Pathological, Bio-chemical and Molecular diversity amongst the isolates of *Xanthomonas axonopodis* pv. *citri* causing Citrus canker in acid lime from different agro-climatic region of India

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**Abstract**— In present investigation isolates of *Xanthomonas axonopodis* pv. *citri* (Xac) causing citrus canker were collected from fourteen agro climatic regions of India. The pathogenic variability of Xac was studied on four different varieties of acid lime viz. Sai sarbati, Phule sarbati, Pramalini and PKM-1 by using detached leaf assay. The isolates showed varied reaction in the symptoms development. The isolates viz. Xac- III, Xac- V, Xac- VII, Xac- XI, Xac- XIII and Xac- XIV found highly virulent and showed of typical symptoms at the point of inoculation within 7 to 9 days. The isolates Xac- I, Xac- II and Xac- IV were found less virulent, developed symptoms after 13 to 16 days of inoculation. The isolates Xac- I and Xac- II failed to develop symptom on variety PKM-1. Further all 15 selected isolates were subjected to biochemical characterization; all isolates were found rod shaped, gram –ve, with colony colour ranging from pale yellow to dark yellow. The isolate were positive for Catalase, KOH and H$_2$S production, hydrolyse starch and gelatin liquefaction. All isolates produce acid from Trhalose. Whereas all isolates fails to produce Indol. The Random Amplified Polymorphic DNA (RAPD) was used to study the variation amongst the 15 isolates of Xac. A total of 27 RAPD primers were screened. Off which 19 primers showed amplification and produced scorable bands with high degree of polymorphism. A total 220 amplicons were obtained of which 218 amplicons were polymorphic with 99.52% level of polymorphism. The banding profile varied from minimum 5 band types (OPB-1) to maximum 21 band type (REP) indicating the high molecular variability amongst all the fifteen isolates of Xac. The similarity coefficient ranged from 0.27 to 0.68. The maximum genetic similarity was found amongst the isolate from Uttar Pradesh (Xac- V) and Shriganganagar (Xac- XIV) i.e. 0.68

**Keywords**— Biochemical test, ERIC, Detached leaf assay, REP, *Xanthomonas axonopodis* pv. *Citri*.

I. **INTRODUCTION**

The Acid lime is an important fruit crop and grown in varying tropical or subtropical regions in the world. It has enormous therapeutic values (Chaudhry *et al.*., 1992). It belongs to family Rutaceae. The worldwide production of acid lime is threatened by a number of biotic and abiotic factors. The citrus canker which is one of the major constraints in cultivation was first reported from Punjab (Luthra and Sattar, 1942). Its occurrence was further recorded in Tamil Nadu (Ramakrishnan, 1954), Andhra Pradesh (Rao,G.P.,1954), Karnataka (Venkatakrishnaiah, 1957), Rajasthan (Prasad,1959), Madhya Pradesh (Parsai,1959), Assam(Chowdhury,1951) and Uttar Pradesh (Nirvan, 1960). The bacterium, *Xanthomonas* causes different types of the symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits, results in defoliation, dieback, premature fruit drop and blemished fruit, which consequently decrease fruit production and market value of the fruits both qualitatively and quantitatively (Zekri *et al.*, 2005; Graham *et al.*,2004; Das, 2003). There are many types in citrus canker disease caused by various pathovars and variants of the bacterium *Xanthomonas axonopodis* (Graham *et al.*, 2004). Recently canker has been detected in kinnow mandarin nursery in Punjab state (Anonymous, 2000). In India, occurrence of strains (pathotypes) of the pathogen has been reported by Rangaswami and Soumini (1957) and Hamlin(1967). Khan and Hingorani (1970) grouped 15 isolates of the pathogens into 3 strains by their reaction on *Murraya exotica*. Kishore

