

Cultural Characterization, Biochemical Profiling and PCR Based Assay for the Detection of *Xanthomonas axonopodis* pv. *punicae*, Causing Bacterial Blight of Pomegranate

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The bacterial blight pathogen of pomegranate *Xanthomonas axonopodis* pv. *punicae* was isolated from the infected plant material collected from the field. The pathogen was confirmed by proving Koch's postulates and PCR detection method. Growth characteristics of the pathogen were studied by using differential and semi-selective culture media viz., XAN-D Agar, MSSXAN Agar, Yeast extract Sucrose Peptone Agar (YSPA), Nutrient Broth Yeast extract Agar (NBYA), Nutrient Glucose Agar (NGA), XTS Agar, NSCAA medium, Luria Bertani Agar (LBA) and Yeast Nutrient Agar (YNA). A considerable range of variations were observed with respect to colony colour, appearance and Fuscan pigment production on different media tried. Negative reaction for gram staining and positive for KOH solubility test indicated the gram negative nature of the bacteria. The bacteria was positive for catalase test, gelatin liquefaction, starch hydrolysis, asculin hydrolysis, acid production from glucose, H₂S production from cysteine hydrochloride, and negative for gas production from glucose, urease production, citrate production and indole production. The PCR amplification of *gyrB* gene resulted in production of an amplicon size of approximately 500 bp which confirmed the pathogen as *Xanthomonas axonopodis* pv. *punicae*.

Key words: Pomegranate, Bacterial blight, *Xanthomonas axonopodis* pv. *punicae*, Cultural characterization, bio-chemical profiling, PCR based assay.

Pomegranate (*Punica granatum* L.) is an ancient fruit crop belong to the family Punicaceae. It is regarded as the "Fruit of Paradise". Apart from its juicy arils delicious nature, each and every part of the plant has got medicinal property used to treat many human diseases.

The fruit crop is a native of Iran. It is cultivated over the whole Mediterranean countries like, Spain, Morocco, Egypt, Iran, Afghanistan and Baluchistan since ancient times. It is widely cultivated in India and drier part of south-east Asia, Malaya, Myanmar, China, Japan, USA (California), East Indies and tropical America.

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In India it is grown as a commercial crop in Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Madhya Pradesh and Uttar Pradesh grown in an area of 1,13,000 ha with a production of 7,45,000 MT with an average productivity of 6.6 MT (Anon., 2013). Pomegranate is prone to a number of diseases, among which the bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh, 1959; Vauterin *et al.*, 1995) is a serious problem. Disease is characterised by the appearance of small, irregular water soaked, dark coloured spots on leaves resulting in premature defoliation. Pathogen also infects stem and branches causing girdling and cracking symptoms. Spots on fruits are dark brown, irregular slightly raised with oily appearance, which splits opens

with 'L'/'Y' shaped cracks at final stages. Under severe conditions it destroys the entire orchard and causes heavy economic losses upto 70-80 per cent (Yenjerappa, 2009 and Benagi *et al.*, 2011). The infected plant residues of pomegranate, such as leaf, stem and fruit, which are left out in the field after the harvest of the crop serve as a primary source of inoculum for subsequent season to initiate the disease. Spread of the disease takes place through rain, spray splashes, windblown rain splashes, contaminated tools and insects. Earlier studies revealed that under favourable temperature and humid conditions, disease incidence increased after every shower (Anon., 2008). Pomegranate "the boon commercial fruit crop to the farmer turned as a big bane" after the severe outbreak of bacterial blight. Recently this disease has been reported even outside Indian subcontinent (Peterson *et al.*, 2010).

In the present study an attempt was made to know cultural character and biochemical profile of the pathogen which will support further studies on this dreaded pathogen in future. Apart from this, PCR detection of the pathogen was also performed using specific primers which will help in avoiding the selection of yellow nonxanthomonads associated with plant tissues, which may interfere and complicate identification. So, this PCR based assay helps in selection colonies actual pathogen before performing the time-consuming pathogenicity test.

MATERIAL AND METHODS

This study was conducted at Department of Plant Pathology, University of Agricultural Sciences, Dharwad, Karnataka.

