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ISOLATION, CHARACTERIZATION AND EVOLUTION OF WILD VIRULENT STRAINS OF *AGROBACTERIUM* FOR THEIR POTENTIAL TRANSFORMATION THROUGH USE OF POTATO DISCS

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Abstract

The present study was aimed to investigate the Crown galls of the trees and plants caused by *Agrobacterium*. For the purpose, the Crown galls were collected from Botanical and Horticultural Gardens of Sindh Agriculture University, Tandojam, Pakistan and examined for *Agrobacterium* species. In all 90 Crown galls were examined for *Agrobacterium* species. The Crown galls extracts were obtained and streaked on PYGA (Peptone, Yeast extract, Glycerol, Agar) and Luria–Bertani (LB) media supplemented with rifampicin (30 µl L^{-1}) antibiotic to inhibit the growth of other bacterial organisms and the plates were incubated at 28°C for 48 hours. Only thirty (33.333%) wild isolates of the Agrobacterium *tumefaciens* were isolated from Crown galls. However, nineteen (19) isolates out of 30 were confirmed as *Agrobacterium tumefaciens.* Among these 19 isolates, At224 was found to be the most virulent strain which showed 80% transformation efficiency (TE) while other three isolates i-e At213-TE: 70%; At222-TE: 70% and At230-TE:70% were induced solid green cell masses/tumors/ galls on the potato discs vigorously. Of the tested isolates, the isolate At224 (*Mangifera indica*) was the most virulent strain that caused 80% transformation as compared to other isolates. Furthermore, when comparison was made among isolates recovered from Crown galls and that of controlled for phenotypical characteristics, the majority of isolates showed similar characteristics as demonstrated in the controlled *(*At 2441- EHA101). Moreover, Ti plasmid was found visible in by using 0.8% in 50 ml of TAE buffer solution. Thus Ti plasmid that helped in for the characterization of wild *Agrobacterium* by molecular system.

Key words: *Agrobacterium* wild strains, Isolation, Characterization, Disease assay, Transient transformation.

Introduction

The genus *Agrobacterium* belongs to the family *Rhizobiaceae*. The species of *Agrobacterium* are phyto-pathogenic in nature and cause various kinds of tumors such as cane gall, crown gall and hairy roots. The virulent strains of the genus *Agrobacterium* harbor tumor or root inducing plasmids (Gelvin, 2009). The *Agrobacterium tumefaciens* are aerobic, gram-negative rods, measuring 0.6-1.0 µm in width and 1.4-3.0 µm in length. It is motile with the help of 2-6 peritrichous flagella and cells are arranged in singles and/ or pairs. It produces smooth and non-pigmented colonies on nutrient agar at temperature ranges from 25–28ºC. Originally, the genus *Agrobacterium* was classified on the basis of pathogenic behaviour of bacterial species, host range and disease symptoms (Lassale *et al*., 2011). Now, the genus *Agrobacterium* have been classified on the basis of phenotypic, physiological, chromosomal characteristics, growth patterns at different temperatures and sensitivity behaviour towards sodium salts (Chebil *et al*., 2013). The most common characteristic difference in these bacterial organisms is their host range. They can produce diseases in more than 600 species, belongs to more than 90 different families of dicots and four families of monocots (Sarker *et al*., 2011; Mattyias, 2006). The deep damages/wounds on dicot trees result in larger galls but the growth depends on the type of Ti plasmid. The Ti plasmid possesses the genes for formation of unusual amino acids, these compounds are considered to be substrate for *Agrobacterium* and therefore the galls are beneficial for bacterial activities (Suzuki *et al*., 2009).

However, the IAA and cytokinins genes on Ti plasmid produce a large amount of auxin and cytokinins which are accountable for the proliferation of plant transformed cells (Suzuki *et al*., 2009). The genus *Agrobacterium* is widely considered to be genetic engineer because it causes disease in host plant by transferring its genetic material in the form of a DNA segment, called as T-DNA or transposon DNA or transfer DNA. This transposon DNA piece is located on tumor inducing (Ti) plasmid. Ti is a circular form of extra chromosomal DNA piece and it measures 190-240 kb in size and always located in number of 1-3 copies per cell. Moreover, that virulent strains of genus *Agrobacterium* usually possess tumor inducing plasmid while some of the non-tumor inducing *Agrobacterium* also have plasmid (Lang *et al*., 2013; Pulawska, 2010). Even though there are differences among the Ti plasmids; each consists of five divergent areas including three regions necessary for tumourogenesis. The regions are *vir* region, T-DNA region and opine catabolism region plus, a region for conjugation containing tra, trb loci and the rep region for replication (Zhao *et al*., 2010). The plasmid also contains genes of conjugation and susceptibility against bacteriocin in the Nopaline and Succinam opine plasmids. Nopaline plasmids like pTiC58 have a T-DNA independent locus tzs that codes an enzyme which produces the phytohormonecytokinins in the bacteria under *vir* inducing conditions (Kondo *et al*., 2011). Further that T-DNA has genes for auxin and cytokinins. The cytokinins regulate several characteristics of plant development, like organization of stem cells in roots and shoots(Tokunaga *et al*., 2012), root development (Ishida e*t al.*, 2008; Kondo *et al*., 2011) and nodule organogenesis (Tirichine *et al.*, 2007).