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and Chand (1972) studied the reaction of eight isolates on C. aurantfolia, C. sinensis and C. jambhiri and showed the presence of more than one strain of the pathogens in Harayana. Recently Das (2002) reported the existence of pathogenic variability within the 'A' strain of X. axonopodis pv. citri. The molecular variability amongst the X. axonopodis pv. Citri can be detected by the Serology (Alvarez et al., 1991), plasmid fingerprints(Pruvost et al., 1992), DNA-DNA homology(Egel et al.,1991) and by various RFLP (Restriction Fragment Length Polymorphism) and PCR (Polymerase Chain Reaction) analyses (Miyoshi et al., 1998; Cubero and Graham, 2002). When the DNA-based assays were unavailable, strains of X. axonopodis pv. citri can be distinguished from other pathogens by infecting a panel of susceptible and resistant citrus hosts or as a bioassay on detached-leaves or leaf-disks (Gottwald et al., 1993). Such pathogenecity test is an essential component in diagnostic programmes for regulation of citrus canker diseases (Schubert et al., 2001). Genetic diversity analyses were performed using two marker systems; Repetitive Polymerase Chain Reaction (Rep-PCR) and Random Amplified Polymorphic DNA (RAPD), (Rezaei, et al., 2012). Hence the present study aimed to understand the Pathogenic and molecular variability amongst the Xanthomonas axonopodis pv. citri in different agro-climatic regions of India

II. MATERIAL AND METHOD

Collection of symptomatic samples and Isolation of causal agent

The symptomatic samples of Citrus canker were collected from the 14 Agro-climatics region of the India (Table-1). The different plant parts like infected leaves, twigs and fruits were used for isolation of pathogen by tissue isolation method. The isolation of Xanthomonas axonopodis pv. citri was done on Nutrient Agar (NA) medium. The typical bacterial colonies showing characteristics of Xanthomonas spp. were maintained on the slant containing Yeast Extract Glucose Chalk Agar (YGCA) medium and subsequently sub cultured at regular intervals. The fourteen pure bacterial isolates of Xanthomonas axonopodis pv. citri were inoculated on NA medium. The cultures were incubated at 27±2°C for 48 hrs. after 48 hrs bacterial suspension of 1 x 10⁸ cfu/ml was prepared for each isolate. The pathogenic variation amongst all fifteen isolates of Xanthomonas axonopodis pv. citri; there reaction were tested by detached leaf method on four different cultivars of acid lime viz. Sai Sarbati, Phule Sarbati, Pramalini and PKM-1 and the isolates were catagerized on the basis of days required for the development of symptoms. The fully expanded leaves of all four cultivars of acid lime were collected separately and washed under running water for about 10 min to remove the dirt on the leaves then leaves were soaked in 1% sodium hypochloride for 1 min., after that leaves were washed for 3 times with sterilized distilled water to remove the traches of chemical and leaves were kept for air drying. For –ve control 10µl of sterilized distilled water was placed aseptically onto three leaves of each cultivar at six different sites on each leaf with the help of sterilized syringing. For +ve control 10µl of each bacterial suspension was placed onto three leaves of each cultivar at six different sites on each leaf with the help of sterilized syringing. Separate syringing was used for each isolate. Plate incling was wrap and plates were placed at 27±2°C in a growth cabinate equipped with white light for 12 hrs exposure to white light and 12 hrs for dark. Observations were recorded from 4th day of inoculation upto 25th day of inoculation to record development of symptoms.

Biochemical characterization of Xanthomonas axonopodis pv. citri:

All the isolates of X. axonopodis pv. citri were characterized on the basis of their biochemical reactions as per by Aneja (2003). The different biochemical tests performed viz. Gram staining, KOH test, Starch hydrolysis, Gelatin liquefaction, H₂S Production, Indol Production, Acid and gas production, Catalase test.

Molecular differentiation among the different isolates of Xanthomonas axonopodis pv. citri.

The Random amplified polymorphic DNA (RAPD) analysis was used to detect the variations among the different isolates of Xanthomonas axonopodis pv. citri. The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel containing ethidium bromide @ 0.5 mg/ml in a horizontal gel electrophoresis system. Genomic DNA (2 µl) of each isolate + 3µl loading dye + 5µl sterile water loaded in each well. After completion of 5 cm run, the gel was observed under UV light and the DNA yield and quality was confirmed. The master mix for each primer was prepared by dissolving PCR Reaction 10X without Mgcl₂ 2.0 µl, MgCl₂ 25 Mm 2.0 µl, DNTPs 10 mM each 1 µl Taq DNA Polymerase 5 unit µl⁻¹ 0.50 µl, Primer 10 µM 1.0 µl, DNA (10ng)10ng/ µl 1.0 µl, Sterile milli Q water 12.50 µl to make the final volum 20 µl. The PCR was performed in Thermo cycler (Applied Biosystem) using a programme for the RAPD primer. The master mix was distributed to PCR tubes and later 10 ng of template DNA of each isolate was added separately to each of the master mix tube. Final volume was made upto 20 µl. The PCR programme consist
of initial denaturation at 94°C for 5 min for one cycle, followed by denaturation at 94°C for 1 min. Anneling at 37°C for 1 min and extension at 72°C for 2 min for a total of 40 cycle, with the final elongation at 75°C for 5 min and retention of PCR-RAPD product at 4°C. The PCR-RAPD product analysis was carried out in horizontal gel electrophoresis. The PCR products were separated electrophoretically in 1.5% agarose gel using 1X TBE buffer. The gel was stained with ethidium bromide (Sambrook et al. 2001). The gel was run for 2 hrs. at 80v. After the run, the gel was removed carefully from the unit and observed under Gel Doc instrument to visualize the amplification. The amplified profiles for all the primers were compared with each other and bands of DNA fragments were scored as ‘1’ for presence and ‘0’ for absence, generating ‘0’ and ‘1’ matrix. Per cent polymorphism was calculated by using the formula.

