

Isolation of *Agrobacterium Tumefaciens* Strains from Crown Gall Disease on Imported Roses Plants in Qazvin Province

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Cultivar of roses (*Rosa* spp.) has been grown in greenhouses in Qazvin region of Iran for local markets. *Agrobacterium tumefaciens* strains were isolated and identified from six different samples of roses plants imported from the Netherland to Iran. During August and September of 2012, nearly 2-5% of rose plants in two different greenhouses in the province of Qazvin were observed with crown gall symptoms on various parts, particularly in the stem. A non-fluorescent gram-negative bacterium was consistently isolated from diseased tissues on to King's B medium. The isolated strains were confirmed as *Agrobacterium* using different biochemical and pathogenicity (tumor forming ability) tests. Four representative strains isolated were aerobic, non-sporing, non-pigmented, motile, rod-shaped, oxidase negative and catalase positive. All of four isolates were positive for tumor forming ability in pathogenicity tests on carrot disc and tomato stem.

Abstract

Keywords: Rose cultivars, *Agrobacterium tumefaciens*, Crown gall, Qazvin.

INTRODUCTION

Agrobacterium tumefaciens is the causal agent of crown gall formation on many dicot plants, including ornamental species (De Cleene and De Ley, 1976). The plant tumour, crown gall, can be induced on many dicotyledonous plants after wounding and subsequent infection with *Agrobacterium tumefaciens*. This bacterium is a soil borne micro-organism has worldwide distribution (Furuya *et al.*, 2004). *A. tumefaciens* is a member of Rhizobiaceae family. These are Gram negative, rod-shaped and motile bacteria that grow aerobically without forming endospores (Collins, 2001). 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 (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 of our knowledge, there is no report on isolation of *Agrobacterium tumefaciens* strains from natural habitat in Bangladesh before this. *Agrobacterium tumefaciens* has immense 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 cut flower rose plants in aspect of local markets.

MATERIALS AND METHODS

Plant Samples

Crown gall tissues were collected from two different greenhouses in Qazvin province (Fig. 1). Samples were immediately transferred to the laboratory. Special care was taken to avoid contamination. The experimental period was from August and September of 2012.

Isolation and Maintenance of *A. tumefaciens*

The surface of the galls were removed by a handy blade and sterilized in 100 ml of 10% commercial bleach containing 4 drops of Tween-20 for 20 min. After sterilization, the galls were washed three times with sterile water (SW). They were then finely chopped and immersed in sterile water for 3 h or overnight at room temperature (27-30°C). One loopful of the gall extract was

streaked onto the Clark's selective medium designated as NASA (Serfontein and Staphorst, 1994). The medium contains nutrient broth, 50 mg/l selenite, 250 mg/l cycloheximide and 15 mg/l sigma agar. Plates were incubated at 28-30°C for 24 to 48 h and examined for growth and color development. Bacterial colonies were selected based on colonies form, elevation, surface, color etc. Putative brick red colonies from NASA were streaked on the same medium to purify single colonies. The purified colonies were cultured on YM medium (0.04% yeast extract, 1% mannitol, 1.7 mM sodium chloride, 0.8 mM magnesium sulfate and 2.2 mM dipotassium phosphate, pH 7.0, 1.5% sigma agar) and stored at 4°C for further experimentation.

Characterization of *A. tumefaciens*

Diagnostic Tests

Diagnostic tests for biochemical and physiological characterizations of the isolates were conducted according to Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994) in addition to Moore *et al.* (1988). Under this following biochemical tests were carried out: (i) Gram stain at room temperature; (ii) catalase and oxidase production; (iii) utilization of lactose, mannitol; (iv) production of 3-ketolactose; (v) salt tolerance (2%); (vi) H₂S production; (vii) utilization of L-tyrosine; (viii) citrate utilization; (ix) action on litmus milk; (x) growth and pigmentation in ferric ammonium citrate and other tests (Table 1).

Pathogenicity Test

Pathogenicity tests were done using both carrot disk (Chen *et al.*, 1999) and tomato stem bioassays (Aysan *et al.*, 2003).

Carrot Disc Bioassay

Collected carrot samples were sterilized with commercial bleach followed by washing with DDW for three times. Each disc was overlaid with 100 µL of appropriate inoculums (10⁸ CFU mL⁻¹). The 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.

