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Isolation and characterization of *Xanthomonas axonopodis* pv. *punicae* from bacterial blight of pomegranate

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Abstract
Successful pomegranate cultivation in recent years has met with different constraints such as pest and diseases. One of the most important diseases of pomegranate in India is Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (*X. axonopodis* pv. *punicae*), which brings down the domestic and export production by 80 per cent. Pomegranate bacterial blight pathogen was isolated from nine different locations of Tamil Nadu and their morphological characters were studied. Based on the morphological and biochemical characterization of the pathogen was identified as the *X. axonopodis* pv. *punicae*. The DNA of *X. axonopodis* pv. *punicae* isolates were extracted and amplified by polymerase chain reaction (PCR) by ITS forward primer 799 and reverse primer 1193.Based on the expression of amplicon at 491 bp, all isolates of the pathogen was confirmed as the *X. axonopodis* pv. *punicae.*

Keywords: Pomegranate, *Xanthomonas axonopodis* pv. *punicae*, Bacterial blight

Introduction
Pomegranate area under cultivation is increasing worldwide because of its hardy nature, wider adaptability and drought tolerance, higher yield levels with excellent keeping quality and remunerative prices in domestic as well as export market. Successful pomegranate cultivation in recent years has met with different constraints such as pest and diseases. Bacterial blight caused by *X. axonopodis* pv. *punicae* is one of the most devastating diseases of pomegranate in India, which brings down the domestic and export production by 80 per cent (Poovarasan *et al*., 2013) [14]. A yield loss up to 70-90 per cent was recorded in India due to bacterial blight of pomegranate (Gargade, 2014) [5]. Petersen *et al*. (2010) [13] first reported the occurrence of bacterial blight of pomegranate in pomegranate orchards in the Western Cape and the Limpopo provinces of South Africa. In the present investigation was carried out with an objective to isolate the bacterial blight of Pomegranate and assess the cultural and morphological characters and pathogenic variability of *X. axonopodis* pv. *punicae.*

Isolation and purification of the bacterial culture
The different parts of the pomegranate plant showing characteristic symptoms of bacterial infection were collected from different locations and subjected to isolation. The infected parts were washed in running tap water and an infected portion along with a small portion of healthy tissue was cut into 5 mm bits. These bits were surface sterilized with 0.1 per cent $HgCl_2$ for about 1-1½ minute and washed three times in sterile distilled water to remove traces of mercuric chloride ($HgCl_2$). The bits were then crushed in 2 to 3 ml of sterile distilled water and allowed to diffuse for 5 to 10 min at room temperature. A loop ful of crushed leachate was streaked on Nutrient agar media (NA) / Yeast Dextrose Calcium Carbonate Agar media (YDCA) plates aseptically and incubated at the temperature (30 ±1 °C) for 48 hours. Colonies grown within 72 hours were picked and again streaked on nutrient agar plates, the discrete colonies were sub cultured on nutrient agar slants for further studies (Raghuwanshi *et al*., 2013) [15]. The isolated bacterial colonies were picked up with the help of sterilized inoculation loop and streaked onto the surface of sterilized Petri plates contain NA media (Schaad and Stall, 1988) [18]. The inoculated plates were incubated at 30 ºC for 72hours. Observations were made for the development of well-separated typical, bright yellow, mucoid colonies. Such pure colonies were further streaked onto the agar slants containing the nutrient agar medium and incubated at 30 ºC for 72 hours. The pure cultures were stored in the refrigerator at 5ºC, which served as a stock culture for further studies. For long-term storage, the bacterial culture added to 70 per cent glycerol and kept at -4 ºC.

Identification of the pathogen
The bacterial blight pathogen was identified based on morphological, biochemical and molecular characters of the pathogen as per standard microbiological and molecular characters.

Morphological characterization
The morphological characteristics of the pathogen such as cell shape, gram reaction, pigmentation, characters were studied as per the standard procedures described by Bradbury (1970) and Schaad (1992) [17].

Biochemical characterization
The bacterial isolates isolated from the infected plant parts from different areas were subjected to various biochemical tests such as Citrate utilization test, Starch hydrolysis, Lysine utilization, Urease production, Phenylalanine deamination, Nitrate reduction, H2S production, and Carbohydrates utilization by following the standard protocol given by the Hi media KB 002 Hi Assorted TM biochemical test kit. The KOH solubility test, in dole production test, and Gelatin liquefaction test were conducted by following procedure given by Schaad, (1992) [17].

