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Cultural and Biochemical characterization of *Xanthomonas axonopodis* pv. *punicae,* causing bacterial blight disease of Pomegranate

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Abstract
The *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate was isolated from diseased leaves and fruits samples collected from Farmers field. Cultural characteristics *viz.* colour of colony, growth on the media, growth appearance, elevations and texture and consistency and biochemical tests helps to identification of bacteria. Different cultural media were Use to study the cultural characteristics *viz.* Nutrient Agar (NA), Nutrient Sucrose Agar (NSA), Glucose Yeast Chalk Agar (GYCA) and Yeast Extract Dextrose Calcium Carbonate Agar (YDCA). Gram staining test, potassium hydroxide test, starch hydrolysis and catalase test were performed to characterized the *X. axonopodis* pv. *punicae.* The results of cultural, morphological and biochemical tests were confirmed the *X. axonopodis* pv. *punicae* a Gram negative bacterium.

Keywords: Pomegranate, *X. axonopodis* pv. *punicae,* cultural characters and biochemical characters

Introduction
Pomegranate (*Punica granatum* L.) is one of the ancient and highly priced favourite table fruits of tropical and sub-tropical regions. Cultivation of high yielding varieties of pomegranate with intensive care and management in the recent past under irrigated condition with early stage exploitation of plants has lead to certain severe pest and disease problems. Among the major diseases, bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae,* causes major threats in pomegranate cultivation (Madhukar and Reddy, 1989) [3].
Bacterial blight disease of pomegranate caused by *X. axonopodis* pv. *punicae,* mostly produces disease symptoms on leaves, stems and fruits. The isolation was made from diseased leaves and fruits samples were collected from farmers' fields. Conformation of bacterium is very important for the application of right management strategies. Therefore, studies on the isolation of bacterium *X. axonopodis* pv. *punicae,* its cultural and biochemical characterization of this bacterium were carried out.

Materials and Methods
Isolation and purification of *X. axonopodis* pv. *punicae*
Pomegranate plants showing typical bacterial blight symptoms on fruits, leaves and twigs were collected for isolation from the field. The infected plant parts were washed in running tap water to remove the other particles before isolation to avoid contamination. The infected part were cut into small bits of the size 2.5 mm with sterilized scalpel. These bits were then surface sterilized with 0.1% sodium hypochlorite solution for two minutes and washed with three successive changes of sterilized water to remove the traces of sodium hypochlorite. The bits were blot dried and four bits each were placed on the solidified nutrient agar media (NA media) plates. These plates were then incubated at 27±1 °C for four days. The pure bacterial growth was transferred to the NA plates for further studies.

Cultural and biochemical characteristics of *X. axonopodis* pv. *punicae*
Cultural characteristics *viz.* colour of colony, growth on the media, growth appearance, elevations and texture and consistency were studied on different culture media *viz.* Nutrient Agar (NA), Nutrient Sucrose Agar (NSA), Glucose Yeast Chalk Agar (GYCA) and Yeast Extract Dextrose Calcium Carbonate Agar (YDCA) and incubated at 27±2 °C temperature. The observations *viz.* colour of colony, growth on the media, growth appearance, elevations and texture and consistency on different media were recorded after 72 Hrs. of incubation.

Biochemical characters of *X. axonopodis* pv. *punicae* pathogen were studied by subjecting to various biochemical tests, *viz*. Gram staining test, potassium hydroxide test, starch hydrolysis and catalase test.

Gram staining

A loopful of bacterial suspension was transferred at the center of slide with the help of wire loop. The drop was smeared over slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with crystal violet for 30 seconds, washed in the tap water. Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 Second's and washed under tap water then decolorized slide with 95% alcohol and rinsed with water. Slide was counter stained with safranin for 10 second and washed with tap water and air dried. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lens.

Potassium hydroxide (KOH) test

Two drops of Potassium hydroxide was placed on a glass slide. A colony of culture was picked up from the medium with the help of inoculating needle and mixed with KOH drops for 10 seconds and raised the needle for 0.5 to 2 cm to from thread to check the test.

Starch hydrolysis test

Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylose, a straight chain polymer of 200- 300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. The positive test indicates by the presence of amalyse enzyme, an exoenzyme that hydrolyses (cleaves) starch, into maltose (disaccharide) and some monosaccharides such as glucose. Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis was observed for clear reddish zone.

Catalase test

Using a sterile inoculating loop, small amount of organism was collected from a well isolated 18- to 24-hours colony and placed it on the slide. Using a droppler, placed 1 drop of 3% H_2O_2 on the organism over the slide. Observed for immediate bubble formation (O2 + water = bubbles). Observed for the formation of bubbles against a dark background enhances readability.

Results and Discussion

Cultural and biochemical characteristics of *X. axonopodis* pv. *punicae*

The results presented in Table 1 and photograph shown in Plate 1 revealed that colony colour of bacterium shows variation on different media i.e., light yellow on NA media, dark yellow on NSA media, dark yellow on GYCA media and whitish yellow on YDCA media. Then growth of bacterium on different media showed variation from excellent to good growth. Excellent growth was observed in NA media followed by NSA media and GYCA media while good growth in YDCA media. Growth appearance were observed slightly raised on NA media and YDCA media, whereas highly raised and glistering on NSA and GYCA media. Growth elevation convex were observed on all media. Later on, texture and consistency showed differentiation on different media i.e., mucoid on YDCA media, slightly mucoid on NA media and highly mucoid on GYCA media and NSA media.

