Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 173-176
Received: 14-07-2020
Accepted: 20-08-2020

Pradeep Manyam
Department of Plant Pathology, University of Agricultural Sciences, Dharwad, Karnataka, India

Nargund VB
Department of Plant Pathology, University of Agricultural Sciences, Dharwad, Karnataka, India

Corresponding Author:
Pradeep Manyam
Department of Plant Pathology, University of Agricultural Sciences, Dharwad, Karnataka, India

Diagnosis of *Xanthomonas axonopodis* pv *citri* isolates, the causal agent of citrus canker by pathogenicity and PCR based methods

Pradeep Manyam and Nargund VB

Abstract
Citrus canker caused *by Xanthomonas axonopodis* pv *citri* (*Xac*) is one of most destructive diseases of citrus in terms of damage to trees and fruit quality and remained as one of the important quarantine disease. Under field conditions, though Asiatic citrus canker disease is relatively easy to diagnose, the accurate detection of pathogen for various pathological studies is necessary. In present study, a total of 13 plant samples collected from various parts of Karnataka state were diagnosed by an integrated approach using pathogenecity, isolation and molecular techniques. Five isolates were able grow on nutrient glucose agar medium characteristic of *Xanthomonas axonopodis* pv. *citri*.. The pathogenicity of all the above five isolates (*XAC* 1, 2, 3, 4 and 5) was proved with typical canker lesions on leaves of citrus after five to eight days of incubation period. All five isolates of *Xac* were confirmed at molecular level by amplifying highly conserved nucleotide sequence *est* A region (777 bp) using *Xc-lip* primer pair that distinguishes *Xanthomonads* from bacteria. The study revealed that integrated approach as reliable technique for detection of *Xac* for laboratory analysis.

Keywords: Citrus canker, pathogenicity, isolation, PCR, xanthomonads, *Xanthomonas axonopodis* pv. *citri*

1. Introduction
In India, citrus species occupies third position among fruits after mango and banana in term of production and Asiatic citrus canker is one of the major constraints of its cultivation. Among five pathotypes of cirus canker pathogen that are geographically distributed in different continents except Europe, cankerous form A which is also known as true or Asiatic canker disease is the most devastating form and can infect most commercial citrus varieties and citrus relatives (Vauterin *et al.*, 1995) [12].

The diseased plants are characterized by the occurrence of conspicuous raised necrotic lesions that develop on leaves, twigs and fruits. Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop (Stall and Seymour, 1983). Canker causes fruit losses ranging from premature fruit drop due to abscission to non-marketable quality due to lesions.

The bacterium *Xac*is rod-shaped measuring 1.5-2.0 x 0.5-0.75 mm, Gram-negative and has a single polar flagellum. Colonies on culture media were usually yellow as a result of *Xanthomonadin* pigment production (Chand and Pal, 1982 and Goto, 1992) [2, 4].

Xac survives primarily in naturally occurring lesions. Cankerous leaves, twigs and branches constitute the main source of inoculum (Nirvan, 1963) [9]. Bacterial cells ooze from existing lesions during wet weather to provide inoculum for further disease development. Infection by *Xac*occurs, like many other bacterial diseases, primarily through stomata and wounds produced during strong winds and by insects.

Methods of detecting *Xac* from natural habitats include leaf-infiltration, bacteriophage, fluorescent antibody and ELISA (Goto, 1992) [4]. The polymerase chain reaction and dot blot immunobinding assay (DIA) were developed for rapid, sensitive, and specific detection of the pathogen. (Wang and Liu, 2004; Mavrodieva *et al*., 2004) [7]. Golmohammadi*et al*. (2007) [3] and Yin *et al.* (2007) [14] found real-time PCR to be more effective at detecting *Xac*, and up to 100-1000 times as sensitive. Since the nucleotide sequence of *est*A is highly conserved in *Xanthomonads*, the sequence was used to design a specific PCR primer set, *Xc-lip*-F2-*Xc-lip*-R2.

2. Materials Andmethods
2.1 Isolationandidentification of the pathogenic bacteria
A total of 13 samples of citrus diseased leaves were collected for the study from eleven places

of northern Karnataka. Characteristic symptoms associated with citrus canker disease such as cankerous lesions that are raised with cracking eruptions, spongy, light yellow in colour surrounded by oil yellow halo on the surface of leaves were used for isolation. Colonies were grown on nutrient agar plates containing glucose and incubated for 72 h.

