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Evolutionary Relatedness and Identification of *Xanthomonas axonopodis* pv. *punicae* Causing Bacterial Blight in Pomegranate

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Abstract

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* has become a serious threat in Southern Asian countries. The prime step in the management of this disease is the characterization of the pathogen. Thus, isolation and identification of pathogen through various methods was performed. Further, *Xap* strain was studied for evolutionary relatedness by comparing with various *Xap* strains across the countries representing various geographical origins deposited in National Center for Biotechnology Information (NCBI) Genbank, targeting 16 rRNA loci. It is worth mentioning that the *Xap* strain IIHR-1 from Indian Institute of Horticulture Research, Bengaluru, India showed a close proximity with strain MS41287 of Himachal Pradesh, India by sharing 98% identity. Thus, 16S rRNA gene aid to unravel the evolutionary relatedness of the *Xap* strains across the countries.

***Key words*:** Pomegranate, *Xanthomonas,* Identification, Evolutionary relatedness

A large shrub that bears fruit, the *Punica granatum*, commonly known as the pomegranate, is a nutritious food with rich history and belongs to family Punicaceae. It is widely cultivated in drier part of southeast Asia, Malaya, Myanmar, China, Japan, USA (California), East Indies, Tropical America and India [1]. The area under pomegranate is 2.32 lakh hectares with production 28.96 lakh tonnes and productivity 12.48 MT/HA in India [2]. Pomegranate cultivation gained a momentum among growers due to its elevated market value. However, from the year 2002, growers are in dire straits due to severe outbreak of bacterial blight caused by *Xanthomonas axonopodis pv. punicae*, which was once deemed as a minor disease and presently became a serious threat for pomegranate production resulting in severe yield losses both in terms of quality and quantity. In India, this disease was first reported by Hingorani and Mehta [3]. Later on, Hingorani and Singh [4] took a thorough investigation on the disease as well as causal organism and designated the pathogen as *Xanthomonas punicae sp. de nov*. Further, Vauterin *et al.* [5] on the basis of DNA homology, renamed pathogen as *Xanthomonas axonopodis* pv. *punicae*.

The disease is characterized by the appearance of one or more water-soaked lesions on leaves, which causes defoliation on stern circumstances. The pathogen also instigates girdling and cracking symptoms on stems while dark brown, irregular slightly raised oily spots noticed on fruits which split opens and cause yield reduction on progression of disease. The disease spreads to long distance via infected plants, twigs and fruits and under severe condition it destroys the entire orchard causing heavy economic losses. Further, it was reported that disease hampered the expansion of pomegranate cultivation, production and export in India [6-7]. The disease has also been recorded in Pakistan, South Africa and Turkey [8-10] and poses a threat of unrestricted spread across the countries in ensuing years. Considering the deleterious effect of pathogen, there is every need of early identification and containing of disease. Hence, the present investigation majorly focused on isolation and identification of causal organism through various methods.

MATERIALS AND METHODS

Isolation of pathogen

The bacterium was isolated from the infected leaves of pomegranate. The infected leaves were washed, disinfected and macerated gently using sterile blade for bacterial diffusion. The presence of bacteria in suspension was confirmed as outlined by Sharma *et al*. [11], isolated and multiplied on LB (Luria Bertani) & 523/YDCA (yeast extract dextrose calcium carbonate agar) medium respectively by incubating at 28±2ºC for 24 -72 h [12].

Identification of pathogen

The morphological, biochemical, physiological and molecular characterization was done as depicted by Mondal and Kumar [13] to identify the pathogen.

Morphological characterization

Colony morphology: Single colony of bacteria was dissolved in sterile water to prepare the suspension. A loopful of bacterial suspension was streaked on various media viz. 523,

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LB and YDCA, then incubated at 28±2°C for 24 -72 hours. After 72 hours, morphology of colony was studied.

Fuscan pigment production: Single colony of bacteria was dissolved in sterile water to prepare the suspension. A loopful of bacterial suspension was streaked on LB medium, simultaneously inoculated in LB broth and incubated at 28 °C. Growth of pathogen was examined for ten days to observe the fuscan or brown pigment development.

