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Isolation and Screening of Canker-Causing Bacteria in Citrus limetta: A Morphological, Cultural, and Biochemical Approach

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A B S T R A C T

The present investigation was designed to isolate and screen bacteria in Citrus limetta canker disease. Bacteria were isolated on YDCA medium from infected leaves and fruits collected from various places in the Beed district of Maharashtra State which showed yellow, circular, and mucoid colonies. Purification of bacteria was done by using the streak plate method. Bacteria were identified as Gram-negative based on Gram's staining. Biochemical tests and Cultural characteristics viz., colony shape, margin, elevations, and pigmentation help to identification of bacteria. The isolated bacterium was found to be gram-negative, small in size, rod-shaped, motile, and pink in color. The biochemical tests showed catalase, oxidase, indole, MRVP-negative, cellulase, and nitrate reduction tests were positive. The present study would be helpful for the biological control of canker disease.

Introduction

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 attack citrus crops, citrus canker is caused by Xanthomonas citri pv. citri is one of the most devastating diseases and has great economic importance. 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).

Citrus canker caused by bacterial pathogen, Xanthomonas citri pv. citri of the 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). 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).

Considering the importance of the pathogen, this experiment was taken to isolate, screen, and biochemical characterization to identify the pathogen Xanthomonas citri pv. citri causing citrus canker disease. Therefore, the main objective of this study was to characterize the causal agent of canker disease and establish a suitable biological control method. Confirmation of bacterium is very important for the application of right management practices.

Materials and Methods

Plant materials

Infected plant parts showing typical symptoms of bacterial canker of acid lime were collected from the different fields of Beed districts, where acid lime fields were infected with bacterial canker. Bacterial canker samples were collected separately in polyethylene bags and labelled. The different parts of the acid lime plant showing characteristic symptoms of bacterial canker infection were collected and subjected to isolation by following the method given by Schaad (1992). The infected plant parts like leaves and fruits showing typical symptoms of citrus canker were collected.

Isolation and culture of bacteria

Small pieces of canker-infected tissues like leaves, twigs, and fruit pericarp were cut with the help of a sterile surgical blade 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 streaking out on a Yeast extract-dextrose-CaCO3 (YDCA) medium. The inoculated plates were incubated at 28 ± 1 °C in an incubator for 24 hours.

After incubation yellow colony will be selected and transferred on a slant medium to obtain the isolate in pure form on YDCA Medium and were incubated at 27 °C for 2 days. Pure cultures of each isolate were kept in the refrigerator for further use.

Morphological and biochemical characterization

Gram staining

The bacterial sample was smeared on a slide and carefully fixed by heat. One drop of crystal violet was placed on the smear held for 1 minute and rinsed with distilled water. One drop of Gram's iodine was placed on a smear for 1 minute and rinsed with distilled water. Added decolorized reagents to 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 a 100X microscope along with one drop of immersion oil.

Kovac's oxidase test

A sterilized loop picked a well-isolated single colony for Kovac's oxidase test. Then the inoculum was smeared over the centre of the filter paper containing a single drop of 1% Kovac's oxidase reagent. The change of color was obtained within 10 seconds (Mubeen *et al.,* 2015a).

Methyl Red and Voges Proskauer Test

Test tubes containing the MR-VP broth were inoculated with the test bacterium and incubated at 28°C for 48 hours. Five drops of methyl red indicator were added to the first set of tubes. The tubes were observed for any color change, indicating a positive or negative reaction.To the second set of tubes, 12 drops of V-P reagent 1 and 2-3 drops of V-P reagent 2 were added. The tubes were shaken for 30 seconds with the caps off to expose the media to oxygen. The tubes were left to react for 15-30 minutes. Observations were recorded for any color change, indicating a positive or negative reaction (Verniere *et al.,* 1998)

Catalase test

A loop full of 24-28 hours old culture of test bacterium was placed on the clean glass slide, and to this, a drop of 3 % hydrogen peroxide (H_2O_2) was mixed and allowed

to react for a few minutes and observed for the production of gas bubbles**.**

Starch Hydrolysis Test

Starch hydrolysis was tested for isolated strains to determine the capability of using carbon sources like starch as described by De Oliverira (2007).

The medium was inoculated with bacterial strains in signal Petri plate four bacteria were steak with the help of lop and incubated at 25±2°C for 172 hours. After full growth of the deferent bacterial strains, the drop of the Iodine was applied on the surface and again incubated at 25±2°C for 24 hours. A color change was observed.

