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ISOLATION AND CHARACTERIZATION OF WILD STRAINS OF AGROBACTERIUM FROM CROWN GALL INFECTED PLANTS

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A B S T R A C T

Agrobacterium tumefaciens is a plant pathogen that has immense importance in plant genetic engineering.Its pathogenic strains have a large tumor inducing plasmid that can transfer and integrate transfer DNA segment in the plant genome andhelp in generation of transgenics. The highly virulent bacteria can transform plant cells more efficiently as compared to non-virulent strains. Keeping in view this fact twenty-five wild strains of *Agrobacterium* were isolated from infected plants of Mango, Sitaashok, Chikoo, Neem, Eucalyptus and Gulmohar growing in the Horticulture Garden, Sindh Agriculture University, Tandojam. Fifteen isolates out of twenty-five were designated as *A.tumefaciens* on the basis of morphological, biochemical and phyto-pathological tests. Among these fifteen isolates, three strains were found to be nonpathogenic whereas the remaining revealed tumor forming ability on carrot discs in an *in vitro* disease assay. Isolate AtMi003 of *A. tumefaciens*was the most virulent strain on the basis of tumor quantity generated and analyzed through ANOVA and LSD at p= 0.05. It showed 80% transformation efficiency and 23.67 mg tumor weight on carrot discs.

Keywords: *Rhizobiaceae, Agrobacterium,*phytohormone, *Manilkarazapota,Delonixregia*

INTRODUCTION

Agrobacterium is the one of the most significant bacterial genus of the family *Rhizobiaceae.Agrobacterium*species are phytopathogens that cause diseases, like crown gall tumors, hairy roots and cane galls. The diseased plants become, stunted, loss vigor, and cause great losses to production. On the other hand it is also a well-known natural genetic engineerdue to its ability to cause the disease in the host plant by transferring its genetic material into plant cell in the form of DNA piece, called as T-DNA or transfer DNA. This T-DNA is present on a tumor inducing (Ti) plasmid. Ti plasmid is a piece of circular chromosomal DNA that is generally 190-240 kb in size and usually present in low copy number (1-3 copies) per cell (Lang *et al,* 2013). This plasmid is lost when *Agrobacterium* is grown above 28ºC and consists of five distinct areas including three regions essential for tumorgenesis(Gelvin, 2012). These regions are vir region, T-DNA region and opine catabolism region plus a region for conjugation containing tra and trbloci, and the rep region for replication. The T-DNA has genes for the auxin and cytokinins production. Although direct cloning intoTi plasmid is impossible particularly due to its large size and presence of oncogenes, it is first disarmed i.e. phytohormone production genes are disabled to allow regeneration of normal plants.

The transformation efficiency of *Agrobacterium*depends on a compatible interaction of the bacteria and the host. Some *Agrobacterium* strains are more virulent on specific host as compared to others. On the other hand some host genotypes are also more or less tolerant to *Agrobacterium* infections (Gelvin, 2012). Such variation is due to the deficiency in either *Agrobacterium* or host machinery for the transfer of T-DNA. When there is incompatible reaction between the bacteria and plant than sometimes a hypersensitive reaction is elicited by the host. (Van der Hoorn *et al,* 2000).Initially

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Agrobacterium was believed to infect dicotyledonous pathogenicity to monocotyledonous plant, fungi, yeast, other prokaryotes etc. Recently, investigations have shown the transfer of T-DNA to even human cells using *Agrobacterium* (Pitzschke and Hirt 2010).

Numerous studies demonstrated isolation of the pathogenic wild strain of *Agrobacteriumtumefaciens*from infected leaves, stems and crown galls of*Viciafaba* (Tiwary*et al.,* 2007), tobacco (Furuya*et al*., 2004), rose (Islam *et al.,* 2010), apricot (AysanandSahin, 2003) and aster (Chen *et al*., 1999). This shows the immense possibility of isolating various *Agrobacterium tumefaciens* strains from different plant species of our surroundings too.Keeping in view the importance of *Agrobacterium* in plant biotechnology, the present study aimed to isolate and characterize wild virulent strains of *A. tumefaciens* from locally infected hosts for multipurpose future uses such as transformation technology or antitumor studies.

