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Biochemical characterization of *Xanthomonas axonopodis* pv. *citri*; A major impediment to Citrus orchards

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Abstract

Citrus is a significant crop playing an important role in Pakistan's economy. However, its production is under constant threat by numerous pathogens resulting in considerable economic losses. Bacterial canker caused by *Xanthomonas axonopodis* pv. *citri* is a major limiting factor in the successful production of citrus. Isolates of the pathogen were collected from infected citrus orchard of Bahawalpur region. Bacterial pathogen was characterized by morphological evaluation, pathogenicity and biochemical tests including gram staining, catalase test, KOH test, simmons citerate test, starch hydrolysis test, methyl red and voges proskauer test, gelatin liquefaction test, casein hydrolysis test, kovacs's oxidase test, nitrate reduction test and tween 80 hydrolysis test were performed which confirmed that citrus canker is caused by *Xanthomonas axonopodis* pv *citri*.

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Introduction

Citrus (*Citrus sinensis*) ranked the third most significant crop of the world (Sing, 2001). In Pakistan, its total production is 2267447 thousand tonnes and cultivated area is 195052 hectares (FAO, 2017). Citrus is delectable, juicy and seedless fruit having abundant nutritional importance (khan *et al*., 1922). Several abiotic and biotic factors influence its yield throughout the citrus-producing regions of the world (Burhan *et al*., 2007). Citrus is attacked by numerous diseases such as citrus canker, gummosis, CTV, citrus decline and greening. Canker is the most devastating disease of citrus caused by *Xanthomonas campestris* pv. *citri* (Sahi *et al*., 2007). It is the most destructive disease of citrus including Asia, Africa, Australia, South America and USA (Graham and Gottwald, 1991). The disease was first reported from Punjab in Pakistan and then spread in all citrus-growing areas as a major problem of the country. The pathogen has wide host range to infect almost all species and cultivars of citrus. Sour orange shows some resistance to pathogen while Grapefruits and sweet orange are susceptible (Civerolo, 1984).

The bacterium is rod-shaped, gram negative having a single polar flagellum and grows as an obligatory aerobic pathogen (Whiteside *et al*., 1988). Symptoms of the disease include lesions that developed on young leaves, fruits and twigs. At initial stage these lesions are round, small, light green and slightly raised spots. After that, colour changes to greyish white, appear corky and ruptured with brown sunken centre and edges encircled by yellowish holes. Diameter of the lesions ranges from 1 to 9-millimeter on leaves and upto 1cm on fruits and twigs. Highly infected plants produced fruit is deformed and scabbed (Agrios, 2005). Symptoms, type and severity based on the kind of cultivar, age of the plant, environmental conditions and type of the pathogen prevalent in a geographic area. Pathogen produced lesion depending on the level of resistance of the cultivar and lesion size (Graham *et al*., 1992). Bacterium Xanthomonas has cosmopolitan occurrence, various aspect of the disease is studied in different areas of the world to manage the disease (Akhtar *et al*., 1996). Proper identification and characterization of the pathogen strain through biochemical tests is useful for the management of the bacterium that causes the significant losses of the yield in terms of quality and quantity (Khalid *et al*., 2010). However, the objective of our study was to characterize the Xanthomonas isolates on the basis of morphological, biochemical and pathogenicity tests. For this purpose, the samples were collected from different citrus orchards of Bahawalpur division.