Nitrate Reduction Test

The test medium was prepared by using Peptone 5g, NaCl 2.5g, KNO3 (nitrate-free) 1g, ager 1.5g and distil water 500ml. Dispense the medium into the test tubes to a depth of a few cm and autoclave. Inoculated the sterilized medium tubes with test bacterium XC1 and incubated at 27 C.

After 15 days few drops of sulfanilic acid (0.8% in 5N acetic) and dimethyl alpha naphthylamine (0.5% in 5N acetic acid), was added to check the nitrate reduction (Lelliott and Stead, 1987).

Cellulose hydrolysis

Bacterial isolates were individually inoculated on CMC agar plates and incubated for 2 days. The plates were flooded with 0.1% Congo red for 20 min and washed with 1 M NaCl for 15 min. The clear zone formed by the isolates indicated their cellulase activity.

Results and Discussion

Isolation of bacteria from diseased specimens

Bacterial pathogen Xanthomonas spp. was isolated on YDCA Medium from infected leaves and fruits (Fig 1a) collected from various places of Beed 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. This result showed YDCA positive (Fig 1b). Visual observation of Morpho-cultural characters, viz., cell shape, flagellation, colony edge, elevation, pigmentation and surface appearance. (Table 1 and Fig 1b).

Gram staining

The isolate XC1 failed to retain the violet color of the primary stain (crystal violet) and showed reddish-pink colour which confirmed the Gram-negative characteristics of the isolated bacteria (Fig. 1c)

Biochemical characteristics of isolated bacteria

Kovac's oxidase test

The principal of Kovac's oxidase test is based on the presence of the cytochrome oxide which is a feature of saprophytic bacteriaIn Kovac's test, the medium containing filter paper and oxidizing agent reagent did not produce any color (Table 2 and Fig 2a).

Isolate (XC1) of our study was oxidase negative.

Methyl Red and Voges Proskauer Test

In the MR test, the test bacteria were grown in a broth medium containing glucose and the color of methyl red not changed from yellow to red. Bacteria showed negative results against Methyl Red test.XC1 did not produce enough acid to change the pH significantly.

A lack of color change after adding VP reagents indicates a negative result of XC1. So, VP test is negative.

Catalase test

Catalase mediates the breakdown of hydrogen peroxide H2O2 into oxygen and water. To find out if a particular bacterial isolate was able to produce catalase enzyme, a small inoculum of bacterial isolates was mixed into hydrogen peroxide solution (3%) and was observed for the rapid elaboration of oxygen bubbles.

The lack of catalase was evident by a lack of or weak bubble production. Catalytic activity of XC1 Isolate was found negative when the culture did not produce bubbles of oxygen within one minute after the addition of H2O2. (Table 2 and Fig 2b).

