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**RESEARCH ARTICLE**

# EVALUATION OF CONVENTIONAL AND 16S r DNA IDENTIFICATION METHODS FOR DETECTION OF XANTHOMONAS CAUSING LEAF BLIGHT IN RICE

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**ABSTRACT**

Leaf blight of rice is a disease of major concern in rice cultivation. This disease caused by bacterial pathogen *Xanthomonas oryzae* pv *oryzae* creates major issues during export and import of food material between countries. World loss due to this disease is the major concern of today's pathologists and food technologists. Conventional methods used for determining the disease consume time, not viable for accurate pathogen identification and thereby hinder the implementation of apt control measures. Molecular methods for identification of pathogen are often reliable, econometric, less time consuming and flexible for easy usage. The regulatory measures levied for controlling the movement of pathogen from one country to other country can be addressed by molecular identification methods determining pathogenic and non-pathogenic Xanthomonads. In the present paper an attempt was made to identify wild xanthomonads using conventional and molecular methods (16S r DNA).



## INTRODUCTION

Rice (*Oryza sativa*) is consumed worldwide and mostly in Asia as staple food. It gains importance being supplier of 1/5 th of the total calorie consumed by humans (Smith, 1988). Rice cultivation is severely affected particularly in tropical Asia by bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv o*ryzae* (XOO) leading to yield losses of up to 50% (Adhikari *et al*., 1994). The disease leads to poor grain development, lower quality / under developed grains with reduced weight resulting in high proportion of broken rice (Ou, 1985). BB disease management concentrates on methods that reduce the initial inoculums and subsequent development of the pathogen on host plants and this can be accomplished through chemical protection, host plant resistance, and biological control. The presence of the pathogen in infected seeds and disease transmission from these infected seeds has been demonstrated (Mew, 1987; Hsieh *et al*., 2001). Importation and movement of this pathogen infected plant material is regulated by plant protection and quarantine program of USDA and several other countries. In view of global seed trade, phyto-sanitary regulations enforced by several countries entail need for reliable, sensitive and less time consuming diagnostic tools for XOO. Traditional methods employed for detection and identification of xanthomonads, such as biochemical (Mooter and Swing, 1990) serological and pathogenecity tests (Benedict *et al*., 1990; Berthier *et al*., 1993) are been extended by molecular methods (Donald and Graham, 1989).Traditional methods are tedious, time consuming and sometimes with non reproducible results. While molecular methods are less time consuming, accurate and with reproducible results. Accordingly an attempt was made identify and analyze wild Xanthomonads isolated from rice plant materials of different locations using conventional and molecular methods.

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## MATERIALS AND METHODS

### Plant Samples

Forty leaf and seed samples of rice from different locations, representing most of the Mahabubnagar, Warangal and Krishna districts of Andhrapradesh were obtained. Plants of age group of near seeding plants were observed for presence of pathogenic lesions, carefully separated by a sharp blade and scissors and carried to laboratory in an ice-box and stored in a refrigerator. Isolation of Xanthomonads was completed within 12 hrs of sample collection.

### Isolation of Xanthomonads

From Seeds: Sixty grams of rice seeds were blended in sterile salt solution (Phosphate-Buffered Saline pH 7.2) and incubated at $5^0$ C for 2 hrs. From this 25 ml of soaking suspension was centrifuged (10,000 RPM, 10min) and the precipitate obtained is again resuspended in salt solution (1/10 in 2.5 ml). Three different concentrations ($10^{-1}$, $10^{-2}$, $10^{-3}$) of the above salt solution was inoculated (100 micro l/plate) on to nutrient agar

+ cycloheximide (semi-selective media) and *Xanthomonas oryzae* selective medium (XOS)(Sucrose 20g, Sodium glutamate 5g, $Ca(NO_3)$ 0.2g, K2HPO4 2g, Fe-EDTA 1mg, Agar 17g, Tetrazolium chloride 10mg, cycloheximide 100mg, kasugamycin 20mg, Cephalirin 20mg, methyl violet 0.3mg, water 1lt, pH 6.8-7.0). Plates were incubated at $28^0$C for 5-7 days. Appearance of straw yellow coloured colonies of 1-2 mm diameter on nutrient agar and rose pink, mucoid raised colonies on XOS agar indicate Xanthomonads and *Xanthomonas oryzae* respectively. These were picked, isolated on to peptone sucrose broth for maintaining.