Gram staining: The bacterial cells were first fixed with heat on the glass slides and then stained with crystal violet. The slides were then treated with an I_2-KI mixture (mordant) to fix the stain, de-stained briefly by washing with 95% alcohol and finally counter stained with safranin, for observation.

Pathogenecity test

The infected leaf samples were collected and pathogen was isolated as pure culture from the diseased tissues. A bacterial suspension containing 10^6 *CFU*/ml was used for pathogenicity test and the control with sterile water inoculation was also maintained.

Attached leaf assay: The healthy plants of the susceptible variety were selected, sprayed and kept in glasshouse at 28 – 30°C for observations.

Detached leaf assay: Matured leaves of susceptible variety were collected, cleaned with sterile distilled water and placed on moistened filter paper in sterile petri-plate. Leaves were inoculated with inoculum by pin prick method and then incubated in a growth chamber at 28±2 °C.

Physiological characterization

Temperature sensitivity: The optimum temperature required for pathogen was standardized by subjecting the petri-plates containing isolate to two temperatures like 28°C and 37°C. Further, the growth of the pathogen was observed after 72 hours of inoculation.

pH sensitivity: LB broth was prepared and 5 ml each of the prepared solution was dispensed to the eight screw cap bottles of 15ml capacity. Few drops of prepared 1N NaOH or 1N HCl was added to the LB broth to adjust the pH to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 respectively. Invariably, 50 µl of bacterial suspension was added to all the bottles and incubated at 28 °C in shaker incubator at 200 rpm for 72 hours. Under each pH, three replications were maintained. After 72 hours, turbidity was measured at 600 nm in spectrophotometer and analyzed.

Salt sensitivity: Stock solution of sodium chloride (20%) was prepared. LB broth was also primed and 5 ml each of the prepared solution was dispensed to the nine screw cap bottles of 15ml capacity. Adequate amount of NaCl stock solution was amended with LB broth, so as to obtain NaCl concentrations of 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% respectively. Subsequently, bacterial suspension (50 µl) was added to all the bottles irrespective to the concentration of NaCl and kept at 28 °C in shaker incubator at 200 rpm for 72 hours, three replications for each concentration of NaCl were maintained. Growth of the pathogen was observed after 72 hours and turbidity was measured at 600 nm in spectrophotometer.

Biochemical characterization

Bacterial isolate was also subjected to various biochemical tests according to Bergey's manual of Determinative Bacteriology for confirmation of pathogen, as *Xanthomonas.* Here, various tests viz., hydrogen sulphide, catalase and oxidase production, starch hydrolysis, methyl red, citrate utilization, gelatin liquefaction, indole and Voges Prauskeur test were employed for identification of pathogen.

Molecular characterization

GyraseB primer: Colony PCR analysis was done using GyraseB primer, where a colony of bacteria was picked with tooth pick and dissolved in a tube containing PCR reaction mixture of 25 µl volume. A PCR reaction volume was made by mixing 1 µl template (100 ng µl-1), 2.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs, 5 µl 5X buffer, 1 µl forward primer (10 pmol µl-1), 1µl reverse primer (10 pmol µl-1), 0.25 µl TaqDNA polymerase (5 units µl-1) (Fermentus Co.), and 14.75 µl nuclease free sterile water. The PCR condition followed consisted of initial denaturation at 94 °C for 5', then 30 cycles comprising denaturation at 94 °C for 30 Sec, annealing at 55 °C for 1', extension at 72 °C for 1' followed by a final extension cycle at 72 °C for 3'and final shock at 4 °C.

Universal primer: A colony of bacteria was allowed to grow in LB broth for 24 hours. Then, DNA was extracted using CTAB method. The extraction of total genomic DNA was carried out following a modified protocol as described by Adachi and Takashi [14].

