

Original Research Article

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Molecular Diversity among the Isolates of *Xanthomonas axonopodis* pv. *citri* Causing Bacterial Canker in Citrus

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ABSTRACT

Keywords

Molecular diversity, RAPD, ISSR, Cluster analysis, *Xanthomonas axonopodis* pv. *citri*

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Genetic variability study of five isolates of *Xanthomonas axonopodis* pv. *citri* was carried out using molecular markers. The isolates were collected from five places in Maharashtra state, India. Morphological and Biochemical tests viz. gram's reaction, starch hydrolysis, indole production, catalase test, KOH test, Gelatin liquefaction and Acid production test were carried out to identify and confirm the purity of the culture. The genetic variability was studied by using 14 RAPD and 8 ISSR primers out of which 7 RAPD primers produced 47 scorable bands and 2 ISSR primers produced 13 scorable bands. The similarity coefficient value ranged from 0.13 to 0.40 for RAPD and from 0.00 to 1.00 for ISSR primers across five isolates indicating high degree of genetic variation.

Introduction

Citrus as a fruit crop is having a great importance as it is delectable, juicy with great nutritional significance and therapeutic qualities as well (Chaudhry *et al.*, 1992; Khan *et al.*, 1992).

It is grown on an area about 9.08 million hectare with the production of around 139.80 million tones. In India it is grown on 1.02 million hectare area with the production of around 11.15 million tones (FAOSTAT, 2017). Citrus canker disease caused by *Xanthomonas axonopodis* pv. *citri* is an important threat to citrus crops produced all over the world. Symptoms of the disease include necrotic

lesions on leaves, twigs and fruits which subsequently can cause leaf abscission, twig die-back and premature fruit drop (Gotwald *et al.*, 1993; Schoulties *et al.*, 1987).

Controlling the disease is a difficult task as the pathogen has different pathovar and strains within the pathovar across the locations which differ genetically. Genetic diversity determination is done on basis of morphological, biochemical and molecular types of information. Molecular markers show genetic differences on a more detailed level without interferences from environmental factors, and where they involve techniques that provide fast results detailing genetic diversity. Several molecular techniques have

been used to study the population structure of different plant pathogens, including RAPD, Rep and Eric-PCR, RFLP, plasmid profile analysis, PCR amplification, SDS-PAGE, sequencing of the 16S rDNA, and pulsed field gel electrophoresis (Pooler *et al.*, 1996; Hauben *et al.*, 1997; Rademaker *et al.*, 2000; Kolliker *et al.*, 2006; Simoes *et al.*, 2007).

The objective of this study was to study the genetic diversity of *X. axonopodis pv. citri* strains collected from different geographic regions of Maharashtra state, India by using RAPD and ISSR markers.

Materials and Methods

Collection of disease samples

The leaf samples showing typical symptoms of citrus canker were collected from five different locations (Table 1) during October-November, 2015.

Isolation of *X.axonopodis pv. citri*

Nutrient Agar (NA) medium composed of 0.3% Beef extract, 0.2% Yeast extract, 0.5% Peptone, 0.5% Sodium chloride and 1.5% Agar agar was used for isolation of *X.axonopodis pv. citri*. The selected diseased leaves were washed thoroughly with tap water and surface sterilized with 0.1 % sodium hypochlorite solution for 5 minutes and washed again with sterile distilled water to remove the traces of sodium hypochlorite. Leaves were allowed to dry on sterilized tissue paper. The infected portion of leaf along with healthy part was excised carefully on sterilized slide using scalpel blade. A drop of water was added on the tissue to macerate it gently using glass rod. Bacterial cells were allowed to ooze out until the water became slightly turbid. A small volume of exudates was collected and streaked on NA medium and incubated at 25 to 28 °C temperature.

