays (Hussain *et al*., 2007).

Carrot Disc Bioassay

Carrot (*Daucas carota* L.) was collected from local market in Rajshahi city, Bangladesh. Collected carrot samples were sterilized with commercial bleach (Savlon, ACI limited, Bangladesh) followed by washing with DDW for three times. Each disc was overlaid with 100 μL of appropriate inoculums (10^8 cfu mL^{-1}). Petri dish was sealed by parafilm and incubated in growth chamber (control environment; 25-30°C). After 3 weeks, the disks were checked for young galls (tumors) developing from the meristematic tissue around the central vascular system.

Potato Disc Bioassay

Red skin potato (*Solanum tuberosum* L., Solanaceae) was also collected from local market. Here also potatoes were sterilized with savlon and 0.1% $HgCl_2$. Sliced potato discs (5×8 mm) were placed on water agar plates (1.5 g/100 mL). Each disc was overlaid with 50 μL of appropriate inoculums (10^8 cfu mL^{-1}). Petri dishes were sealed by parafilm and incubated at room temperature (25-30°C) for 3 weeks. After 3 weeks, discs were stained with 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).

Here it is noted that in every cases of test, commercially available *Agrobacterium tumefaciens* strain named ATCC23308^T was used as standard. Necessary aseptic conditions were taken whenever it is necessary.

RESULTS

Isolation of *A. tumifaciens*

Eight bacterial colonies were observed and screened, isolated from 6 crown gall samples on the basis of their color development on selective medium. After 24 h of inoculation, bacterial colonies were visible in necked eyes on MacConkey agar plate and after 48 h, colonies were turned into pink to brick-red color. Colonies cultured on NASA medium turned into putative brick color after 2 days of incubation. From these initial results, the isolated bacteria were tentatively identified as *Agrobacterium* stains.

Characterization of *A. tumifaciens*

Biochemical Test

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

Test of Antibiotics Resistance on Isolates

Results showed that isolates were susceptible to kanamycin and cefuroxime (showing zone of inhibition) and resistant against rifampicin and tetracycline (showing no zone of inhibition) Table 1.

Pathogenicity Test

In this case out of eight only six isolates (Fig. 1A-F, 2A-F) including standard sample show positive (tumor forming ability) for pathogenicity test (Table 1). Isolates having accession No. AtSl0104 and AtRc0108 were negative for tumor formation (Table 1). Isolates namely AtAh0116, AtTg0117, AtTa0112, AtAc0114, AtSl0105 and AtRc0107 (accession No.

Table 1: Phenotypic characteristics of the selected isolates

	Selected isolates								Standard *Agrobacterium*
Biochemical test	AtAh0116	AtTg0117	AtTa0112	AtAc0114	AtSl0104	AtSl0105	AtRc0107	AtRc0108	ATCC23308^T
Gram stain	-	-	-	-	-	-	-	-	-
Motility test	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Utilization of carbohydrates									
Lactose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+
3-keto lactose production	+	+	+	+	+	+	+	+	+
Salt tolerance	+	+	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+	+	+
L-tyrosine utilization	-	-	-	-	-	-	-	-	-
Resistance									
Kanamycin	S	S	S	S	S	S	S	S	S
Cefuroxime	S	S	S	S	S	S	S	S	S
Tetracycline	R	R	R	R	R	R	R	R	R
Rifampicin	R	R	R	R	R	R	R	R	R
Pathogenicity test	+	+	+	+	-	+	+	-	+