The conversion efficiency of the cells from normal to the diseased one depends upon the attuned interaction of bacteria and the host cells. A few of the *Agrobacterium* strains are highly virulent to the susceptible hosts as compared to none or less susceptible. Furthermore, that some host genotypes are highly or some extent tolerant to *Agrobacterium* (Gelvin, 2012). Such deviation may be due to insufficiency in the bacterium or host machinery or the transfer of T-DNA. Sometimes such incompatible reaction takes place in between bacteria and host plants; a hypersensitive reaction is elicited by the host against many Laboratory strains of *Agrobacterium* (Van der *et al*., 2000). Through scientific method it is observed that the most of the developed world is cultivating several transgenic crops, like cotton, potatoes, canola, soyabean, *Solanumly copersicumes*, corn etc., (Evenson, 2005).

When the results are correlated by biochemical, virulence and PCR analysis suggested that the PCR could be used efficiently in detection of tumorigenicity of *Agrobacterium vitis* obtained from grape vines (Genov *et al*., 2006). Ali *et al*. (2010) conducted an extensive survey and examined the plants for typical symptoms to determine the incidence and severity of Crown gall disease on cherry, apple and apricot trees. *Agrobacterium tumefaciens* strains have been isolated from dicot plants i.e., Jack Fruit (*Artocarpus heterophyllus*), Teak (*Tectona grandis*), Kahua bark (*Terminalia arjuna*), Kadam (*Anthocephalus codomb*a), *Solanum copersicum* and China rose (*Rosa chinenses*). The isolated strains were characterized on the bases of biochemical, antibiotic resistance and pathogenicity testing. *Agrobacterium* strains AtSlOl 05 and AtAcOl 14 have been observed as the most virulent strains compared to *Agrobacterium tumefaciens* (ATCC233087) (Islam *et al*., 2010). *Agrobacterium tumefaciens* strains such as AtTp0120, AtTp0120, AtMo0122 and AtMi0123 were isolated from *Tagete spatula, Tagete serecta, Moringa oleifera* and *Mangifera indica* plants respectively; the isolates were confirmed as *A. tumefaciens* on the basis of morphological, biochemical, phytopathogenicity and analysis of plasmid DNA (Sarker *et al*., 2011). The wild strain of *Agrobacterium tumefaciens* were isolated from infected leaves, stems and Crown galls of Vicia faba (Tiwary *et al*., 2007). China Rose, Jack Fruit, *Solanum lycopersicum*, Teak and Kadam (Islam *et al*., 2010; Davoodi & Hajivand, 2013), stone fruit plant (Gupta *et al*., 2010), almond (Benali & Bencheikh, 2013), grapes (Genov *et al*., 2006), plants (Otten *et al*., 2008), cherry, apple and apricot (Ali *et al*., 2010).

Materials and Methods

Bacterial isolates: Crown gall was observed on the plants of Botanical and Horticultural Gardens of Sindh Agriculture University, Tandojam, Pakistan. A total of 90 Crown gall samples were collected and processed. The average rainfall in the area is 415 mm/ year and average temperature is about 46ºC during the months of May to August while the temperature is about 10ºC in December and January. Crown gall tissues were collected from different infected plant species such as, Neem (*Azadirachta indica*), Eucalitus (*Eucaipus civiodoras*), Amaltas (*Cassia fistuia*), Peepal (*Fiscus religrosa*), Gul Mohar (*Delonix regia*), Kachnar (*Bauhinia variegate*) Mango (*Mangiafera indica*) Lignum (*Guaiacum officinae*), Sunflower (*Helianthus annuus*), Tomato (*Solanum lycopersicum*) and China rose (*Rose chinensis*). Then galls were processed according to (Aliullah *et al.,* 2016) and sterilized in 10% household bleach for 20 minutes followed by rinsing the galls three times in Sterile-Distilled Water (SDW). Further galls were chopped into small pieces of 2-3 g by sterilized surgical blade and were soaked in SDW overnight at room temperature. The crown gall extracts were collected and streaked on PYGA and Luria–Bertani (LB) media. The plates were incubated at 28°C for 48 hours. As colonies appeared on the agar plates, the pure bacterial colonies were picked-up from mixed culture and transferred to the fresh plates to get pure culture. After obtaining pure culture, the purified colonies were cultured on LB medium containing rifampicin (30 µl L^{-1}) to inhibit the growth of other contaminating bacterial organisms.

Phenotypic characteristics of *Agrobacterium* species: For phenotypic characterization, a positive controlled species was brought in use in the present study i.e. *Agrobacterium tumefaciens* (At 2441- EHA101). Identification of bacterial isolates was carried out on the basis of morphological, cultural, Gram staining reaction, bacterial motility, and growth characteristics in liquid and solid media according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Bacterial growth characteristics in liquid and solid media were determined by spreading a loop full of purified isolates over on LB solid and inoculation was made in LB liquid medium. The cultures were allowed to grow for 48 hours at 28ºC without shaking under aseptic conditions. The colony characteristics of the bacteria on solid medium were recorded by examining a single colony of an isolate. In this regard, the shape, size, margin, surface, elevation, opacity and chromogenesis were recorded. The motility of isolates was observed by placing a drop of overnight grown bacterial suspension using bacteriological loop on a cavity slide and a glass cover-slip was placed over the suspension and then slide was examined under microscope by using both, low and high power objectives. Gram staining was performed using standard method as described by Hans (1884).

Biochemical tests: Biochemical tests of the isolates were carried out followed Bergey's Manual of Determinative Bacteriology (Holt*et al*., 1994) which included: catalase production, NaCl (2% and 5%) tolerance, temperature tolerance (28ºC and 37ºC), sugar fermentation, 3-ketolactose production, oxidase, and antibiotic sensitivity test.