Table 1 Various strains of *Xanthomonas axonopodis* and their sources

S. No.	Pathovar and strains of *Xap Xanthomonas axonopodis*	Accession number	Source of strain	S. No.	Pathovar and strains of *Xap Xanthomonas axonopodis*	Accession number	Source of strain
1.	Xa pv. punicae strain Serik4	KM007073	Serik, Turkey	51.	Xa strain S53	AB101447	MG, Brazil
2.	Xa pv. punicae strain AP-5	JQ067629	AP, India	52.	Xa strain S54	AB101446	Tochigi, Japan
3.	Xa pv. punicae strain AP-4	JQ067628	AP, India	53.	Xa strain S11	AB101445	Tochigi, Japan
4.	Xa pv. punicae strain AP-3	JQ067627	AP, India	54.	Xapv.poinsettiicolastrainBACTu l	EF093138	Tlalnepantl a, Mexico
5.	Xa pv. punicae strain AP-2	JQ067626	AP, India	55.	Xapv. punicae strain ISO26	HM590466	Karnataka, India
6.	Xa pv. punicae strain AP-1	JQ067625	AP, India	56.	Xapv. punicae strain ISO27	HM590465	Karnataka, India
7.	Xa pv. punicae strain 85	JN109175	Maharashtra, India	57.	Xapv. punicae strain ISO2	HM590464	Karnataka, India

8.	Xa pv. punicae strain 84	JN109174	Maharashtra, India
9.	Xa pv. punicae strain 82	JN109173	Maharashtra, India
10.	Xa pv. punicae strain 81	JN109172	Maharashtra, India
11.	Xa pv. punicae strain 80	JN109171	Maharashtra, India
12.	Xa pv. punicae strain 16 IARI	JN036629	New Delhi, India
13.	Xa pv. punicae strain 15 IARI	JN036628	New Delhi, India
14.	Xa pv. punicae strain 14 IARI	JN036627	New Delhi, India
15.	Xa pv. punicae strain 13 IARI	JN036626	New Delhi, India
16.	Xa pv. punicae strain 12 IARI	JN036625	New Delhi, India
17.	Xa pv. punicae strain 11 IARI	JN036624	New Delhi, India
18.	Xa pv. punicae strain 6 IARI	JN036623	New Delhi, India
19.	Xa pv. punicae strain 5 IARI	JN036622	New Delhi, India
20.	Xa pv. punicae strain 4 IARI	JN036621	New Delhi, India
21.	Xa pv. punicae strain IIHR 1	KT222897	Karnataka, India
22.	Xa strain LMG538	AF209753	SP, Brazil
23.	Xa strain MTCC5435	JF838178	Gujarat, India
24.	Xa strain KNUC393	EU239097	Daegu, Korea
25.	Xa strain SBANHCu14	KT020945	Tamil Nadu, India
26.	Xa strain BPFR20	KC869695	Maharashtra, India
27.	Xa strain YPH-1	KC841470	Guangdong, China
28.	Xa strain KSI1432	KC113174	Ahvaz, Iran
29.	Xa strain PSBNRA-1	HQ393866	Rajasthan, India
30.	Xa strain PB28	JF430872	CA, USA
31.	Xa strain Xac-2	GU071116	Haryana, India
32.	Xa strain Xac-1	GU071115	Haryana, India
33.	Xa strain Sdau08-29	GU129691	Shandong, P. R. China
34.	Xa strain MST07	EF101980	MG, Brazil
35.	Xa strain MSF	EF101979	MG, Brazil
36.	Xa strain IP2-23	EF101978	MG, Brazil
37.	Xa strain AR-PINLBH3	HM582883	WP, Malaysia
38.	Xa strain ESS01	EF101970	MG, Brazil
39.	Xa pv. punicae strain 3IARI	JN036620	New Delhi, India
40.	Xa pv. punicae strain MS41287	KR181933	HP, India
58.	Xapv. punicae strain ISO12	HM590463	Karnataka, India
59.	Xapv. punicae strain ISO9	HM590462	Karnataka, India
60.	Xapv.poinsettiicola strain HN-18	GU144262	Zhejiang, China
61.	Xapv. syngonii strain X191	AY576651	FL, USA
62.	Xapv. malvacearum	DQ414745	Maharashtra, India
63.	Xapv. punicae strain MTCC5606	JN036619	New Delhi, India
64.	Xapv.manihotisstrain MXAHN01	FJ196285	Hainan, China
65.	Xapv. phaseoli strain KASC1126	KT283577	Rajasthan, India
66.	Xapv. dieffenbachiae strain CE6	KT758341	Kaduna, Nigeria
67.	Xa pv. phaseoli strain KASK1178	KR005845	Rajasthan, India
68.	Xa pv. dieffenbachiae strain AC1	KP247494	Guelph, Canada
69.	Xa pv. punicae strain Xavp1	KP168824	Maharashtra, India
70.	Xa pv. cyamopsidis strain xac-xj2	KF563928	Xinjiang, China
71.	Xa pv. cyamopsidis strain xac-xj1	KF563927	Xinjiang, China
72.	Xa pv. cyamopsidis strain xac-xj3	KF563926	Xinjiang, China
73.	Xa pv. phaseoli strain YX3	HQ670695	Beijing, China
74.	Xa pv. phaseoli strain YX2	HQ670694	Beijing, China
75.	Xa pv. aurantifolii strain X341	AF442740	FL, USA
76.	Xa pv. aurantifolii strain X84	AF442739	FL, USA
77.	Xa pv. poinsettiicola strain XAP1	DQ414814	Taiwan, China
78.	Xa pv. punicae strain ISO41	HM590468	Karnataka, India
79.	Xa pv. glycines strain C12	KM593178	Jiangsu, China
80.	Xa pv. glycines strain C5	KM593177	Jiangsu, China
81.	Xa pv. glycines strain B523	KM593176	Jiangsu, China
82.	Xa pv. glycines strain A224	KM593175	Jiangsu, China
83.	Xa pv. dieffenbachiae strain XADK	KM576803	Kerala, India
84.	Xa pv. desmodiirotundifolistrain KNU28189	GU969139	Gangwon, Korea
85.	Xa pv. vasculorum strain KNU28186	-	-
86.	Xa pv. vasculorum strain KNU28187	-	-
87.	Xa pv. alfalfae strain KNU 28188	GU969136	Gangwon, Korea
88.	Xa pv. poinsettiicola strain HN-20	GU144263	Zhejiang, China
89.	Xa pv. poinsettiicola strain HN-1	GU144261	Zhejiang, China
90.	Xa pv. poinsettiicola strain LMG849	GU144258	Zhejiang, China