Pathogenicity test

Healthy leaves were washed with water, sterilized with 70% ethanol for 1 minute and rinsed twice with sterile distilled water. Three leaves were placed on four layers of sterile blotting paper towel in covered Petri plates. These were inoculated *in vitro* with pin prick method (Di *et al.*, 1991) in which sterile pins dipped in inoculum were used to prick the leaves on sides and in the center. The pathogen was re-isolated from the infected leaf samples after it showed the typical symptoms of canker to ensure the infection by *X. axonopodis pv. citri*. The identification of the pathogens was determined by conducting studies on morphological and biochemical tests *viz.* gram's reaction, starch hydrolysis, indole production, catalase test, KOH test, Gelatin liquefaction and Acid production test.

DNA extraction from *X.axonopodis pv. citri*

Total genomic DNA of *X. axonopodis pv. citri* strains was extracted by using the protocol described by Cooksey and Graham (1989) and Egel *et al.*, (1991), with few modifications. Pure culture was multiplied in nutrient broth in 100 ml flask at 28 °C for 72 hours with vigorous shaking at a speed of 120 rpm. About 1.5 ml aliquots of broth culture were taken in 2.0 ml of micro-centrifuge tube and spun at 13000 rpm for 5 minutes. The supernatant was discarded and 200µl of lysis buffer was added to the tubes containing pellet and was mixed well. 66µl of 5 M NaCl was added and contents were centrifuged at 13000 rpm for 10 minutes. Supernatant (250µl) obtained was transferred to a new tube, to which 1 µl RNase (20mg/ml) was added, mixed well and incubated at 37 °C for 30 minutes. An equal volume of chloroform isoamyl alcohol was added, mixed gently by inverting the tubes and centrifuged at 13000 rpm for 6 minutes. The upper aqueous phase was transferred to a clean tube and 1 ml of 95% ethanol was added.

The tubes were then kept in deep freezer at -20 °C for overnight. After incubation tubes were spun at 13000 rpm for 6 minutes. Ethanol was poured off, DNA pellet was air dried and re-suspended in 200µl of 1X TE buffer and stored in deep freezer at -20 °C.

Genotyping

Genotyping of five isolates of pathogen was carried out using fourteen RAPD markers and eight ISSR markers (Table 2). The PCR amplification was performed according to Williams *et al.*, (1990) with certain modifications and amplicons were separated on 1% agarose gel. Scoring of bands in each lane was done by comparing with 100 bp ladder of biotechnology grade from Hi-media laboratory. The data was recorded from each line as the bands were scored (0) for absence and (1) for the presence for the band. A similarity matrix was generated from the binary data using Jaccard's similarity coefficient in the SIMQUAL program of the NTSYS-pc package.

Cluster analysis was performed with the unweighed pair group arithmetic mean method (UPGMA) in the SAHN program of the NTSYS-pc package. The graphic dendrogram of the genetic relatedness among the five isolated was produced by means of UPGMA (Un-weighted Pair Group Method with Arithmetic average) cluster analysis of averaged similarity index.

Results and Discussion

Isolation of *X. axonopodis pv. citri*

Creamy or pale yellow colonies of *X. axonopodis pv. citri* were observed on NA medium (Fig. 1). Straight and rod shaped bodies containing single, polar flagellum was observed under microscope, which confirmed the purity of the culture.

Pathogenicity by detached leaf method

The symptoms on leaves started to develop from 10th day as pinpoint spots which eventually turned into water soaked lesions with sunken central spot. Isolate *XacV* showed largest water soaked lesions surrounded by yellow hallow zone followed by *XacII*, *XacIII*, *XacI* and *XacIV* in contrast with control plate inoculated with sterilized water instead of inoculum (Fig. 2). These symptoms confirm the purity of *X. axonopodis pv. citri* cultures.

Morphological and biochemical characteristics of *X. axonopodis pv. citri*

All the isolates were studied with respect to their colony color, shape and Grams staining reaction. Bacterial cells appeared short rod and Gram negative. All five isolates were positive for starch hydrolysis, forming a clear hallow around the colony by hydrolyzing the starch. In KOH test all the isolates formed thread like slime after addition of KOH. All isolates were catalytically active as they produced bubbles of oxygen within one minute after addition of H₂O₂. All five isolates development of cherry red color after addition of Kovac's reagent into culture supernatant confirmed Indole production. Bacteria utilized Dextrose as carbon source and were observed as positive for production of acid and gas. All isolates were observed to start liquification of the gelatin after seven days (Fig. 3; Table 3).