\[
\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100
\]

The data was used to generate similarity coefficient using simple matching coefficient based on RAPD bands scoring. The Dice similarity coefficient between each pair of accessions were then used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetic Average (UPGMA).

III. RESULT AND DISCUSSION

Collection of diseased samples

A total of fifteen symptomatic samples of acid lime infected with citrus canker were collected from fourteen agro climatic regions of India as listed in Table -1. The isolation of causal agent was done from various infected plant parts viz. leaf, twig and fruit. The causal agent thus isolated from each location was designated as an ‘isolate’ viz., Xac- I, Xac- II, Xac- III, Xac- IV, Xac- V, Xac- VI, Xac- VII, Xac- VIII, Xac- IX, Xac- X, Xac- XI, Xac- XII, Xac- XIIIB, Xac- XIII and Xac- XIV. Two isolates were taken from 12th agro climatic region of India (West Cost Plane and Ghat Region) so they are designated as Xac- XII and Xac-XIIIB (Table 1). Valenchia et al. (2004) obtained 123 isolates of Xanthomonas axonopodis pv. dieffenbachiae (Xad) from Los Banos, Islam et al. (2014) who collected 9 disease samples of Citrus canker from different regions of Bangladesh and the isolates were identified based on morphological, cultural and biochemical characteristics. Similarly;

The pathogenic variability among Xanthomonas axonopodis pv. citri

The pathogenic variability amongst the fifteen isolates Xanthomonas axonopodis pv. citri, was studying (Table-2, Plate-3) inoculating on four different varieties of acid lime viz. Sai sharbati, Phule sharbati, Pramalini and PKM-1 by using detached leaf assay technique (Tuite, 1969). All varieties were found susceptible to all the fifteen isolates Xanthomonas axonopodis pv. citri. The isolates Xanthomonas axonopodis pv. citri showed varied reaction in the symptoms development. The isolates viz. Xac- III, Xac- V, Xac- VII, Xac- XI, Xac- XIII and Xac- XIV found highly virulent in development of typical symptoms i.e. white crystalline callus formation at the point of inoculation within 7 to 9 days. The isolates Xac- I, Xac- II and Xac- IV were found less virulent as they developed symptoms after 13 to 16 days of inoculation. The isolates Xac- I and Xac- II failed to develop symptom on variety PKM-1. The categorization of isolates of Xac was done on the basis of symptoms development on leaves and days taken for appearance of the symptoms as No canker (-), Weak canker (+), Moderate canker (+++) and Strong canker (+++) as presented in Table. 2. Atiq et al. (2007) who screened fifteen citrus cultivars for resistance against citrus canker disease incited by X. axonopodis pv. citri and reported that no immune response was exhibited by any variety in the experiment. Ismail et al. (2014) studied the reaction of Xac on 5 different host of Rutaceace family by detached leaf assay and reported that the pathogen also produced water soaking, followed by necrosis around the wound inoculated surface on grape fruit, Rough lemon followed by Lime.

