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ORIGINAL RESEARCH

Isolation of *Agrobacterium tumefaciens* strains from stem gall disease on *Manilkara zapota* tree in Padappai, Tamil Nadu, India.

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ABSTRACT
Stem gall induced by *Agrobacterium tumefaciens* was observed, identified and isolated from an unusual host plant - *Manilkara zapota* at the Agricultural Farm, International Institute of Biotechnology and Toxicology, at Padappai, Tamil Nadu. The galls also occurred on leaves wounded by insect bites or mechanical shearing. The isolated strain was confirmed as *A. tumefaciens* by different biochemical, antibiotic resistance and pathogenicity (tumor forming ability) test. The isolate was positive for tumor forming ability in pathogenicity tests on *Kalanchoe pinnata,* Bryophyllum leaves and Tomato stem. In summary, the isolated strain is newly reported regarding host plant in aspect of Padappai, Tamil Nadu province. This strain could be used for construction of genetically engineered strains, *in vitro* antitumor studies of plant extracts and other biological aspects.

KEY WORDS: *Agrobacterium tumefaciens, Manilkara zapota, Kalanchoe pinnata, pathogenicity*

Introduction

The Gram negative soil bacterium *A. tumefaciens* having a worldwide distribution (Furuya *et al*., 2004) are known to induce crown gall tumors, by the insertion of a part of the plasmid DNA into the plant genome (Mattysse, 2006), After its integration into the plant genome, the T-DNA genes encodes enzyme responsible for the uncontrolled synthesis of the plant hormones such as auxin and cytokinin which account for the appearance of abnormal tissue proliferation and gall formation on the crown, roots and in some cases on stem (Rhouma *et al*., 2006). Since the genes located between the ends of the transformed DNA are expressed in plant cells, plasmids carrying this DNA can be used as a vector for genetic engineering. A number of plant species has so far been successfully transformed, and transgenic plants of several species are reported to carry new trait that improve their economic values (Fraley *et al*., 1986; Zambryski *et al.,* 1989). Although most dicotyledonous plants are susceptible to Agrobacterium, numerous examples indicates that tumourigenesis specifically depends both on the type of plasmid harbored by the bacteria and on the plant genome (Neskovic *et al*., 1990). The different degree of host specificity was observed and studied by Anderson and Moore (1979) among different isolates of agrobacteria which also amplifies the fact that speciation in Agrobacterium based on Pathogenicity is of little taxonomic value.

All over the world many *A. tumefaciens* species exist which remain unexplored. Tamil Nadu is the third largest state in the production of *Manilkara zapota* (nhb.gov.in), there is no report on infection of *A.tumefaciens* strains to *M. zapota* in Tamil Nadu. The present study was undertaken to isolate and characterize virulent *A. tumefaciens* strain from an unusual host *M. zapota*.

Materials and Methods

Collection of stem gall tissue

In the present study, Stem Gall tissues were collected from Agricultural Farm, International Institute of Biotechnology And Toxicology (IIBAT) (Fig. 1). Samples were immediately transferred to the laboratory and special care was taken to avoid contamination.

Figure 1:.Stem Gall on *Manikara zapota* tree

Isolation of Agrobacterium

The surface of the galls were removed using a blade and sterilized in 100 ml of 10%Commercial bleach containing 4 drops of Tween-80 for 20 min. After sterilization, the galls were washed thrice with sterile water (SW). They were then finely chopped and immersed in sterile water for overnight at room temperature (27-30^{0}C). A loopful Overnight incubated stem gall extract was streaked on selective media i.e., MacConkey agar plates. Inoculated plates were incubated at 28-30^0 C for 24 hrs and examined for growth, fermentation and color development (*Soriful et al*., 2010)

Characterization of A. tumefaciens

Biochemical test

Biochemical test of the isolate was done according to Bergey's manual of Determinative Bacteriology (Moore *et al*., 1988).Based on the manual the following test were carried out: Gram Stain, Motility at room temperature, Catalase and Oxidase production, Utilization of Lactose, Mannitol, Production of 3-ketolactose, Salt Tolerance (2%), H_2S Production, Utilization L-tyrosine, Growth on MacConkey and LB agar, Growth and pigmentation in ferric ammonium citrate.

Test for Antibiotics Resistance on Isolate

The antibiotic sensitivity of selected isolate was determined according to the method of Bauer-Kirby (Bauer *et al.,* 1966).The following antibiotics i.e., Kanamycin (30 µm 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 (6mm in diameter) were impregnated with 10µL 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^{0}C for 24h. Antibiotic susceptibility was determined by measuring the size of the inhibition zone.