Table.1 Cultural and morphological characteristics of the isolate on YDCA Medium

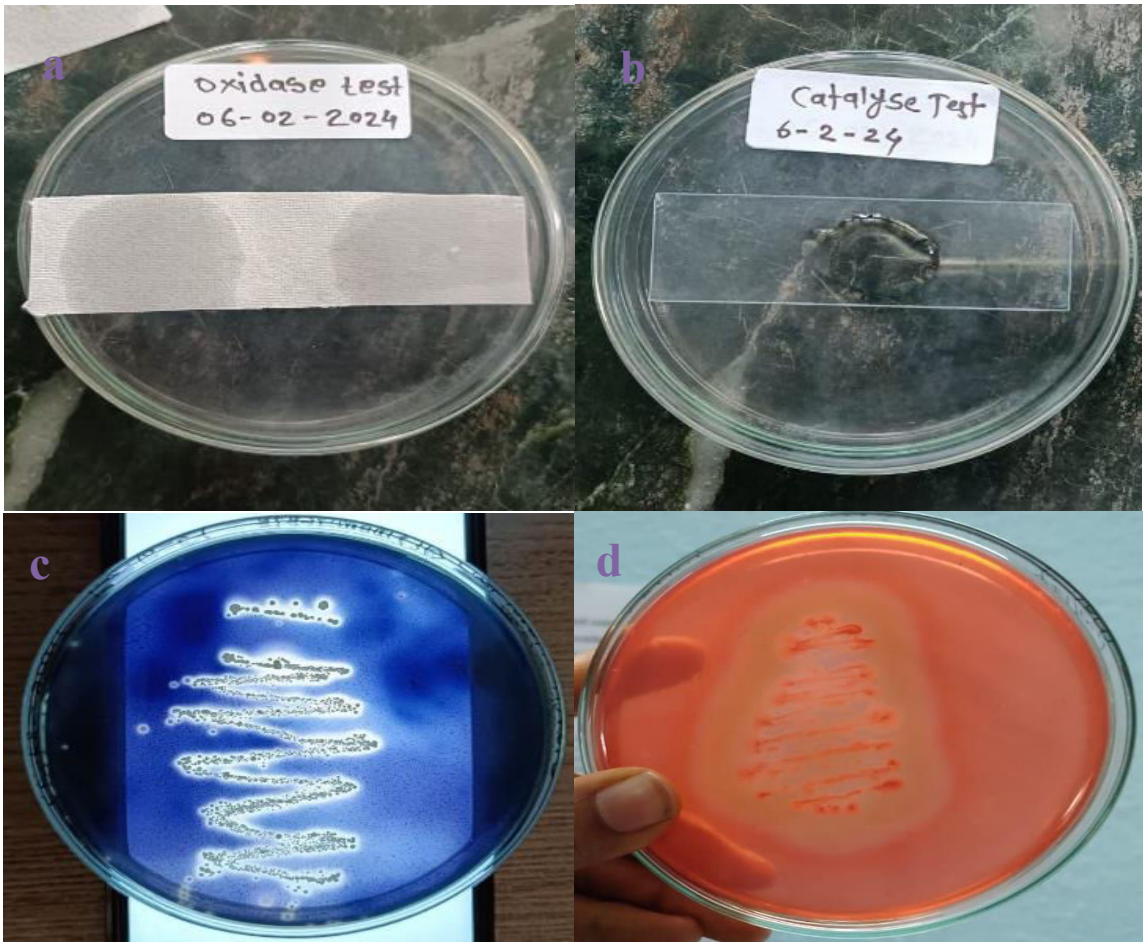
Name of isolate	**XC1**
Pigmentation	Yellow
Colony shape	Filiform
Elevation	Convex
Margin	Entire margin
Texture	Smooth
Gram's nature	Gram-negative

Fig.1 Showing the plant sample, causal organism isolation and gram staining (a) Bacterial Canker disease of Citrus, (b) Bacterial colony (creamy) of isolated Xanthomonas on YDCA Medium, (c) Gram-negative bacteria

Table.2 Biochemical characterizations of bacterial XC1 strain

Sr. No.	Name of Test	Result
1	Gram staining	Negative
2	Kovac's oxidase test	Negative
3	Methyl Red and Voges Proskauer Test (MRVP)	Negative
4	Catalase test	Negative
5	Starch Hydrolysis Test	Positive
6	Nitrate Reduction Test	Positive
7	Cellulose hydrolysis	Negative

Fig.2 Photographs showing biochemical tests of XC1 isolate **(a)** Kovac's oxidase test (b) Catalase test (c) Starch Hydrolysis Test (d) Cellulose hydrolysis



Starch Hydrolysis Test

Results presented in Table 2 and photograph shown in Fig 2 the test bacterium produced colourless zone around bacterial growth on starch agar medium flooded with iodine and showed positive for starch hydrolysis test. Similar results were also reported earlier by many workers (Al – saleh *et al.,* 2014; Mubeen *et al.,* 2015; Katkar *et al.,* 2016; Abhang *et al.,* 2018; Bhure *et al.,* 2019)

Nitrate Reduction Test

After 15 days few drops of sulfanilic acid (0.8% in 5N acetic) and dimethyl alpha naphthylamine (0.5% in 5N acetic acid), were added to check the nitrate reduction in culture media of XC1 isolate. Isolate XC1 was able to reduce the nitrate because the bacterium was able to produce pink or red color indicating that the XC1 was positive to nitrate reduction test (Table 2 and Fig 2c).

Cellulose hydrolysis

A cellulase activity test measures the rate at which an enzyme, cellulase, breaks down cellulose into smaller sugar molecules, typically quantified by measuring the amount of reducing sugars released from a cellulose substrate like carboxymethyl cellulose (CMC) or filter paper. The isolated bacterium XC1 forms a clear zone indicating that bacterium has ability for cellulose production (Table 2 and Fig 2d).

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Author Contributions

Suresh V. Dhere: Investigation, formal analysis, writing—original draft. Sarjerao B. Mali: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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