INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 16, No. 3, p. 70-79, 2020
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Biochemical and cultural characterization of *Xanthomonas axonopodis* pv. *citri* isolated from infected citrus plants from Bahawalpur, Pakistan

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Article published on March 30, 2020

Key words: Bacterial canker, *Xac*, Biochemical assays, Culture tests; YDCA medium

Abstract

Citrus is an important fruit grown all over the world, but it is vulnerable to attack of *Xanthomonas axonopodis* pv. *citri* (*Xac*), causative agent of citrus canker. It is documented as most devastating disease leading to great economic losses by affecting overall fruit production in citrus. In contemporary study *Xac* was isolated from the infected plant samples which were collected from Bahawalpur by using nutrient agar medium. The bacterium was purified on nutrient agar medium by streaking method. Confirmation of *Xac* was done by its morphological, biochemical and cultural features. It showed positive result in KOH solubility test, starch hydrolysis test, casein hydrolysis, catalase test, and citrate utilization test while it expressed negative result in fluorescent pigmentation, Staining test, indole production and Kovac's oxidase test. The bacterium colonies appeared as mucoid, convex, yellow to orange color on NA medium; flattened or slightly raised, bright to pale yellow color, mucoid colonies on YDCA medium. This study is helpful for researchers and scientist for accurate identification of *Xac* which is necessary for *in-vitro* evaluation of different chemicals to find out most affective chemical against canker disease in field.

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Introduction

Citrus includes important species like oranges, grapefruit, lemons, pomelo and lime. It is cultivated throughout the world in both tropical as well as subtropical climates (Bhure, 2019). It is one of the important export products of Pakistan with total cultivated area of 195 thousands hectares while production is 2.3 million tons (Jabeen *et al.*, 2016). Pakistan is the 13th largest producer and 6th largest exporter of citrus in the world (Naseer *et al.*, 2019). Citrus contained beneficial nutrients and phytochemicals that are essential for health e.g., carbohydrates, fibre, folate, niacin, vitamin C, vitamin B6, thiamine, magnesium, copper, calcium were present in citrus fruit and juices (Al-Snafi, 2016).

In Pakistan, variety of pests are attacking citrus which includes citrus psylla, butterfly, fruit flies white flies and scale insects (Mahmood *et al.*, 2014). Citrus leaf miner (CLM) is major insect pest of citrus nursery as well as mature orchard and affects its production (Ullah *et al.*, 2019). Many plant diseases caused by fungi are dominated followed by bacteria and virus (Muthukumar *et al.*, 2017). Different pathogens threatened the citrus production that causes economic losses and severe social impacts e.g., citrus tristeza virus, citrus canker, Huanglongbing, citrus variegated chlorosis (Mendonça *et al.*, 2017).

Citrus canker is one of the most destructive disease which causes substantial damage and its severity of infection varies with the different species, variety and climatic conditions. Except Europe, it is endemic in India, Japan and Southeast Asian countries spreading to all citrus producing continents (Prakash *et al.*, 2012). It has been eradicated from some areas of the world including Australia, the Fiji Islands, Mozambique, New Zealand and South Africa while eradication programs have been applied in infected areas such as Argentina, Brazil, Florida and Uruguay with high rate of success (Niphadkar *et al.*, 2013).

Xanthomonas axonopodis pv. *citri* (*Xac*) is the causal agent of citrus canker. It belongs to the family Xanthomonadaceae, the largest and the most important group of phytopathogens. *Xac* has been used as model organism for the study of pathogenesis, phylogeny and host-bacterium interaction (Ference *et al.*, 2018). It enters the host through the natural opening (stomata and hydathodes) or by wounds and spread by windblown rain (Li *et al.*, 2011). It multiplies in intracellular spaces to cause disease (Gottwald *et al.*, 2002).

Xac uses specific protein named adhesins, is one of the important mechanisms to colonize its host. Once the bacteria attacked to the plant, it delivers its effector protein inside the plant via type III secretion system that influences the plant defense response in favor of the pathogen. Later, formation of biofilms helps in bacterial population in adherence involving the adhesins and exopolysaccrharide xanthan (Gottig *et al.*, 2010).