Biochemical Characterization of Xanthomonas axonopodis pv. citri

All the fifteen isolates of Xac were rod shaped produce typical mucoid colonies with the color variation among all the isolates from pale yellow to dark yellow. They are Gram’s positive and showed string formation in KOH test (Table-3). All the fifteen isolates of Xac were found positive for hydrolysis of starch in variable degree. The isolates Xac- I, Xac- III, Xac- IV, Xac- V, Xac- VII, Xac- IX, Xac- XIV, Xac- XIIIB and Xac-XIII showed strong reaction with clear zone around hydrolyzed area when lugol iodine was poured. The isolates Xac- II, Xac- VI, Xac- VIII, Xac- X, Xac- XI, Xac- XII and Xac- XIV showed moderate reaction, showed slow zone formation around the area of hydrolysis when lugol iodine was poured. For the liquefaction of gelatin by fifteen isolates of Xanthomonas
**axonopodis pv. citri**, showed variable reaction. The isolates Xac- V and Xac- XHA showed strong reaction for gelatin liquefaction as they produced strong proteolytic exo-enzyme due to which gelatin was hydrolyzed. The isolate Xac- III, Xac- IV, Xac- VI, Xac- VIII, Xac- IX, Xac- XI, Xac- X, Xac- XIIB showed moderate reaction for gelatin liquefaction. The isolate Xac- I, Xac- II, Xac- VII, Xac- XIII and Xac- XIV showed weak reaction for gelatin liquefaction (Table-3). All the fifteen isolates of Xac showed variable reaction for H₂S Production. The isolates Xac- I, Xac- II, Xac- III, Xac- IV, Xac- V, Xac- VI, Xac- IX, Xac- X, Xac- XII, Xac- XIIB, Xac- XIII and Xac- XIV were strong H₂S producer, showed black coloration along the line of stab inoculation within 3 to 4 days after inoculation. The isolate Xac- VII, Xac- VIII and Xac- XI were found weak H₂S producer as the black coloration along the line of stab inoculation was formed after 7 days of inoculation. Similarly all isolates produce acid from carbon source Trahalose but they are fails to produce gas from the same carbon source. All isolates were negative for Indol production. All the fifteen isolates of Xac were found positive for Catalase test as bubble formation was observed after addition of 3% hydrogen peroxide in the 48 hrs old incubated bacterial culture. All the isolates were rod shaped, Gram negative showed circular pale yellow colonies on nutrient agar as earlier reported by Patel (1950) who observed bacterial colonies of X. malvacearum as flat glistening pale yellow on nutrient agar also identified the organism is short rod with rounded ends, Gram negative. The results obtained are in confirmation with those reported by Manjula (2002) who reported that, the bacterium Xanthomonas axonopodis pv. punicae causing oily spot pomegranate were small rods, appeared singly, Gram negative. Gottwald et al. (2002) who reported that Xanthomonas axonopodis is a rod shaped Gram negative bacterium. The cultures showed variable reaction among the isolates of Xanthomonas axonopodis pv. citri. Similar variation among the isolates has been earlier noted by Raut (1990) he studied 15 isolates of Xanthomonas axonopodis pv. mangiferae indicae for different physiological and biochemical properties viz. H₂S production , action on carbohydrates, gelatin test, KOH test etc. Das (2003) reported that the bacterial cells of Xanthomonas citri are positive for hydrolysis of starch, liquefaction of gelatin, catalase. Das (2005) studied different isolates of Xanthomonas axonopodis pv. citri for different physiological and biochemical properties viz. H₂S production, gelatin liquefaction, KOH test, catalase test, acid production from different sugars.