Molecular characterization of *X. axonopodis* pv. *punicae*
Isolation of DNA from a bacterial pathogen
The genomic DNA was extracted from the bacterial pathogen by following lys is method. Bacterial culture was prepared by inoculating a loop ful of culture into the nutrient broth and incubated in the shaker for 28± 2° C at 120 rpm for 48 hours. About 1.5 ml of 48hours old culture was taken in Eppendorf tube and centrifuged at 12000 rpm for 3minutes.The supernatant was discarded and the pellet was re suspended in 200 µl of lysis buffer by vigorous shaking. To this suspension, 5µl of RNase was added to remove the RNA contamination by incubating at 37° C for 30 minutes. After incubation, 60 µl of 5M NaCl was added, mixed well and centrifuged at 1200 rpm for 10 minutes at 4°C.After centrifugation, clear supernatant was transferred to new Eppendorf tubes. To this supernatant, an equal volume of chloroform was added and mixed well by inverting the tubes 5-6 times to attain a milky turbid solution. Again it was centrifuged at 12000 rpm for 3 minutes and the supernatant was transferred to new tubes. To this supernatant, 100%ethanol (2 volumes) was added and incubated at – 40 °C for 30 minutes to precipitate DNA. After incubation, centrifugation was done at 12000 rpm for 10 minutes. The supernatant was discarded and the pellet was made to float in 70 per cent ethanol inside the tube for 5 minutes and centrifuged at 10000 rpm for 10 minutes at 28 °C to dry the pellet. The supernatant was discarded and the pellet was allowed to dry. The dried pellet was reuses pended in 50 µl of TE buffer or double sterile water and stored in - 20° C for further use. Total DNA from the extracted product was checked by loading 5 µl of DNA mixed with 1µl of loading dye into 0.8 per cent agarose gel stained with ethidiumbromide. Electrophoresis was carried out and a photograph was taken under the UV light (Birnboim, *et al*., 1979)

Molecular confirmation of *X. axonopodis* pv. *punicae* through ITS primer
ITS primers 799 F (5'- AACMGGATTAGATACCCKG-3'), 1193 R (5'-ACGTCATCCCCACCTTCC-3') 3' were used to amplify the 400 bp gene of ITS region. The 10µl reaction mixture contained 2 µl of DNA (50 ng concentration), 20 pmol of 1 µl of each forward and reverse primers and 5 µl of red dye PCR master mixer (Genei Bangalore, India) along with 1 µl of sterile water. PCR amplification was performed in thermo cycler (Eppendorf Master cycler) using the conditions: Initial denaturation at 94 ºC for 2 min, 35 cycles consisting of 94 ºC for 50 sec (denaturation), 55ºC for 30 sec (annealing), 72 ºC for 1 min (primer extension) and final extension of 72 ºC for 10 min (Lee *et al.,* 2000) [8]. Agarose gel electrophoresis was performed based on the method described by Sambrook*et al.* (1989) to check the products amplified through PCR. 1X TAE tank buffer in 500ml quantity was prepared to fill the electrophoresis tank and to prepare the gel. In a separate conical flask, agarose (1.2 per cent for PCR product) was added to 1XTAE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature (40) ºC. Ethidium bromide was added at the rate of 2.5 µl/ 100ml of agarose solution and was allowed to mix completely. Then it was poured into the gel mould; the comb was placed properly and allowed to solidify for half an hour at room temperature28±2°C.After solidification, the comb was removed carefully. The casted gel was placed in the electrophoresis tank containing 1X TAE buffer with the well near the cathode and submerged to a depth of 1cm. Ffifteen micro litres of the PCR product mixture was loaded into the wells with the help of the micropipette. Four micro liters of 100 bp DNA ladder (Genei) was loaded in one of the wells as a standard marker. The cathode and anode were connected to power pack using power cord and the gel was run at a constant voltage of80 volts. The negatively charged DNA molecules move towards the anode and got separated according to their molecular weight. The power was turned off when the marker reached the anode end and the gel was viewed in an UV trans illuminator and the banding pattern was observed and analyzed. The sizes of the PCR products were determined by comparison with a standard 100bp molecular marker (Bangalore Genei, India).

Molecular confirmation of *X. axonopodis* pv. *punicae* through *gyrB* genus-specific primer
Genus-specific primers *viz*., KKM-5 Forward 5'GTTGATGCTGTT CACCAGCG3', KKM-6 Reverse 5'CATTCATTT CGCCCAAGCCC 3' were used to amplify the gyrB gene product of 491bp. The 10 µl reaction mixture contained 2 µl of DNA (50 ng concentration), 20 pmol of 1 µl of each forward and reverse primers and 5 µl of red dye PCR master mixer (Genei Bangalore, India). PCR amplification was performed in thermo cycler (Eppendorf Master cycler) using the conditions: Initial denaturation at 94ºC for 5 min, 30 cycles consisting of 94ºCfor 30 sec (denaturation), 58ºC for 1 min (annealing), 72 ºC for 1 min (primer extension) and final extension 72 ºC for 3 min. Final PCR product was electrophoresed on 1.2 per cent agarose gel stained with Ethidium bromide and was analyzed using a gel documentation system (Kalyan *et al.,* 2012) [7].