Antibiotic sensitivity: The sensitivity of selected isolates against antibiotics was detected using the method of Bauer-Kirby (Bauer *et al.,* 1966) also known as Disc

Diffusion Method. The antibiotics used in this study were: Kanamycin (30 µg mL^{-1}), Cefuroxime (30 µg mL^{-1}), Tetracycline (30 µg mL^{-1}) and Rifampicin (10 µg mL^{-1}). Whatmann No. 1 filter paper was used to prepare discs (5.5 mm in diameter). When a clear zone of no growth was recorded as zone of inhibition, when zone appeared around the disc then the isolates were regarded as susceptible to the particular antibiotic.

Characterization of phyto-pathogenicity: The degree of transformation efficiency depends on the virulence of pathogen. Thus, virulence of pathogen was studied on the basis of a disease assay on potato discs. Initially the isolates were inoculated in LB broth and allowed to grow at 28ºC for 2 days.

Potato disc assay: The potatoes (*Solanum tuberosum* L.) were obtained from local market of Tandojam, Pakistan. The whole potato was cut into three pieces and the surfaces of the potato were sterilized using 20% commercial bleach for 10-15 minutes and were rinsed twice in Double Distilled Water (DDW). The potato discs were sliced by 5 mm thick aseptically using a sterilized scalpel or borer. The potato discs were then loaded with 30 µl suspension containing bacterial cells of OD_{600}= 2.0 and dried under clean bench. The test tubes and plates were sealed with para-film and incubated at 25 to 28ºC in incubator. After 25 to 30 days of incubation, the changes in the potato tissues towards the formation of tumour was noted. The results were incorporated for tumour frequency.

Isolation of Ti plasmid: Three different protocols were used to isolate Ti plasmid. The Standard Alkaline Lysis Method was used for the isolation of plasmids by (Sambrook & Russell, 2001). According to the second protocol (Kiran *et al*., 2010) overnight grown bacterial cells were centrifuged and medium was removed. The pelleted bacterial cells were suspended in TE buffer, boiled in a water bath for 05 minutes and immediately transferred on ice for 05 minutes. Then this suspension was loaded on 0.8% gel for visualization. In third protocol (Scortichini *et al*., 2002) 5ml LB medium was inoculated with various isolates and grow over night at 28ºC. After 2 days of growth, 3 ml of bacterial culture was pelleted in Ependroff tube (1.5 ml) and centrifuged at 13500 rpm for 2 minutes and supernatant was discarded. The pellet was washed 2-times in 100 µl of 0.85% NaCl solution. After washing, the pellet was re-suspended in 30 µl of 0.85% NaCl solution and/ or molecular grade water and boiled for 10 minutes at 95ºC in a water bath. The boiling suspension was then immediately placed on ice for 20 minutes. A 3 µl of loading dye was added to cooled bacterial suspension.

Agarosegel analysis: A 0.8% agarose gel was prepared by mixing and boiling 0. 4 g agarose gel in 50 ml of TAE buffer solution. After dissolving the agarose particles in TAE, the suspension was allowed to cool until temperature reached to 50-55ºC.

Results and Discussion

Ninety samples of Crown galls from 11 different plant species were collected and examined, 30 isolates of wild *Agrobacterium tumefacien*s were isolated from Crown galls of infected plants & trees were characterized by different morphological, biochemical, physiological, antibiotic sensitivity and phyto-pathogenicity tests (tumor inducing capability on potato discs). From 90 galls, the overall prevalence of the species was noted in 30 Crown galls, thus the percentage prevalence was recorded as 33.33% while negative galls were noted as 60 in numbers, the percentage was observed as 66.66%. Genov *et al*. (2006) carried out a survey against the Crown galls in Apple, Apricot, and Cherry plants of 5 villages of Gilgit-Baltistan, Pakistan. A total of 6100-Cherry, 6900-Apple and 8000-Apricot plants gave the mean prevalence of Crown galls as 87.87, 87.96 and 00.00% respectively. Although the causative agent of this neoplastic disease is *A. tumefaciens*, has been isolated from tumors of Aster (Chen *et al*., 1999), *Tageteserecta*, *Tagetspatula*, *Mangifera indica* and *Moringa oleifera* (Sarker *et al*., 2011), tobacco (Furuya *et al.*, 2004), rosa (Aysan & Sahin, 2003), apricot (Aysan *et al*., 2003), root nodules of *Viciafaba* (Tiwary *et al*., 2007) and *S. lycopersicum*, *A. hetero-phyllus*, *T. arjuna*, *R. chinensis*, A. *codomba* and *T. grandis* (Islam *et al*., 2010), in many countries. The present work was carried out for first time at Sindh Agriculture University Tandojam in particular and general in Pakistan on isolation and characterization of virulent wild strains of *A. tumefaciens*.