Materials and methods

Diseased leaf samples of lime citrus having typical lesion surrounded with a yellow hole were collected based on the symptomology, from the citrus orchard. The Islamia University of Bahawalpur and from the citrus orchard of Yazman. Three samples were collected from each orchard in plastic envelops and brought in the laboratory for further processing. Small pieces of 5mm of samples were cut and surface sterilized by using 70% ethanol (10ml of 70% preparing using 7ml of ethanol and 3ml of water using micropipette) in an Eppendorf tube and subsequently washed with other three Eppendorf tubes containing distilled water. Then take another Eppendorf tube place the sample in it and chopped to make fine suspension of the sample. The resulting suspension was streaked on Petri plate contain YPGA media (yeast extract 3.5g, Peptone 3.5, Glucose 3.5, Agar 9g, Cephalexin 0.5microliter, Propiconazole 40 microliter, Kasugamycin 0.5microliter, distil water 500 ml, NaOH 1.5ml) and at 28°C for 24 hours petri plates were incubated. Purified colonies of the pathogen were streaked on a nutrient slant and incubated at 28°C. One slant was preserved at 4°C in the refrigerator for further use.

Biochemical characterization

Biochemical characters of the bacterium were studied using the bacterial isolates to various biochemical test viz. Gram staining, KOH,H_2O_2, Starch hydrolysis, Simmons citrate test, Methyl red test, Vogues Proskauer's, Gelatin liquefication test, Kovac's oxidase test, Casein hydrolysis test, Tween 80-hydrolysis test and Pathogenicity test.

Gram Staining

Gram reagent was made using crystal violet, Lugoliodine, Acetone and counterstain of Safranin. Isolates were made by taking pure culture with the help of sterilized needle and make smear on clear slide with distilled water. The drop of crystal violet was placed, smear on and kept for 30 seconds followed by washing with sterilized water. Afterwards drop of Lugol's iodine was placed by following the washing with DAW and then permanently washed with pure acetone. Finally a drop of safranin was added, kept for 30 seconds and washed with water. The mounts were dried using blotter paper, a drop of Canada balsam was placed on the stained and observed under microscope at 100X.

KOH Test

3% potassium hydroxide was dropped on the glass slide and 24-hour old culture was mixed with a sterilized needle and stirred for a few seconds and observed for slim threads. When needle raised if the loop is seen then the bacterium is gram-negative. (Ryu, 1940).

Catalase Test

24 hours old fresh culture was settled on the clean glass slide and mixed with a drop of 3% hydrogen peroxide and allowed to react for a few minutes and observed for bubbles formation (Lelliott and Stead, 1987).

Starch Hydrolysis Test

Starch agar medium was prepared (Starch of potato is used) and pour into the Petri plate and allow them to solidify and test bacterium was streaked and incubated for 96 hours at 28°C in inverted position. After the incubation period, iodine solution was flooded on the surface of plates with a dropper for 30 seconds and observed for colour change of medium around the line of the growth, (Fahy and Persley, 1983).

Simmons citrate Test

Simmons citrate agar was prepared by using the ammonium dihydrogen phosphate 1g, dipotassium phosphate 1g, Sodium chloride 5g, magnesium sulfate 0.2g, agar 15g, bromothymol blue 0.8g, distil water 1000ml and excluding phosphate which was dissolved separately in 100ml of H_2O. Mixed these to make the volume of 1000ml and maintain pH to 6.9 then autoclaved then poured it in plates for solidification. Streaked the bacterium on it and incubate for 28hours at 28°C and observed the coloration and growth of the culture (Simmons, 1926).

Methyl Red and Voges Proskauer Test

MR-VP broth was prepared by using the components; Peptone 7g, dextrose 5g, Potassium phosphate 5g, distil water 1000ml. Pour 5ml broth in each test tube and autoclave them. Incubate the test tubes inoculated with test bacteriumat 28°C for 48 hours and make two sets of tubes. In one set of tubes, added 5 drops of methyl red indicator. Observe the colour change. Add 12 drops of V-P reagent 1 and 2-3 drops of V-P reagent 2 to the tubes of set2. For 30 seconds, shake the tubes with the caps off to expose the media to oxygen. The reaction takes 15-30 minutes to complete. Observe the colour change. (Verniere *et al.*, 1998)

