

# Characterization of *Xanthomonas axonopodis* pv. punicae (Hingorani and Singh) Vauterin et al. and Isolation of Xap Specific Bacteriophage

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Bacterial blight (BLB) caused by Xanthomonas axonopodis pv. punicae (Xap) has emerged as a potential threat in pomegranate cultivation in India. To know the present disease scenario and to identify the root cause for its epidemic, survey was conducted in five different localities of Tamil Nadu. Per cent disease severity (PDS) ranged from 1 to 36 per cent on leaves and zero to 40 per cent infection on fruits. Bacteriophage or bacteria killer specific to Xap-1 (Sennampatty) isolate was isolated from pond water after enrichment of water sample. Phage produced plaques of 2 mm, 1 mm and < 1mm size that coalesced after 18 h of plating which shows virulence and rapid phage multiplication. Disease severity on test plants were studied to know the degree of virulence and variability among the isolates with highest severity of 37.31 per cent documented in Xap-1 followed by Xap-3 (28.7%) and Xap-2 (24.3%). Physiological factors such as temperature, pH and their role in growth of Xap-1 were accessed. Maximum growth was observed at 28°C (0.86 OD) followed by 30°C (0.68 OD) and pH 7 (1.83 OD) and optimum at 7-8 pH for virulent (Xap-1) isolate. Molecular confirmation of Xap through 16S rRNA sequencing gave the amplicon of 1500 bp size and amplification through KKM5 and KKM6 further confirmed specific identity.

Key words: Pomegranate, Bacterial blight, Bacteriophage, Physiological and molecular characterization

Pomegranate (*Punica granatum L.*) belongs to the *Punicaceae* family which is one of the economically important fruit crops of India. Place of origin is believed to be Iran. In India it is cultivated over an area of 208.73 thousand ha with production of 2442.39 thousand metric tonnes (MT) and productivity of 11.70 MT. h<sup>-1</sup>. Maharashtra occupying 2/3<sup>rd</sup> of area followed by Karnataka and Gujarat, but highest productivity is recorded in Tamil Nadu with 27.43 t. ha<sup>-1</sup> ("Horticulture statistics at a glance"). In Tamil Nadu the crop is cultivated in Erode, Dindigul, Coimbatore and Tirunelveli districts with major markets in Coimbatore and Chennai. Popular varieties extensively cultivated are Bhagwa, Ruby and Ganesh.

In spite of these the fruit crop is attacked by several diseases such as anthracnose, *Cercospora* leaf spot and bacterial blight (BLB). Bacterial blight was first reported in Delhi by Hingorani and Mehta during 1952. Hingorani and Mehta (1952) first identified the causal organism as *Xanthomonas campestris*, later it was reclassified and renamed as *Xanthomonas axonopodis* pv. *punicae* (*Xap*). Symptomatology of BLB on leaf appears as small (2 to 5mm), irregular, prominent water soaked spots, which later develops necrotic center surrounded by water soaked margins and in the progressive stage of the disease the individual spots coalesce giving an eventual blighted appearance, lesions on stem are dark brown to black that leads to cracking and the breaking of branches. On fruits spots appears as irregular brown to black with Y or L shaped cracking or splitting of pericarp (Mondal *et al.*, 2012). With this background information, the present study was carried out to isolate and study the *Xanthomonas axonopodis pv. punicae* pathogen in pomegranate from different regions of Tamil Nadu and also an attempt was made to isolate *Xanthomonas axonopodis pv. punicae* specific bacteriophage from pond water.

#### **Material and Methods**

For collection of symptoms and to know the bacterial blight of pomegranate severity in the field, survey was conducted from five different localities of Tamil Nadu. Survey was done from Sennampatty (12.1206°N and 78.3814°E) of Erode district, Thondamuthur (10.9899°N and 76.8409°E) of Coimbatore district, Tiruchengode (11.3782°N and 77.8969°E) of Nammakal district, Udumalpet (10.5846°N and 77.2514°E) of Tirupur district, TNAU orchard (11.0045°N and 76.961632°E) of Coimbatore district, where symptoms were collected from Bhagwa variety pomegranate plants and the per cent disease severity was determined using 0-6 disease severity scale given by ICAR-NRCP.

### Xanthomonas axonopodis pv. punicae culture

Symptoms like blightening with oily ring on the leaves were selected and surface sterilized with 70 per cent ethanol further sterilized with 0.1 per cent mercuric chloride for 1 min. Followed by transferring

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the bits serially in sterile distilled water, then sterilized bits transferred in to Eppendorf tube containing sterile water and crushed; which allows the bacteria to diffuse in water for 10 min finally with the help of inoculation loop the diffused bacteria is streaked on the nutrient agar, plates incubated at 29-30° C for 48-72 h. Appearance of yellow convex or mucoid colonies can be observed on nutrient agar after 48 h. of incubation and the isolates were designated as *Xap*-1, *Xap*-2, *Xap*-3, *Xap*-4 and *Xap*-5, respectively (Raghuwanshi *et al.*, 2013).