MATERIALS AND METHOD

Crown gall tissues were collected from heavily infected plants of Mango (*Mangiferaindica),* Sitaashok*(Saracaasoca),* Chikoo*(Manilkarazapota),* Neem(*Azadirachtaindica),* Eucalyptus (*Eucalyptus spp)* andGulmohar (*Delonixregia*).Species growing in the Horticulture Garden, Sindh Agriculture University, Tandojam.The collected galls were surface sterilizedin 20% bleach solution and soaked in Sterile-Distilled Water (SDW) overnight at room temperature. Next day the obtained suspensions were streaked on PYGA media (5gPeptone-Oxoid, 3gyeast extract-Oxoid, 10mlglycerol-Sigma, 2gK_2HPO_4-Sigma and 20gagar-Oxoid) and Luria–Bertanimedia (10gBacto-tryptone-Oxoid,5gyeast extract-Oxoid,10gNaCl-Merk, and 20g agar-Oxoid) and incubated at 28°C for 48 hours. Morphological and biochemical data was collected for the form of colony, bacterial motility, bacterial growth in liquid media, gram staining, catalase production, tolerance to 2% and 5% NaCl, tolerance to temperatures 28ºC and 40ºC, utilization of sugars, production of 3-ketolactose and sensitivity to antibioticsas described by Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) for the identification of unknown bacteria.All the isolates with a positive reaction in above described tests were considered as *A. tumefaciens*biovar 1 (Bouzar, 1993).

Four different antibiotics were used to demonstrate the sensitivity or resistance pattern of *Agrobacterium* isolates namely Kanamycin (30 µg mL^{-1}), Cefuroxime (30µg mL^{-1}), Tetracycline (30 µg mL^{-1}) and Rifampicin (10 µg mL^{-1}).The degree of transformation efficiency was determined on the basis of an *in vitro* disease assay optimized on carrot discs. Carrots were sterilized in 20% bleach, discs (1x0.5 cm^2) were prepared and inoculated by 30 µl of bacterial suspensions of various isolates normalized to OD_{600}= 2.0 using a spectrophotometer. The inoculated discs were cultured on slants containing 15% water agar in test tubes and incubated at 21±3 ºC. After 25 to 30 days the discs revealed tumor initiation from meristimatic tissues of the carrot surrounding the central vascular system. Data were collected for tumor frequency and tumor weight. ANOVA and LSD (p= 0.05) was applied to find out the most virulent isolate.In this study apreviously characterized wild strain of *Agrobacterium tumefaciens*was used as a positive control(Yasmin, 2010).

RESULTS AND DISCUSSION

The main focus of this research work was to isolate thevirulent strains of *Agrobacterium tumefaciens* that can be utilized in transformation experiments. For this purpose, twenty five pure colonies of *Agrobacterium spp.* were isolated from six crown gall samples collected from heavily infected plants on two semi selective media i.e. PYGA and Luria–Bertani (LB). Initial colony purification decreased the number of isolates to nineteen. These isolates were given identification names based on bacterium name i.e. At for *Agrobacterium tumefaciens* followed by initials of host plant/ source plant and a number (Table1) for example AtMi001 means *Agrobacterium tumefaciens*(At) wild strain isolated from *Mangiferaindica*(Mi) and first isolate (001).

The isolated *Agrobacterium spp.* revealed small to medium sized, translucent/ opaque, raised colonies with entire marginson solid LB media after 48 hours of bacterial growth at 28ºC. The color of colonies was found as the different shades of yellow/ cream or golden. The control strain At2441 also showed the same colony characters. The growth pattern of isolates was also evaluated in LB liquid medium andall isolates including the control At2441showed uniform turbidity without pellet and sediments in broth whereas AtMz010, AtDr018 and AtDr019 showed in-significant powdery sediments. All theisolates were found gram negative, scattered motile rods under oil immersion lens and catalase positive indicating them as aerobic bacteria. In agreement to present study, there are many reports on