Pathogenicity studies
Pomegranate Ganesh variety was raised in polythene bags by planting apparently healthy layered cuttings. One and half months 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. To prove the pathogenicity, the pathogen was multiplied in nutrient broth for two days at 28 ºC. Pomegranate plants were first provided with water spray and then covered with a polyethene bags for 24 hours before

inoculation. Pre-incubated pomegranate plants were slightly injured with a sterilized pin. Twenty-five to thirty leaves are injured perplant and each leaf was pricked with 12-15pricks (injuries) and bacterial suspension (5×10^8cfu per ml) was sprayed on to the surface of the leaves with a small baby sprayer. The inoculated plants were covered with polythene bags and kept in glasshouse 3 days for build up the humidity and constant spraying of water at 25 to 30 ºC. Plants sprayed with sterilized distilled water served as a control. The inoculated plants were regularly observed for development of symptoms. After 10 days of inoculation, symptoms were observed. From the infected portion, the pathogen was re isolated and culture was compared and confirmed with the original culture.

Results

Isolation and maintenance of pomegranate bacterial blight pathogen

A bacterial pathogen was consistently isolated from the infected leaf and fruits by tissue segment method on Nutrient Agar (NA) medium. The bacterial colonies were purified on NA medium by streak plate technique. The individual colonies observed as bright yellow and mucoid. These colonies were maintained on the NA medium for further studies (Plate.1a and 1b).

Characterization of pomegranate bacterial blight pathogen

Pomegranate bacterial blight pathogen was isolated from 9 different locations of Tamil Nadu and their morphological characters were studied. The colour of the colony varies from pale yellow to dark lemon yellow with moderate to profuse in growth. The colonies were slimy in nature and smooth in texture. All bacterial isolates showed a negative reaction for gram staining, Indole production, Gelatin hydrolysis, Phenylalanine deamination, nitrate reduction and urease production and positive reaction to citrate utilization, starch analysis, KOH solubility, Gelatin hydrolysis, Lysine utilization, Ornithine utilization, Nitrate reduction and H2S production. Based on the morphological and biochemical characterization of the pathogen was identified as *X. axonopodis* pv. *punicae*. These isolates were named as *X. axonopodis* pv. *punicae* TMR, SVR, TMP, MNPT, ODSDR, AMBK, SMPT, VMPT and TNAU (Table 1, Table 2, Plate 2).

Table 1: Morphological characters of *X. axonopodis* pv. *punicae* isolates

S. No.	*X. axonopodis* pv. *punicae* isolates	Colony colour	Growth	Appearance	Texture
1	TMR	Light yellow	Profuse	Slimy	Smooth
2	SVR	Pale yellow	Moderate	Less slimy	Smooth
3	TMP	Pale yellow	Profuse	More slimy	Smooth
4	MNPT	Medium yellow	Profuse	More slimy	Smooth
5	ODSTR	Dark lemon yellow	Profuse	More slimy	Smooth
6	AMBK	Dark lemon yellow	Profuse	More slimy	Smooth
7	SMPT	Pale yellow	Moderate	Slimy	Smooth
8	VMPT	Pale yellow	Profuse	Slimy	Smooth
9	TNAU	Medium yellow	Profuse	Slimy	Smooth

Table 2: Biochemical characters of different isolates of *X. axonopodis* pv. *punicae*

Biochemical tests	*X. axonopodis* pv. *punicae* isolates								
	TMR	SVR	TMP	MNPT	ODSTR	AMBK	SMPT	VMPT	TNAU
Gram's staining	_	_	_	_	_	_	_	_	_
Citrate utilization	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	++	+	+	+	+	+
KOH solubility	+	+	+	+	+	+	+	+	+
Indole production	_	_	_	_	_	_	_	_	_
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+
Lysine utilization	+	+	+	+	+	+	+	+	+
Ornithine utilization	+	+	+	+	+	+	+	+	+
Urease	_	_	_	_	_	_	_	_	_
Phenylalanine Deamination	_	_	_	_	_	_	_	_	_
Nitrate reduction	+	+	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+	+	+
Glucose	++	+	+	+	+	++	+	+	+