+: Positive, - : Negative, R: Resistant, S: Susceptible

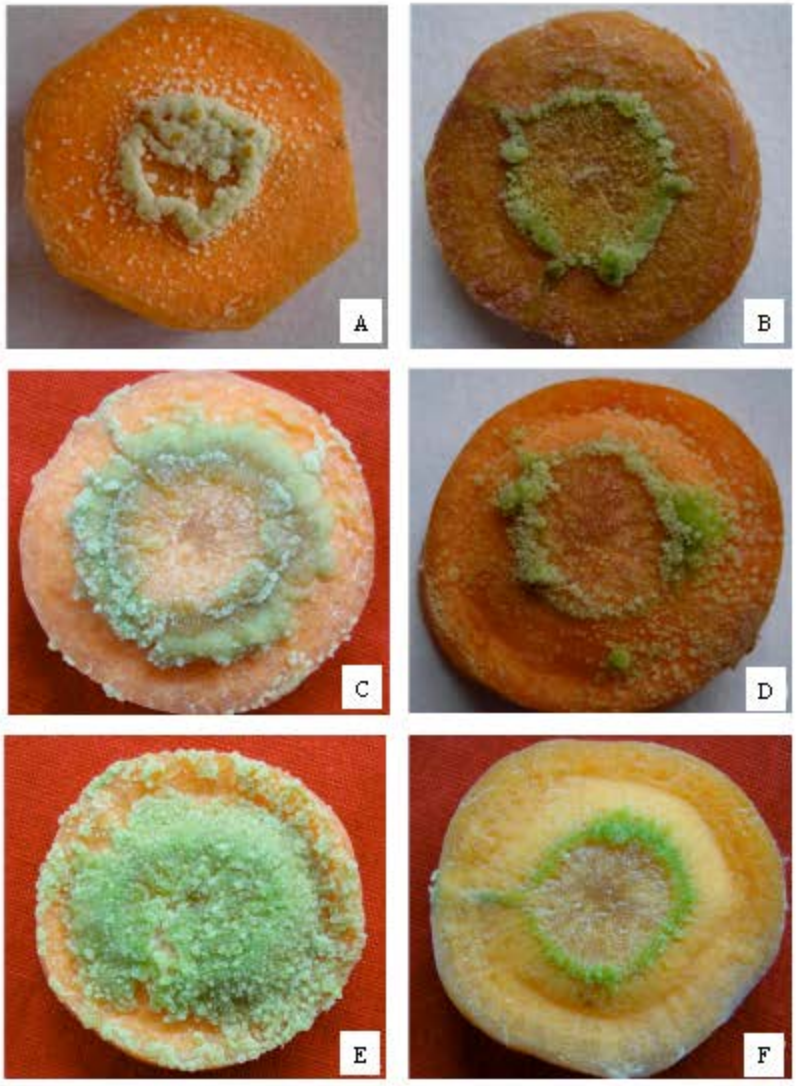

Fig. 1: Figure shows tumor forming ability of selected isolates on carrot disc. (A) AtAh0116, (B) AtTg0117, (C) AtTa0112, (D) AtAc0114, (E) AtSl0105 and (F) AtRc0107

was given according to the respective host plants) were finally identified as *Agrobacterium tumefaciens* strains. Here, At for *Agrobacterium tumefaciens*, Ah for *Artocarpus heterophyllus*, Tg for *Tectona grandis*, Ta for *Terminalia arjuna*, Ac for *Anthocephalus codomba*, Sl for *Solanum lycopersicum*, Rc for *Rosa chinensis*. Statistical analysis showed that isolated strains are significantly different with each other for their tumor formation ability on potato discs ($p<0.001$) (Table 2). Highest mean No. of tumor formation was observed in AtSl0105 (16.21±0.72) followed by AtAc0114 (14.83±0.90), whereas the standard *Agrobacterium tumefaciens* ATCC23308^T showed 12.19±0.79. Lowest No. (5.40±0.66) tumor formation was observed in AtTg0117 (Fig. 3).

Fig. 2: Figure shows tumor forming ability of selected isolates on potato disc. (A) AtAh0116, (B) AtTg0117, (C) AtTa0112, (D) AtAc0114, (E) AtSl0105 and (F) AtRc0107

Table 2: Statistical analysis on tumor induction by the studied six strains of *A. tumefaciens* on potato discs

Source of variation	df	Sum of squares	Mean square	F-value	Prob.
Replication	2	6.44	3.22	2.03	0.1742
Strains	6	335.48	55.91	35.23	0.0000
Error	12	19.05	1.59		
Total	20	360.97			

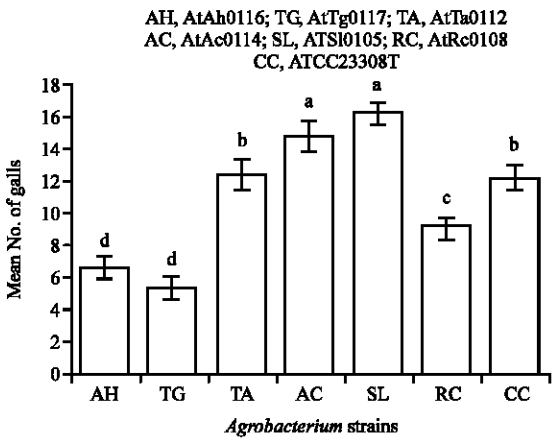

Fig. 3: The differential significant gall formation caused by six *Agrobacterium* strains on potato discs. Values are represented as Mean±SE of triplicate experiments. Here, LSD value = 2.433, any two means having a common letter are not significantly different at the 5% level of significance