Pathogenicity test

Citrus kagzi lime plants highly susceptible to citrus canker infection were heavily pruned and kept in glass house to induce a new flush of growth (3-4weeks) and to avoid infection of the disease. High humidity (75-90%) was maintained by spraying water and subsequently covering the plant with polythene bag for two days prior to inoculum spray. Bacterial cells grown for two days on NGA medium were harvested and resuspended in sterile distilled water to a concentration of approximately 10^8 CFU/ml and mist sprayed on foliage. After inoculation, the plants were placed inside the polythene bags for an additional 24 h of high moisture. The inoculated plants were observed for cankerous symptoms. Control was maintained with water spray only.

Molecular confirmation of isolates by PCR technique

i) **DNA isolation:** The extraction of total genomic DNA from bacterial isolates was carried out following a modified protocol as described by Mondal and Kumar (2011) [6].

ii) **Primers:** Primer sequences for PCR as described by Yung *et al.*, 2009 was used in this study

Primersequence (5'-3')	Annealing (°C)	Productsize (bp)	Target gene
F- TATGTGATGGTCCCGACCATTC	68	777	*Xc-lip*
R- GGACTTCGCGGTCCACGTCGTAG			

iii) **PCR amplification:** The PCR reaction was carried out in a 20 µl volume containing Template DNA (25 ng/µl):1.0 µl**,** 10X assay buffer with 15 mM $MgCl_2$ 2.0 µl**,** dNTPs mix (10 mM): 1.0 µl**,** Primer Forward (5 pM/ µl): 1.0 µl, Primer Reverse (5 pM/ µl): 1.0 µl**,** Sterile distilled water: 13.50 µlandTaq DNA polymerase (3.0 U/ µl): 0.5 µl.

iv) **Gel analysis:** The PCR fragments were analyzed on 1.5% agarose at 75 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (10 mg/ml). The gel was analyzed using gel documentation system under UV (302 nm).

Stage	Steps	For *Xac*		
		Temperature(°C)	Duration	Number of cycles
I	Initial denaturation	94	4 min	1
II	Denaturation	94	1 min	35
	Annealing	68	1 min	
	Extension	72	2 min	
III	Final extension	72	10 min	1
	Hold temperature	4	30 in	-

3. Results

3.1 Isolation and identification of the pathogenic bacteria

The pathogen on nutrient agar plates containing glucose showed characteristically creamy-yellow coloured colonies with copious slime, raised and convex. Such colonies, after 72 h of incubation were picked and preserved for further use. A total of five isolates characteristic to *Xac* colonies were separated (Table1, Figure1). *Pantoea agglomerans* was the contaminating bacterium most frequently found in citrus canker during isolation of *Xac*.

3.2 Pathogenicity test on brinjal seedlings

Five isolates of *Xac* were tested on citrus kagzi lime plants for pathogenicity under glasshouse conditions to prove Koch's postulates. All the five isolates (XAC 1, 2, 3, 4 and 5) induced typical erumpent canker lesions on leaves of citrus after five to eight days of incubation period. Typical canker lesions of slight yellow, raised eruptions were observed on the surface of leaves and twigs in the initial stages. In later stages, as the lesions enlarged from pin point size to different lesion sizes of 5-10 mm, the spongy eruptions began to crack several times at the centre portion and slowly turning the lesion to brown colour, forming a crater-like appearance. The pathogen was re-isolated from diseased tissue that showed typical *Xac* colonies.

3.3 Characterization of *Xanthomonas Axonopodis* pv.*citri* using DNA-based techniques

The extraction of total genomic DNA from bacterial isolates of *Xac* was carried out following a modified protocol as described by Mondal and Kumar (2011) [6] resulted in sufficient DNA yield and was confirmed by running on 1.0 per cent agarose gel.

The *Xc-lip*primer pair gave a single product of 777 bp with DNA of all five isolates (XAC-1, 2, 3, 4 and 5) of *Xac* at an annealing temperature of 68^0C through polymerase chain reaction (PCR**)** (Figure 2).

4. Discussion

Graham and Gottwald (1990) reported that Citrus canker caused by *Xanthomonas axonopodi*s pv. *citri* is known to be the most destructive disease of citrus spreading across the world including all continents of Africa, Asia, Australia, South America and USA. In the present study, isolates were identified as *Xac* on the basis of phenotypic characters as well as genotypically based on PCR amplification. Colonies of five

isolates of *Xac* appeared lemon yellow and very mucoid on NGA and had a "sticky" texture. Colonies were slow growing, visible clearly only after four days and such five isolates were picked and preserved. The similar observations were made previously by Young *et al.* (2008) [15] and Nitish *et al.* (2014) [10].