- - Negative; + -Normal; ++ - Strong

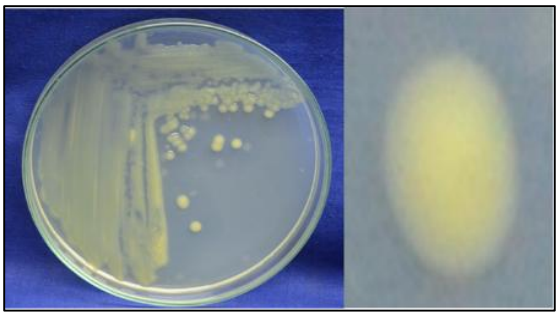

Plate 1a: Pure culture of *X. axonopodis* pv. *punicae*

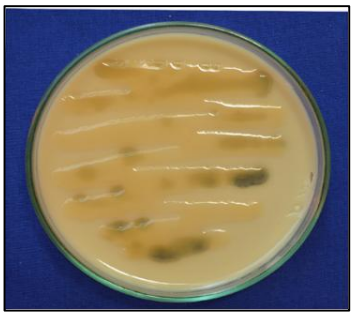

Plate 1b: 72 hours culture on YDCA media

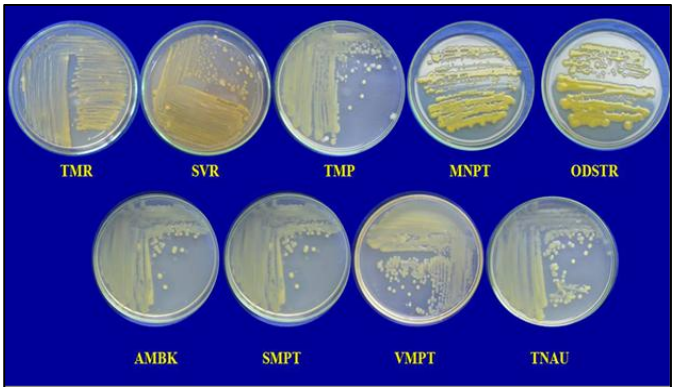

Plate 2. Different isolates of *X. axonopodis* pv. p*unicae*

Molecular characterization of *X. axonopodis* pv. *punicae*

The DNA of *X. axonopodis* pv. *punicae* isolates were extracted and amplified by polymerase chain reaction (PCR) by ITS forward primer 799 and reverse primer 1193.The amplified product is compared with the 100 bp molecular marker. Polymerase chain reaction (PCR) yielded an amplicon of the size of 400 bp for all the *X. axonopodis* pv. *punicae* isolates. Similarly, polymerase chain reaction (PCR) was performed by using gyr B genus-specific primers KKM 5 and KKM 6 which yielded an amplicon size of 491 bp in all isolates of *X. axonopodis* pv. *punicae.* Based on the expression of amplicon at 491 bp, all isolates of the pathogen was confirmed as *X. axonopodis* pv. *punicae* (Plate 3a and 3b).

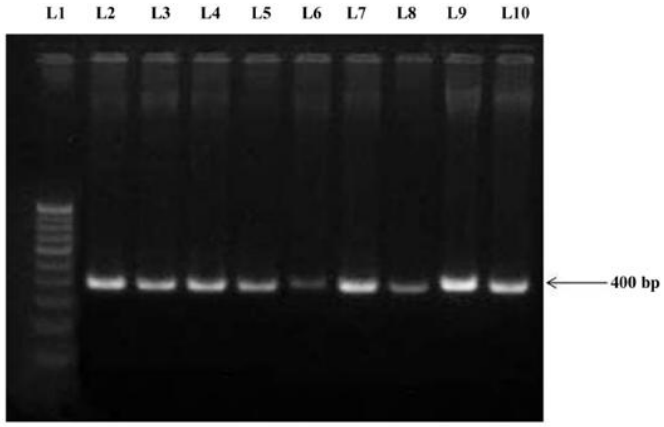

Plate 3a. Molecular confirmation of *X. axonopodis* pv. *punicae* by using ITS primer