**Pathogenecity Test**

Isolates are tested on susceptible rice cultivars. For *X. oryzae* pv. *oryzae* 30-45 days old TN1 (Taichung native 1) rice plants were used. Local popular rice varieties from the Andrapradesh region with known susceptibility to the disease *viz.,* Sonamasuri, Tellahamsa, Vijeta MTU 1001, Swarna MTU 7029 and Samba masuri BPT 5204 are included in the tests.

**The clip-method (Kauffman *et al*., 1973)**

The tips of 30-40 leaves of rice plants were cut (2-3 cm) with a scissors immersed in the bacterial suspension ($10^8$ CFU $mL^{-1}$). The inoculated plants were covered for 24 h with a polythene bag, and incubated at 30°C with 12 h light cycle. Plants were observed for symptoms after 48-72 h up to 14 days ie., checked for water-soaked areas in the inoculated leaves, usually beginning from the inoculated ends as water-soaked stripes, which is an indication of *X. oryzae* pv. *oryzae*. Plants are observed for milky exudates, extension of wavy lesions (red stripes) symptoms. The rice plants were grown in potting mixture with a weekly fertilization of 1–2 g urea $L^{-1}$. Inoculated plants were kept under high moisture conditions with 12 h light/dark cycle at an optimum temperature of 28–32°C. A negative control was maintained (plants inoculated with sterile saline solution alone) to monitor false positive reactions caused.

**Identification**

**Conventional methods (Biochemical tests)**

All the Forty isolates of Xanthomonas were identified using conventional biochemical methods. Strains were identified basing the tests- Grams reaction, Indole-Methyl red-Voges proskauer-Citrate tests (IMVIC), Oxidase, Nitrate reduction, Arginine hydrolase, Pectinase, Catalase and Urease. Standard methods were followed as described by Bergey's manual for determinative bacteriology (Bradbury, 1984). Further they were checked for their ability to utilize different sugars Viz., D-Arabinose, L- Arabinose, D-Galactose, D-Glucose, D-Fructose, Cellobiose, Adonitol, Dulcitol, Inositol, Mannitol, Salicin, Sorbitol, Lactose, Manose, Maltose, Melibiose, Rhammose, Raffinose, Sucrose, Inulin, Xylose, and Trehalose. Readymade sugar discs (Himedia) were incorporated in to the sterile medium ($NH_4H_2PO_4$-0.5g, $K_2HPO_4$- 0.5g, $MgSO_4.7H_2O$- 0.2g, NaCl- 5g, Yeast extract-1g, Distilled water- 1 lt, Bromocresol purple- 0.7ml (1.5%alcohol sol) containing test tubes which were aseptically inoculated with respective bacterial isolate. Tubes incubated for 48 hrs at $28\pm2^o$ C were checked for presence of growth and formation of yellow color indicating acid production (Schaad, 1980).

**Molecular analysis**

**DNA extraction**

Overnight grown culture (1.5ml) was centrifuged at 13000 rpm for 2 minutes. Supernatant was discarded and pellet was obtained. To the pellet 100 µl of 1x TE buffer was added and mixed properly so that pellet comes into solution. This suspension was added to enzyme coated tubes (lysozyme 5µl (10mg/ml) + lyticase100 units/sample) and incubated in water bath at $37^0$ C for 30 minutes. To this 100 µl of 2% SDS and 5 µl (20 mg/ml) of proteinase K was added. The tubes were incubated at $55^0$ C for 1 and 1/2 hours. To this 200 µl of Guanidium HCl (PH-5.2) solution was added and centrifuged at 10000 rpm for 10 min. The pellet was discarded and to the supernatant equal volume of isopropanol was added and loaded on to the column. The elute obtained by centrifugation at 10,000 rpm for 2 min was discarded. To the column 500 µl of 70% alcohol was added and centrifuged at 10,000 rpm for 2 minutes, elute discarded. The column was spun with caps open at 10000rpm for 3 min (drying). The column was placed in eppendorf tube and 100 µl of Elution buffer was added