Isolation of the pathogen

Infected leaves showing typical symptoms of bacterial blight were subjected to isolation of *Xanthomonas axonopodis* pv. *punicae*. The diseased leaves were washed thoroughly with tap water and allowed to dry. The infected portion along with healthy part was cut into small pieces and was surface sterilized with one per cent sodium hypochlorite solution for 60 seconds and washed three times serially in sterile distilled water to remove the traces of Sodium hypochlorite. The diseased bits were then suspended in a Petri plate containing 1 ml of

sterilized distilled water and crushed with sterilized scalpel and allowed for 2-3 minutes. When the sufficient oozing of bacterial masses were released from the crushed tissues, a loop-full of sap was taken and streaked on to Petri plate containing Nutrient Glucose Agar medium (NGA). The inoculated plates were incubated at $28\pm 1^\circ\text{C}$ for 72-96 h. After the 72 h of incubation, *Xap* usually appeared as pinhead sized colonies. The young and fast growing colonies were saprophytic ones. Thus observations were made for the development of well separated, typical, light yellow coloured bacterial colonies resembling that of typical *Xanthomonas* sp. at 96 h.

Purification and preservation of bacterial culture

The suspected bacterial colonies were picked up with the help of sterilized inoculation loop and streaked onto the surface of sterilized NGA Petri plates. The inoculated plates were incubated at $28\pm 1^\circ\text{C}$ for 72-96 h. Observations was made for the development of well separated, typical, bright yellow, mucoid colonies. Such pure colonies were further streaked onto the agar slants containing the NGA medium and incubated at $28\pm 1^\circ\text{C}$ for 72 h. Then the cultures were stored in the refrigerator at 4°C . A loop full of pure cultures were stored in sterile propylene culture tubes and stored at 4°C in refrigerator for further use as stock culture. For long term preservation, a loopful of bacterium was suspended in 600 μl of nutrient glucose broth in 1.5 or 2 ml eppendorf tube and added with 600 μl of glycerol (40%) and stored at -80°C for further use.

Proving Koch's postulates

Test plants

Susceptible pomegranate variety (Bhagwa) to bacterial blight was raised first in earthen pots by planting apparently healthy layered cuttings. One month old saplings were then transplanted into the pots containing sterilized soil amended with necessary nutrients. The seedlings were regularly watered and exposed to sufficient sunlight.

Inoculum preparation and inoculation

To prove the pathogenicity, bacteria was multiplied in nutrient glucose broth (200 ml) in Erlenmeyer flask by inoculating a loopful of bacterial culture. The inoculated flask was incubated for three days at $28\pm 1^\circ\text{C}$. Two months old pomegranate susceptible variety (Bhagwa)

plants were first sprayed with water and then covered with a polythene sheet for 24 h before inoculation. The pre-incubated pomegranate plants were sprayed with 4×10^7 cfu/ml of bacterial suspension (200ml: 800ml; bacterial suspension: sterilized distilled water) with hand sprayer on three branches. The sprayed plants were covered with polythene sheets and kept in humid chamber for the next 48 h, in which humidity (>95%) was maintained. Plants sprayed with sterilized distilled water served as control. Inoculated plants were sprayed twice a day with distilled water to maintain humidity. Observations were recorded every day for the appearance and development of symptoms. When artificially inoculated leaves expressed symptoms, re-isolation of the pathogen was done and compared with the original culture. The experiment was repeated twice.

Cultural characterization of the pathogen

Growth characters of the pathogen were studied by using differential and semi-selective media developed by the different workers. The media used for the cultural study includes, XAND agar medium (Lee *et al.*, 2009), MSSXN agar medium (Dezordi *et al.*, 2009), Yeast extract Sucrose Peptone Agar (YSPA), Nutrient Broth Yeast extract Agar (NBYA), Nutrient Glucose Agar (NGA), XTS Agar, NSCAA medium, Luria Bertani Agar (LBA) and Yeast Nutrient Agar (YNA). A culture suspension of *X. axonopodis* pv. *punicae* was prepared in sterile distilled water by using 72 h old culture grown on NGA plates which was streaked on above mentioned media to study the morphology of the colonies. The observations regarding colony colour, shape and margin, appearance and Fuscan pigment were recorded at 120 h after inoculation.