DISCUSSION

The purpose of this study is to isolate different virulent indigenous *Agrobacterium tumefaciens* strains from different host dicot plants and confirm their characteristics using different biochemical, antibiotic resistance and pathogenicity test (tumor forming ability on carrot and potato disc). Crown gall is a neoplastic disease of plants causative agent of this disease is the Gram-negative bacterium *A. tumefaciens* (Galsky *et al*., 1980). It is a common disease of dicot plants, including many woody shrubs and various herbaceous plants including mainly stone and pome fruit-trees, grapevines, roses and some ornamental plants (Rhouma *et al*., 2006). Crown galls are often found at or just below the soil surface on the roots or crown region of plants (Ogawa *et al*., 1995). *Agrobacterium 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). According to the information, galls were collected from different dicot plants species namely *A. heterophyllus*, *T. grandis*, *T. arjuna*, *A. codomba*, *S. lycopersicum* and *R. chinensis* found in different places of Rajshahi University campus for isolating virulent *A. tumefaciens* strains. On the basis of color development, desecrate eight colonies were isolated from selective media (MacConkey agar and Clark's selective medium). Isolates grew as pink to brick-red colonies on MacConkey agar and putative brick colonies on NASA medium, tentatively identified as Gram negative and *Agrobacterium* 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 us. Chen *et al.* (1999) also supported our results to confirm *Agrobacterium* strains because they also cultured crown gall extracts of aster on NASA medium and putative brick colonies were obtained that was similar to us. For further confirmation of *Agrobacterium tumefaciens* strains, several biochemical tests were conducted according to Moore *et al.* (1988) and the following results were attained i.e., isolates are negative for Gram test and L-tyrosine utilization and positive for motility, catalase, oxidase, lactose, mannitol, 3-keto lactose production, H_2S production. Several

workers (Chen *et al*., 1999; Koivunen *et al*., 2004) used series of biochemical tests (Moore *et al*., 1988) and their obtained results are good agreement with our results.

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 *Agrobacterium tumefaciens* strains. Antibiotic resistance means, the ability of a microorganism to withstand the effects of an antibiotic (Karthy *et al*., 2009). In gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or Topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Robicsek *et al*., 2006).

Tumor forming ability of the six selected isolates finally confirmed them as *Agrobacterium tumefaciens* strains. Significant difference among the isolates for tumor forming ability on potato discs shows that there are distinct variations for virulence. Among the locally isolated indigenous strains of *Agrobacterium tumefaciens* (AtAc0114, AtTa0112, AtRc0108, AtAh0116 and AtTg0117) including standard strain (ATCC23308^T), AtSl0105 was comparatively more virulent. These findings may be attributed to the nature of host plant, internal physiology of *Agrobacterium tumefaciens* and also environmental condition. Chen *et al*. (1999) isolated *Agrobacterium tumefaciens* strains from different types of gall tissue of aster and presented diversified virulence stains. Reports are not available on *Agrobacterium tumefaciens* strains isolation from *A. heterophyllus*, *T. grandis*, *T. arjuna* and *A. codomba* in our country (Bangladesh) and also over the world whereas Chen *et al*. (1999), Aysan and Sahin (2003) and Aysan *et al*. (2003) proved only tumor forming ability of *S. lycopersicum* and Aysan and Sahin (2003) reported crown gall disease of *Rosa* sp.

CONCLUSION

On the basis of *in vitro* tumor inducing capability, different biochemical and antibiotic resistance tests, six selected isolates with the accession No. AtAh0116, AtTg0117, AtTa0112, AtAc0114, AtSl0105 and AtRc0107 were finally identified as indigenous virulent *Agrobacterium tumefaciens* strains. First four strains are newly reported regarding host plants and other two strains are also newly reported in aspect of our country (Bangladesh). High virulent strains could be used for construction of genetically engineered strains, *in vitro* antitumor studies of plant extracts and other biological aspects.

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