**Quantitation of DNA by Spectrophotometric method**

10ml of DNA solution was diluted with 990ml of TE. Mixed well and absorbance at 260nm and 280nm was measured. The absorbance at 260nm can be used to calculate the concentration of DNA as follows:

**Calculations**

OD260 of 1= 50 micro g/ml DNA; Dilution factor =100
Concentration of DNA in a given solution (micro g/ml) =50 X OD X Dilution factor / 1 X 1000

**PCR amplification (16s rDNA)**

PCR Set up

A 1.0µl of Sample DNA (approximately 100 ng/µl) was added to PCR Mixture containing 100mM Tris HCl (pH 8.3), 500mM KCl (pH 8.3), 2.5µl $MgCl_2$ (25mM), 2.0µl dNTP's (2.5mM), 1.0µl Primer Forward & Reverse (each of 10pm/µl) and 1u /µl of Taq Polymerase (Bioserve Make) and the final volume made to 25 µl with nuclease free water. The primer set FD1 Fw and RP2 Rw designed in the conserved region was used for the amplification of the 16S region of the test organisms and the primer sequences are

FD1 Fw: 5'- AGAGTTTGATCCTGGCTCAG -3'
RP2 Rw: 5'-ACGGCTACCTTGTTACGACTT-3'

**PCR condition**

The amplification conditions for amplification of bacterial 16S region is: one cycle of Initial denaturation at $95^0$C for 5 min

followed by 35 cycles of denaturation at $95^0$C for 30 Sec, annealing at $52^0$C for 90 Sec; extension at $72^0$C for 60Sec and final extension at $72^0$C for 10 min and held at $4^0$C

**Electrophoresing the amplicons**

The PCR Products (6 - 10µl) were separated by electrophoresis in 2% Agarose gels containing ethidium bromide (1µg $ml^{-1}$).The electropherogram obtained after electrophoresis of the PCR amplicons. In the electropherogram the bands of the size ~1500bp (for Xoo samples 1-40) was observed against 1Kb DNA ladder (Fermentas make). About 3µl Ethidium Bromide was used as staining dye and 5µl of Bromothymol blue was used as tracking dye. About 100V DC was maintained between the electrodes of gel apparatus for 20 minutes. Following electrophoresis the gel was analyzed and pictured in gel doc system (BIOSERVE Hyderabad).

**Gel Elution**

DNA band of ~1500bp was cut from gel with sterile blade and taken in 1.5 ml eppendorf tube; elution was done using Bioserve gel extraction kit following the manufacturer's instructions.

**DNA sequencing**

QIAGEN QIA quick TM kit was used for sequencing reaction. The sequencing PCR was done to amplify one strand of gene employing the primers under standard PCR conditions. Primers used are: 16 Seq 4 R- TGCTGCCTCCCGTAGGAGT; 16 Seq 2 R- TACGCATTTCACCGCTACAC; 16 Seq 2 R 2- TACGCATTTCACTGCTACAC; 16 Seq 4 F- TTAGATAC CCTGGTAGTCCA; 16 Seq 3F- AAGTCCCGCAACGAGC GC; 16S 1 REV- TGCGCTCGTTGCGGGACTTAACC. The samples were precipitated and suspended in 40µl of loading solution provided with the kit. Sequencing was done with Mega Bace sequencer- Bioserve India, Hyderabad. Sequences thus obtained were edited and aligned using commercial Gene tool Lite (version 1.1) software