41. Xa strain IP1-36	EF101977	MG, Brazil
42. Xa strain ECLCAF03	EF101969	MG, Brazil
43. Xa strain BSC23	EF101968	MG, Brazil
44. Xa strain BSC475a	EF101967	Kampala, Uganda
45. Xa strain NCPPB2965	KP033430	Kampala, Uganda
46. Xa strain DSHC17	KP033429	Kampala, Uganda
47. Xa strain NCPPB638	KP033428	Kampala, Uganda
48. Xa strain NCPPB457	KP033423	Kampala, Uganda
49. Xa strain NCPPB210	KP033417	Kampala, Uganda
50. Xa strain NCPPB410	KP033415	Tochigi, Japan
91. Xa pv. spondiae	EF989734	Reunion, France
92. Xa pv. poinsettiicola strain LMG5401	GU144255	Zhejiang, China
93. Xa pv. allii	-	-
94. Xa pv. vesicatoria	AY613946	Chicago, USA
95. Xa pv. citri strain XCW	DQ991194	Taichung County, Taiwan
96. Xa pv. passiflorae strain PA6.1	FJ828867	SP, Brazil
97. Xa pv. passiflorae strain LP1.1a	FJ828866	SP, Brazil
98. Xa pv.citrumelo strain F1	AF442741	FL, USA
99. Xa pv. vesicatoria strain CNPH411	AY288081	SP, Brazil
100. Xa pv.vitians strain IBSBF1553	FJ828870	SP, Brazil

Data analysis

A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) Version 6.0 [15] to the set of *Xanthomonas axonopodis* sequence analogs of present and previous studies (Table 1). Cluster analysis was carried out using CLUSTALX [16] based on the neighbour-joining tree [17] with default settings. Statistical analyses were performed using SPSS package (SPSS Inc. version 16.0) for all sets of data.