Study was launched to screen out the morphological physiognomies of wild strains *of A.tumefaciens*. The isolates from At201 to At230 (30 isolates) of wild strains of *A.tumefaciens* recovered from Crown galls of shrubs and trees were Gram-negative, pink/red in colour, curved and small coccobacilli rods. The cells were cocco-bacillary with rounded ends. No obvious influences of the shurbs and trees on the morphological characteristics of *A. tumefaciens* isolates were observed. Gelvin (2009) defined the wild strain of *A.tumefaciens* as aerobic, Gram-negative, bacillary rods, motile with the help of 2-6 peritrichous flagella, measuring 0.6-1 um in width and 1.4-3.0 µm in length. Whereas Benali & Bencheikh (2013) demonstrated that *Agrobacterium* isolates recovered from galls of stone fruit trees were rod shaped, with rounded ends and having single and sometimes paired flagella at pole. *A. tumefaciens* isolates were Gram-negative and motile. *A. tumefaciens* isolates from plants, trees and nurseries were analyzed for morphological properties (Kuzmanovic & Obradovic, 2013; Ali *et al*., 2016 Davoodi & Hajivand, 2013). The isolates were aerobic, motile, Gram-negative, non-spore forming curved and rod shaped. The findings about the morphological characteristics of *A.tumefaciens* isolates in the present study were similar. All 30 isolates of *A.tumefaciens*, isolated from Crown galls of 11 different plant species were cultured on LB (Luria Bartani) solid

and liquid media. The isolates of *A.tumefaciens* showed similar patterns of growth on both, the solid and liquid media, although the isolates were recovered from different species of plants, amazingly no obvious influence of tree and shurbs cells on the behaviour of *A. tumefactions* isolates' growth patterns was detected. However, on solid LB medium, the isolates of *A.tumefactions* produced rough, yellowish, raised, opaque, smooth, pinpoint, convex, spherical, sometimes golden, translucent, shiny mucoid, creamy color, even and plane colonies, while on liquid medium, the isolates of *A.tumefactions* produced uniform turbidity but not any powdery sedimentation, pellet and even pellicle colonies were manifested. Similar cultural characteristics were represent by (Gelvin, 2009,Tiwary *et al*., 2007;Benali & Bencheikh, 2013) who stated that the *Agrobacterium* isolates recovered from galls of stone fruit trees which produced circular, smooth, non-pigmented, translucent, mucoid, convex, and easily suspendable in water colonies on MacConkeys and YMA analysed media. Davoodi & Hajivand (2013); Kuzmanovic & Obradovic (2013); Ali *et al*., (2016) the isolates of *A. tumefaciens* obtained from different plants for cultural properties. The isolates produced pink, smooth, circular, mucoid, translucent and shiny colonies on MacConkeys and YMA agar media. They also described that the cells of plants had no influence on the pattern of growth of *A.tumefaciens* isolates on different culture media. Generally, the animal and *plant* cells in which the bacteria grow and multiply could contribute a role in the genetic behavior of the bacteria, ultimately the growth patterns of the bacteria would be changed if one culture on a suitable or alternative media. However, it could mislead the researchers for proper identification of the bacterial species (Ali *et al*., 2016).

All the isolates identified from Crown galls of the trees and plants consumed D-mannitol, L-arabinose, Rhamnose, Glucose, Lactose, and hydrolyzed Starch and liquefied gelatin. Furthermore, they also formed acid and gas during utilization of sugars. When the sugar fermentation characteristics of the recovered isolates were compared with controlled isolate, it presented the similar behaviour as noted for the isolates of *A. tumefaciens.* Benali & Bencheikh (2013) reported that all strains of *A.tumefaciens* obtained from almond nurseries in Algeria oxidized sucrose, D-mannitol, L-arabinose, D-lactose, Rhamnose, Lactose and Glucose and also reported that all the isolates transformed the gelatin and starch. Whereas Islam *et al*., (2010) also investigated sugar fermentation property of the isolates of *A.tumefaciens*. The isolates were found positive for catalase, oxidase, lactose, mannitol, and produced H_2S. The sugar fermentation tests applied to the isolates for the confirmation of the sugar properties were also applied by the above workers and found similar characteristics as noted in this survey. It meant the results of fermentation of sugars demonstrated were very close to the other workers. Therefore we believe that the variation in the properties of sugar utilization achieved by various isolates of *A.tumefaciens* are the same as observed by other scientists the minor differences could be due to the differences in the sources of isolates.

Agrobacterium tumefaciens isolates recovered from Crown galls of trees and plants were tested for physico-biochemical properties (Table 1; Fig. 1). Only the isolates At202, At216, At227 and At228 isolated from *Azadirachta indica*, *Helianthus annuus* and *Rosa chinensis* were recorded as negative for oxidase property, respectively. The isolates At202, At204, At208, At209, At216, At225 and At228 obtained from *Azadirachta indica*, *Solanum lycopersicum*, *Helianthus annuus* and *Rosa chinensis* did not survive at 2% salt concentration. Whereas the isolates At202, At204, At208, At215, At 220, At221 and At225 could not grow at 37ºC. *A.tumefaciens* isolates were tested to observe the properties of 3-Ketolactose and Catalase. The isolates, At202, At208, At209, At216, At220, At225, At227 and At220 obtained from *Mangiafera indica*, *Helianthus annuus*, and *Rosa chinensis* no 3-Ketolactose characteristics respectively whereas others were noted to have 3-Ketolactose property. Whereas all 30 isolates had Catalase property. Benali & Bencheikh (2013) demonstrated that all 10 isolates exhibited the properties of catalase, and oxidase and also oxidized the lactose to 3-keto-lactose. Davoodi & Hajivand (2013) isolated. *Agrobacterium* isolates from rose plants were detected as catalase positive while oxidase negative. On the other hand (Gelvin, 2009) examined the heat tolerance and growth of *A. tumefaciens* on nutrient agar at temperature ranging from 25-37ºC; *A. tumefaciens* strains grew very well and tolerated the temperature range but beyond this temperature the isolates did not survive and even no evidence of growth was noted. *A.tumefaciens* isolates recovered from the galls of different trees and plants were tested for sugar utilization. The data regarding sugar fermentation properties of the isolates are given in Tables 2.