Molecular characterization of Xanthomonas axonopodis pv. citri

Out of 27 primers screened, 19 primers showed amplification and produced scorable bands with high degree of polymorphism. A total 220 amplicons were obtained of which 218 amplicons were polymorphic with 99.52% level of polymorphism was observed (Table-4). The banding profile varied from minimum 5 band types (OPB- 1) to maximum 21 band (REP) indicating the high molecular variability amongst all the fifteen isolates of Xac. The amplification profile of selected primer on 1.5% agarose gel were showed in Plate-1and 2. The information on banding pattern of all primers was used to determine genetic distance between the fifteen isolates of Xac and the dendrogram was constructed by using Un-weighted Pair Group Arithmetic Mean method (UPGMA). Based on simple matching coefficient a genetic similarity matrix was constructed to access the genetic relatedness amongst the fifteen isolates of Xac. The genetic similarity coefficient of fifteen isolates given in Table 5. The similarity coefficient ranged from 0.27 to 0.68 showed high genetic diversity. The maximum genetic similarity was found between the isolate from Uttar Pradesh (Xac- V) and Shriganganagar (Xac- XIV) i.e 0.68 and both isolates in a same cluster B₁. The least similarity was found between the isolates from Uttarakhand (Xac- I) and Dhule, Maharashtra (Xac- IX) 0.27. The dendrogram (Fig-1) showed that both isolates falls in different cluster i.e. Xac- I in cluster A and Xac- IX in sub cluster B₂ of cluster B. Further the dendrogram constructed by UPGMA clearly showed the main two clusters viz. Cluster A and Cluster B. The Cluster B was again divided into two sub clusters namely Cluster B₁ and Cluster B₂. Two isolates Xac- I and Xac- III were falls under cluster A as they are more similar to each other and much differs with other isolates of Xac. Isolate Xac- II, Xac- IV, Xac- V, Xac- XIV, Xac- XIII, Xac- XIA ,Xac- VI, Xac- VII, Xac- XI, Xac- VIII and Xac- X were falls under same cluster in cluster B₁ and remaining two isolates Xac- IX and Xac- XIIB were falls under same cluster B₂. The dendrogram showed matched results with similarity coefficient values; as the isolates showed high similarity coefficient value falls.
under same cluster (Xac- V and Xac- IVX) and the isolates showed less similarity coefficient values (Xac- I and Xac- IX) falls in a different cluster (Fig. 1). The size of banding pattern was found variable for each primer shown in Table - 5. The present findings corroborates with the findings of Yenjerappa (2009) who studied molecular variation amongst the 20 isolates of Xanthomonas axonopodis pv. punicae, by using Random Amplified Polymorphic DNA (RAPD) technique by using primers belonging to OPA, OPB and OPF series. Among the 20 primers used 11 primers exhibited amplification. The primers, OPA- 20, OPB- 03, OPF- 07 and OFP- 10 showed 100 per cent polymorphism that helped to identify the isolates and served as a basis for identification of specific primers. Arshiya et al. (2014) studied the rep-PCR fingerprint profiles, obtained with the REP, ERIC and BOX primers confirmed that all 20 isolates of X. axonopodis pv. citri were amplified with the Rep- PCR by using the universal primers of REP, ERIC and BOX to identify genetic diversity in Xanthomonas axonopodis pv. citri. REP, ERIC and BOX primers sets gave reproducible genomic PCR profiles consisting of approximately 100- 3kb bands. The bands were clearly differentiated by Agarose gel electrophoresis. These profiles were complex and revealed polymorphic bands among Xanthomonas axonopodis pv citri also the results obtained in the present investigation were in accordance with the results of Rezaei et al. (2012) who reported different RAPD marker was to determine the genetic relationship between Iranian strains of Xcc. Primers 211,220,230,232 and OPA11 generated different fingerprints among Xcc strains. PCR products of these primers ranged from 100 to 7000 bp. Similarly Rouhrazi (2012) reported that four races of Xcc were found in northern Iran (1, 4, 5 and 6), to examine the distribution of dispersed repetitive DNA, Enterobacterial Repetitive Intergenic Consensus (ERIC), BOX, Repetitive Extragenic Palindromic (REP) and Random Amplified Polymorphic DNA (RAPD) sequences in the genome of Xcc using conserved primers. The different markers produced characteristic banding patterns and the similarity matrices from binary banding data was derived with the similarity for qualitative data program (SIMQUAL). Present findings corroborates with the findings of Lin et al. (2008) reported the similarity coefficient of both a typical symptoms-inducing strains XL16 and XL38 was 0.9 – 1.0 to Xac. The present investigation clearly concludes that there exist the pathological, biochemical and molecular variation amongst the different isolates of Xanthomonas axonopodis pv. citri collected from the different agro-ecological regions of India.

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Table 1: Details of disease samples collected from Agro climatic regions of India