Molecular variability

RAPD marker

Out of fourteen randomly selected primers screened seven primers produced reproducible and scorable bands with high degree of polymorphism (Fig. 4). Forty seven amplicons were obtained with the seven primers. All were found to be polymorphic and the level of polymorphism was 100 % (Table 4).

Table.1 Details of locations of isolate collection

Sl. No	State	District	Place of collection	Geographic coordinates	Code
1	Maharashtra	Ahmednagar	Vadgavlandga	19° 34' N, 74° 5' E	<i>XacI</i>
2	Maharashtra	Pune	Pharandwadi	18° 41' N, 73° 39' E	<i>XacII</i>
3	Maharashtra	Solapur	Pandharpur	17° 40' N, 75° 19' E	<i>XacIII</i>
4	Maharashtra	Nashik	Muldondri	19° 59' N, 73° 47' E	<i>XacIV</i>
5	Karnataka	Belgaon	Belgaon	15° 50' N, 74° 29' E	<i>XacV</i>

Table.2 RAPD and ISSRprimers selected for PCR

Sr.no	Marker type	Primer screened	Sequence (5'-3')
1	RAPD	ERIC 1R	ATGTAAGCTCCTGGGGATTAC
2		ERIC 2	AAGTAAGTGACTGGGGTGAGCG
3		a211	GAACCGCGAT
4		a230	CGTCGCCCAT
5		a232	CGGTGACAT
6		REP	CGCGCATCGGC
7		OPA 18	AGGTGACCGT
8		OPF 4	GGTGATCAGG
9		OPF6	GGAATTCGG
10		OPF9	CCAAGCTTCC
11		OPF10	GGAAGCTTGG
12		OPF12	ACGGTACCAG
13		OPB01	GTTTCGCTCC
14		BOX	CTACGGCAAGGCGACGCT
15	ISSR	ISSR820	GTGTGTGTGTGTGTGTC
16		ISSR827	ACACACACACACACACACACACACACACACACACG
17		ISSR816	CACACACACACACACAT
18		ISSR811	GAGAGAGAGAGAGAGAC
19		ISSR8	CACACACACACACAGC
20		ISSR857(101)	ACACACACACACACYC
21		UBC8932800	AGCAGCAGCAGCGT
22		UBC8932814	CGAGAGAGAGAGAGA

Table.3 Morphological and Biochemical characteristics of *Xanthomonas axonopodis* pv. *citri* isolates

Sr. No.	Morphological & Biochemical Tests	<i>XacI</i>	<i>XacII</i>	<i>XacIII</i>	<i>XacIV</i>	<i>XacV</i>
1.	Colony colour	Yellow	Yellow	Pale Yellow	Yellow	Dark yellow
2.	Gram's reaction	-ve	-ve	-ve	-ve	-ve
3.	Starch hydrolysis	+++	+++	+++	+	+
4.	Indole production	++	+	+	++	+++
5.	Catalase test	+++	+++	++	+++	+
6.	KOH test	+++	+	++	++	+++
7.	Gelatin liquefaction	+	++	+++	+	+++
8.	Acid production	+	++	+++	++	+

-ve: Negative, +:Positive, Varying degree of + ve reaction: : + poor; ++ moderate; +++ strong

Table.4 Per cent polymorphism observed in RAPD primers

Sr. No.	Primer	Total bands	Polymorphic bands	% Polymorphism
1.	ERIC2	5	5	100
2.	REP	9	9	100
3.	BOX	4	4	100
4.	OPF04	6	6	100
5.	OPB01	7	7	100
6.	OPA18	8	8	100
7.	a211	8	8	100

Table.5 Binary similarity matrix for RAPD primers

	<i>XacI</i>	<i>XacII</i>	<i>XacIII</i>	<i>XacIV</i>	<i>XacV</i>
<i>XacI</i>	1.0000000				
<i>XacII</i>	0.1304348	1.0000000			
<i>XacIII</i>	0.2727273	0.2727273	1.0000000		
<i>XacIV</i>	0.1481481	0.3823529	0.2250000	1.0000000	
<i>XacV</i>	0.1363636	0.3793103	0.4000000	0.3055556	1.0000000