### Isolation of bacteriophage specific for Xanthomonas axonopodis pv. punicae

The isolation procedure of bacteriophage from pond water was done as described by (Chae et al., 2014). pond water (76.92°E and 11.006°N) of 5 ml was collected, to that equal volume of phage broth (peptone 10 g, yeast extract 5 g, sodium chloride 2.5 g, dipotassium hydrogen orthophosphate 8 g, water 1000 ml) and 4 to 5 ml of 48 hours old virulent Xap-1 broth was added and incubated overnight at room temperature. Consecutively, few drops of chloroform was mixed in the sample by keeping them in rotating shaker for 20 min followed by centrifugation of the incubated sample at 13,000 rpm for 10 min, supernatant was passed through 0.22 µm syringe filter. Phage filtrate was stored at 4°C in complete darkness. Enumeration of phages was done by double agar overlay method proposed by Lark and Adams, 1953. Later for long term storage of the phages, single phage plaque were taken in the form discs and added to phage buffer, centrifuged and filter sterilized.

# Enumeration of phages by double-layer plaque assay

Enumeration is done by double agar overlay method as proposed by Lark and Adams (1953). Nutrient glucose agar (beef extract 3 g, peptone 5 g, sodium chloride 5 g, glucose 2.5 g, water 1000 ml) medium supplemented with 10 mM CaCl, and kept in the water bath at 5560°C. Nutrient glucose agar (Bottom agar) of 15 to 20 ml was poured on the sterilized plates and allowed to solidify. Sterilized nutrient glucose agar at 7 per cent agar (Top agar) concentration was amended with 10 mM CaCl and top agar was maintained at 45-50° C. After the incubation 100 µl of phage filtrate and 100 µl of overnight mass multiplied Xap-1 broth was mixed and poured above the bottom agar, plates were allowed to solidify for 20 min and incubated overnight at room temperature. Number and morphology of plaques was accessed after incubation of plates at room temperature.

### Pathogenicity test

Pathogenicity test was conducted in greenhouse conditions where one and half year old seedlings of susceptible variety "Bhagwa" were pruned 45 d before inoculation and well irrigated. Five isolates was tested for pathogenicity, one d prior to inoculation the pots were covered with polythene covers with holes to maintain more than 80 per cent relative humidity required for successful infection (OD at 600nm = 0.3, equivalent to 10<sup>8</sup> cfu/ml). Bacterial isolates streaked on the medium was collected with the help of inoculation loop and mixed with sterile water and final volume was made to 100 ml; later the inoculum was sprayed on pomegranate plants during evening hours using hand sprayer (Peerajade et al., 2017) and finally covered with polythene cover, whereas the control plants were sprayed with sterile distilled water. Isolates showing high disease severity in test plants was considered as virulent and virulent isolate was tested for standardization of inoculation technique by three methods such as spray inoculation, infiltration and pin prick. Pathogenic variability among isolates was determined by symptom expression and lesion development up to 45th d after inoculation (Peerajade et al., 2017).

### Influence of temperature and pH on Xap-1 growth

The Xap-1 inoculated in Nutrient glucose broth (NGB) was placed in constant temperature at 10°C, 28°C, 29°C, 30°C, 35°C, 37°C and room temperature for 48 h with three replications. Mass multiplied broth was spectrophotometrically measured at 600 nm and minimum, maximum and optimum growth with varying temperatures was recorded. To study the optimum pH for the growth of the Xanthomonas axonopodis pv. punicae, the pH of the NGB medium was adjusted to a range of 2 to 9 with an increase value of one, inoculated with virulent isolate (Xap-1) and incubated at 28°C for 48 h. OD values were spectrophotometrically measured and the pH showing highest growth was optimized as favorable pH.

# Amplification by 16S rRNA primers

Confirmation of conserved 16S rRNA region of *Xanthomonas axonopodis* pv. *punicae* by using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') with PCR conditions at initial denaturation of 96°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min in nexus gradient Mastercycler, shows the amplicon size of 1537 bp (Mondal *et al.*, 2013).

# Amplification of gyrB specific region by specific primers

Specific primer amplification was carried out at 94°C for 7 min, followed by 30 cycles of 94°C for 30 sec, annealing of 57°C for 60 sec and extension 72°C for 1 min. Reactions were completed with a final extension step of 10 min at 72°C in nexus gradient Mastercycler by KKM5F (5'-GTTGATGCTGTTC ACCAGCG-3') and KKM6R (5'-CATTCATTTCGCCCAAGCCC-3') primers (Mondal *et al.*, 2013).