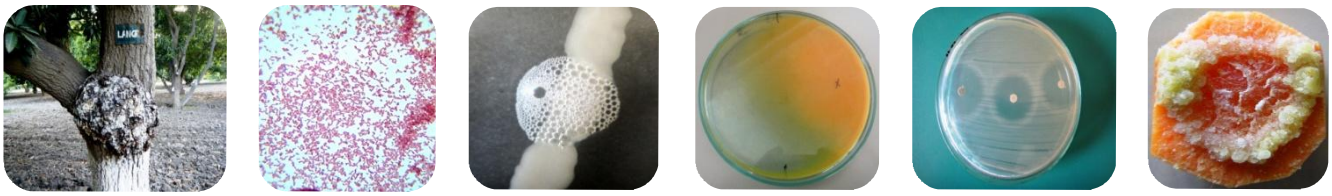
the isolation of *Agrobacterium tumefaciens*strains from different hosts following the same scheme of identification of *Agrobacterium* spp. as followed in this study. Islam *et al*. (2010) and Sarker*et al*. (2011) isolated *Agrobacterium tumefaciens*from crown gall tissues and followed the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994; Moore *et al*.,1988) to identify these bacteria. Most of the strains of *A. tumefaciens* are included in Biovar 1. These bacteria can grow without growth factors in media, can tolerate 2% NaCl, can tolerate 40ºCtemperature and can produce 3-ketolactose when grown on lactose sugar (Sarker*et al*., 2011). All thebiovars can utilize mannitol, sucrose, glucose and lactose by producing acid. Biovar 2 (*A. rhizogenes*) cannot tolerate 0.5% NaCl or 37°C and require biotin for growth in media whereas biovar 3 (*A. vitis*) can grow on 2% NaCl but cannot tolerate 37°C. Additionally biovars 2 and 3 cannot produce 3-ketolactose (Moore *et al*.,1988). In thepresent study only those isolates were designated as *A. tumefaciens*that showed results of all the tests in accordance with the typical pattern of *A. tumefaciens*as described above.

All the bacterial spp. showed tolerance to 2% NaClexcept AtMi004, AtAi012, AtAi014 and AtDr017 whereas none of the isolates was able to tolerate 5% concentration of NaCl. It is well documented that *A. tumefaciens* can grow on 2% salt concentration but cannot grow on 5% salt. The same isolates (AtMi004, AtAi012, AtAi014 and AtDr017) which cannot tolerate 2% NaCl were also not able to grow at 40ºC and did not produce 3-ketolactose. All the isolates including the control were able to grow at 28ºC and also were able to ferment glucose, sucrose, mannitol, and lactose with acidproduction. Antibiotic test results showed that all the nineteen isolates including the control are resistant to rifampicin and susceptible to Oxytetracycline, Kanamycin and Cefuroxime. This data is in good agreement with by Islam *et al*., (2010) and Karthy*et al.,* (2009).The phyto-pathogenicity test was performed to finally confirm the isolates as *Agrobacteriumtumefaciens* by evaluating their virulence or pathogenic potential.

Table 1. Morphological, biochemical and phyto-pathological characteristics of Agrobacteriumtumefaciensisolates.

Source Plant	ID of the isolates	Salt tolerance		Temp. tolerance		Catalase testing	3-ketos testing	Tumor related features				
		2%	5%	28ºC	40ºC			+/-	days	%	type	weight
Mango *Mangiferaindica*(Mi)	AtMi001	+	-	+	+	+	+	+	25	40	G, S	12.7ef
	AtMi002	+	-	+	+	+	+	+	20	50	G, S	10.7g
	AtMi003	+	-	+	+	+	+	+	18	80	G, S	23.7a
	AtMi004	-	-	+	-	+	-	-	N/A	00	N/A	0.0 i
Sitaashok *Saracaasoca*(Sa)	AtSa005	+	-	+	+	+	+	+	22	50	G, S	13.0ef
	AtSa006	+	-	+	+	+	+	-	N/A	00	N/A	0.0i
	AtSa007	+	-	+	+	+	+	+	26	30	C, V	8.4h
	AtSa008	+	-	+	+	+	+	-	N/A	00	N/A	0.0i
Chikoo *Manilkarazapota*(Mz)	AtMz009	+	-	+	+	+	+	-	N/A	00	N/A	0.0i
	AtMz010	+	-	+	+	+	+	+	18	70	G, S	15.0cd
Neem *Azadirachtaindica*(Ai)	AtAi011	+	-	+	+	+	+	+	21	50	G, S	15.0cd
	AtAi012	-	-	+	-	+	-	-	N/A	00	N/A	0.0i
Eucalyptus *Eucalyptus spp*(Es)	AtEs013	+	-	+	+	+	+	+	20	60	G, S	15.7c
	AtEs014	-	-	+	-	+	-	-	N/A	00	N/A	0.0i
	AtEs015	+	-	+	+	+	+	+	25	70	G, S	15.1cd
Gulmohar *Delonixregia*(Dr)	AtDr016	+	-	+	+	+	+	+	22	40	C, V	7.8h
	AtDr017	-	-	+	-	+	-	-	N/A	00	N/A	0.0i
	AtDr018	+	-	+	+	+	+	+	21	70	G, S	13.7de
	AtDr019	+	-	+	+	+	+	+	25	70	G, S	11.4fg
Control (Yasmin, 2010)	At2441	+	-	+	+	+	+	+	20	80	G, S	20.3b