| Sr  | Agro climatic regions          | State                | Location                                      | Designation | Plant part used for isolation |
|-----|--------------------------------|----------------------|-----------------------------------------------|-------------|------------------------------|
| 1   | Western Himalayan Region       | Uttarakhand          | G.B.Pant University of Agriculture and Technology Pantnager | Xac- I      | Leaf                         |
| 2   | Eastern Himalayan Region       | Meghalaya            | Barapani                                      | Xac- II     | Leaf                         |
| 3   | Lower Gangentic Plane          | West Bengal          | B.C. K. V., Nodia                            | Xac- III    | Leaf                         |
| 4   | Middle Gangentic Plane         | Bihar                | Aria                                          | Xac- IV     | Leaf                         |
| 5   | Upper Gangentic Plane          | Uttar Pradesh        | BHU, Varanasi                                 | Xac- V      | Leaf                         |
| 6   | Trans Gangentic Plane          | Panjab               | Ludhiana                                      | Xac- VI     | Leaf                         |
| 7   | Eastern Plateau Hill Region    | Nagpur               | Dahigoan, Parseoni                           | Xac- VII    | Fruit                        |
| 8   | Central Plateau Hill Region    | Rajasthan            | Bikaner                                       | Xac- VIII   | Leaf                         |
| 9   | Western Plateau Hill Region    | Maharashtra          | College of Agriculture, Dhule                 | Xac- IX     | Fruit                        |
| 10  | Southern Plateau Hill Region   | Andhra Pradesh       | Anantpur                                      | Xac- X      | Twig                         |
| 11  | East Cost Plain and Hill Region| Tamil Nadu           | Vamban, Pudukkottai                          | Xac- XI     | Leaf                         |
| 12  | West Cost Plane and Ghat Region| Maharashtra          | Walawa, Sangali                              | Xac- XIA    | Leaf                         |
| 13  | West Cost Plane and Ghat Region| Maharashtra          | MPKV, Rahuri                                  | Xac- XIIB   | Fruit                        |
| 14  | Gujarat Plane and Hill Region  | Gujrat               | Anand Agriculture University, Anand           | Xac- XIII   | Fruit                        |
| 15  | Western Dry land               | Rajasthan            | Shriganga nagar                              | Xac- XIV    | Leaf                         |

Table 2: Pathogenicity reaction of isolates Xac on different cultivars of acid lime (By Detached Leaf Assay)

| Sr. | Isolates | Location | Reaction on leaves of different cultivars after 20 days of inoculation |
|-----|----------|----------|------------------------------------------------------------------------|
|     |          |          | Sai sarbati | Phule Sharbati | Pramalini | PKM- I |
| 1   |          |          |             |                |           |        |
| 2   |          |          |             |                |           |        |
| 3   |          |          |             |                |           |        |
| 4   |          |          |             |                |           |        |
| 5   |          |          |             |                |           |        |
| 6   |          |          |             |                |           |        |
| 7   |          |          |             |                |           |        |
| 8   |          |          |             |                |           |        |
| 9   |          |          |             |                |           |        |
| 10  |          |          |             |                |           |        |
| 11  |          |          |             |                |           |        |
| 12  |          |          |             |                |           |        |
| 13  |          |          |             |                |           |        |
| 14  |          |          |             |                |           |        |
| 15  |          |          |             |                |           |        |
| No. | Xac  | Location                  | Days to initiation of symptoms | Symptoms | Days to initiation of symptoms | Symptoms | Days to initiation of symptoms | Symptoms | Days to initiation of symptoms | Symptoms |
|-----|------|---------------------------|--------------------------------|----------|--------------------------------|----------|--------------------------------|----------|--------------------------------|----------|
| 1   | Xac-I| Pantnagar, Uttarakhand    | 16                             | +        | 16                             | +        | 15                             | +        | -                              | -        |
| 2   | Xac-II| Barapani, Meghalaya       | 14                             | ++       | 15                             | +        | 15                             | +        | -                              | -        |
| 3   | Xac-III| West Bengal              | 7                              | +++      | 8                              | +++      | 8                              | +++      | 7                              | +        |
| 4   | Xac-IV| Aria, Bihar               | 13                             | ++       | 12                             | +++      | 13                             | ++       | 13                             | +        |
| 5   | Xac-V | BHU, Varanasi, U.P.       | 9                              | ++       | 8                              | ++       | 9                              | ++       | 8                              | +        |
| 6   | Xac-VI| Ludhiana, Panjab          | 12                             | ++       | 11                             | ++       | 11                             | +++      | 13                             | +        |
| 7   | Xac-VII| Nagpur, Maharashtra      | 7                              | +++      | 7                              | +++      | 8                              | +++      | 9                              | +        |
| 8   | Xac-VIII| Bikaner, Rajasthan     | 10                             | ++       | 11                             | ++       | 9                              | +++      | 10                             | +        |
| 9   | Xac-IX| Dhule, Maharashtra        | 12                             | +++      | 12                             | ++       | 13                             | +++      | 12                             | +        |
| 10  | Xac-X | Anantpur, A.P.            | 12                             | ++       | 13                             | ++       | 11                             | ++       | 12                             | +        |
| 11  | Xac-XI| Vamban, Tamilnadu         | 7                              | +++      | 8                              | ++       | 7                              | +++      | 8                              | +        |
| 12  | Xac-XII A| Sangali, Maharashtra   | 10                             | ++       | 11                             | ++       | 13                             | +        | 11                             | +        |
| 13  | Xac-XII B| MPKV, Rahuri, Maharashtra | 10                             | ++       | 12                             | ++       | 11                             | ++       | 10                             | +        |
| 14  | Xac-XIII| AAU, Anand, Gujarat      | 8                              | +++      | 7                              | +++      | 7                              | +++      | 7                              | +        |
| 15  | Xac-XIV| Shriganganagar, Rajasthan| 8                              | ++       | 9                              | ++       | 7                              | +++      | 8                              | +        |

