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

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Abstract

Cultivar of roses (*Rosa* spp.) has been grown in greenhouses in Qazvin region of Iran for local markets. *Agrobacterium tumefaciens* strains were identified and isolated from six different samples of roses plants imported from the Netherlands 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 onto 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 (AtRA1, AtRA2, AtRQ1 and AtRQ2) were positive for tumor forming ability for pathogenicity tests on Carrot Disc and Tomato stem.

Keywords: Roses, *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 tumor, 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 is often brought to a garden on the stems or roots of an infected plant and spread with contaminated pruning tools and soils. This bacterial disease causes the formation of large corky galls up to several inches in diameter. They appear at the base of the plant and on stems and roots, and commonly on the bud union. The galls are rounded with rough, irregular surfaces and may be dark and cracked. Plants with numerous galls are weak; growth is slowed and leaves turn yellow. Branches or the entire plan may die back. Plants with only a few galls often show no other symptoms. Crown gall is not specific to roses and can affect apples, raspberries, honeysuckle, euonymus, and many vegetables. For this reason, roses should not be planted where plants susceptible to crown gall have been removed because of the disease (Londeree, 2013). Crown gall affects both woody and herbaceous plants, attacking several hundred different plants belonging to at least 142 genera within 61 widely separated families. This is really the only serious bacterial disease of Rose. The bacteria is found worldwide wherever roses are grown and is especially severe in loose sandy or sandy loam soils. Crown gall is economically important on only a relatively small number of young, rapidly growing plants. These include almond, apple, apricot, blackberry, cherry, cottonwood, crabapple, euonymus, fig, grape, honeysuckle, nectarine, peach, pecan, pear, plum, prune, poplar, pyracantha, raspberry, rose, sugar beet, turnip, walnut, and willow (Forsberg, 1999).

This bacterium changes the DNA in the plant's cells around the wound, and a tumor starts to grow. The abnormal cells proliferate rapidly. Galls usually begin as green, pliable tissue; then develop into dark, crusty growths. The galls may disrupt the flow of water and nutrients up the roots and stems. Following gall formation, growth is stunted, foliage is sparse, and bloom production is reduced. Most often galls grow at the soil level or just below it. Many rose plants can live and perform beautifully for many years with crown gall. Or gall can kill a plant – it is unknown why this is the case, but it's usually a good idea to destroy galls, or even the plant once they are discovered (Londeree, 2013). 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; Islam *et al.*, 2010), 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. 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. One loopful of the gall extract was streaked onto the Clark's selective medium designated as NASA (Serfonteinand 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 to48 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 choloride, 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 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 conducted using both carrot disk (Chen *et al.*, 1999; Islam *et al.*, 2010) and tomato stem bioassays (Aysan *et al.,* 2003).

Carrot disc bioassay

Collected carrot samples were sterilized with commercial bleach followed by washing with sterilized distilled water (SDW) for three times. Each disc was overlaid with 100 µL of appropriate inoculums (10^8 cfu/ml). 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 (Lycopersicum 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. Here it is noted that in every cases of test, reaction of commercially available *Agrobacterium tumefaciens* strain named ATCC23308T was used as standard. Necessary aseptic conditions were taken whenever it is necessary.

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 and screened, isolated from 6 crown gall samples from 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.

CharacterizationofA.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 lactoseproduction 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 show positive (tumor forming ability) for pathogenicity test on Carrot disc (Fig. 2: A-D) and Tomato stem (Fig. 3). 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 (Fig.4).

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.. 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 aim of this study 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 of cut flower 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. 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) indicates that Gram negative bacteria generally grow as putative brick red colonies on NASA selective medium which was similar to us.

However, our group (Islam *et al.*, 2010; Sarker *et al*., 2011) and Chen *et al*. (1999) supported our present results to confirm Agrobacterium strains. 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 uti-

lization 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; Islam *et al.*, 2010 and Sarker *et al.*, 2011) used series of biochemical tests according to Moore *et al.* (1988) and their obtained results are good agreement with our present 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 Agrobacterium showed pathogenecity on both hosts. Isolation of *Agrobacterium tumefaciens* strains from different hosts were reported by many of 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 us.

CONCLUSION

On the basis of *In Vitro* tumor inducing capability and different biochemical tests, four selected isolates with the accession 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 *A. tumefaciens*	
	AtRA1	**AtRA2**	**AtRQ1**	**AtRQ2**	**ATCC23308^{T}$_{a}$**	**Biovar 1$_{b}$**
Gram reaction	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Growth at 35^{o} C	+	+	+	+	+	+
3-Ketolactose production	+	+	+	+	+	+
Citrate utilization	-	-	-	-		-
Utilization from:						
Erythritol	-	-	-	-		-
Melezetose	+	+	+	+		+
Sucrose	+	+	+	+		+
Lactose	+	+	+	+	+	
Mannitol	+	+	+	+	+	
H_2S production	+	+	+	+	+	
Growth in 2% NaCl	+	+	+	+	+	+
L-tyrosine utilization	-	-	-	-	-	-
Litmus milk reaction:						
Alkalin	+	+	+	+		+
Acid	-	-	-	-		-
Ferric ammonium citrate	+	+	+	+		+
Pathogenicity on:						
Tomato stem	+	+	+	+	+	+
Carrot disc	+	+	+	+	+	+

+: Positive, - : Negative, [a]ATCC23308^{T} was kindly provided by Sarker *et al*. As a reference for *A. tumefaciens*, [b]Data from Moore *et al.,* 1988.

Figures

Fig.1. Gall samples in roses plants.

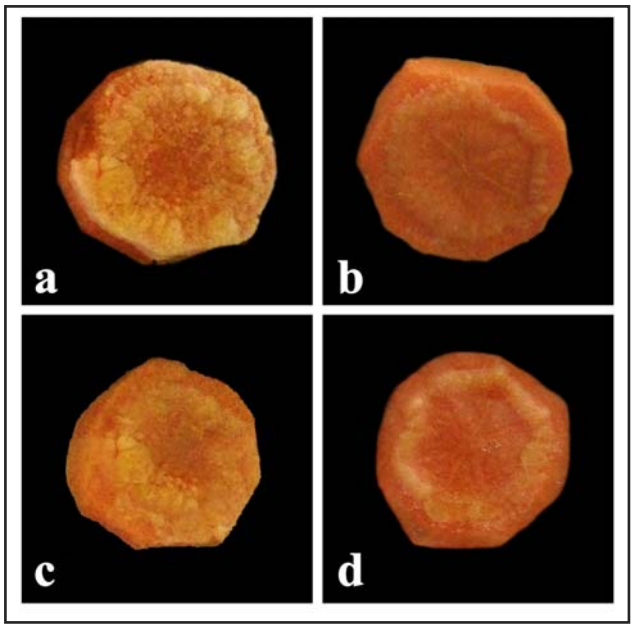

Fig. 2. Figure shows tumor forming ability of selected isolates on carrot disc. (A) AtRA1, (B) AtRA2, (C) AtRQ1, (D) AtRQ2.

Fig.3. Gall formation in tomato stem in pathogencity test.

Fig.4. No gall formation in Rose leaves after re-inoculation of Agrobacterium.