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Isolation, pathogenicity and identification of *Xanthomonas citri* pv. *citri* causing citrus canker disease in Gujarat

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Abstract
In India, Citrus is one of the major grown fruit crops after banana and mango which is known for its high nutritive and refreshing value, distinct aroma, delicious taste and also for its medicinal properties. Among all the diseases that attacks citrus crops, citrus canker caused by *Xanthomonas citri* pv. *citri* is one of the most devastating disease and of great economic importance. In present study, bacteria were isolated on Nutrient agar medium from infected leaves and fruits collected from the various places of Gujarat which showed yellow, circular and mucoid colony. Purification of bacteria was done by using streak plate method. Bacteria were identified as Gram negative based on Gram's staining. Pathogenicity test was successfully carried out using syringe inoculation method. The initial symptoms appeared on leaves in the form of lesions as dark green and later become thickened brown and corky which become raised and blister-like, growing into white or yellow spongy pustules. The pathogen was identified through Indian Type Culture Collection (ITCC), ICAR-Indian Agricultural Research Institute (IARI), New Delhi as well as by marker assisted identification using specific primers XACF and XACR. From the results of identification, the pathogen was identified as a *Xanthomonas citri* pv. *citri*.

Keywords: *Xanthomonas citr*i pv. *citri*, Nutrient agar media, Nutrient broth

Introduction
The genus Citrus is one of the most important group of fruit crops worldwide, belongs to the family *Rutaceae* comprising 140 genera and 1300 species distributed throughout the world (Saunt, 1990, Savita *et al.*, 2012) [28, 29]. Among the various fungal, bacterial and viral diseases, few diseases in any citrus growing areas cause significant damage and require due attention for their effective management. Citrus canker is one of the most feared of citrus diseases, affecting all types of important citrus crops. The disease causes extensive damage to citrus and severity of this infection varies with different species and varieties and prevailing climatic conditions (Pitino *et al*., 2015) [24]. The disease is endemic in India, Japan and other South- East Asian countries, from where it has spread to all other citrus producing continents. In India, citrus canker was first reported from Punjab (Luthra and Sattar, 1942) [18]. Then its occurrence was further recorded in Assam (Chowdhury, 1951) [7], Tamil Nadu (Ramakrishnan, 1954) [26], Andhra Pradesh (Govinda Rao, 1954) [12], Karnataka (Venkatakrishnaiah, 1957, Aiyappa, 1958) [32, 1], Rajasthan (Prasad, 1959) [25], Madhya Pradesh (Parsai, 1959) [23], Uttar Pradesh (Nirvan, 1960) [21] and several others have reported the incidence of canker from different state. The causative pathogenic bacterium was first isolated by Hasse in 1915 from diseased samples of Florida, Texas and Mississippi and he named it *Pseudomonas citri* following its characterization and pathogenicity in citrus (Hasse, 1915) [13]. Subsequently it was placed under different genera by different workers and in 1939 it was named as *Xanthomonas citri* (Doidge, 1916, Dowson, 1939) [10, 11]. *X. citri* was divided into different strains where strain A was assigned to those associated with Asiatic citrus canker, B to strains with wider host range and strains C to those causing canker only in Key lime (*Citrus aurantifolia*) (Namekata and Oliveira, 1972, Rossetti, 1977) [20 27]. In 1978 the bacterium was again placed in *X. campestris* pv. *citri* to preserve *citri* at the intra-sub-specific level (Young *et al*., 1978) [33]. Citrus canker caused by bacterial pathogen, *Xanthomonas citri* pv. *citri* of *Xanthomonadaceae* family is rod-shaped measuring 1.5- 2.0 × 0.5-0.75 µm, Gram-negative and has a single polar flagellum with obligate nature (Das, 2003) [8]. Colonies on culture media are usually yellow due to the production of xanthomonadin pigment.

The diseased plants were characterized by the occurrence of conspicuous raised necrotic lesions that develop on leaves, twigs and fruits. First appearance is as oily looking 2-10 mm circular spots which become raised and blister-like, growing into white or yellow spongy pustules. These pustules then darken and thicken into a light tan to brown corky canker, which is rough to the touch. Often a water-soaked margin develops around the necrotic tissue and easily viewed with transmitted light. Sunken centres are especially noticeable on fruits. Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop. (Chand and Pal, 1982) [6]. Naturally, bacterial infection is elevated by wounds or mechanical damages and is disseminated by wind and rainfall splashes which assist infection much faster. Citrus leaf miner is also one of the vital aspects causing infestation of citrus plants and indirectly raises the disease to many fold (Kumar *et al*., 2019) [17].