Xac causes a variety of symptoms. Circular spots are easily visualized on the dorsal surface of leaves. Bacteria colonize the apoplast of the cell. By bacterial colonization, hyperplasia takes place in the cell and epidermis of the leaf cells break down. Dark necrotic corky lesion rise on the leaves, stems and fruits (Gottig *et al.*, 2010). It can cause premature fruit drop, defoliation, twig dieback and tree decline (Niphadkar *et al.*, 2013). On susceptible variety, high severity of disease results in disfig.d fruit, dieback, immature fruit drop and defoliation which as a result decreases the production of fruit and also reduces the market value (Graham *et al.*, 2004).

Five races of *Xanthomonas* have been reported yet i.e., A, B, C, D and E based on level of aggressiveness, geographical origin and host range (Schaad *et al.*, 2005). All five races can be differentiated through pathogenicity and growth on differential media. Common and most threatening of citrus canker is Asiatic canker or canker A, first reported in Florida (Schubert *et al.*, 2001). Many scientists documented this strain as citrus canker disease.

Several approaches have been employed to identify and discriminating the various strains of *Xanthomonas*. Different biochemical and cultural tests are used as identification marker that facilitate in the identification of bacteria such as gram staining, potassium hydroxide test, casein hydrolysis, starch hydrolysis, indole production, kovac's oxidation, fluorescent pigment, citrate utilization test and bacterial growth on nutrient agar and yeast extract carbonate agar medium, respectively.

Biochemical and cultural analysis of the bacterium is essential for the management of the disease. It is also important to check the aggressiveness of the pathogen and screening the susceptible varieties of the citrus. Keeping in view the above mentioned discussion the present study was conducted to isolate, identify and analyzed the local strain of *Xac* infecting citrus in Bahawalpur, Pakistan.

Materials and methods

Plant Material

Diseased leaf samples of *citrus sinensis* were collected showing typical symptoms from Citrus Research Orchard, The Islamia University of Bahawalpur.

Isolation and Purification of Xanthomonas axonopodis pv. citri

Symptomatic leaves were cut down into 2×2 mm disc. Using 70% ethanol, the samples were surface sterilized and giving subsequent washing with distilled water in the 1.5ml eppendorf tubes. Under sterile conditions nutrient agar medium (Nutrient broth 13g, Agar 15g in 1000ml distilled water) was prepared. Direct streaking was done using sterilized platinum loop and smear was streaked on medium and incubated for 24 hrs. at 30°C. After incubation, the purified single colonies of bacterium were streaked on another nutrient agar plate and incubated. One set of purified bacteria were stored in refrigerator at 4°C for further processing. Under this storage temperature, the bacterial culture remains viable for 4-6 weeks (Simione *et al.*, 1991).

Pathogenicity Test

Detached leaf assay

To evaluate the confirmation of pathogenicity of *Xac*, detached leaf assay was performed. By using 70% ethanol leaves surface was disinfected then washed in distilled water and placed on 1% water agar with their abaxial surfaces facing upwards. Ten wounds with entomological sterilized needle (110 - 0.5mm) were performed on one leaf and then on each wound bacterial suspension of 10^6 $CFUml^{-1}$ was placed which are done by serial diluted method and leaves were incubated at 28°C for 3 weeks in incubator. Sterile water was used as negative control (Verma *et al.*, 1976).

Biochemical tests

Biochemical tests *viz.,* gram staining, KOH test, Kovacs oxidase test, catalase test, starch hydrolysis test, fluorescent pigment test, casein hydrolysis test, citrate utilization test, voges proskauer test, indole production test and methyl red test were carried out for the confirmation of *Xac* according to laboratory manual by Aneja (Aneja, 2003).