No canker (-), Weak canker (+), Moderate canker (++) Strong canker (++++)

*Table 3: The biochemical reaction of the fifteen isolates of Xac*
| Parameter | Xac-I | Xac-II | Xac-III | Xac-IV | Xac-V | Xac-VI | Xac-VII | Xac-VIII | Xac-IX | Xac-X | Xac-XI | Xac-XIIA | Xac-XIIIB | Xac-XIIV |
|-----------|-------|--------|---------|--------|-------|--------|---------|----------|--------|-------|--------|----------|-----------|---------|
| Shape     | Rod   | Rod    | Rod     | Rod    | Rod   | Rod    | Rod     | Rod      | Rod    | Rod   | Rod    | Rod      | Rod       | Rod     |
| Colony colour | Pale yellow | Pale yellow | Pale yellow | Dark yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Dark Yellow |
| Gram reaction | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| Catalase | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Indole production | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| KOH | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| H₂S production | +++ | +++ | +++ | +++ | +++ | + | + | +++ | +++ | + | +++ | +++ | +++ | +++ |
| Acid and gas production | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Hydrolysis | Geletine | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Starch | +++ | + | +++ | +++ | + | +++ | +++ | + | +++ | +++ | + | +++ | +++ | +++ | +++ |

- : Negative Reaction
+ : Weak Reaction
++ : Moderate Reaction
+++ : Strong Reaction
### Table 4: Per cent polymorphism observed in RAPD, BOX, ERIC 1 REP primers

| Sr. No. | Primer Name | Total Number of Bands | Polymorphic Bands | Monomorphic Bands | Per cent Polymorphism | Maximum band size (bp) | Minimum band size (bp) |
|---------|-------------|-----------------------|-------------------|-------------------|-----------------------|------------------------|------------------------|
| 1       | OPA-1       | 15                    | 15                | 0                 | 100%                  | 7227                   | 364                    |
| 2       | OPA-2       | 10                    | 10                | 0                 | 100%                  | 2777                   | 236                    |
| 3       | OPA-3       | 10                    | 9                 | 1                 | 90%                   | 3070                   | 337                    |
| 4       | OPA-4       | 10                    | 10                | 0                 | 100%                  | 2278                   | 231                    |
| 5       | OPA-7       | 11                    | 11                | 0                 | 100%                  | 6401                   | 263                    |
| 6       | OPA-9       | 12                    | 12                | 0                 | 100%                  | 5994                   | 444                    |
| 7       | OPA-10      | 8                     | 8                 | 0                 | 100%                  | 3792                   | 236                    |
| 8       | OPA-11      | 12                    | 12                | 0                 | 100%                  | 3361                   | 396                    |
| 9       | OPB-1       | 5                     | 5                 | 0                 | 100%                  | 4476                   | 426                    |
| 10      | OPB-4       | 7                     | 7                 | 0                 | 100%                  | 3539                   | 390                    |
| 11      | OPB-5       | 10                    | 10                | 0                 | 100%                  | 4367                   | 236                    |
| 12      | OPB-6       | 7                     | 7                 | 0                 | 100%                  | 2943                   | 677                    |
| 13      | OPB-7       | 11                    | 11                | 0                 | 100%                  | 5247                   | 436                    |
| 14      | OPF-2       | 13                    | 13                | 0                 | 100%                  | 3060                   | 335                    |
| 15      | OPF-4       | 14                    | 14                | 0                 | 100%                  | 2900                   | 271                    |
| 16      | OPF-6       | 11                    | 11                | 0                 | 100%                  | 3709                   | 113                    |
| 17      | BOX         | 17                    | 16                | 1                 | 94.11%                | 3999                   | 109                    |
| 18      | ERIC 1      | 18                    | 18                | 0                 | 100%                  | 2785                   | 375                    |
| 19      | REP         | 21                    | 21                | 0                 | 100%                  | 4221                   | 122                    |