Table.6 Per cent polymorphism observed in ISSR primers

Sr. No.	Primer	Total bands	Polymorphic bands	%Polymorphism
1	ISSR820	11	11	100
2	UBC8932800	02	02	100
3	ISSR816	06	06	100

Table.7 Binary similarity matrix for ISSR primers

	<i>XacI</i>	<i>XacII</i>	<i>XacIII</i>	<i>XacIV</i>	<i>XacV</i>
<i>XacI</i>	1.0000000				
<i>XacII</i>	0.0000000	1.0000000			
<i>XacIII</i>	0.1666667	0.5000000	1.0000000		
<i>XacIV</i>	0.3333333	0.5000000	0.2727273	1.0000000	
<i>XacV</i>	0.1818182	1.0000000	0.5833333	0.1818182	1.0000000

Fig.1 Pale yellow colonies of *X. axonopodis* pv. *citri*



Fig.2 The symptoms of Citrus canker on leaves

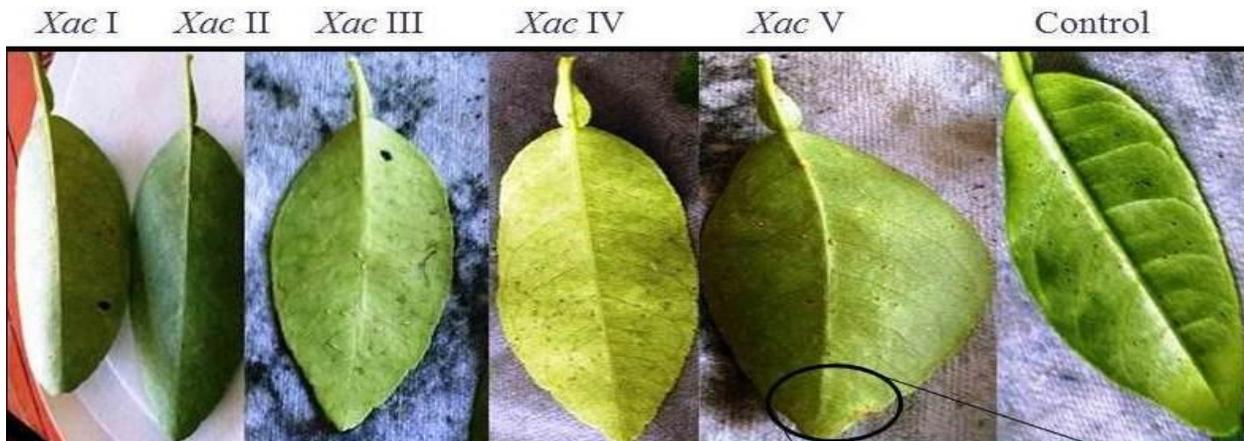


Fig.3 Morphological and Biochemical characteristics of *Xanthomonas axonopodis* pv. *citri* isolates (a) Gram test, (b) Catalase test, (c) Acid production test, (d) Indol production test, (e) KOH test, (f) Starch hydrolysis test, (g) Gelatin liquefaction test



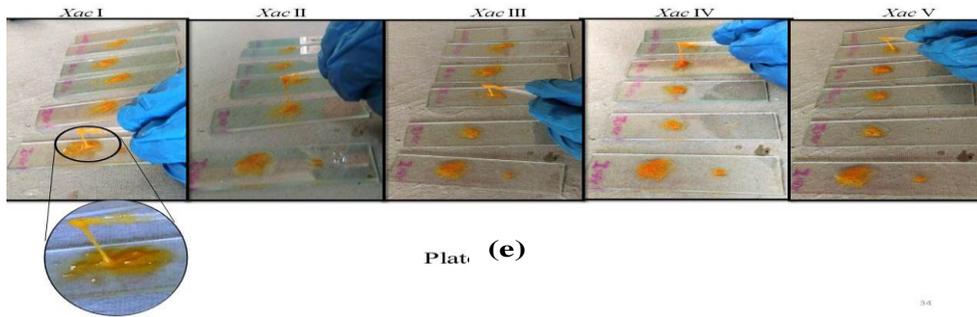
(a)