Similar results were also reported earlier by Vauterin *et al.*, (1995) [5] and Patil *et al.*, (2017) [4].

Plate 1: Photograph showing cultural characteristics of *X. axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate

Table 1: Cultural characteristics of *X axonopodis* pv. *punicae* on different growth media

Sr. no.	Colony character	NA media	NSA media	GYCA media	YDCA media
1.	Colour	Light Yellow	Dark Yellow	Dark Yellow	Whitish Yellow
2.	Growth on the media	Excellent	Excellent	Excellent	Good
3.	Growth appearance	Slightly raised	Highly raised, glistering	Highly raised, glistering	Slightly raised
4.	Elevation	Convex	Convex	Convex	Convex
5.	Texture and consistency	Slightly mucoid	Highly Mucoid	Highly Mucoid	Mucoid

*NA=Nutrient Agar, NSA= Nutrient Sucrose Agar, GYCA= Glucose Yeast Chalk Agar and YDCA= Yeast Extract Dextrose Calcium Carbonate Agar

Biochemical tests

The studies take on biochemical characterization of *X. axonopodis* pv. *punicae* showed their positive reactions for potassium hydroxide (KOH) test, starch hydrolysis test and catalase test whereas, negative response for Gram staining as shown in photograph of Plate 2, and presented in Table 2.

Gram staining

A loopful of bacterial suspension was transferred at the center of slide with the help of wire loop. The drop was smeared over slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with crystal violet for 30 seconds, washed in the tap water. Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 seconds and washed under tap water then decolorized slide with 95% alcohol and rinsed with water. Slide was counter stained with safranin for 10 second and washed with tap water and air dried. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lens. The bacterial pathogen showed Gram negative reaction i.e. rod shape red/ pink cell were observed under oil immersion lens.

Table 2: Biochemical characterization of *X. axonopodis* pv. *punicae*

Sr. No.	Biochemical test	Reaction
1.	Gram staining test	Negative
2.	Potassium hydroxide (KOH) test	Positive
3.	Catalase test	Positive
4.	Starch hydrolysis test	Positive

Potassium hydroxide (KOH) test

Formation of slime threads or loop is an indication of being gram-negative because gram negative bacteria have relatively fragile cell walls, bounded by an outer membrane. This is readily disrupted by exposure to 3% KOH releasing the viscous DNA. The *X. axonopodis* pv. *punicae* were showed to form mucoid thread after added KOH and found positive test.

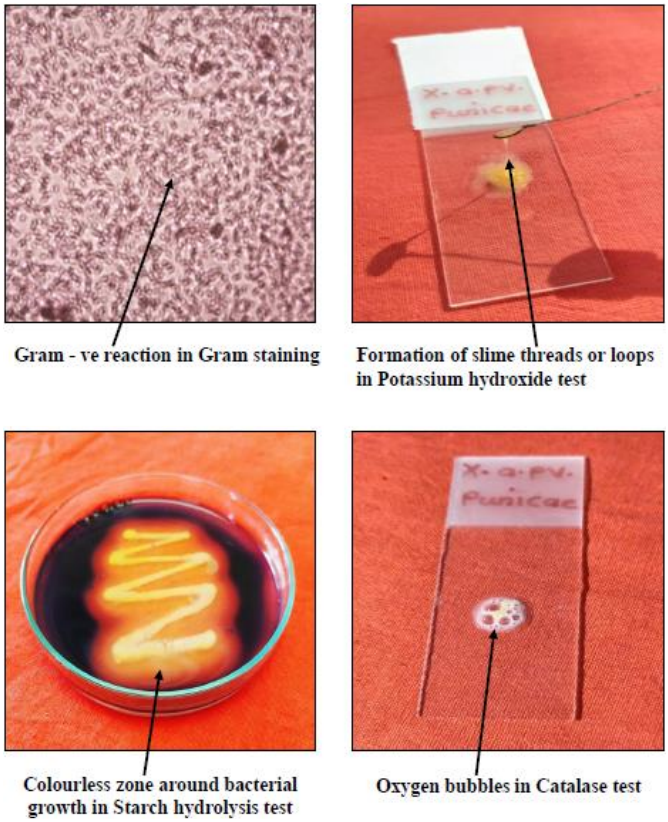

Plate 2: Photograph showing results of biochemical tests of *X. axonopodis* pv. *punicae* causing bacterial blight disease of pomegranate

Starch hydrolysis test

X. axonopodis pv. *punicae* produced colourless zone around bacterial growth on starch agar medium flooded with Lugol's iodine and showed positive for starch hydrolysis test. The *X. axonopodis* pv. *punicae* were showed that hydrolyzed starch by exoenzyme amylase and broken down to dextrins, maltose, and glucose/alpha amylase.

Catalase test

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. A small inoculum of bacterial suspension was mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production. Catalytic activities of *X. axonopodis* pv. *punicae* were found positive, when culture was produced bubbles of oxygen within one minute after addition of H_2O_2.

Similar results were also reported earlier by Abhang (2015) [1] and Katkar *et al.*, (2016) [2].

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