**Total No. of Bands:** 220 218 2 99.52% (Avg.) - -

### Table 5- Similarity coefficient for RAPD analysis

|       | Xac-I | Xac-II | Xac-III | Xac-IV | Xac-V | Xac-VI | Xac-VII | Xac-VIII | Xac-IX | Xac-X | Xac XI | Xac XII A | Xac XII B | Xac XIII | Xac XIV |
|-------|-------|--------|---------|--------|-------|--------|---------|----------|--------|-------|---------|-----------|-----------|-----------|---------|
| Xac-I | 1     |        |         |        |       |        |         |          |        |       |         |           |           |           |         |
| Xac-II| 0.396 | 1.000  |         |        |       |        |         |          |        |       |         |           |           |           |         |
| Xac-III | 0.418 | 0.363 | 1.000   |        |       |        |         |          |        |       |         |           |           |           |         |
| Xac-IV | 0.455 | 0.547 | 0.521   | 1.000  |       |        |         |          |        |       |         |           |           |           |         |
| Xac-V  | 0.301 | 0.598 | 0.533   | 0.535  | 1.000 |       |         |          |        |       |         |           |           |           |         |
| Xac-VI | 0.478 | 0.470 | 0.461   | 0.532  | 0.578 | 1.000  |         |          |        |       |         |           |           |           |         |
| Xac-VII | 0.514 | 0.545 | 0.369   | 0.365  | 0.482 | 0.531  | 1.000   |          |        |       |         |           |           |           |         |
| Xac-VIII | 0.318 | 0.513 | 0.252   | 0.389  | 0.500 | 0.500  | 0.442   | 1.000    |        |       |         |           |           |           |         |
| Xac-IX | 0.271 | 0.464 | 0.329   | 0.403  | 0.361 | 0.339  | 0.370   | 0.300    | 1.000  |       |         |           |           |           |         |
| Xac-X  | 0.325 | 0.454 | 0.314   | 0.347  | 0.400 | 0.341  | 0.487   | 0.522    | 0.474  | 1.000 |         |           |           |           |         |
| Xac-XI | 0.375 | 0.403 | 0.410   | 0.386  | 0.440 | 0.446  | 0.585   | 0.417    | 0.37   | 0.464 | 1.000   |           |           |           |         |
| Xac-XII A | 0.447 | 0.516 | 0.360   | 0.480  | 0.588 | 0.488  | 0.436   | 0.496    | 0.378  | 0.360 | 0.365   | 1.000     |           |           |         |
| Xac-XII B | 0.387 | 0.380 | 0.368   | 0.436  | 0.440 | 0.392  | 0.421   | 0.320    | 0.434  | 0.485 | 0.429   | 0.376     | 1.000     |           |         |
| Xac-XIII | 0.22  | 0.46  | 0.311   | 0.431  | 0.461 | 0.491  | 0.450   | 0.464    | 0.387  | 0.422 | 0.460  | 0.536     | 0.500     | 1.000     |         |
| Xac-XIV | 0.333 | 0.592 | 0.406   | 0.446  | 0.683 | 0.590  | 0.567   | 0.619    | 0.392  | 0.536 | 0.519   | 0.540     | 0.423     | 0.59 6    | 1.000    |
Fig. 1: The RAPD UPGMA dendrogram of fifteen isolates of Xanthomonas axonopodis pv. citri based on Jaccards similarity coefficient.
Plate 11 – RAPD banding pattern of primer OPA-1 and OPA-4.

M- Marker (Size of marker in bp.)
1. Xac- V  2. Xac- VI  3. Xac- VII  4. Xac- VIII  5. Xac- IX  6. Xac- X  7. Xac- XI
8. Xac- XII  9. Xac- XII A  10. Xac- XII B
11. Xac- XIII  12. Xac- XIV  13. Xac- XV

Plate-1 : The RAPD banding pattern of primer OPA-1 and OPA-4 against 15 isolates of Xanthomonas axonopodis pv. citri
Plate-2: The banding pattern of BOX and ERIC R1 primer against 15 isolates of Xanthomonas axonopodis pv. citri
**Plate 3:** Variation in symptoms development on Phule and Sai Sharbati by Detached leaf Assay

**Plate 4:** Pathogenicity on the cultivar Sai Sharbati by Attached Leaf Assay