## RESULTS

Selective isolation of Xanthomonas resulted in 40 (Xanthomonas strains (XS)) isolates. All these isolates were initially checked for pathogenecity on different rice varieties (Table 1). Taichung native (TN-1) rice variety was observed to be susceptible to all the 40 isolates (XS 1-40). Strains which were strongly positive /negative are represented in the Table 1. Among the 40 isolates of Xanthomonas, that was found positive in causing infection in at least one of the rice variety were further evaluated for biochemical tests in order to evaluate them on physiological basis. From Table 1 it can be inferred that all the isolates were able to cause the infection in one or more rice varieties. A critical perusal of the Table 2 indicates that all the isolates were gram negative. They were negative also for oxidase test, egg yolk reaction, tetrazolium salt resistance, fluorescent pigment production, nitrate reductase, arginine hydrolase, pectinase and growth under anaerobic conditions. For KOH test, aesculin hydrolysis and resistance to cupric nitrate (0.001%) all the tested isolates were found positive. Remaining tests shown in the Table 2 represented varied results (indole, MR, VP, catalase, starch hydrolysis, casein hydrolysis)

**Table 1. Test for pathogenecity of 40 isolates, XS (1-40) on Rice plants**

| S.No | Rice variety | Negative Isolates |
|---|---|---|
| 1 | TN 1 | XS (1-40) |
| 2 | Sonamasuri | XS-13, XS-17, XS-27, XS-28, XS-30, XS-31, XS-38, XS-39 |
| 3 | Tellahamsa | XS-19, XS-21, XS-23, XS-30, XS-37, XS-38, XS-39 |
| 4 | Vijetha MTU 1001 | XS-17, XS-31, XS-36, XS-40 |
| 5 | Swarna MTU 7029 | XS-17, XS-19, XS-25, XS-27, XS-40 |
| 6 | Sambamasuri BPT 5204 | XS-13, XS-17, XS-28, XS-30, XS-36, XS-37 |

**Table 2. Biochemical tests pertaining to 40 Xanthomonas isolates**

| S.No | Tests | Isolates |
|---|---|---|
| 1 | Gram reaction negative | XS (1-40) |
| 2 | KOH test positive | XS (1-40) |
| 3 | Fluorescent pigment negative | XS (1-40) |
| 4 | Indole test positive | XS-34, XS-14, XS-16 |
| 5 | Methyl red positive | XS-34 |
| 6 | Vogues prouskaur positive | XS-14, XS-16 |
| 7 | Oxidase negative | XS (1-40) |
| 8 | Nitrate reductase negative | XS (1-40) |
| 9 | Arginine dehydrolase/Pectinase | XS (1-40) |
| 10 | Catalase negative | XS-14, XS-27, XS-31 |
| 11 | Urease/Cellulase negative | XS (1-28), 34, 39 |
| 12 | Starch hydrolysis positive | XS-34, XS-39 |
| 13 | Aesculin hydrolysis positive | XS (1-40) |
| 14 | Casein hydrolysis negative | XS (15-40), XS-8 |
| 15 | Resistance to cupric nitrate positive | XS (1-40) |
| 16 | Tetrazolium salt resistance (0.02%) negative | XS (1-40) |
| 17 | Anaerobic growth negative | XS (1-40) |
| 18 | Egg Yolk reaction negative | XS (1-40) |

**Table 3. Utilization of sugars by the 40 Xanthonmonas isolates**

| S.No | Acid Production from | | Isolate |
|---|---|---|---|
| 1 | D-Arabinose | Positive | XS (1-40) |
| 2 | L- Arabinose | Negative | XS (1-40) |
| 3 | D-Galactose | Positive | XS (1-40) |
| 4 | D-Glucose | Positive | XS (1-40) |
| 5 | D-Fructose | Positive | XS (1-40) |
| 6 | Cellobiose | Positive | XS (1-40) |
| 7 | Adonitol | Negative | XS- 13, XS-16 |
| 8 | Inositol | Negative | XS-31, XS-40, |
| 9 | Salicin | Negative | XS-34,XS-40 |
| 10 | Sorbitol | Negative | XS-40 |
| 11 | Lactose | Negative | XS-40 |
| 12 | Manose | Positive | XS (1-40) |
| 13 | Maltose | Negative | XS (1-40) |
| 14 | Melibiose | Negative | XS-40 |
| 15 | Rahmmose | Positive | XS (1-40) |
| 16 | Raffinose | Negative | XS-13, XS-40 |
| 17 | Sucrose | Negative | XS-34,XS-37,XS-40 |
| 18 | Inulin | Negative | XS-34, XS-40 |
| 19 | Xylose | Positive | XS (1-40) |
| 20 | Trehalose | Negative | XS (1-40) |