RESULTS AND DISCUSSION

Isolation and identification of Xanthomonas axonopodis pv. punicae

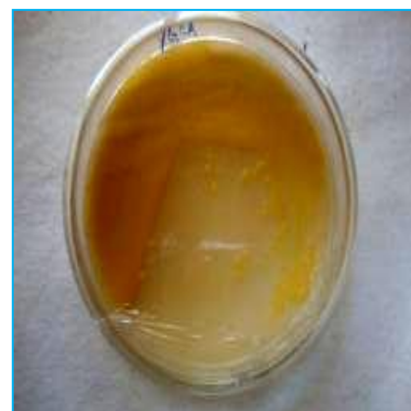
The microscopic observation of suspension revealed the association of pathogen with the bacterial blight disease in pomegranate by exhibiting the inherent characteristics like appearance as single or in pairs, capsulated, rod shaped and Gram negative in nature. Isolation made from the bacterial suspension exhibited colonies as circular, convex, mucoid, shiny and yellow on agar plates confirming pathogen. Nevertheless, further confirmation studies are essential to distinguish from other *Xanthomonas* species.

Morphological characteristics: It was observed that morphology of the colonies showed varied phenotype on different culture media evaluated. The colony was minute, yellow in colour, slightly raised, convex surfaced and translucent on LB medium, while on YDCA and 523 medium, luxuriant growth of bacteria was noticed and colony appeared deep yellow color, shiny and more mucous. Similar phenotypic observations of *Xanthomonas axonopodis* pv. *punicae* were made [18-20]. The fuscan production was observed in all the media after 7 days, irrespectively (Plate 1). The diffusion of brown pigmentation was also noticed in LB broth (Plate 2). The secretion and subsequent oxidation of homogentisic acid might be rationale for diffusible brown pigment in the media as suggested by Goodwin and Sopher [21] with *Xanthomonas campestris* pv. *phaseoli.*

Pathogenecity test: The plants sprayed with the cell suspension exhibited symptoms after 10 days of inoculation in attached leaf assay. Initially oily spots were observed, which later appeared as yellow haloes, turned brown and finally formed typical necrotic lesions on progress of disease. In detached leaf assay, similar phenotypic observation was made during the development of disease; however, early onset of necrotic lesion was noticed after 2 days and rapidly infected whole leaf within 10 days of inoculation in susceptible variety (Plate 3). In contrast, no symptoms were seen in the control treatment. The pathogen was re-isolated from the inoculated plant and found similar with the original culture. Thereby, Koch postulates were demonstrated and results corroborated with the previous findings [22-23] for pathogen conformity studies.

LB medium

523 medium



YGCA medium

Plate 1 Fuscan production in various medium by *Xanthomonas axonopodis* pv. *punicae*

Attached leaf assay

Detached leaf assay

Plate 3 Pathogenicity test to prove Koch's postulates

Physiological characteristics: The luxuriant growth of bacteria was observed in LB plates kept at the temperature of 28 °C as against no growth in 37 °C. Pathogen propagation was abundant at a pH ranged from 6 to 9 and no growth evinced below 6 and above 9 (Table 2, Plate 4). Salt concentrations 1% & 2% were found optimum for multiplication of pathogen, beyond which no growth was noticed. It was also evident that plenty of growth recorded in control than any other treatment under salt assay sensitivity test (Table 2, Plate 5). The similar results were presented in isolates of Maharashtra [24].

Table 2 Effect of pH and NaCl on the growth of *Xanthomonas axonopodis* pv.*punicae* isolates in LB broth medium

pH	Turbidity (absorbance at 600 nm)		NaCl concentration (%)	Turbidity (absorbance at 600 nm)	
	IIHR Isolate	NRCP Isolate Reference isolate		IIHR Isolate	NRCP Isolate Reference isolate
1.	0.000	0.001	0	1.522	1.423
2.	0.000	0.001	1	1.442	1.435
3.	0.000	0.001	2	1.168	1.024
4.	0.001	0.002	3	0.307	0.313
5.	0.003	0.002	4	0.047	0.013
6.	0.085	0.091	5	0.013	0.012
7.	0.091	0.092	6	0.003	0.002
8.	1.206	1.152	7	0.003	0.003
9.	0.091	1.021	8	0.000	0.000
10.	0.001	0.001	-	-	-
SE(m)	0.001	0.001	SE(m)	0.001	0.001
CD	0.002	0.002	CD	0.003	0.002

Plate 4 pH sensitivity test for the growth of *Xanthomonas axonopodis* pv. *punicae*