Gelatin liquefication Test

The medium was prepared (Beef extract 1.5g, Peptone 2.5g, Gelatin 60g in 500ml distilled water) was poured into 5ml test tubes, plugged and autoclaved. Test tubes were inoculated with fresh bacterium culture of 24-hours old and incubated at 27°C. After 72 hours the tubes were placed at 4°C for 25 to 30 mints prior to the recording of the result. The same procedure was continued after 7, 14 and 21 days. Test tubes were gently tipped the gelatin flowed easily which indicates hydrolysis had taken place by the bacterium otherwise unhydrolyzed gelatin was unable to flow (Cowen, 1974)

Casein Hydrolysis Test

Prepared casein hydrolysis medium by using skim milk powder 50g, peptone 2.5g, agar 7.5g in distil water maintain pH at 7.2 and autoclaved the solution. Poured the plates with the medium allowed it to solidify. After that, streaked the 24hours old culture bacterium on the medium and incubated for 48 hours in an inverted position and check for clear zone along the line around the growth of bacterium (Verniere *et al.*, 1998)

Kovac's oxidase test

1% Kovac reagent was poured in the middle of Whatman filter paper No.1 and used fresh bacterium culture for the test and platinum needle was used to gently rubbed the bacterium on filter paper. After that results observed within 30 to 60 seconds (Kovac, 1956).

Nitrate reduction Test

The test medium was prepared by using Peptone 5g, NaCl 2.5g, KNO_3 (nitrate-free) 1g, ager 1.5g and distil water 500ml. Dispense the medium into the test tubes to a depth of few cm and autoclave. Inoculate the sterilized medium tubes with test bacterium and incubate at 27°C. After 15 days added few drops of sulfanilic acid (0.8% in 5N acetic) and dimethyl alpha naphthylamine (0.5% in 5N acetic acid), to check the nitrate reduction (Lelliott and Stead, 1987).

Tween 80-Hydrolysis Test

Tween 80-hydrolysis test (NaCl 2.5g, $CaCl_2.2H_2O$ 0.50g, peptone 5g, 7.5g agar and 500ml distilled water) autoclave the media by adding the 1% Tween 80 to this molten medium and poured it into plates. After solidification of media we streaked the bacterium and incubate the plates at 27°C for seven days and observed the results (Sierra, 1957).

Pathogenicity Test

Pathogenicity test is the baseline to confirm the pathogen. For this purpose, a test was conducted on the lime citrus to inoculate with freshly purified isolates of the Xanthomonas bacterium to confirm the pathogen. All isolates of the bacterium were scraped off from 24 hours streaked culture on ager medium and suspended into sterile distil water for inoculation into citrus. Leaves were inoculated with bacterium suspension, injected by applying pressure. The symptom was observed after seven days and then 14 days.

Result and discussion

Cultural characteristic includes colony, shape, margin, size, elevation and pigmentation of 6 isolates (3 samples collected from one orchard) were studied by using YPGA as a basel cultural medium. Biochemical characters *Xanthomonas campestris* p.v *citri* was studied by subjecting following different biochemical test. In gram staining results indicated the pathogen is negative bacteria. The unique opaque yellow colour colonies were obtained in the medium. The yellow colour was due to the production of Xanthin produced by the genus Xanthomonas. Colony colour of the bacterium was pale yellow to yellow while the shape and size of the colony were medium, convex and mucoid.

Table 1. Morphological characteristic of Xanthomonas Colonies.