Note: Table 1 only represents the key tests which helped in identify *A. tumefaciens* isolates among all the isolates. In the table '+ & -' indicate positive or negative reaction to the indicated test respectively. In column tumor related features '+ & -' shows tumor formation or absence, whereas days mean days taken to initiate tumor; % represent transformation or tumor forming efficiency; tumor types include G, S= green solid mass, C, V= colorless vitrified mass, N/A= not applicable because tumor was not formed; tumor weight is in milligrams and different letters in front of the numbers show presence of significant differences among means.



A B C D E F

Figure 1. Different morphological, biochemical and phyto-pathological tests carried out to identify Agrobacterium isolatesA: mango plant with crown gall; B: Gram negative rods; C: Catalase test; D: 3-Ketos test; E: Antibiotic resistance test; and F: Phtopathological test

In total 12 bacterial isolates with ID no AtMi001, AtMi002, AtMi003, AtSa005, AtSa007, AtMz010, AtMz011, AtAi013, AtAi015, AtDr016, AtDr018 and AtDr019 showed tumor forming ability and positive pathogenicity. All these isolates induced green solid cell mass on discs except AtSa007 and AtDr017 which induced colorless vitrified cell mass on discs. Inoculated discs were also observed for tumor induction frequency (%). The highest tumor frequency was revealed by isolate AtMi003 that was 80% equal to control strain. Remaining 07 isolates did not produce tumors namely AtMi004, AtSa006, AtSa008, AtMz009, AtAi012, AtEs014 and AtDr017. Among these AtMi004, AtAi012, AtEs014 and AtDr017 were also not able to tolerate 2% salt and 40ºC temperature (table 1). Additionally, these isolates did not produce 3-ketolactose. On the basis of these observations it could be suggested that these four isolates are not *Agrobacterium tumefaciens*that is why they did not produced tumor on carrot discs. Nevertheless, these could be a part of *Agrobacterium* group that can be confirmed by molecular characterization of these isolates. Three isolates out of the seven non-tumor forming isolates namely AtSa006, AtSa008 and AtMz009 were nonpathogenic strains of *Agrobacterium tumefaciens*as these were able to tolerate 2% salt, 40ºC temperature and also produced 3-ketolactoses on lactose medium (table 1) and did not induce tumor. Virulent strains usually possess Ti plasmid but many non-tumorigenic *Agrobacterium* also have plasmids (Gelvin, 2012).

It is quite obvious from the data presented in table 1 for tumor frequency and the time indays taken to initiate tumor that the isolated bacterial strains have different virulence potential as compared to each other. Some strains are more virulent in pathogenicity than others. This difference could be due to the environmental conditions, nature of the host plantand/ or genetics of isolates (Sarker*et al*., 2011). Chen *et al*. (1999) demonstrated the same level of differences in agro-virulence by isolating *Agrobacterium* strains from different tumors of aster. On the basis of tumorigenic ability in vitro disease assay confirmed these strains as *A. tumefaciens*Biovar 1.To identify the most virulent strain, tumors were weighted after 40 days of post inoculation. The results of mean tumor weights induced by isolated *Agrobacterium* spp. are presented in table1. The results showed that the isolate AtMi003 is the most virulent strain that produced tumor weighting 23.67 mg. The mean weight of tumor produced by this isolate is significantly different from other isolates on LSD p=0.05. The mean tumor weights of the remainingisolates were below the range of standard isolate.

In conclusion, this is a novel research and first report on the isolation of virulent wild strains of *A. tumefaciens* from Mango (*Mangiferaindica*), Sitaashok(*Saracaasoca*), Chikoo(*Manilkarazapota*), Neem(*Azadirachtaindica*), Eucalyptus (*Eucalyptus spp*) and Gulmohar (*Delonixregia*) in Sindh, Pakistan.

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