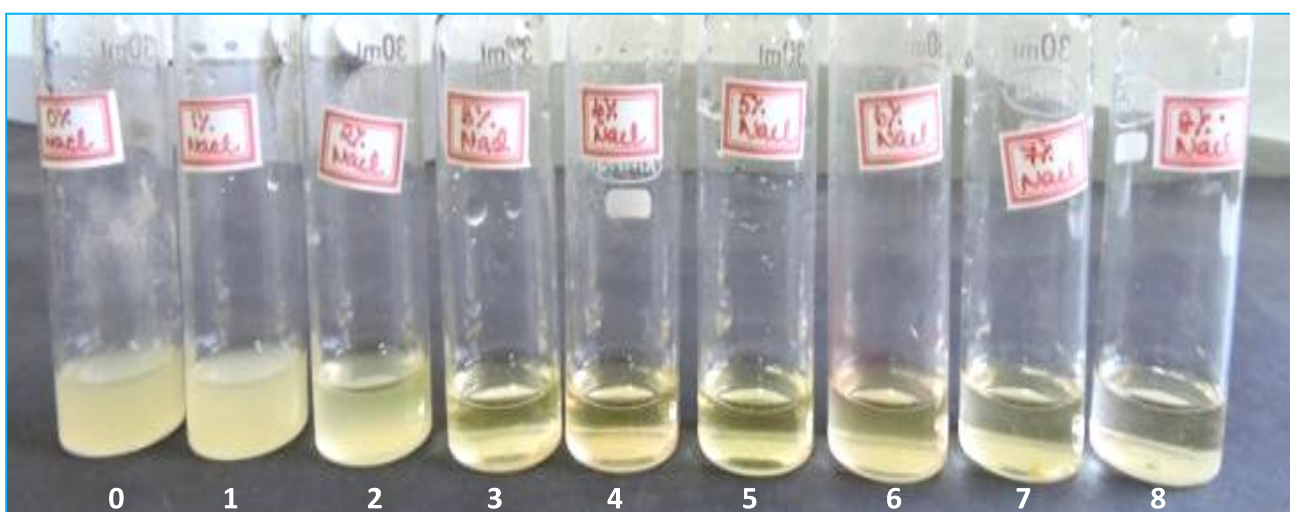


Plate 5 Salt sensitivity test (%) for the growth of *Xanthomonas axonopodis* pv. *punicae*

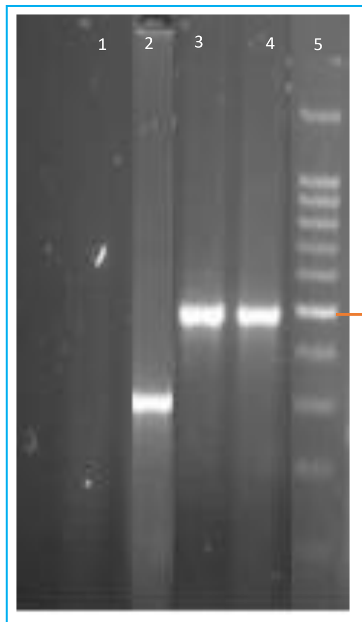
Biochemical characteristics: Biochemical properties vary between different groups of pathogens. Here, pathogen reaction was positive to starch hydrolysis, hydrogen sulphide production, catalase, oxidase production, methyl red and citrate utilization test, while negative to gelatin liquefaction, indole and Voges Prauskeur test. Biochemical characterization was accomplished and corroborated with previous findings [25-27].

Molecular characteristics: The rapid and reliable method of molecular characterization using GyraseB primer gave amplification of template DNA with amplicon size 491bp confirming pathogen as *Xanthomonas axonopodis* pv. *punicae* (Plate 6) as illustrated [28]. Further authentication of pathogen was made using universal primers (27F and 1492R) as the 16S rRNA sequence is a highly conserved region and aids discrimination between the species and genera of various pathogens [29]. Subsequently, DNA was sequenced, subjected to phylogeny analysis and deposited in NCBIGen bank as *Xanthomonas axonopodis* pv. *punicae* isolate IIHR-1 with an accession number KT 222897 (Plate 7). Similar results were presented from Tamil Nadu, India [30].

It was also note-worthy that the isolate IIHR-1showed similar characteristics of reference isolate collected from National Research Centre on Pomegranate, Solapur at morphological, biochemical and molecular level identification of pathogen.