(b)

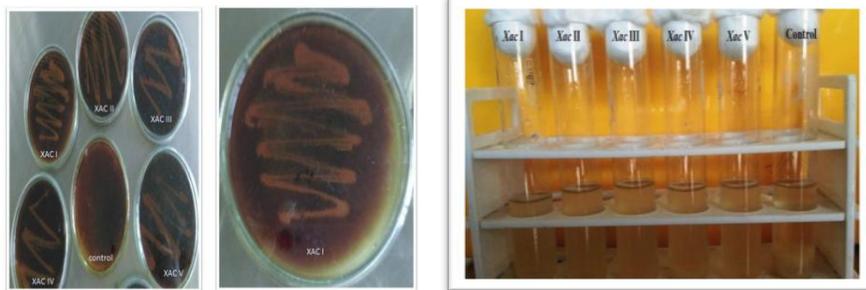


(c)

(d)



Plat (e)



(f)

(g)

Fig.4 Banding pattern of (a) RAPD and (b) ISSR primers

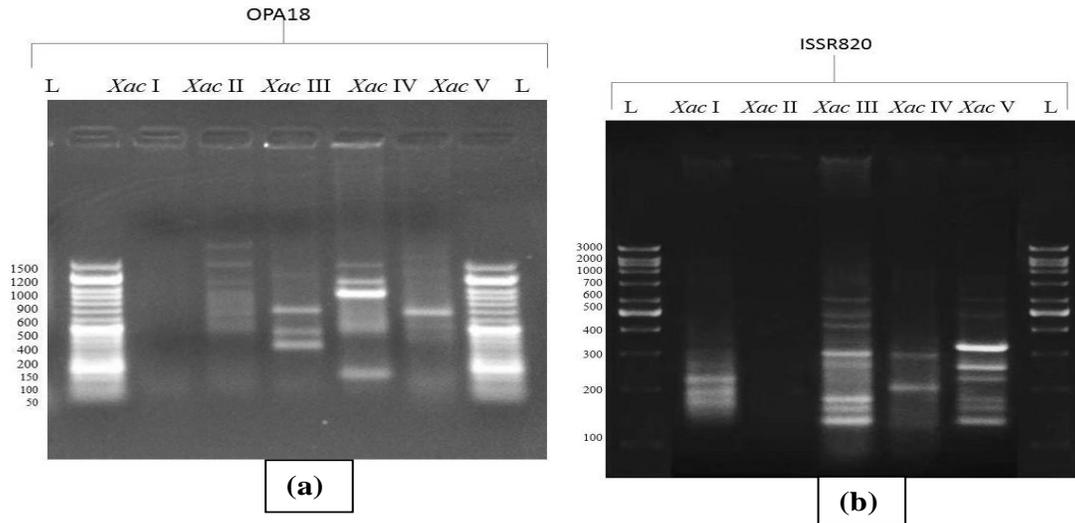
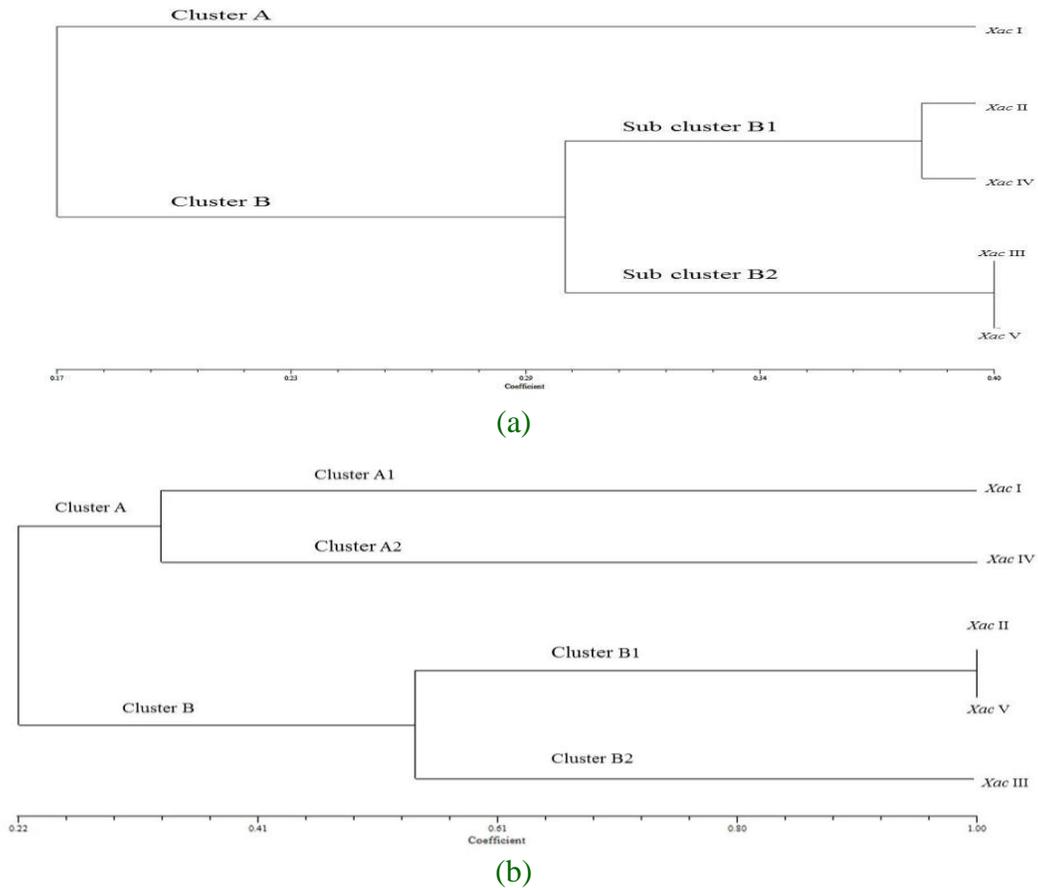