Fig. 1. Shows physico-biochemical and phyto-pathogenicity properties of *Agrobacterium tumefaciens* recovered from Crown galls of trees and plants. A: Neem with Crown gall; B: Gram staining; C: Catalase test; D: 3-ketolactose production test; E: Green masses on potato disc; F: Green masses on potato discs stained by Lugol's iodine solution.

Table 1. Characterization of the wild *Agrobacterium* strains isolated from trees and plants.

Isolates	Sci. name	L./English name	D-Mannitol	L-Arabinose	Rhamnose	Glucose	Lactose	Starch	Liquefied gelatin	Acid & H_2S
AT201	*A. indica*	Neem	+	+	+	+	+	+	+	Acid &gas
AT202	*A. indica*	Neem	+	+	+	+	+	+	+	Acid& gas
AT203	*E. citrodora*	Eucalyptus	+	+	+	+	+	+	+	Acid &gas
AT204	*E. citrodora*	Eucalyptus	+	+	+	+	+	+	+	Acid& gas
AT205	*E. citrodora*	Eucalyptus	+	+	+	+	+	+	+	Acid& gas
AT206	*B. variegata*	Kachnar	+	+	+	+	+	+	+	Acid& gas
AT207	*B. variegata*	Kachnar	+	+	+	+	+	+	+	Acid& gas
AT208	*B. variegata*	Kachnar	+	+	+	+	+	+	+	Acid& gas
AT209	*C. fistula*	Amaltas	+	+	+	+	+	+	+	Acid& gas
AT210	*C. fistula*	Amaltas	+	+	+	+	+	+	+	Acid& gas
AT211	*C. fistula*	Amaltas	+	+	+	+	+	+	+	Acid& gas
AT212	*C. fistula*	Amaltas	+	+	+	+	+	+	+	Acid& gas
AT213	*D. regia*	GulMohar	+	+	+	+	+	+	+	Acid& gas
AT214	*D. regia*	GulMohar	+	+	+	+	+	+	+	Acid& gas
AT215	*D. regia*	GulMohar	+	+	+	+	+	+	+	Acid& gas
AT216	*S.lycopersicum*	Tomato	+	+	+	+	+	+	+	Acid& gas
AT217	*S.lycopersicum*	Tomato	+	+	+	+	+	+	+	Acid& gas
AT218	*S.lycopersicum*	Tomato	+	+	+	+	+	+	+	Acid& gas
AT219	*F. religrosa*	Peepal	+	+	+	+	+	+	+	Acid& gas
AT220	*F. religrosa*	Peepal	+	+	+	+	+	+	+	Acid& gas
AT221	*F. religrosa*	Lignum	+	+	+	+	+	+	+	Acid& gas
AT222	*F. religrosa*	Lignum	+	+	+	+	+	+	+	Acid& gas
AT223	*M. indica*	Mango	+	+	+	+	+	+	+	Acid& gas
AT224	*M. indica*	Mango	+	+	+	+	+	+	+	Acid& gas
AT225	*M. indica*	Mango	+	+	+	+	+	+	+	Acid& gas
AT226	*H. annuus*	Sun flower	+	+	+	+	+	+	+	Acid& gas
AT227	*H. annuus*	Sun flower	+	+	+	+	+	+	+	Acid& gas
AT228	*R. chinensis*	China Rose	+	+	+	+	+	+	+	Acid& gas
AT229	*R. chinensis*	China Rose	+	+	+	+	+	+	+	Acid& gas
AT230	*R. chinensis*	China Rose	+	+	+	+	+	+	+	Acid & gas
Control AT2441			+	+	+	+	+	+	+	Acid & gas

Note: Sci. = Scientific, L. + Local, At = *Agrobacterium tumefaciens*, + = indicates Positive reaction, D-Mannitol, L-Arabinose H2S = Hydrogen sulphide

Table 2. Physico-biochemical properties and Phyto-pathogenicity potential of the wild *Agrobacterium* strains isolated from trees and plants.