Construction analysis of phylogeny

The 16S rRNA ribosomal PCR of the isolate (IIHR-1) amplified 661 bp product of *Xanthomonas axonopodis* pv. *punicae*. The sequence of 16S rRNA gene obtained with the universal primers 27F and 1492R was analyzed using NCBI BLAST as described [31], it showed 98% similarity with other bacterial strains of same species, existing in National Center for Biotechnology Information (NCBI) database. A total of 100 strains collected from National Center for Biotechnology Information (NCBI) database were compared with the bacterial 16S rRNA gene sequence of isolate IIHR-1, constructed a phylogenetic tree and depicted the evolutionary relatedness of IIHR-1 strain using maximum likelihood method along Jones Taylor Thornton (JTT) model with 500 bootstrap replications. Considering the bootstrap value 65, phylogenetic tree formed two major clades, where the strain *Xanthomonas axonopodis* pv. *punicae* IIHR-1 was integrated in the first clade, which includes maximum strains of *Xanthomonas axonopodis* pv. *punicae* from various states of India (Fig 1). It was also noticed that the strain IIHR-1 showed close proximity with the strain MS41287 from Himachal Pradesh, India with the accession number KR181933. Similar genetic relationship with various strains of *Xanthomonas axonopodis* was also established [32]. The pathogen isolated was thus confirmed at the morphological, biochemical and molecular levels and was further used for various studies.



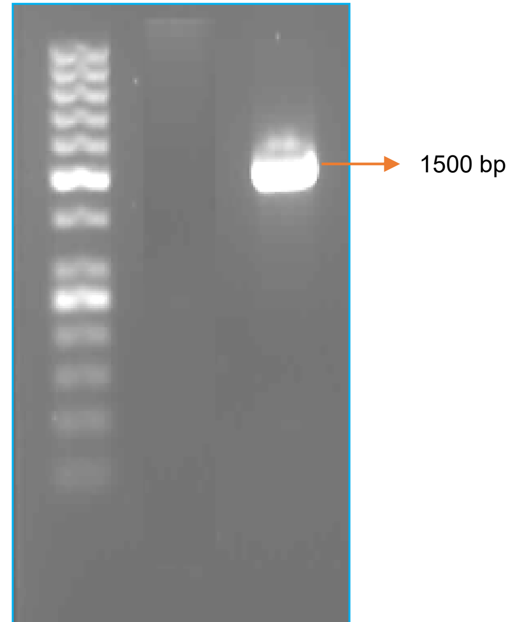


Plate 6 Appearance of 491bp amplicon indicates the presence of pathogen in the sample using primer gyrBF & gyrBR

Lane 1 - Control without sample
Lane 2 - Negative control
Lane 3 - *Xap* from NRCP, solapur
Lane 4 - *Xap* from IIHR, Bengaluru
Lane 5 - Ladder

Plate 7 Appearance of 1500bp amplicon indicates the presence of pathogen in the sample using universal primer

Lane 1 - Ladder
Lane 2 - Control without sample
Lane 3 - *Xap* from IIHR, Bengaluru