Gram Staining

Staining chemicals such as crystal violet, iodine, ethanol and safranin were prepared as Gram's reagent. On the slide, bacterial smear was made by mixing pure *Xac* culture in a drop of sterile water. Smear was stained with primary stain and holds it for 30 seconds, then washed the slide with distilled water to remove unbound crystal violet. Two drops of iodine were stained on the slide for 30 seconds and then washed with distilled water followed by ethanol. In the last, counter staining, one drop of safranin and holds it for 30 seconds then washed with water. Slide was dried with blotter paper and observed under light microscope at 100X. Gram positive bacteria will retain the crystal violet and look violet/purple while Gram negative bacteria will loss the crystal violet stain and looks red (Schaad *et al.*, 1988).

Potassium Hydroxide (KOH) Test

Two drops, approximately 50µl of 3% (w/v) KOH solution (30g potassium hydroxide dissolved in one litre) were placed on the clean slide.

Mix a loop full of bacterial culture and stirred that solution in quick circular motion for 10 seconds. Formation of thread indicated a positive result while no thread formation showed negative (Ryu, 1940).

Kovac's Oxidase Test

One drop of 1% Kovac's reagent (1g Tetramethyl-p-phenylenediamine Dihydrochloride in 100ml distilled water) was poured on filter paper. Using sterile flat wooden toothpick, inoculum of bacteria was rubbed on it. Purple color developed in 10-30 seconds showed positive test while no coloration showed negative test. Positive test showed an increase in drop viscosity and string occurred within 15 seconds (Kovacs, 1956).

Catalase Test

A loop full of 24 hours old bacteria was placed on the clean slide. A drop of hydrogen peroxide was placed and allowed to react for few minutes. Formation of gas bubbles indicates the positive result (Salle, 1961).

Starch Hydrolysis Test

Starch agar medium (Meat extract 3g, peptone A 5g, starch 2g and Agar 15g in 1000ml distilled water) was prepared and sterilized. Bacterial culture was streaked out on plate and incubates it for 24 hours until the growth appears. After incubation, cultural plates were flooded with iodine. The appearance of yellowish, clear zone around the growth showed positive reaction while no formation of zone showed negative reaction (Cowan *et al.*, 1974).

Fluorescent Pigment Test

King's medium B (Protease peptone 20g, K_2HPO_4 1.5g, MgSO4.7H2O 1.5g, Agar 15g, Glycerol 10g dissolved in 1000ml distilled water) was prepared. Glycerol provides energy and also enhances the production of pigments. Inoculate the bacterial culture on the medium and incubate it for 24 hours until the bacterial growth appears. Under UV light, plates were observed for fluorescent. Use *Pseudomonas aeruginosa* culture as a positive control (King *et al.*, 1954).

Casein Hydrolysis Test

Skim milk agar (Powder of skim milk 28g, Casein enzymic hydrolysate 5g, Yeast extract 2.5g, D-Glucose 1g, Agar 15g dissolved in 1000ml distilled water) was prepared. Overnight culture was inoculated and incubated it for 24 hours till the bacterial colonies appear. Clear zone around the bacterial growth showed a positive test while no zones showed negative test (Cappuccino, 2008).

Indole Production Test

Tryptophan broth medium (Casein enzymic hydrolysate 10g, sodium chloride 5g, Tryptophan 1g dissolved in 1 litre of Distilled water) was prepared. Inoculate the bacterium and incubate it for 24 hours at 28°C. After incubation, add 0.3ml of Kovac's reagent. Ring formation of pink color expressed a positive result while no color change showed negative result (Isenberg *et al.*, 1958).

Methyl Red Test

MR-VP medium (Peptone 7g, Dextrose 5g, Dipotassium phosphate 5g dissolved in one litre of distilled water) was prepared and sterilized. Bacterial culture was inoculated on the medium and incubated for 48 hours. Indicator (Methyl red) was added. Changes in color indicate the positive reaction (Aneja, 2003).