Isolates	Sci. name	L./Eng. name	28ºC	37ºC	Salt 2%	Salt 5%	Oxides test	3-ketolactose	Catalase	T F days	% tumor	G C tumour
AT201	*A. indica*	Neem	+	+	-	+	+	+	+	23	60	S G
AT202	*A. indica*	Neem	-	-	-	+	-	-	+	NA	0	No G S
AT203	*E. citrodora*	Eucalyptus	+	+	-	+	+	+	+	22	40	C less
AT204	*E. citrodora*	Eucalyptus	+	-	-	+	-	+	+	NA	0	No G S
AT205	*E. citrodora*	Eucalyptus	+	+	-	+	+	+	+	22	60	S G
AT206	*B. variegata*	Kachnar	+	+	-	+	+	+	+	23	40	S G
AT207	*B. variegata*	Kachnar	+	+	-	+	+	+	+	23	50	S G
AT208	*B. variegata*	Kachnar	+	-	-	+	-	-	+	NA	0	No G S
AT209	*C. fistula*	Amaltas	+	-	-	+	+	-	+	NA	0	No G S
AT210	*C. fistula*	Amaltas	+	+	-	+	+	+	+	22	40	S G
AT211	*C. fistula*	Amaltas	+	+	-	+	+	+	+	24	30	S G
AT212	*C. fistula*	Amaltas	+	+	-	+	+	+	+	28	20	C less
AT213	*D. regia*	GulMohar	+	+	-	+	+	+	+	22	70	S G
AT214	*D. regia*	GulMohar	+	+	-	+	+	+	+	24	40	C less
AT215	*D. regia*	GulMohar	+	+	-	+	-	+	+	NA	0	No G S
AT217	*S.lycopersicum*	Tomato	-	-	-	+	+	-	+	NA	0	No G S
AT218	*S.lycopersicum*	Tomato	+	+	-	+	+	+	+	23	60	C less
AT218	*S.lycopersicum*	Tomato	+	+	-	+	+	+	+	26	40	C less
AT219	*F. religrosa*	Peepal	+	+	-	+	+	+	+	23	50	S G
AT220	*F. religrosa*	Peepal	+	+	-	+	-	-	+	NA	0	No G S
AT221	*F. religrosa*	Peepal	+	+	-	+	-	+	+	NA	0	No G S
AT222	*F. religrosa*	Peepal	+	+	-	+	+	+	+	21	70	G S
AT223	*M. indica*	Mango	+	+	-	+	+	+	+	22	50	G S
AT224	*M. indica*	Mango	+	+	-	+	+	+	+	19	80	G S
AT225	*M. indica*	Mango	+	-	-	+	-	-	+	NA	0	No
AT226	*H. annuus*	Sun flower	+	+	-	+	+	+	+	27	50	G S
AT227	*H. annuus*	Sun flower	-	-	-	+	+	-	+	NA	0	No G S
AT228	*R. chinensis*	China Rose	-	+	-	+	+	-	+	NA	0	No G S
AT229	*R. chinensis*	China Rose	+	+	-	+	+	+	+	23	60	G S
AT230	*R. chinensis*	China Rose	+	+	-	+	+	+	+	21	70	G S
Control AT2441			+	+	-	+	+	+	+	28	60	G S

Note: Sci.= Scientific, Eng. English, L. Local At = *Agrobacterium tumefaciens*+ = Positive - = Negative T F.= Tumor formation G. = Green, S. = solid, C = Colour NA = Not applicable, G C = General Character

During current investigation, the inoculation of the isolates of wild *A.tumefaciens* was made on the discs of potato to demonstrate the phyto-pathogenicity of the wild strains of *A.tumefaciens* obtained from different plants and trees (Table 2). Nineteen (19) isolates out of 30 were identified as *Agrobacterium tumefaciens*. Among these 19 isolates, At224 was found to be the most virulent strain showing 80% transformation efficiency while three other isolates (At213-TE: 70%; At222-TE: 70% and At230-TE:60%) were also induced solid green cell mass/ tumor/ gall on potato discs from 60-70% transformation efficiency. Crown gall disease is detected in many dicot plants such as fruit trees, grapes, roses and some ornamentals (Rhouma *et al*., 2008). Among the tested *Agrobacteria*, At202, At204, At208, At209, At215, At216, At220, At221, At 225, At227 and At228 isolates detected from *Azadirachta indica*, *Eucaipus civiodoras*, *Bauhinia variegate*, *Cassia fistula*, *Delonix regia*, *Solanum lycopersicum*, *Fiscus religrosa*, *Guaiacum officinae*, *Mangiafera indica*, *Helianthus annuus* and *Rosa chinensis* did not respond and did not produce any tumor like green cell mass on the potato discs while all other isolates produced solid green cell mass with variable degree of intensity and showed their capability to cause Crown galls in the inoculated discs. Phyto-pathogenicity test is an important parameter to confirm isolates as virulent strains of *A. tumefaciens*. Bauer *et al*., (1966) isolated 15 *A. tumefaciens* strains and inoculated on carrot discs to evaluate the pathogenicity of isolates. In contrast to present study, many researchers used intact plants to observe pathogenicity of isolates such as Tomato, Sunflower, *Datura* spp., Kalanchoedai gremontiana, tobacco, and *Nicotiana glauca*. It was suggested that *virulent* and avirulent also be identified through generating lipid and fatty acid profile by using Polymerase Chain Reaction (Ponsonnet & Nesme, 1994) against carrot and potato discs to evaluate the pathogenicity of Agro-strains. During present study, among the tested isolates, At224 of *Mangiafera indica* was the most virulent strain showing 80% transformation efficiency. The results of present study are comparable to earlier represents. Using this disease assay by different workers indicated that the isolates are genetically different from each other and possess different levels of virulence. These differences could be due to the environmental conditions, nature of the host and/ or genetics of isolates (Sarker *et al*., 2011). Aysan & Sahin (2003) demonstrated the same level of differences in Agro-virulence by isolating *Agrobacterium* strains from different tumors of aster. On the basis of this tumorigenic *in- vitro* disease assay one can confirm these strains as *A. tumefaciens* Biovar 1.