Cultural and physiological factors required for *Xanthomonas axonopodis* pv. *punicae*, disease severity from pathogenicity test were analyzed by generalized univariate linear model. Effect of pH, temperature and per cent disease severity was analyzed using SPSS 16.0. The treatment means

were separated using the Fisher's protected least significant difference (DMRT) ( $\alpha$  = 0.05).

# **Results and Discussion**

The survey report mentioned revealed that among the five locations, highest per cent disease severity

of blight was observed in *Xap*-1 (Sennampatty) showing maximum disease severity (PDS) of 36 per cent on leaves and 40 per cent on fruits, followed by Thondamuthur (*Xap*-2) region (13 % on leaves and 2 % on fruits). Third isolate (*Xap*-3) was collected from Tiruchengode field (8 and 2 PDS on leaves and fruits).

Table 1. Pathogenicity test of *Xanthomonas axonopodis* pv. *punicae* and symptom development on leaves up to 45 days

Isolates	DAI for symptom expression*	Disease severity (%)	5 DAI	15 DAI	25 DAI	35 DAI	45 DAI	SE	CD(0.05)
Xap-1	6	37.3 (6.11)ª	0.0 (0.71) <sup>d</sup>	3.5 (2.00)°	5.00 (2.35)⁵	5.50 (2.45)ª	5.52 (2.45)ª	0.02	0.05
Xap-2	7	24.3 (4.93)°	0 (0.71) <sup>e</sup>	2.00 (1.58) <sup>d</sup>	3.00 (1.87) <sup>°</sup>	4.5 (2.24) <sup>b</sup>	4.97 (2.34)ª	0.01	0.03
Xap-3	8	28.7 (5.35)⁵	0 (0.71)⁰	3.50 (2.00) <sup>b</sup>	3.50 (2.00) <sup>b</sup>	3.99 (2.12)ª	4.00 (2.12)ª	0.01	0.04
Xap-4	8	10.2 (3.19) <sup>d</sup>	0 (0.71) <sup>d</sup>	3.00 (1.87)⁰	3.50 (2.00) <sup>b</sup>	3.50 (2.00) <sup>ь</sup>	4.00 2.12)ª	0.01	0.03
Xap-5	8	6.8 (2.60) <sup>e</sup>	0 (0.71) <sup>d</sup>	2.00 (1.58)⁰	3.00 (1.87) <sup>b</sup>	3.50 (2.00)ª	3.50 (2.00)ª	0.01	0.03
	SE	0.04							
	CD (0.05)	0.1							

Mean of three replications; \* DAI - Days after inoculation; values within parenthesis are square root transformation. Means in a column followed by same superscript letters are significantly different according to DMRT.

In Udumalpet (*Xap*-4) region disease severity was very low (2%) on leaves and no symptoms were recorded on fruits. *Xap*-5 isolate was collected from TNAU orchard plants with only few spots on leaves.

Lowest PDS was recorded from TNAU orchard because of fewer populations of plants, wider spacing and regular chemical sprays.



Fig. 1. a- Minute oily spots seen in pomegranate susceptible variety "Bhagwa" inoculated plants; bdevelopment of necrotic center with oily ring on leaves; c- lesion with yellow halo on upper surface; d- lesion on lower surface; e- shedding or defoliation of infected leaves.

### Xap specific phages from pond water

Pond water phage sample designated as PE-1 contained circular plaques of 2 mm, 1 mm and <1 mm size appeared after 4-5 h of plating and merging of plaques were noticed after 18 h of plating. Plaques are the lytic zone produced by virulent bacteriophages on Xap-1 bacterial lawn, genomics behind this phage lytic principle is "endolysins" which are phage encoded peptidoglycan hydrolases that works in harmony with holins and together cause phage lysis after maturation of phages. Single plaque disc was separated with inoculation loop and added to phage buffer ( NaCL 5.8 g, MgSO<sub>4</sub>.7 H<sub>2</sub>O 2 g, 1M Tris HCl (pH 7.4) 50 ml, 2% gelatin 5 ml and water 1000 ml) and preserved at 4° C in complete darkness. Haq et al. (2012) isolated phage from stream water of plaque size ranging from 0.1 to 0.5 mm in diameter and with unique pinpoint morphology and well defined boundaries against Aeromonas punctata which is a gram negative, rod shaped bacterial cells. Phages are highly host specific and their efficacy against other bacteria can be tested by spot testing on respective bacterial lawn.