Considering the importance of the pathogen, this experiment was taken to isolate, prove the pathogenicity and identify the pathogen *Xanthomonas citri* pv. *citri* causing citrus canker disease.

Materials and Methods

Collection of samples

The infected plant parts like leaves, twigs and fruits showing typical symptoms of citrus canker were collected from the various places of Gujarat and brought to the laboratory for microscopic examination followed by isolation.

Isolation and purification of pathogen

Small pieces of canker infected tissues like leaves, twigs and fruit pericarp were cut with the help of sterile surgical blade along with healthy tissue and surface sterilized with 1 percent sodium hypochlorite disinfectant. The diseased samples were placed in distilled water for 6 hours. Direct streaking was carried out by taking a sterilized wire loop full of bacterial suspension and streaked out on nutrient agar (NA) medium. The inoculated plates were incubated at 28 ± 1 °C in incubator for 24 hours.

The single purified colonies of that bacterium were streaked with the help of a sterilized wire loop to Nutrient agar slants and were incubated at 27 °C for 2 days. Pure cultures of each isolate were kept in refrigerator for further use.

Table 1: Isolates of citrus canker collected from different region of Gujarat

Sr. No.	Name of isolates	Place of collection	Affected plant parts collected
1	Xcc1	Kheralu	Leaf, Fruit
2	Xcc2	Modasa	Leaf, Fruit
3	Xcc3	Himmatnagar	Leaf
4	Xcc4	Dhoraji	Leaf, Fruit
5	Xcc5	Arvalli	Leaf, Fruit
6	Xcc6	Vadali	Leaf
7	Xcc7	Morbi	Leaf
8	Xcc8	Kadi	Leaf
9	Xcc9	Kheda	Leaf
10	Xcc10	Rajkot	Leaf, Fruit
11	Xcc11	Junagadh	Leaf
12	Xcc12	Anand	Leaf, Fruit
13	Xcc13	Bayad	Leaf, Fruit
14	Xcc14	Halol	Leaf
15	Xcc15	Ider	Leaf

Morphological characterization of bacteria

Gram staining

Gram staining was performed on each isolate by taking crystal violet, Gram's iodine, decolorizes (alcohol) and stain safranin (Aneja, 2007) [2]. Bacterial sample was smeared on a slide and carefully fixed by heat. One drop of crystal violet was placed on smears and holds it for 1 minutes and rinsed with distilled water. One drop of Gram's iodine was placed on smears for 1 minutes and rinsed with distilled water. Added decolorize reagents on the sample for 30 seconds. The specimen was counter stained with safranin for 30 seconds, washed and air dried for several minutes. The slide was observed under 100X microscope along with one drop of immersion oil to examine shape, arrangement and staining reaction of bacterial isolates.

Motility test

Motility test of bacterial isolates was carried out by Agar stabbing method described by Islam *et al.* (2017) [15]. Each isolated colony was picked from the culture and inoculated separately by stabbing the center of the nutrient agar media to a depth of 1 inch.

Pathogenicity

The seedlings of acid lime were sufficiently watered and exposed to sunlight. To prove the pathogenicity, bacterial suspension of Anand isolate was prepared in nutrient broth (200 ml) in conical flasks by inoculating a loopful of bacterial culture. The inoculated flask was incubated for three days at 28±2 °C. Inoculation was done by syringe inoculation method. The plants were maintained under humid condition. Seedlings were regularly examined for symptoms development after inoculation. The uninoculated seedling were placed as a control.

Identification

Pure culture of re-isolated bacteria was submitted to Indian Type Culture Collection (ITCC), ICAR-Indian Agricultural Research Institute (IARI), New Delhi for identification. Alternatively, genetic identification is progressively used to identify the pathogen, so the PCR assay using a primer set XACF and XACR designed from the sequence of hrpW gene of *Xanthomonas axonopodis* pv. *citri* would be used for the detection and identification of *Xanthomonas* spp. causing citrus canker (Park *et al*., 2006) [22].

Results and Discussions

Isolation of bacteria from diseased specimens: Bacterial pathogen *Xanthomonas* spp. was isolated on Nutrient agar medium from infected leaves and fruits collected from various places of Gujarat and incubated at 28 ℃. After 24 hrs of incubation, yellow, smooth, convex and circular colonies were observed which became somewhat irregular after 72 hrs due to viscous fluid secreted by the bacteria.