Highly effective drugs observed against the isolates of wild strain *A.tumefaciens* in this survey were Rifampicin, Oxytetracycline, Kanamycin and Cefuroxime are presented (Table 3). Generally, the drug Cefuroxime was found to be highly effective drug against At220, At216, At206, At213, At218, At224 and At227 isolates recovered from *Ficus religiosa*, *Solanum lycopersicum*, *Bauhinia, variegata*, *Delonix regia*, *Mangiafera indica* and *Helianthus annuus* and its efficacy against the isolates of *A.tumefaciens* was 100, 98.18, 90.91, 90.91, 90.91, 90.91, 90.91 and 90.91% respectively. The second highly effective antibiotic against the *A. tumefaciens* isolates was noted as Kanamycin and its action against At220, At204, At206, At203 and At207 which were isolated from *Fiscus religrosa*, *Eucalyptus citriodora and Bauhinia variegate;* was 78.18, 76.36, 74.55, 72.73 and 72.73% respectively. According to Genov *et al*., (2006) who detected 30 *A.tumefaciens* strains from soil samples, apple and cherry plant galls and tested for their susceptibility against 06 different antibiotics. All strains were resistant to Lincomycin, Amoxicillin, Ampicillin and Cloxacillin whereas the strains exhibited intermediate sensitivity to drugs Cephradine, Tetracycline and Doxycycline. Similar antibiotic susceptibility test against *A.radiobacter* was conducted by Gupta *et al*., (2010) who reported *A. radiobacter* isolates UHFBA-8 and 11 as Rifampicin resistant mutants and those were able to colonize the root system of peach colt. They suggested that the *A.tumefaciens* infections could be controlled through biologically only by using agrocin, colonizing the root system andor by physically blocking of the infection sites using *A. radiobacter* mutants. In another study Ali *et al*., (2010) performed antibiotic susceptibility test against *Agrobacterium tumefaciens* isolates recovered from peach tree galls. The bacterial isolates did resist to ampicillin and tetracycline and no zone of inhibition was developed around the discs. But on the other hand, the *A.tumefaciens* isolates showed susceptibility to Kanamycin and Cefotaxime by forming a clear zone of inhibition around the antibiotic discs. In this study the drug Cefuroxime was recorded as a highly effective drug against the isolates of *A.tumefaciens* recovered from trees and plants while the second most highly effective drug demonstrated was Kanamycin. The results of the present survey are some extent in line to the antibiotic susceptibly against isolates determined by Ali *et al*., (2016) who observed Cefotaxime and Kanamycin as intermediately effective against *Agrobacterium tumefaciens* isolates obtained from peach tree galls. However, the difference between pathogenic and non-pathogenic *Agrobacteria* is due to the presence and absence of Ti plasmid. The Ti plasmid produces particular compounds those are being utilized by *Agrobacteria* for their growth. Thus, the characterization of Ti plasmid (size 200-250 kb) is most important. Nevertheless, before characterization there was a need to optimize its rapid and cost effective isolation. In this regard different isolation methods are evaluated based on literature survey and a modified method was recorded as optimized method. Initially, Standard Alkaline Lysis method (Sambrook & Russell, 2001) and a Modified Protocol (Kiran *et al*., 2010) were utilized for mini preparation of plasmid to isolate Ti plasmid. These methods did not result Ti plasmid isolation because Ti plasmid is some extent bigger (200-250 Kb) and these methods could be sued to isolate smaller plasmids (>100 kb). Then a method for Ti plasmid was optimized based on (Scortichini *et al*., 2002). In the original protocol of Scortichini *et al*., (2002) the bacterial suspension was boiled in 0.85% NaCl whereas in Modified Protocol, the bacterial suspension was only washed in 0.85% NaCl and boiled in molecular grade water. This modification dramatically improved results and after electrophoresis at 100 volts for 3 hours, Ti plasmid was found visible in gel, the pictures were obtained by using Gel Documentation System of Biometric (Fig. 2.). Thus a cost effective protocol was optimized for the isolation of Ti plasmid that would help in future research for the characterization of Ti plasmid by molecular approach.

Table 3. The zone of inhibition (mm) and percentage of susceptibility of *Agrobacteriumtumefaciens* isolates against selected antibiotics.