Citrus kagzi lime plants were used for pathogenicity testing of five isolates earlier identified phenotypically under glasshouse conditions. According to Nirvan (1961) [8], the Kagzi lime is most susceptible citrus species followed by grape fruit and sweet oranges. Hence, Kagzi lime was used for pathogenicity and all the five isolates induced typical erumpent canker lesions on leaves of citrus after five to eight days of incubation period. The pathogen was re-isolated from diseased tissue, showed typical *Xac* colonies. Broadbent *et al.* (1992) [1] and Mohammadi *et al.* (2001) [5], have also obtained similar inoculation results with *Xanthomonas axonopodis*pv. *citri* and they observed symptomatic plants after two-three weeks following inoculation.

Since *est*A is highly conserved in Xanthomonads, one option for reliable identification of xanthomonads is the detection of *est*A by a PCR assay. In present study, using the specific primer set *Xc-lip* derived from *est*A, a specific DNA fragment of 777 bp was amplified from all five isolates of *Xac* that was earlier culturally identified. The primer worked well in distinguishing Xanthomonads from yellow non-xanthomonads commonly isolated from plant, seeds and leaves and from other plant-pathogenic bacteria tested, such as *Erwinia, Pseudomonas, or Ralstonia* strains in a study by Yung *et al.* (2009) [16]. The major contaminant *Pantoea agglomerans* was found to be negatively reacted in polymerase chain reaction.

The above two studies enabled to detect reliably the citrus canker causing pathogen *Xanthomonas axonopodis* pv. *citri* from group of bacterial contamination in an integrated manner. These techniques will be highly useful for pathological studies of *Xanthomonas axonopodis* pv. *citri* in Laboratory conditions and at quarantine stations.

Table 1: Isolates of *Xanthomonas axonopodis*pv. *citri*used in this study

S. No.	Name of the Isolate	Location
1	XAC1	Jubilee Gardens, Dharwad
2	XAC2	Vijayapur
3	XAC3	Vijayapur
4	XAC4	Dharwad
5	XAC5	Bagalkot

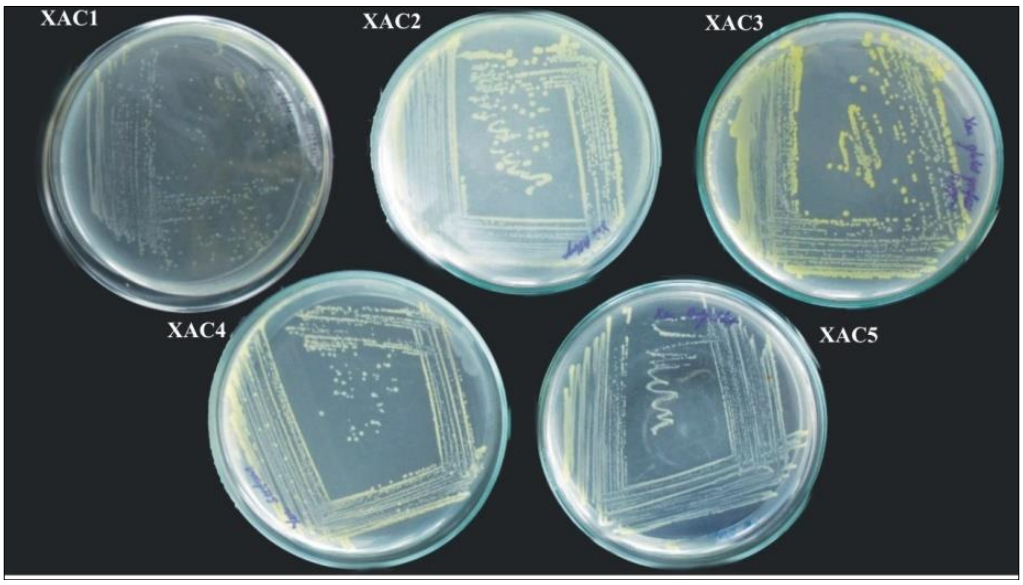

Fig 1: Characteristic colonies of *Xanthomonas axonopodis*pv. *citri*isolates (XAC1, XAC 2, XAC 3, XAC 4 and XAC 5) on nutrient glucose agar media