Isolates	Location	Shape	Size	Colour	Elevation	Margin	Surface
Xac1	IUB	Rod	Medium	Yellow	Convex	Even	Mucoid
Xac2	IUB	Rod	Medium	Yellow	Convex	Even	Mucoid
Xac3	IUB	Rod	Medium	Pale yellow	Convex	Even	Mucoid
Xac4	Yazman	Rod	Medium	Yellow	Convex	Even	Mucoid
Xac5	Yazman	Rod	Medium	Pale yellow	Convex	Even	Mucoid
Xac6	Yazman	Rod	Medium	Pale yellow	Convex	Even	Mucoid

In KOH's test, the formation of the loop was an indication that the bacterium was gram-negative because it has fragile cell wall-bounded with outer membrane due to the disrupted exposure to 3% KOH releasing viscous DNA. The present study revealed that bacterium shows a positive reaction. Catalase test shows the production of bubbles when pathogen mixed with hydrogen peroxide which revealed that the bacterium is catalase positive. Bacterium produced a clear colourless zone around bacterial growth on starch agar medium when Lugol's iodine solution applied to it after 96 hours. It indicates the pathogen was starch hydrolysis positive and break the exoenzyme amylase into maltose, glucose etc. Simmons citrate test used to check the ability of the bacterium to utilize citrate as the sole carbon source. It depends on the presence of enzyme citrate that disintegrates the citrate into acetic acid and oxaloacetic acid. After that, change into pyruvic acid and carbon dioxide. Colour changes from green to blue shows that the result of the test was positive to the bacterium. The bacterium was negative to methyl

red and Voges- Proskauer tests. It was used to test the bacterium either facultative anaerobic or enteric bacteria indicate that the pathogen produces large number of acids and those that produce neutral products acetone as an end product. In gelatin liquefication test, the conversion of the semisolid gel into liquid due to hydrolysis by bacteria. Gelatin medium starts to flow when tubes were tilting showed that the result is positive. All the isolates showed positive result. In case of casein hydrolysis test bacterium show positive result by forming the clear area around the bacterial growth having the ability to deteriorate the casein protein by exoenzyme called proteinase which break the peptide bond by addition of water molecule. The principal of Kovac's oxidase test is based on the presence of the cytochrome oxide which is a feature of saprophytic bacteria. If the pink or purple colour is developed in 30-60 seconds the bacterium was positive to test. All isolates of our study were oxidase negative. All isolates were unable to reduce the nitrate because the bacterium was unable to produce any pink or red colour it indicates that the *Xanthomonas campestris* p.v *citri* was negative to the nitrate reduction test. Bacterial isolates able to reduce the tween 80 indicate that the presence of the opaque zone or milky white precipitate zone around bacterial colonies after 72 hours incubated at 27°C. All isolates were positive to this test and it is a unique feature of genus Xanthomonas. In pathogenicity test, the bacterium was inoculated on lime citrus to confirm the pathogen. All isolates formed typical symptoms of bacterium *Xanthomonas campestris* p.v *citri* on the leaves of the lime plant within 14 to 15 days of the inoculation. Symptoms appeared as lesions developed on young leaves. In starting these lesions are round, slightly raised, small, and light green spots. While in later stages colour changed and appear corky with the edges of yellow holes. After that, the bacterium was re- isolated from the infected leaves and grown on the culture medium for Koch's patulates validification. Similar experiments also carried by the Broadbent *et al.*, (1992), Mohammadi *et al.*, (2001) and Verniere *et al.*, (1998) observed that the symptoms developed within 2-3 weeks following the inoculation.

Table 2. Biochemical characterization of *Xanthomonas campestris p.vcitri.*

Name of isolate	KOH test	H_2O_2 test	Starch hydrolysis test	Simmons citrate test	MR- VP test	Gelatin liquefaction test	Casein test	Oxidase test	Tween 80 hydrolysis test	Nitrate reduction test
Xac1	+	+	+	+	+	+	+	-	+	-
Xac2	+	+	+	+	+	+	+	-	+	-
Xac3	+	+	+	+	+	+	+	-	+	-
Xac4	+	+	+	+	+	+	+	-	+	-
Xac5	+	+	+	+	+	+	+	-	+	-
Xac6	+	+	+	+	+	+	+	-	+	-

Table 3. Pathogenicity test.

Isolates	Pathogenicity test
Xac1	+
Xac2	+
Xac3	+
Xac4	+
Xac5	+
Xac6	+

Reference

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