Tomato Stem Bioassay

Pathogenicity of the strains was confirmed on five week-old tomato plants (*Lycopersicon esculentum*) with needle inoculation of bacterial suspensions containing 10⁸ CFU/ml in 0.85% saline. Inoculated and control (saline injected) plants were maintained in the growth chamber for 10-12 days at 25 °C and 70% RH. After 2 weeks, the stems were checked for young galls (tumors) developing from the stem tissue of tomato plant. It is noted that in every cases of test, reaction of commercially available *Agrobacterium tumefaciens* strain named ATCC23308^T was used as standard. Necessary aseptic conditions were taken whenever needed.

Re-inoculation of *Agrobacterium* to Rose Leaves

Healthy rose plants without galls and with lateral shoots were collected from greenhouse. Each leaf was cut twice at both sides from the margin to half of the blade. A pair of sterile scissor was dipped in overnight grown bacterial suspension and use to cut the leaves. Control leaves were cut with sterile scissor without bacteria. Pot-grown rose plants were wrapped in a plastic bag for two days to retain high humidity. They were then grown in greenhouse with ambient temperature during October 2012. Gall formation was scored after two weeks.

RESULTS

Isolation of *A. tumefaciens*

Four bacterial colonies were observed, screened and isolated from 6 crown gall samples in two different greenhouses on the basis of their color development on selective medium. 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. tumefaciens*

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, Citrate and erythritol utilization and positive for catalase, oxidase, lactose, mannitol, melezetose, sucrose, 3-keto lactose production and H₂S production. Similar reaction was also observed for standard sample.

Pathogenicity Tests

In this case, all of four isolates (Table 1) similar to standard sample showed positive (tumor forming ability) for pathogenicity test on Carrot Disc and Tomato Stem (Fig. 2: A-D). Isolates namely AtRA1, AtRA2, AtRQ1 and AtRQ2 (accession No. was given according to the respective host plants and origin) were finally identified as *Agrobacterium tumefaciens* strains.

Re-inoculation of *Agrobacterium* to Rose Leaves

When healthy rose plants were inoculated with 4 *Agrobacterium* strains, they all did not produce visible galls after 10 to 12 days on the wound sites.

DISCUSSION

Rose plants from two greenhouses in Qazvin province were found to have stem and crown gall after the rose plants were planted during the summer of 2012. About 2-5% of the greenhouses grown plants were galled. The underground parts of some plants also had dark brown to gray galls. The bacteria likely was imported and spread by vegetative propagation of rose cuttings by farmers from the Netherlands origin. The goal of this research is to isolate different virulent *Agrobacterium tumefaciens* strains from different rose plants and confirm their characteristics using different biochemical and pathogenicity test (tumor forming ability on carrot disc and tomato stems). Crown gall 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 rose plants in greenhouses for isolating virulent *A. tumefaciens* strains. On the basis of color development, four colonies were isolated from selective media (Clark's selective medium). We were able to isolate smooth, round colonies with dark red centers and light transparent rings in the margin on the NASA selective medium from different gall samples. This selective medium is therefore suitable for *Agrobacterium* isolated from rose galls. Isolates grew as putative brick red colonies on NASA medium, tentatively identified as Gram negative and *Agrobacterium* strains. Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994) indicated that Gram negative bacteria generally grow as putative brick red colonies on NASA selective medium which was similar to our isolates. Chen *et al.*, 1999 and Sarker *et al.*, 2011 also supported our results to confirm *Agrobacterium* strains because they also cultured crown gall extracts of aster and Dicot plants on NASA medium and putative brick colonies were obtained that was similar to our results. 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

were negative -in Gram test and L-tyrosine utilization and positive in motility, catalase, oxidase, lactose, mannitol, 3-keto lactose production, H₂S production. Several workers (Chen *et al.*, 1999; Koivunen *et al.*, 2004 and Sarker *et al.*, 2011) used series of biochemical tests (Moore *et al.*, 1988) and found results in agreement with our results.

Tumor forming ability of the four selected isolates on carrot discs and tomato stems finally confirmed them as *Agrobacterium tumefaciens* strains. When the purified strains were inoculated on the stem surface of tomato. Signs of gall formation were observed after 4-6 days. The galls grew larger after 2-3 weeks. All of 4 isolates were able to induce galls on carrot and tomato stems, suggesting that rose agrobacterium showed pathogenicity on both hosts. This ability among the isolates for tumor forming inducing on carrot discs and tomato stems showed that there were capable in inducing virulence. Isolation of *Agrobacterium tumefaciens* strains from different hosts were reported by many researchers (Chen *et al.*, 1999; Sarker *et al.*, 2011; Furuya *et al.*, 2004; and Aysan *et al.*, 2003). Aysan and Sahin (2003) reported crown gall disease of *Rosa* sp. which was similar to our findings us.