Voges-Proskauer Test

Test tube containing MR-VP medium (Peptone 7g, Dextrose 5g, Dipotassium phosphate 5g dissolved in one litre of distilled water) was inoculated with culture and incubated for 48 hours. After incubation, VP-I (5% alpha naphthol) and VP-II (40% Potassium hydroxide) reagents were added and observe the color change after 2 hours (Aneja, 2003).

Citrate Test

Simmon's citrate agar (Sodium chloride 5g, Sodium citrate 2g, Ammonium dihydrogen phosphate 1g, Dipotassium phosphate 1g, Magnesium sulphate 0.2g Bromothymol blue 0.08g, Agar 15g dissolved in 1 litre of Distilled water) was prepared and bacteria was

inoculated and incubate it for 48 hrs. After incubation, changes in color from green to blue were recorded (Hall, 2013).

Cultural tests

While using different media *Xac* can be differentiated by colony morphology and growth. Pathogen growth characteristics were studied by using differential and semi-selective media (Schaad *et al.*, 2001).

Growth on Nutrient Agar (NA) media

Sterile medium was prepared and poured in the petri plates. Bacterium was streaked and incubated it at 30°C for 24 hours and the colony characters were observed.

Growth on semi-selective Yeast extract calcium carbonate agar (YDCA) media

Medium (Yeast extract 10g, Glucose 20g, Calcium carbonate 20g and Agar 15g dissolved in 1 litre of Distilled water) was prepared. Bacterium culture was streaked and incubated for 24 hours at 30°C and the colony characters were observed.

Results

Pathogenicity Test

Pathogenicity test was performed using pin prick method on one year old citrus healthy leaves. After 3 weeks of incubation symptoms were appeared as spots and eventually converted into pustules with a raised margin surrounding a halo spot. *Xac* was re-isolated from inoculated leaves and re-identified by phenotypic characters.

Biochemical tests

Microscopic examination of Grams stained *Xac* showed that it cannot retain the primary stain and cells appeared pink due to the counter staining (Safranin). Hence the bacterium was Gram negative. Formation of Slimy thread showed a positive reaction. Due to thin cell wall of gram negative bacteria, the viscous DNA of the bacterium released when exposure to the 3% KOH. Gram positive bacteria had thicker cell wall due to this KOH did not hydrolyzed.

Table 1. Biochemical Characteristics of *Xanthomonas axonopodis* pv. *Citri.*

SN	Biochemical Tests	Optimization	Remarks	Results
1	Gram Staining	Small rod shaped, pink color colony	Isolated bacteria was gram negative	-ve
2	Potassium hydroxide (KOH) Test	Viscous and thread like slime	Isolated bacteria was gram negative	+ve
3	Kovac's Oxidase Test	Colorless	Isolated bacteria gave no purple color within 15 seconds	-ve
4	Catalase Test	Oxygen bubbles	Isolated bacteria was capable to produce catalase enzyme	+ve
5	Starch Hydrolysis Test	Clear zone around the bacterial colony	Isolated bacteria hydrolyzed the starch by adding Lugol's Iodine	+ve
6	Fluorescent Pigment Test	No fluorescent of any color	Gram negative bacteria characteristically produced no fluorescent of any color	-ve
7	Indole Production Test	No color change	Isolated bacteria gave no color by adding Kovac's Reagent	-ve
8	Methyl Red Test	Change of color medium	Isolated bacteria was capable to utilize the glucose with the production of stable acids	+ve
9	Voges Proskauer Test	Formation of red cherry color layer	Isolated bacteria has the ability to produce acetoin	+ve
10	Casein Hydrolysis Test	Formation of clear zone	Isolated bacteria was capable to hydrolyze the casein	+ve
11	Citrate Utilization Test	Blue Color	Isolated bacteria was capable to utilize citrate	+ve

Xac did not show the purple/dark blue color which shows a positive reaction. Hence the bacterium was oxidase negative. The bacterium produced bubble when react with the drop of 3% H_2O_2 releasing the oxygen gas. *Xac* shows a catalase positive reaction.