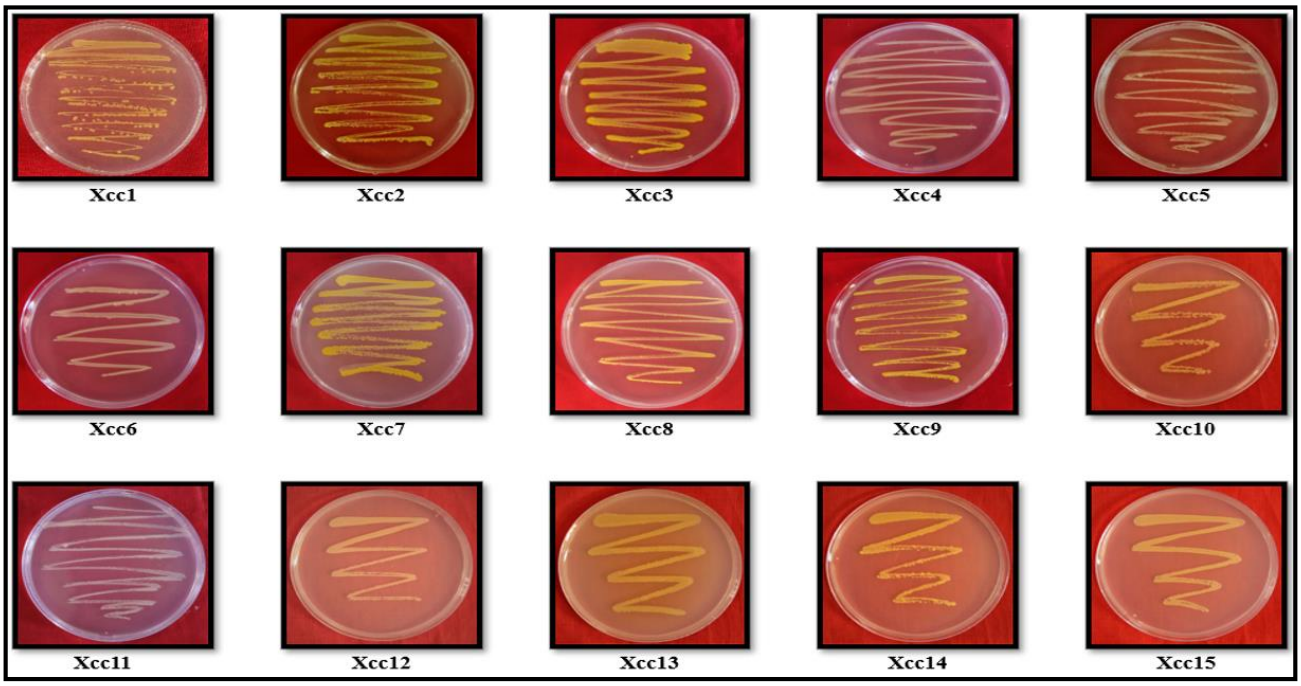

Plate 1: Pure culture of different isolates collected from various places of Gujarat

Sun *et al*. (2004) [30], Islam *et al*. (2014) [14] and Daungfu *et al*. (2019) [9] isolated bacteria from collected diseased samples on NA media and observed yellow, smooth, convex and circular colonies of bacteria.

Gram staining: All the bacterial isolates were failed to retain violet colour of the primary stain (crystal violet) and showed reddish pink colour which confirmed the Gram-negative characteristics of the isolated bacteria.