However, for the conclusion, on the basis of in vitro tumor inducing capability and different biochemical tests, four selected isolates with the accession Cod No. AtRA1, AtRA2, AtRQ1 and AtRQ2 were finally identified as indigenous virulent *Agrobacterium tumefaciens* strains.

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Tables

Table 1. Phenotypic characteristics of the selected isolates.

	Reaction of Isolates				Type strain of <i>A. tumefaciens</i>	
	AtRA1	AtRA2	AtRQ1	AtRQ2	ATCC23308 ^T _a	Biovar 1 _b
Gram reaction	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Growth at 35° C	+	+	+	+	+	+
3-Ketolactose production	+	+	+	+	+	+
Citrate utilization	-	-	-	-		-
Utilization from:						
Erythritol	-	-	-	-		-
Melezetose	+	+	+	+		+
Sucrose	+	+	+	+		+
Lactose	+	+	+	+	+	
Mannitol	+	+	+	+	+	
H ₂ S production	+	+	+	+	+	
Growth in 2% NaCl	+	+	+	+	+	+
L-tyrosine utilization	-	-	-	-	-	-
Litmus milk reaction:						
Alkaline	+	+	+	+		+
Acid	-	-	-	-		-
Ferric ammonium citrate	+	+	+	+		+
Pathogenicity on:						
Tomato stem	+	+	+	+	+	+
Carrot disc	+	+	+	+	+	+

+: Positive, -: Negative, ^aATCC23308^T was kindly provided by Sarker *et al.* As a reference for *A. tumefaciens*,

^bData from Moore *et al.*, 1988.

Figures



Fig.1. Gall samples in roses plants.

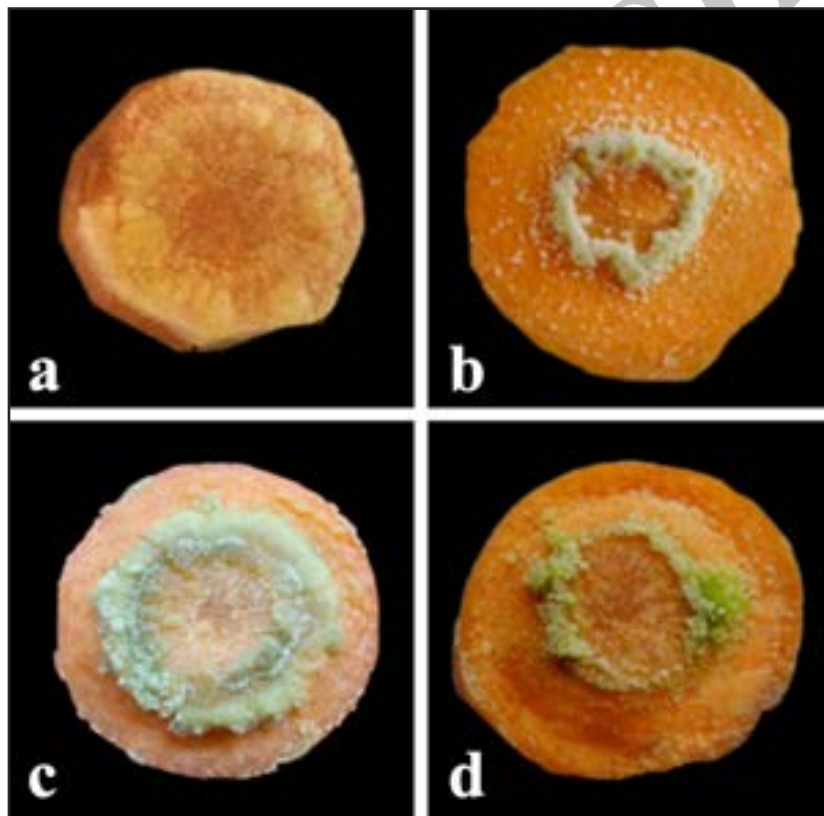


Fig. 2. Tumor forming ability of selected isolates on carrot disc. (A) AtRA1, (B) AtRA2, (C) AtRQ1, (D) AtRQ2.