In this test, colorless clear zone was observed around the bacterial colonies when flooded with iodine solution. The bacteria hydrolyzed the starch by exoenzyme amylase and exhibit unique zone. *Xac* showed no fluorescent of any color under the UV

light. Gram negative bacteria characteristically produced no fluorescent under the UV light. No ring of pink color was formed, showed a negative result. It declared that *Xac* did not hydrolyze the tryptophan to form a compound, indole. Changed color of the medium after the addition of methyl red (indicator) showed a positive result. It means that *Xac* produced the large amount of organic acids from glucose fermentation. Formation of red color of the medium after the addition of VP-I and VP-II (reagents) showed a positive result. It means that *Xac* was produced a specific compound, acetoin. *Xac* showed a clear zone around the growth indicate the positive result. It means bacterium secrete a proteolytic exoenzyme (caseinase) to hydrolyze the casein. Blue color of the medium showed the positive result. It means that Xac utilized citrate as a sole carbon source.

Cultural Characters

Convex, mucoid, yellow to orange colour colonies on NA medium while on YDC agar colonies were viscous, slightly raised, pale yellow and mucoid.

Table 2. Cultural characteristics of *Xanthomonas axonopodis pv. citri* on different solid media.

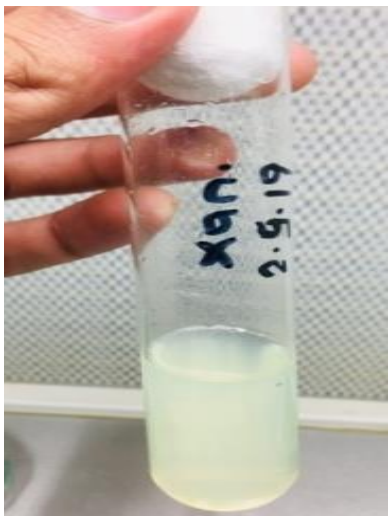
Media	Colony Characters	
	Color	Appearance
NA	Yellow to orange	mucoid, convex
YDCA	Bright to pale yellow	mucoid, flattened or slightly raised

A) KOH Test

B) Kovac's Oxidase Test

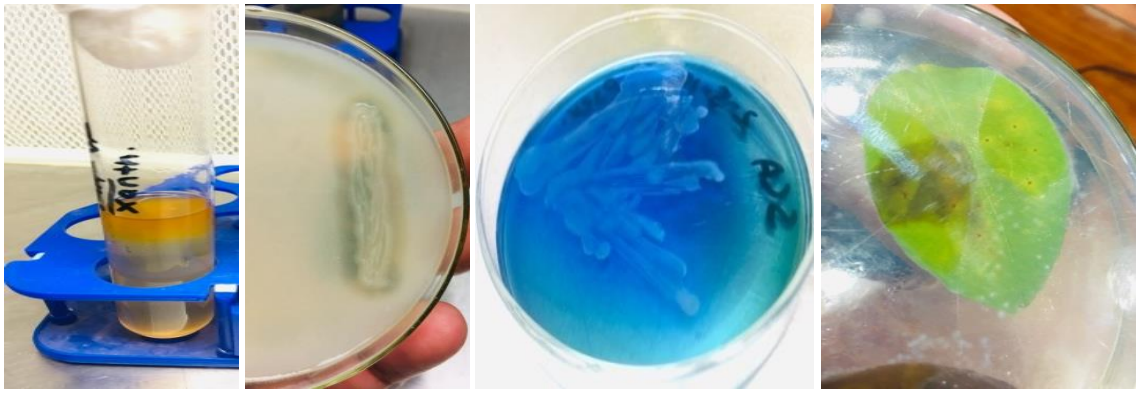
C) Catalase Test

D) Starch Hydrolysis Test



E) Indole Production Test

F) Methyl Red Test



G) Voges Proskauer Test H) Casein Hydrolysis Test I) Citrate Utilization Test J) Pathogenicity Test

Discussion

Citrus is very important fruit grown all over the world, sweet orange (*Citrus sinensis*) is accounting 70% of the total citrus production (Al-Snafi, 2016). It is also vulnerable to numerous diseases caused by bacterial, fungal, and viral pathogens in which citrus canker, *Xanthomonas axonopodis* pv. *citri* (*Xac*) caused heavy economic losses. Erumpent and Conspicuous lesions were formed on leaves, stems and also on fruits.