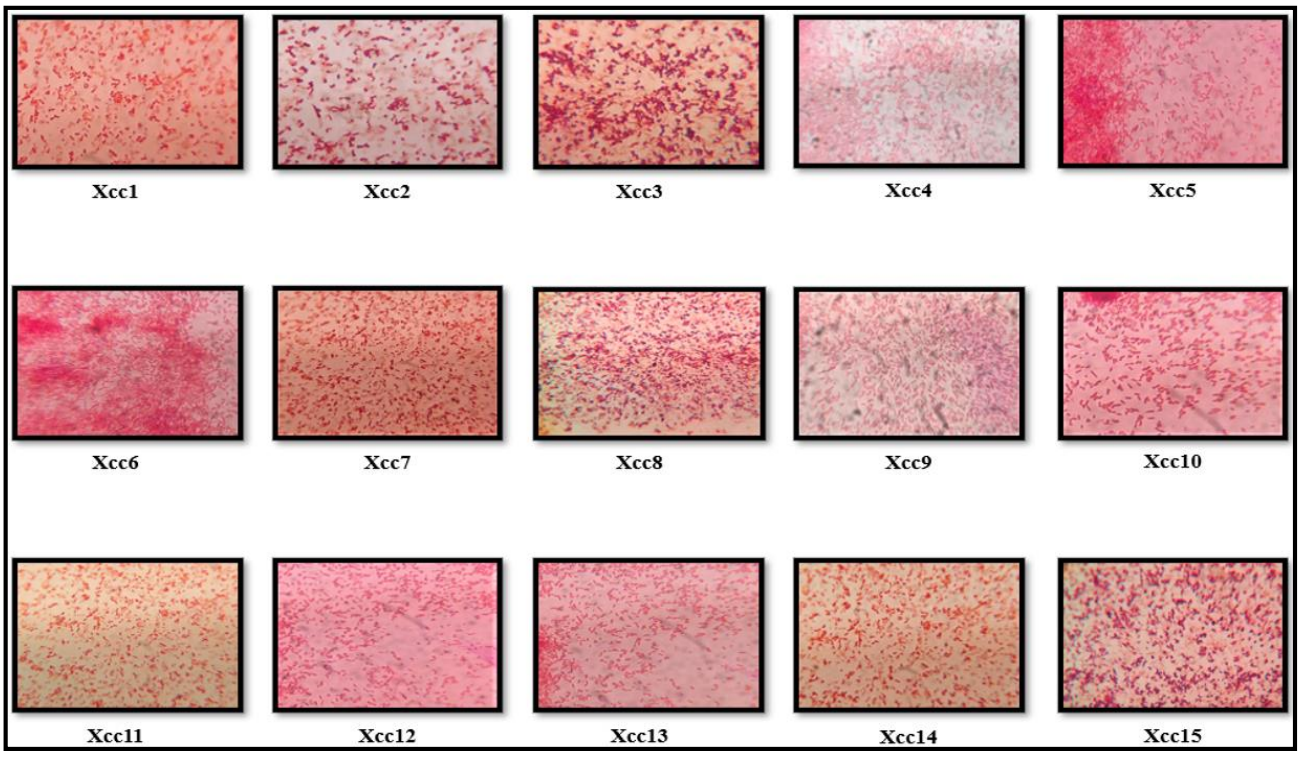

Plate 2: Photomicrograph showing morphological characters of *X. citri* pv. *citri* by Gram staining method

Motility test: Inoculated slants showed slight yellow colour which indicate the positive reaction of this test in all the isolates. Bacteria *Xanthomonas* spp. having a single flagellum which helps them to grow throughout the media.

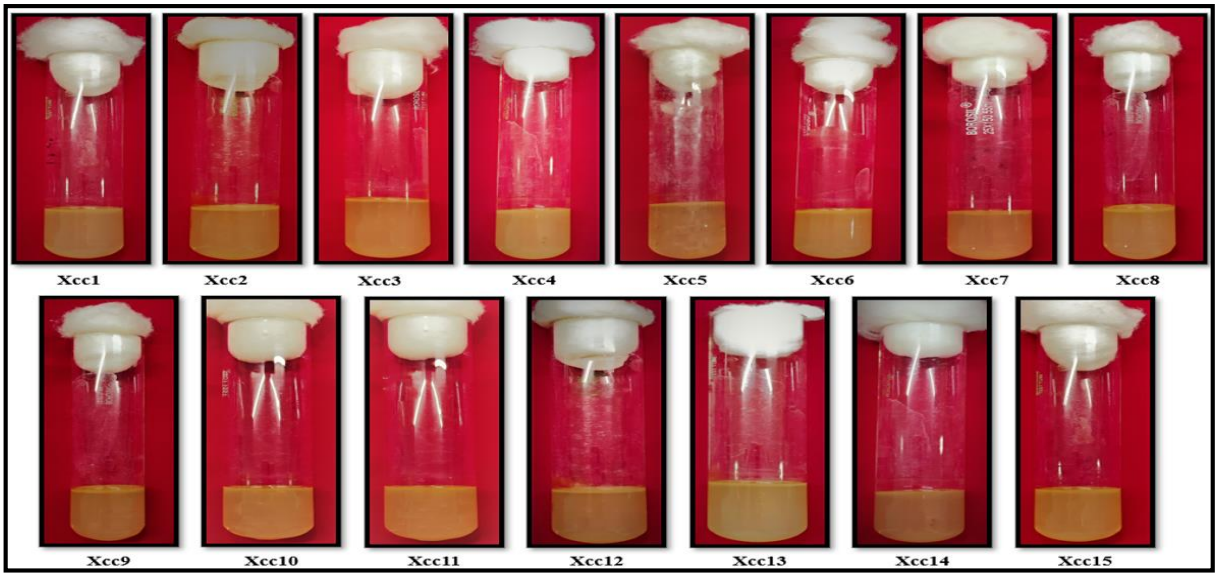

Plate 3: Reaction showing motility test on isolates of *X. citri* pv. *citri*

Morphological characteristics were recorded by performing Gram staining and motility test were consistent with the results reported by Suryawanshi *et al.* (2011) [31] and Arshiya *et al*. (2014) [4].