Biochemical profiling of the pathogen

The biochemical characters of the bacterial pathogen was studied for Gram's staining, KOH solubility test, catalase test, Gelatin liquefaction, Starch hydrolysis, asculin hydrolysis, Acid production from glucose, H₂S production from cysteine hydrochloride, gas production from glucose, urease production, citrate utilization and indole production. The tests were conducted as per the methods described by Schaad (1992). Seventy two hours old active culture of *Xap* grown on NGA plates was used for performing all the biochemical tests.

PCR assay for detection of pathogen

DNA extraction

Well separated fluidal colonies of *Xap* isolates were used for genomic DNA extraction after growing them for 96 h at $28 \pm 1^\circ\text{C}$ on NGA. A loop full of *Xap* culture was mixed in 1.0 ml distilled sterilized water in 1.5 ml micro-centrifuge tube and centrifuged at 14,000 rpm (room temperature i.e., $28 \pm 1^\circ\text{C}$) for 5 min. The supernatant was discarded and the pellet was washed three times with sterile water. About 550 μl of TE buffer was added to the tubes containing pellet and resuspended then added with 76 μl of 10% SDS and proteinase K and incubated at 65°C for 15 min. Then 100 μl of 5 M NaCl was added and mixed by flipping tubes and 80 μl of CTAB was mixed by flipping and incubated at 65°C for 10 min. About 700 μl of chloroform: isoamyl alcohol (24:1) was added, mixed gently by inverting the tubes, and centrifuged at 14,000 rpm for 5 min. The upper aqueous phase was transferred to a new tube without disturbing the middle layer. Equal volume of isopropanol was added and inverted several times to mix properly and centrifuged at 10,000 rpm at 4°C for 15 min. Gently the supernatant was drained off and 1.0 ml of 70 per cent ice cold ethanol was carefully added. DNA was collected by centrifugation for 5 min at 14000 rpm. Supernatant was carefully removed and the remaining ethanol was evaporated in laminar air flow cabinet. DNA was dissolved in 40-50 μl of 10mM Tris (pH 8) and incubated overnight at 4°C . Six μl of RNase was added to DNA solution to remove RNA contamination and incubated for 30 min at 37°C . DNA was stored at -20°C for further use.

PCR amplification of *gyrB* gene

Reaction mixture was prepared in 0.2 ml thin walled PCR tubes. The total volume of each reaction mixture was 20 μl containing 1.0 ml of template DNA (25 ng/ml), 2.0 ml of 10X assay buffer with 15 mM MgCl₂, 1.0 ml of dNTPs mix (10 mM), 1.0 ml of Primer Forward (5 pM/ml), 1.0 ml of Primer Reverse (5 pM/ml), 13.50 ml of Sterile distilled water, 0.5 ml. of *Taq* DNA polymerase (3.0 U/ml).

PCR amplification of *gyrB* gene amplification was performed using the primer set *gyrBF-gyrBR* (Mondal *et al.*, 2012) (*gyrBF*-GTTGATGCTGTTACACGCG and *gyrBR*-CATTTCATTCGCCCAAGCCC) with the following thermal profile: initial denaturation for 5 min at 94

°C; 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; final extension of 72 °C for 10 min; and final cooling at 4 °C. The amplification was performed in a DNA thermal cycler (Corbett Research, Australia). Following amplification, 20 µl of each amplificate was analysed by electrophoresis at 80 Volt (1.2 % agarose gel, 0.2 µg of ethidium bromide ml⁻¹) in TAE buffer.

RESULTS AND DISCUSSION

Isolation of the pathogen showed typical, yellow, mucoid bacterial colonies on NGA medium after 72 hours of incubation at 28±1 °C. Pure culture was maintained in 20 per cent glycerol stocks.

Pathogenicity was proved by inoculating bacterial cell suspension (200:800; culture: sterile water) to the 2 month old susceptible pomegranate plants of Bhagwa variety. The characteristic symptoms of the disease appeared six days after inoculation. Reisolation carried out from artificially inoculated plants yielded the bacterial colonies similar to the original culture. Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity. Infection was readily seen by them on tender leaves artificially inoculated plants in seven to ten days of incubation. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976). He observed the symptoms within four to

Table 1. Growth and cultural characters of *Xanthomonas axonopodis* pv. *punicae* on different solid media