Pathogenicity test

Tomato stem bioassay

Pathogenicity of the strain was confirmed on five week old tomato plants (*Lycopersicum esculentum*) with needle inoculation of bacterial suspension containing 10^8 CFU/ml in 0.85% saline. Inoculated and control (saline injected) plants were maintained in the growth chamber for 10-12 days at 25^0 C and 70% relative humidity. After three weeks, the stems were checked and found with young galls (tumors) developing from the stem tissue of tomato plant. Necessary aseptic conditions were maintained. (Davoodi and Hajivand, 2013)

Kalanchoe pinnata leaf bioassay

The bacteria slurry grown on the MacConkey agar medium was scraped off with a sterile surgery scalped and slash – inoculated on both sides of the upper leaf epidermis of *K. pinnata* (Minnemeyer and Lightfoot, 1991).The inoculated plants were cultivated inside the laboratory. A control slash was made without the bacteria. Gall formation was scored two months after inoculation.

Results

Isolation of *Agrobacterium*

Bacterial colonies was observed, screened and isolated from Stem gall samples on the basis of its color development on selective medium, and identified as *Agrobacterium* strain.

Characterization of *A. tumefaciens*

Biochemical Test

The results of biochemical test are presented in (Table1.).Similar reaction was also observed for standard samples.

Table 1: Result of Biochemical Tests ((+) Positive, (-) Negative)

Characteristics	Response
Gram Stain	-
Motility at room temperature	+
Catalase and Oxidase production	+
Utilization of Lactose, Mannitol	+
Production of 3-ketolactose	+
Salt Tolerance (2%)	+
H_2S Production	+
Utilization L-tyrosine	-
Growth on MacConkey and LB agar	+
Growth and pigmentation in ferric ammonium citrate.	+

Test for Antibiotics Resistance on Isolate

Antibiotic resistance test showed that the isolate was resistant to Rifampicin and Tetracycline and susceptible to Kanamycin and Cefuroxime which was supported by (Koivunen *et al.,* 2004: Karthy *et al.,* 2009). It is a another parameter to confirm *A.tumefaciens* strain. Antibiotic resistance is the ability of a microorganism to withstand the effect of an antibiotic. In Gram–negative bacteria, plasmid – mediated resistance genes produce protein 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.

Pathogenicity Test

After twenty one days of inoculation, the tomato plants were checked for tumor formation at the site of inoculation. Signs of tumor was observed which was completely not seen in the control tomato plants. When the isolated and purified strains were inoculated on the leaf surface of *Kalanchoe*, Signs of gall formation were observed after 10-15 days. This induced small galls on the wound sites after 2 months which were finally identified as indigenous virulent *Agrobacterium tumefaciens* strains.

Discussion

The genus *Agrobacterium* is noted for its broad host range. *A. tumefaciens* prompted the first successful development of a biological control agent and is now used as a tool for engineering desired genes into plants. The purpose of this study is to isolate virulent indigenous *A.tumefaciens* strain from the host plant and confirm their characteristics using different biochemical, antibiotic resistance and pathogenicity test. Bergey's Manual of determinative Bacteriology, indicates that Gram negative bacteria generally grow as pink to brick–red colonies on MacConkey agar which was similar to us. For further confirmation of *A. tumefaciens* strain several biochemical tests were conducted according to Moore *et al.,* (1988). And to investigate the stem gall caused by *A. tumefaciens* which was becoming a big threaten to nursery and fruit production.

On the basis of *in vitro* tumor inducing capability, isolated strain was identified as indigenous virulent *A. tumefaciens* strain. Since the isolated agrobacteria cells were able to induce galls on Tomato and *Kalanchoe* leaf, they supposedly contain tumor inducing genes for, e.g. auxin and cytokinin biosynthesis. They also cause the rapid growth of the galls after inoculation, an indication of strong virulence on herbaceous plants. The *ipt* and *rolC* genes have been cloned from other agrobacteria strains/species and introduced into genome of higher plants to modify their morphology (Brzobohaty *et al.,* 1994). It is possible to plate the same gene homologues from zapota agrobacteria that we reported here. Modified Agrobacterium has also been used as a vector for plant genetic engineering, and we suggest that zapota Agrobacterium has potential as a tool to introduce foreign genes into important crop plants. This strain is newly reported regarding host plant and also newly reported in this region. 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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