Table 3 depicts the ability of the 40 isolates of xanthomonas in utilizing various sugars for acid production by fermentation. Most of the isolates were able to use all the sugars tested. All the 40 isolates were producing acid in the media containing L-Arabinose, D-Galctose, D-Fructose, Cellobiose, Adonitol, Mannose, Maltose, and Rhamnose. While, all isolates were negative for acid production with L-Arabinose, Maltose and

Trehalose as media component. All the 40 isolates showed varied results for biochemical properties representing Xanthomonas (Schaad *et al*., 2001). These isolates however could not be differentiated from other Xanthomonas sp. The pathogenecity assay and biochemical characteristics confirm them as Xanthomonas pathogens (Nayudu, 1972)

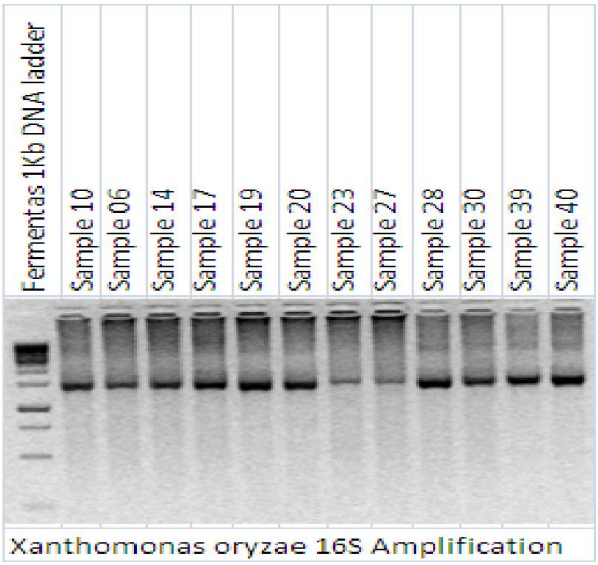


**Fig. 1. Amplification of Xanthomonas 16s rDNA region recognized by band in 1500bp region**

The DNA isolation from all the 40 isolates yielded sufficient and ample amount of DNA in terms of purity as evidenced from $A_{260}/A_{280}$ between 1.80-1.90. Molecular confirmation of the isolates using PCR primers FD2 (AGAGTTTGATCATGGCTCAG) and RP1 (ACGGTTACCTTGTTACGACTT) resulted a 1500 bp band (targeted to 16s rDNA region) on 1.5% agarose gel on electrophoresis. Carefully eluted bands were subjected to sequencing using sequencing primers (16 seq 2R, 16 seq 3F, 16 seq 4F, 16 seq 4R and 16s1Rev) resulting in complete 1500 bp 16 s rDNA sequence. NCBI BLAST performed with the sequences confirmed the isolates as *Xanthomonas oryzae*. Further, few of these sequences were deposited in NCBI gene bank and the accession numbers are HM 747116, HM 747117, HM 747118, HM 747119, HM 125569 and HM 125570.

## DISCUSSION

In the present investigation, biochemical tests for identification of the Xanthomonas pathogen gave varied results indicating existence of genetic variability. For instance, starch hydrolysis has been reported by Swings *et al*., (1990) which was contrasting to the results presented by Guvera and Marsella (1999) who did find this character in their isolates. Our isolates varied, as some of the isolates were positive and other was negative. Overlapping results and cross contamination with other similar isolates for identification on the basis of biochemical characterization is a problem needed to be answered. Traditional methods used for identification of pathogen leaves vague results. Labor intensive, time taking biochemical tests identification of pathogen are needed to be replaced by molecular based methods. Accurate, reliable and econometric molecular methods help in answering the regulatory affairs levied by various countries in export and import of food materials. Molecular methods increase the ease of identification of the pathogen-*Xanthomonas oryzae* pv *oryzae*. Many molecular based methods are recently been explored largely but, there still remains many unoccupied lacuna even in this area. Still investigations are under way for accurate differentiation between pathogenic and non-pathogenic strains of Xanthomonas.

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