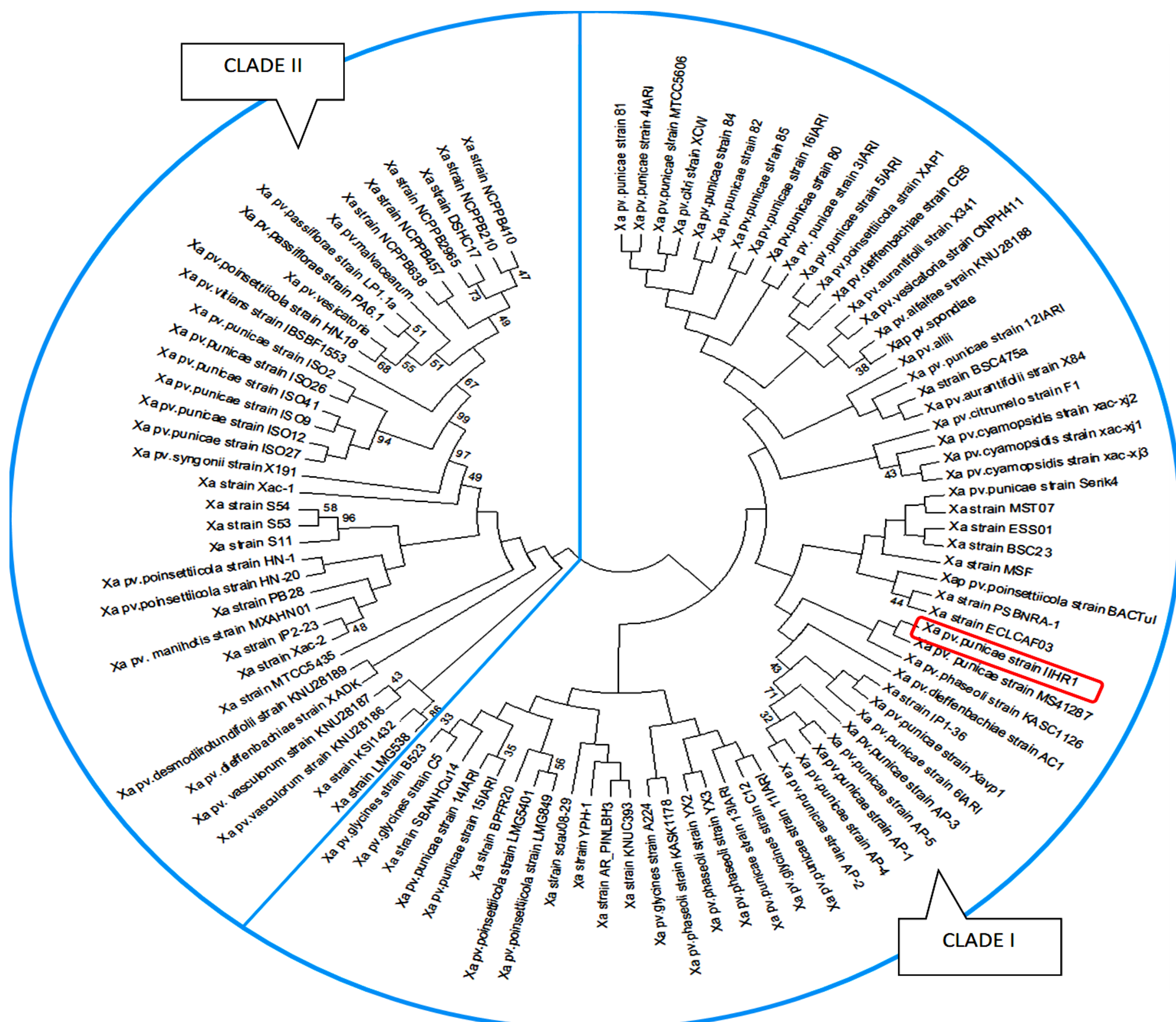


Fig 1 Molecular phylogenetic analysis of *Xanthomonas axonopodis pv. punicae* of current and previous studies by using maximum likelihood method along with jones-taylorthornton (JTT) model. The numbers on the branches of tree are confidence values based on bootstrap method higher than 30% (500 replications). Clade I and Clade II represents the strains of *Xanthomonas axonopodis* pv. *punicae*

CONCLUSION

Characterization of pathogen at morphological, biochemical and molecular level are essential for an accurate identification of unknown pathogen. Thus, the study encompassed disease diagnosis by isolation, sub-culturing and assessment of pathogen. The results revealed that causal organism of bacterial blight isolated from the leaves of pomegranate was *Xanthomonas axonopodis* pv. *punicae*. Further, the DNA (deoxyribonucleic acid) sequence of the isolate was deposited in National Center for Biotechnology Information (NCBI) gene bank with an accession number KT 222897. The phylogenic and evolutionary correlations of concatenated sequences of *Xanthomonas spp*. classified strains into two clades and depicted a close relationship between strains MS41287 and IIHR-1 from Himachal Pradesh and Karnataka of states India, respectively. Additionally, these findings provide a reliable foundation for developing targeted disease management strategies and improving pomegranate crop protection through precise pathogen identification.

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Author's contribution

B. Tanuja Priya: Writing - original draft, methodology; conceptualization, investigation, data analysis; B. N. S. Murthy: Review, editing, Visualization, conceptualization and supervision; Anand C. Reddy: Review & editing; C. Gopalakrishnan: Review and Supervision

Conflict of interest

Authors declare that no conflict of interest exists.

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