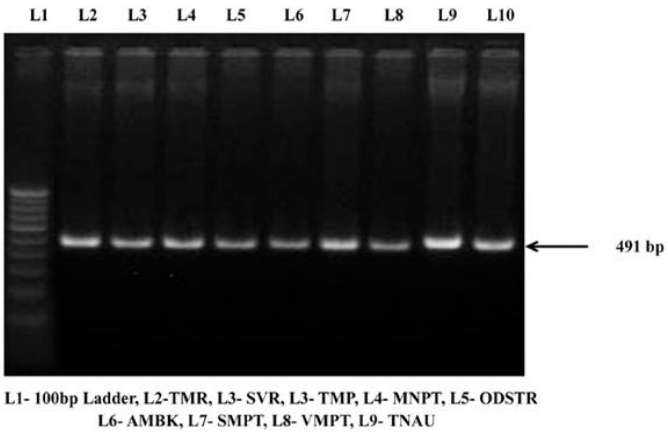

Plate 3b: Molecular confirmation of *X. axonopodis* pv. *punicae* by using ITS primer gyr B genus specific primer

Pathogenicity studies

The pathogenic nature of isolated pathogen was confirmed by establishing pathogenicity. Pathogenicity study was conducted with *X. axonopodis* pv. *punicae* isolate MNPT on the leaves of Ganesh variety by pin prick method. All the inoculated plants produced bacterial blight symptoms on leaves one 10 days after inoculation. A maximum disease incidence of 18 per cent was observed on 20 days after inoculation with the disease severity of 9 per cent. From the infected leaves, the pathogen wasreisolated and confirmed with an original culture (Plate 4).

Plate 4: Pathogenicity test Healthy leaves

Pathogen inoculated leaves

Discussion

Isolation and characterization of pomegranate bacterial blight pathogen

Isolation of the pathogen associated with plant disease is most important to know the etiology spread and its management. A bacterial pathogen was consistently isolated from the infected plant parts like leaf and fruit on Nutrient agar media (NA) and identified as *X. axonopodis* pv. *punicae* through morphological and bio chemical characterization. Further, the pathogen was confirmed as *X. axonopodis* pv. *punicae* through molecular characterization. Raghuwanshi *et al.* (2013) [15] isolated four isolates from different places of western Maharashtra from pomegranate bacterial blight infected plants. All the isolates showed yellow mucoid shiny colonies they have also performed KOH test and Gram staining test to confirm the Gram-negative rod these bacteria further characterized through a biochemical test. Pbasamma *et al.* (2013) [12] isolated twenty-one isolates of *X. axonopodis* pv. *punicae* from the pomegranate and studied their cultural characters like optimum pH, optimum salt concentration, optimum temperature and pigment fuscan production. Chand and Kishun (1991) [4] identified *X. axonopodis* pv. *punicae* based on the morphological, cultural, biochemical and

physiological characters. Hingorani and Mehta (1952) [6] first identified *X. axonopodis* pv. *punicae* in India through a morphological and biochemical test. The result of the present study like mucoid yellow colony negative to Gram reaction test and positive to citrate utilization, Starch hydrolysis, KOH solubility, Gelatin like action, H_2S production etc., were in conformity with other workers. The isolates of *X. axonopodis* pv. *punicae* were further confirmed by molecular characterization ITS region of 16s rDNA was amplified with ITS marker through polymerase chain reaction(PCR) which yielded an amplicon size of 400 bp. PCR reaction with gyr B gene-specific primer *X. axonopodis* pv. *punicae* yielded an amplicon size of 491 bp. Kalyan *et al.* (2012) [7] confirmed the *X. axonopodis* pv. *punicae* eight isolated through polymerase chain reaction with the help of 16s rRNA gene-based universal primers. They designed gyr B gene-based primers set namely KKM-5 forward andKKM-6 reverse which gene an amplicon size of 491. Wayde *et al.* (2015) [19] identified the *X. axonopodis* pv. *punicae* by employing the polymerase chain reaction. They used opb 0.6 and opb 19 primers which yielded an amplicon size of 500 base pairs. In the present study, the pathogen was confirmed by polymerase chain reaction with the help of ITS and gyr B gene primers.

Pathogenicity

Proving the pathogenicity of a pathogen is essential for identifying the pathogenic nature of a microorganism. The results of the pathogenicity study revealed that all the inoculated plants produced diagnostic symptoms on the leaves. Subsequently, the pathogen was reisolated and compared with original *X. axonopodis* pv. *punicae* isolate. The reisolated *X. axonopodis* pv. *punicae* was similar in all cultural, morphological and biochemical characters with that of the original MNPT isolates. Bora and kataki (2014) [2] established pathogenicity test spraying of 48 hours old bacterial culture on pinpricked 40 days old healthy pomegranate leaves and observed the identical symptoms on 23 days after inoculation. The pathogenicity test of *X. axonopodis* pv. *punicae* conducted by several workers *viz.*, Manjula (2002) [9], Peterson *et al.* (2010), Mondal and Mani (2012) [7] and Parkinson *et al.* (2009) [11].

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