Fig. 2. PCR amplification of fragments of approximately 1500bp generated by 27F and 1492R that refers to the gene of region 16s rRNA, from the purified DNA: L1=5kb bp ladder, L2-L6= *Xap-1* to *Xap-5* isolates, L7=Negative control

### Pathogenicity

Five isolates were tested for pathogenicity, among them Xap-1 showed symptom 6th d after inoculation (DAI) as in Fig. 1 followed by Xap-2 on 7th DAI. Xap-3, Xap-4 and Xap-5 displayed oily spots after 8th d of inoculation. Disease severity from pathogenicity test is given in Table 1. Results revealed that highest severity was reported from Xap-1 (37.3%) isolate inoculated plants, followed by Xap-3 (28.7%) and Xap-2 (24.3%) thus Xap-1 was considered as virulent isolate and used for further experiments. Xap-1 was further tested by three inoculation methods expressed symptoms 7<sup>th</sup> d in spray inoculation, 12<sup>th</sup> d in infiltration and 12<sup>th</sup> d in pin prick method and per cent disease severity was recorded as 29.8, 15.2 and 14.6 respectively, whereas Sharma et al., 2017 findings showed that pathogenicity by spray inoculation displayed symptoms 14 d of post inoculation with highest incidence of 71 per cent and severity of 67.8 per cent, 67.5 of disease incidence and 63.2 of disease severity in pin prick method and 19.0 per cent disease incidence and 92.7 per cent disease severity.

Xap-1 isolate showed no symptom up to 5<sup>th</sup> DAI. At 15<sup>th</sup>, 25<sup>th</sup>, 35<sup>th</sup> and 45<sup>th</sup> DAI lesion size was 3.5 mm, 5.00 mm, 5.00 mm and 5.52 mm, respectively. Isolate Xap-2 initially showed min 2 mm sized oily spot at 15 DAI and developed up to 4.97 mm lesion size. Xap-3 and Xap-4 isolates developed 4 mm lesion size at 45 DAI as shown in the Table 1. Xap-5 inoculated plants showed lesion development up to 3.5 mm size at 45 DAI, with increase in days after inoculation, lesion size increased which infers that positive correlation exists between lesion size and DAI.

Patil *et al.*, 2017 reported the progression in the lesion; *Xap*-4 isolate had more lesion size of 4.3 mm after 18 d of inoculation followed by *Xap*-3 which showed 4.1 mm after 18 d and *Xap*-1 isolate showed 3.6 mm after 18 d of inoculation. Pathogenic variability among isolates is determined based on magnitude of infection on test plants, in the present findings *Xap*-1 showed high virulence by producing larger (5.52 mm) followed by *Xap*-3 (4.97 mm).

Colony morphology was yellow, mucoid, glossy and raised with fuscan or brown pigment production in YDCA medium. Patil *et al.* (2017) stated that the blighted leaves and fruits surface of around 2.5 mm size were crushed and loop full of suspension was streaked on nutrient agar plates which were incubated at  $28\pm 2^{\circ}$  C for 72 h. Typical yellow raised colonies were observed.



Fig. 3. PCR amplification of fragments of approximately 491bp generated by KKM5F and KKM6R that refers to the gene of region gyrB from the purified DNA: L1=5kb bp ladder, L2-L6= *Xap-1* to *Xap-5* isolates, L7=Negative control

Best growth was recorded at 28° C (0.86 OD) and second highest growth was observed at 29° C (0.68 OD) which was statistically at par with 28° C, followed by 30° C (0.62 OD). Results inferred that optimum growth range for virulent isolate (*Xap-1*) was observed at 28° C to 30° C. Yenjerappa (2009) recorded the maximum growth of the *Xap* at 30° C. pH studies inferred that 7-8 pH is optimum for growth, with highest growth at pH 7 (1.83 OD) whereas highly acidic and alkaline pH has negative influence on growth of *Xap*-1, least growth at pH 2 (0.02 OD). Similar findings were documented by Ghildiyal (2017) where it was recorded that OD value at pH 6.0 and 7.0 was significantly higher than the OD values at other levels of pH.

# Amplification by 16S rRNA and gyrB specific region

All the Xanthomonas axonopodis pv. punicae isolates (Xap-1 to Xap-5) were subjected to polymerase chain reaction (PCR) for confirmation of pathogen at genus level using universal primers such as 27F and 1492R with reaction mixture of 30 µl. All isolates showed the amplicon size of 1500 bp (Fig. 2). Virulent isolate Xap-1 was further sequenced and the same was submitted to NCBI. Genbank accession number assigned was MH015003.1. 16S rRNA amplification was reported by Sharma et al. (2017) with prokaryotic universal primers 27F and 1492R amplified the ITS region at 1500 bp size Amplification of gyrB region using the primers KKM5 and KKM6 showed the product of 491 bp, further confirmed the identity as Xanthomonas axonopodis pv. punicae as shown in Fig. 3.

### Conclusion

The results of current study would be useful to fix parameters for exact identification of symptoms of bacterial blight in pomegranate. Molecular confirmation could be made with universal primers 27F, 1492R and specific primers KKM5F, KKM6R. *Xap* specific bacteriophages can further be used for the management of *Xanthomonas axonopodis* pv. *punicae*.

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