Isolates	Scintific name	L./Eng. name	Rifampicin (10 µg/ml)		Oxytetracycline (30 µg/ml)		Kanamycin (30 µg/ml)		Cefuroxime (30 µg/ml)	
			Zone of Inhibiti.	% Suscept.	Zone of Inhibiti.	% Suscept.	Zone of Inhibiti.	% Suscept.	Zone of inhibiti.	% Suscept.
AT201	*A. indica*	Neem	14+0.68	25.45	23+1.35`	41.82	32+1.20	58.18	42+0.68	76.36
AT202	*A. indica*	Neem	7.0+1.35	12.73	26+1.20	47.27	31+0.78	56.36	34+0.68	61.82
AT203	*E. citrodora*	Eucalyptus	14+1.15	25.45	31+1.15	56.36	40+0.58	72.73	38+0.58	69.09
AT204	*E. citrodora*	Eucalyptus	16+0.58	29.09	29+0.58	52.73	42+1.00	76.36	45+1.00	81.82
AT205	*E. citrodora*	Eucalyptus	11+0.58	20	29+1.15	52.73	30+0.58	54.55	30+1.00	54.55
AT206	*B. variegata*	Kachnar	10+0.58	18.18	25+1.00	45.45	41+1.00	74.55	50+0.58	90.91
AT207	*B. variegata*	Kachnar	15+1.15	27.27	27+1.15	49.09	40+0.58	72.73	43+1.00	78.18
AT208	*B. variegata*	Kachnar	13+1.00	23.64	29+0.58	52.73	30+1.15	54.55	30+1.52	54.55
AT209	*C. fistula*	Amaltas	13+1.00	23.64	29+0.58	52.73	30+1.15	54.55	30+1.52	54.55
AT210	*C. fistula*	Amaltas	7.0+0.00	12.73	28+1.15	50.91	32+0.58	58.18	44+1.00	80
AT211	*C. fistula*	Amaltas	14+0.58	25.45	28+0.58	52.73	31+0.58	56.36	43+0.58	78.18
AT212	*C. fistula*	Amaltas	16+1.00	29.09	28+0.00	50.91	30+0.58	54.55	40+1.00	72.73
AT213	*D. regia*	GulMohar	14+0.57	25.45	29+1.15	52.73	34+0.58	61.82	50+1.00	90.91
AT214	*D. regia*	GulMohar	10+0.58	18.18	27+1.00	49.09	33+0.58	60	48+0.58	87.27
AT215	*D. regia*	GulMohar	13+0.00	23.64	29+0.00	52.73	32+1.00	58.18	45+1.00	81.82
AT216	*S.lycopersicum*	Tomato	15+1.15	27.27	28+0.58	50.91	31+1.15	56.36	54+1.52	98.18
AT217	*S.lycopersicum*	Tomato	13+0.58	23.64	27 1.15	49.09	31+0.58	56.36	40+1.00	72.73
AT218	*S.lycopersicum*	Tomato	15+0.58	27.27	27+0.58	49.09	34+0.58	61.82	50+0.58	90.91
AT219	*F. religrosa*	Peepal	14+1.15	25.45	28+0.58	50.91	38+1.00	69.09	48+1.15	87.27
AT220	*F. religrosa*	Peepal	15+1.15	27.27	27+1.15	49.09	43+1.15	78.18	55+0.58	100
AT221	*F. religrosa*	Lignum	7.0+0.0	12.73	28+1.15	50.91	32+0.58	58.18	44+1.00	80
AT222	*F. religrosa*	Lignum	14+0.58	25.45	28+0.58	50.91	31+0.58	56.36	43+0.58	78.18
AT223	*M. indica*	Mango	16+1.00	29.09	28+0.00	50.91	30+0.58	54.55	40+1.00	72.73
AT224	*M. indica*	Mango	14+0.57	25.45	29+1.15	52.73	34+0.58	61.82	50+1.00	90.91
AT225	*M. indica*	Mango	10+0.58	18.18	27+1.00	49.09	33+0.58	60	48+0.58	87.27
AT226	*H. annuus*	Sun flower	13+0.00	23.64	29+0.00	52.73	32+1.00	58.18	45+1.00	81.82
AT227	*H. annuus*	Sun flower	13+1.15	23.64	27+0.58	49.09	31+1.15	56.36	54+1.52	98.18
AT228	*R. chinensis*	China Rose	15+0.58	27.27	28+1.15	50.91	31+0.58	56.36	40+1.00	72.73
AT229	*R. chinensis*	China Rose	15+0.58	27.27	27+0.58	49.09	34+0.58	61.82	50+0.58	90.91
AT230	*R. chinensis*	China Rose	14+1.15	25.45	28+0.58	50.91	38+1.00	69.09	48+1.15	87.27
Control AT2441			13+0.58	23.64	28+1.00	50.91	32+0.58	58.18	44+1.00	80
Ave. range			7.0 -16	12.7-29.1	25-31	41.8-56.3	30-43	54.6-78.2	30-55	54.6-100

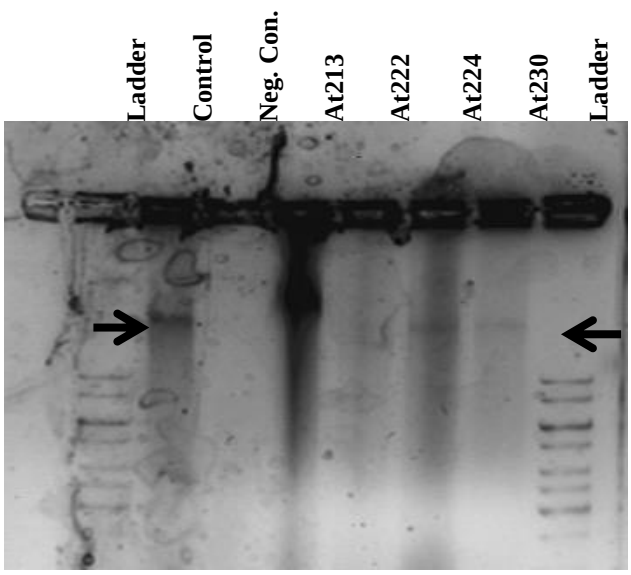

Fig. 2. Shows Ti plasmid isolated from wild strains of *Agrobacterium tumefaciens* recovered from Crown galls of infected trees. Arrows specifically indicate the presence of Ti plasmid in 0.8% agarose gel. 1-kb ladder was used as marker.

Conculsion

Of the 90 Crown galls of plants and trees examined, only 30 wild strains of *Agrobacterium tumefaciens* were isolated. From 30 isolates, 19were identified as *Agrobacterium tumefaciens* and preserved as a genetic resource for future studies. From 19 isolates of *Agrobacterium tumefaciens*, At224 was screened out as the most virulent strain showed 80% transformation efficiency while three other isolates i.e., At213, At222 and At230 exhibited 70, 70 and 70% transformation efficiency and induced solid green galls on potato discs respectively. However, these virulent wild strains are regarded as highly potential and could be used to generate plant transgenic through *Agrobacterium* mediated transformation methods.

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