S. No.	Media	Colony characters		
		Colour	Appearance	Fuscan pigment production
1.	MSSXAN Agar	Pale yellow	Convex, smooth, highly raised, glistening colonies surrounded by whitish hallow	Low
2.	XAN-D Agar	Greenish to brown colour	Convex, smooth, slightly raised, glistening colonies surrounded by translucent zone	High
3.	Luria Bertani Agar (LBA)	Yellowish to pale brown	Convex, smooth, slightly raised, glistening	High
4.	Nutrient Broth Yeast extract Agar (NBYA)	Pale yellow	Convex, smooth, highly raised, glistening	Low
5.	Nutrient Glucose Agar (NGA)	Slightly dark yellow	Convex, smooth, highly raised, glistening	Low
6.	NSCAA	Yellowish to pale brown	Convex, smooth, slightly raised, glistening	High
7.	Yeast extract Sucrose Peptone Agar (YSPA)	Dark yellow	Convex, smooth, highly raised, glistening	High
8.	Yeast Nutrient Agar (YNA)	Yellowish to pale brown	Convex, smooth, slightly raised, glistening	Medium
9.	XTS Agar	Yellowish to pale brown	Convex, smooth, slightly raised, glistening	High

seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts. The observations made pertaining to the isolation and pathogenicity in the present investigation were in conformity with the work of Manjula (2002), Yenjerappa (2009) and Basamma (2013).

The growth of bacterium on different differential and semi-selective media showed considerable differences with respect to colony colour, appearance and Fuscan pigment production (Table 1 and Plate 1).

The shape of the colonies was circular with entire margin on all the media tried. In case of XAN-D medium colonies were greenish to brown coloured, convex, smooth, slightly raised, glistening surrounded by translucent zone and produced high quantity of Fuscan pigment. The development of translucent zone indicates ability of pathogen to hydrolyse skimmed milk and Tween-80 which are added in the medium. The results of present study are in conformity with the studies conducted by Lee *et. al.*, 2009 on use of chromogenic differential medium for the isolation of phytopathogenic *Xanthomonas* spp.

On MSSXAN medium colonies were pale yellow, convex, smooth, highly raised, glistening surrounded by whitish halo and produced low quantity of Fuscan pigment. The development of white halo around colonies indicates the ability of the pathogen to hydrolyse the Tween-80 in the medium. Similar observation was recorded by

Dezordi *et al.*, 2009 while studying on use of semi-selective culture medium for detection of *Xanthomonas axonopodis* pv. *malvacearum* in cotton seeds.

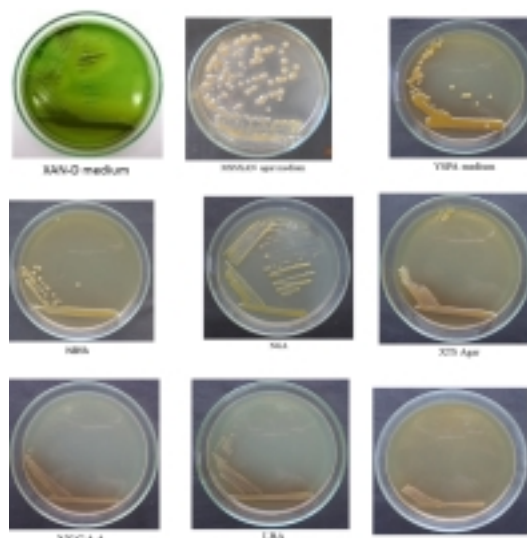


Plate 1. Growth of *Xanthomonas axonopodis* pv. *punicae* on different differential and semi-selective media