It can cause disfig.d fruit, premature fruit drop, twig dieback and tree decline (Niphadkar *et al.*, 2013). In present study, *Xac* was confirmed biochemically through different tests. Under light microscope at 100X with oil immersion, the bacterium was appeared pinkish color. It did not retain crystal violet stain because the amount of peptidoglycans in cell wall is very less and the absence of teichoic acid. Furthermore the production of slimy thread in KOH test that support the result of Gram staining test i.e., the bacterium was Gram negative. Compared to Gram staining reaction, KOH test was rapid and accurate test. Similar results in KOH and Gram staining test were reported in literature (Jabeen *et al.*, 2016; Suslow *et al.*, 1982).

In the present study, *Xac* showed negative response in Kovacs oxidase test that is used to identify the bacteria produces cytochrome oxidase, an endoenzyme used in electron transport chain. Jabeen and his co-workers in 2016 also reported oxidase negative test of *Xac* (Jabeen *et al.*, 2016; Mohammadi *et al.*, 2001). *Xac* showed positive results in catalase and starch hydrolysis test. Bacteria show clear zone in starch test which indicate that the production of exoenzyme amylase which cleaves the starch into sugars while the production of catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide. It facilitates the cellular detoxification.

Jadhav, 2018 and Bhure, 2019 and his colleagues reported the same result (Bhure *et al.*, 2019; Jadhav *et al.*, 2018). In this study, *Xac* was also expressed positive results in methyl red and voges proskauer test. It ferment sugars via butanediol fermentation pathway, this pathway consequence in the low acidification of the culture medium and the pH indicator, methyl red was found to be suitable to measure the hydrogen ions while in the fermentation of sugars, *Xac* also produced acetoin as an intermediate which can be further reduced to 2,3-butanediol. Sujatha and Sai Gopal, 2010 were reported same results (Sujatha *et al.*, 2010).

In fluorescent pigment test, *Xac* did not produced any pigment under the ultraviolet light and in controlled nutritional medium while some genus of bacteria under controlled nutritional conditions excretes water soluble fluorescent pigment into the medium. This ability of excretion is variable and depends on the composition of growth medium. The principal factor which is responsible for the production of fluorescent pigment in the controlled medium is the concentration of iron.

The iron metabolite is a basic requirement for the synthesis of cytochrome system enzymes (Garibaldi, 1967). Other test in which *Xac* showed negative response was indole production test. Tryptophan, an amino acid undergo deamination and hydrolysis of bacteria that express tryptophanase enzyme. By reductive deamination indole was produced. When indole is combined with Kovacs reagent, the solution changed its color. Similar result has been noted by (Bhure *et al.*, 2019; Sujatha *et al.*, 2010; Mohammadi *et al.*, 2001).

In citrate utilization test *Xac* showed positive response. It uses citrate as sole carbon source and inorganic ammonium salts as the sole source of nitrogen. It has the ability to produce citrate permease which converts the citrate to pyruvate and then pyruvate enters the bacteria metabolic cycle for production of energy. When citrate metabolized, the ammonium salts broken down which increases the alkalinity. These results are in line with the previous study by (Sujatha *et al.*, 2010).

In the above study, *Xac* showed variety of growth pattern on different media such as it has mucoid, convex, yellow to orange colour colonies in nutrient agar while viscous, slightly raised, and mucoid colonies were observed on YDCA medium after 48-72 hrs at 28℃ (Jabeen *et al.*, 2012; Patil *et al.*, 2017). Therefore, it is concluded that biochemical and cultural characterization is mandatory for the management of citrus canker. This study is baseline for formulating appropriate management strategy in controlling citrus canker disease which has already become a threat to the citrus production.

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