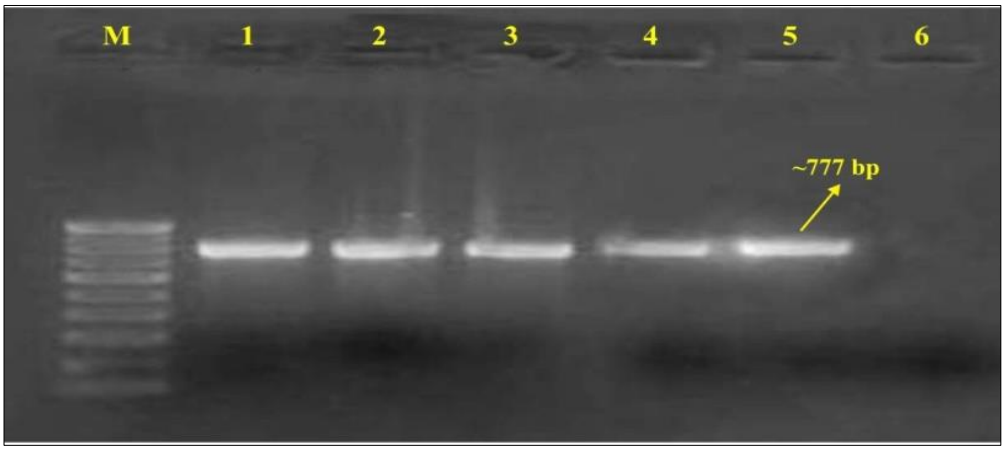

Fig 2: Genomic DNA amplification of *est A* region of *Xac*using *Xc-lip*primer pair Lane(M):DNAmarker (100bp), Lane1,2, 3, 4, 5 :amplified productof fiveisolates XAC1, XAC2, XAC3, XAC4 and XAC5, Lane 6:Buffer control

5. Acknowledgements
Corresponding author is thankful to Dept. of Science and Technology, New Delhi, India for financial assistance in the form of INSPIRE-JRF (professional).

6. References

1. Broadbent P. Perceived vulnerability of citrus to canker in the major citrus growing areas of Australia. Australasian Plant Pathol. 1992; 21:158-162.
2. Chand JN, Pal V. Citrus canker in India and its management. (Cited in: Raychaudhuri, S. P. and Ahlawat, Y. S., Eds) Problems of citrus diseases in India. Surabhi Printers and Publishers, New Delhi, 1982, 21-26.
3. Golmohammadi M, Cubero J, Pealver JM, Quesada JM, Lapez MM, Llop P. Diagnosis of *Xanthomonas axonopodis* pv. *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods. J Appl. Microbio. 2007; 103(6):2309-2315.
4. Goto M. Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego, California, 1992.
5. Mohammadi M, Mirzâee MR, Rahimian H. Physiological and biochemical characteristics of Iranian strains of *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus bacterial canker Disease. J Phytopathol. 2001; 149(2):65-75.
6. Mondal KK, Kumar A. Phenotype based identification of *Xap*. In: Practical Manual on Advances in Phenomics, Genomics and Diagnostics of Xap Causing Bacterial Blight of Pomegranate, 2011, 5-11.
7. Mavrodieva V, Levy L, Gabriel DW. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology. 2004; 94(1):61-68.
8. Nirvan RS. Citrus canker and its control. Hort. Adv., 1961; 5:171-175.
9. Nirvan RS. Citrus canker and its control. *Gardening*, 1963; 4(11):52- 58.
10. Nitish RB, Vinod U, Mamta N. Biochemical characterization of *Xanthomonas axonopodis*pv. *citri*, causal agent of citrus canker. The bioscan. 2014; 9(1):429-431.
11. Stall RE, Seymour CP. Canker: a threat to citrus in the GulfCoast states. Plant Dis. 1983; 67:581-585.
12. Vauterin L, Hoste B, Kersters K, Swings J. Reclassification of Xanthomonas. Int. J Syst. Bacteriol. 1995; 45(3):472-489.
13. Wang Y, Liu JH. Exogenous treatment with salicylic acid attenuates occurrence of citrus canker in susceptible navel orange (*Citrus sinensis*Osbeck). J Plant Physiol. 2012; 169(12):1143-1149.
14. Yin YP, Huang GJ, Zhao Y, Liu H, Wang ZK. Establishment and application of real time fluorescent PCR approaches for detection of *Xanthomonas axonopodis* pv. *citri.* Acta Phytophyl. Sinica. 2007; 34(6):607-613.
15. Yung JM, Park DC, Shearman HM, Fargier E. A multilocus sequence analysis of the genus Xanthomonas. Syst. Appl. Microbiol. 2008; 31:366-377.
16. Yung AL, Ai NS, Tzu FL, Yung SL, Appl. Environ. Microbiol. 2009; 75(21):6831-6838.