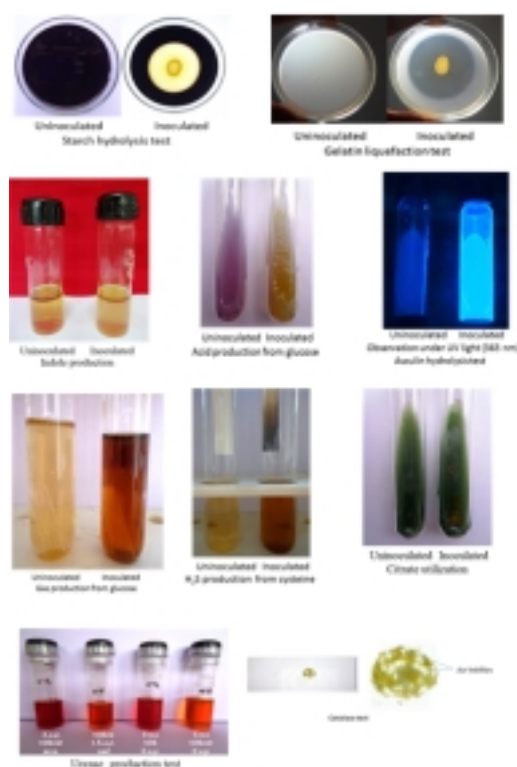


Plate 2. Biochemical profile of *Xanthomonas axonopodis* pv. *punicae*

Table 2. Biochemical profile of *Xanthomonas axonopodis* pv. *punicae*

S. No.	Characteristics	Reaction
1.	Gram reaction	-
2.	KOH solubility test	+
3.	Catalase test	+
4.	Gelatin liquefaction	+
5.	Starch hydrolysis	+
6.	Asculin hydrolysis	+
7.	Acid production from glucose	+
8.	H ₂ S production from cysteine hydrochloride	+
9.	Gas production from glucose	-
10.	Urease production	-
11.	Citrate utilization	-
12.	Indole production.	-

The *X.a.p.* produced dark yellow coloured, convex, smooth, highly raised, glistening colonies with high Fuscan pigment on YSPA while on NBYA it showed pale yellow, convex, smooth, highly raised, glistening colonies with low Fuscan pigment production.

Slightly dark yellow, convex, smooth, highly raised, glistening colonies with low Fuscan pigment production were observed on NGA. Growth on XTS, NSCAA and LBA media resulted in yellowish to pale brown coloured, convex, smooth, slightly raised, glistening colonies with high Fuscan pigment production.

In case of YNA, the pathogen produced yellowish to pale brown, convex, smooth, slightly raised, glistening colonies with medium range of Fuscan pigment production.

The isolated bacterium tested negative for gram reaction and positive for KOH solubility test indicating the gram negative nature of the bacteria. The bacteria was positive for catalase test, gelatin liquefaction, starch hydrolysis, asculin hydrolysis, acid production from glucose, H₂S production from cysteine hydrochloride, and negative for gas production from glucose, urease production, citrate production and indole production (Table 2 and Plate 2). Yenjerappa (2009),

reported that the pomegranate bacterium *X. axonopodis* pv. *punicae* hydrolysed the starch, liquefied the gelatin and was positive for H₂S production, catalase and oxidase enzyme activity. The organism utilized various carbon sources viz., glucose, fructose, dextrose and produced mild acid from these carbon sources, but fail to utilize lactose, mannose and mannitol. Raghuvanshi *et al.* (2013) also observed similar results during characterization of *X. axonopodis* pv. *punicae* isolates collected from western Maharashtra.

The PCR assay performed for *gyrB* gene by using the primer set *gyrBF-gyrBR* produced DNA products of the predicted size *i.e.*, 491 bp or approximately 500bp which confirmed the identity of the pathogen as *Xanthomonas axonopodis* pv. *punicae* (Fig. 1). The result is in accordance with the findings of Basamma (2013) who also got similar result when *gyrB* gene primer set was used for confirmation of all the 18 isolates of *X. axonopodis* pv. *punicae* collected from Karnataka, Maharashtra and Andhra Pradesh. So, *gyrB* gene sequence offered a useful method for the detection of pathogen.

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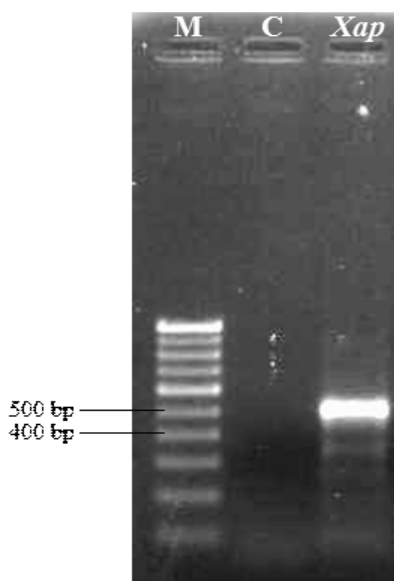


Fig. 1. PCR amplification of the *gyrB* gene in *Xanthomonas axonopodis* pv. *punicae*. Lanes M, 100 bp ladder; C, Water control; *Xap*, PCR amplified product of *gyrB* gene.

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