Fig.5 The UPGMA dendrogram of five isolates of *X. axonopodis* pv. *citri* based on Jaccard's similarity coefficient (a) RAPD (b) ISSR



The similarity coefficient value ranged from 0.13 to 0.40 across five isolates indicating high degree of genetic variation (Table 5).

Isolate *XacI* was found to be highly different from *XacII* (0.1304) followed by all other isolates viz., *XacV*, *XacIV* and *XacIII* with similarity coefficient of 0.1364, 0.1481 and 0.2727 respectively. The highest genetic similarity coefficient to an extent of 0.40 was recorded between *XacIII* and *XacV* isolates followed by 0.38 between *XacII* and *XacIV* isolates. The dendrogram constructed by UPGMA for RAPD analysis shows that isolates can be grouped into two major clusters A and B (Fig. 5). Cluster B divided into two sub clusters namely cluster B1 and cluster B2. The cluster A is containing single isolate *XacI*. Sub cluster B1 shows two isolates *XacII* and *XacIV*, whereas sub cluster B2 having two isolate *XacIII* and *XacV*.

ISSR marker

Out of eight randomly selected markers, two produced reproducible and scorable bands with high degree of polymorphism (Fig. 4). A total thirteen amplicons were obtained with the two primers which were found to be polymorphic and the level of polymorphism was 100% (Table 6).

The similarity coefficient value ranged from 0.0000 to 1.00 across five isolates (Table 7). Isolate *XacI* was found to be highly different from *XacII* (0.0000) followed by all other isolates viz., *XacIII*, *XacV* and *XacIV* with similarity coefficient of 0.1667, 0.1818 and 0.3333 respectively. The dendrogram grouped the isolates into two major clusters A and B (Fig. 5). Cluster A divided into two sub clusters namely cluster A1 and cluster A2. Sub cluster A1 shows isolate *XacI* and sub cluster A2 shows isolates *XacIV*. Sub cluster B1 shows two isolates *XacII* and *XacV* and sub cluster B2 shows isolates *XacIII*.

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