Pathogenicity

Seedlings inoculated with bacterial pathogen *Xanthomonas* spp. exhibited typical symptoms after 14 days of inoculation. Initially, these may appear water-soaked or oily at the edges. The symptoms produced on the artificially inoculated seedlings were identical to those observed in the field. Uninoculated seedlings were not showed any symptoms of canker and remains healthy.

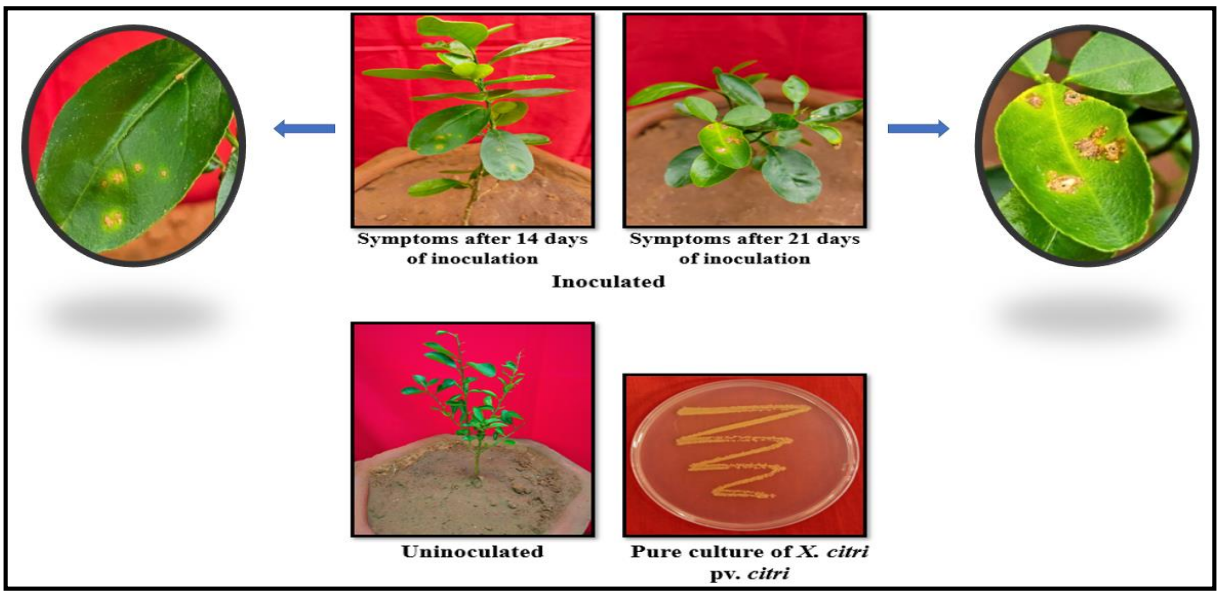

Plate 4: Pathogenicity test of *X. citri* pv. *citri* on citrus seedlings

The pathogenicity test of these pathogen is confirmed with the findings of various scientists *viz.,* Arshiya *et al.* (2012) [3], Bhardwaj *et al.* (2014) [5], Pitino *et al.* (2015) [24], Khan *et al.* (2018) [16] and Manyam and Nargund (2020) [19].

Identification

The identification of pathogen was done through Indian Type Culture Collection (ITCC) (Ref. no. PP/1944), ICAR-Indian Agricultural Research Institute (IARI), New Delhi as well as by marker assisted identification by specific primers. In this investigation, the ITS region of the bacteria was sequenced and resulted strain was identified as *Xanthomonas citri* pv. *citri.*

Conclusions

The bacteria causing citrus canker were isolated from affected leaves and fruits collected from various places of Gujarat on Nutrient agar media which produce smooth, yellow convex, mucoid and circular colony after 24 hrs of incubation. The bacteria were found Gram negative based on morphological characterization. The pathogenicity test of Anand isolate was successfully conducted on citrus seedlings using syringe method which produced clear symptoms after 21 days of inoculation. Isolated bacterium was identified as *Xanthomonas citri* pv. *citri* through Indian Type Culture Collection (ITCC) (Ref. no. PP/1944), ICAR-Indian Agricultural Research Institute (IARI), New Delhi as well as

by marker assisted identification using ITS region of the bacteria (GenBank Accession No. MZ284949.1).

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Conflict of interest: The author declare that they have no conflict of interest.

Statement on human and animal rights: This article does not contain any studies with human participants or animals performed by any of the authors.

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