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Virulence Diversity in *Xanthomonas axonopodis* pv. *Punicae* isolates on Pomegranate Variety cv. Bhagwa

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Abstract

Xanthomonas axonopodis pv. *punicae (Xap*) causing bacterial blight of pomegranate incurs substantial yield loss and reduces the export quality of pomegranate. The disease severitydiffered among geographical regions and the host genotype. In testing virulence efficiency of nine different isolates of *Xap*, the isolates were cultured. Their colonies were yellow pigmented, mucoid, circular, convex, glistening and raised on nutrient agar medium. In Pathogenicity tests of nine different *Xap* isolates all isolates exhibited symptoms of minute water soaked lesions after seven days of post inoculation. Molecular detection by PCR using universal primer16S rRNA and the sequence homology of nine isolates showed high similarity to previously identified *Xap* isolates in the NCBI database. EPS production among the nine isolates varied between 20 mg to 106 mg. Higher EPS was produced in isolate Xap1 and lowest in Xap9. Xap1 isolate proved more virulent and Xap9 showed the least necrotic lesions on pomegranate. This study demonstrates that diversity exists in different isolates of Xap for infection and further, the infection process of different isolates is directly co-relatated with the EPS production.

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

Pomegranate (*Punica granatum*) is a medicinal valued commercial fruit yielding tree species grown mostly in tropical and subtropical parts of the world. India is the world's largest pomegranate grower with cultivable area of 2.3 lakh ha with an annual production of 28 lakh tonnes. Maharashtra covers largest area (1.4 lakh ha) and is the India's largest pomegranate producer, followed by Gujarat, Karnataka and Andhra Pradesh (Reddy *et al*., 2018).

In recent years,bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap*) is one of the most devastating disease of pomegranate resulting in yield loss of 70-90 per cent (Gargade & Kadam, 2014 and Sonyal *et al*., 2015). Associated symptoms irregular water-soaked blackish brown spots of various sizes on leaves, premature defoliation girdling and cracking in stem. On fruits, irregular dark brown spots with an oily appearance, fruit cracking and its split opening were distinct symptoms. In India, this disease has brought down the domestic and foreign export by 80 per cent (Poovarasan *et al*., 2013 and Shantamma & Rangaswamy, 2016).

In India, blight severity increases during June and July and reaches a maximum in September and October (Sharma *et al*., 2017). Bacterial pathogen can survive in the soil without host for not more than 30 days (Sharma *et al*., 2012b). However, they can survive in dormant buds during the off season and are responsible for new infections in the following season (Sharma *et al*., 2017).

Exopolysaccharides (EPS) production plays a major role in the induction of susceptible water soaking reactions on pomegranate (Suryawanshi *et al*., 2018). EPS is responsible for the adhesion of bacterial cells to substrates and favoring the colonization of the bacteria on plants' surface and internal tissues. The xanthan gum, an anionic complex of EPS, suppresses defense genes and consequently inhibits callose deposition in plants and hence it is an important factor in bacterial pathogenicity. With this background information, the present study was carried out to isolate Xap and study its pathogenic variability in pomegranate.

Material and Methods

Collection of Infected Leaf Samples

The blight infected pomegranate leaf samples showing the typical symptoms of bacterial spot were collected from major pomegranate growing areas of India (Table 1) in zip lock polythene covers.

Table 1
Place of isolates collected

Isolates	Place of collection in India
Xap 1	Salehali, Jagalur
Xap 2	Jagalur
Xap 3	New Delhi
Xap 4	New Delhi
Xap 5	Solapur, Maharastra
Xap 6	New Delhi
Xap7	New Delhi
Xap 8	New Delhi
Xap 9	Anantapur

Isolation and Maintenance of *Xanthomonas axonopodis* pv. *punicae*

The collected bacterial blight infected pomegranate leaf samples were washed with running tap water. The infected portion of the sample was surface sterilized with 1 per cent sodium hypochlorite solution and then thoroughly washed three times with sterile distilled water and dried. The infected sample was then cut into small pieces (2 - 3 mm), put in a vial containing 2 mL sterile distilled water and allowed the bacterium to ooze into the liquid medium. After 5 - 10 min, a loopful of bacterial suspension was taken and serially diluted upto10^{-7}. From 10^{-6} dilution, 100 µl aliquot was spread on nutrient glucose agar (per L distilled water: 3 g beef extract; 5 g peptone; 2.5 g glucose; 15 g agar). The Petri plates were incubated at 28±1°C for 48 - 72 h for growth. The isolates were designated as Xap1, Xap2, Xap3, Xap4, Xap5, Xap6, Xap7, Xap8 and Xap9.

Pathogenecity Assay

It was performed with pure culture of Xap. Bacterial blight susceptible pomegranate variety cv. Bhagwa of 120 days old were kept for two days in an environmentally controlled polyhouse for conditioning. The temperature and relative humidity in the polyhouse were maintained at 25° ± 5°C and 70 - 80 per cent, respectively. Fresh single colony of Xap strains was cultured in nutrient broth, amended with glucose at one per cent concentration and incubated at 28°C at 120 rpm for 24 - 48 h.

The bacterial cells were centrifuged at 13,000 rpm for 15 minutes and resuspended in distilled water and adjusted to 0.1 OD (at 600 nm). The pre-conditioned healthy pomegranate seedlings with fully expanded leaves were infiltrated with Xap on the ventral side of the leaves using a syringe without a needle (Soni and Monda, 2018). Reisolation of the pathogen was carried out from the symptomatic diseased leaves from each isolate to confirm the Xap pathogen's pathogenicity with three independent replications.

Confirmation of Pathogen Using 16S rRNA

Genomic DNA of the bacterium was extracted as described by Zhang *et al*. (1998) with some modification. 0.3 g of washed bacterial cells pellet was suspended in 200 µl of Cetyltrimethylammonium bromide (CTAB) buffer (50 mMTris, pH 8.0; 0.7 mMNaCl; 10 mM EDTA; 2 Cetyltrimethylammonium hexadecyltrimethylammonium bromide) followed by 100 µl of 10 per cent sodium dodecyl sulfate and incubated at 65°C for 10 min. DNA was purified with chloroform and precipitated with iso-propanol at -20°C overnight. Purified DNA was washed with 70 per cent ethanol, then the pellet was air dried and resuspended in 30 µl of DNase free sterile distilled water. DNA concentration was measured using NanoDrop (De Novex) at 260 / 280 nm. DNA quality was checked on 0.8 per cent agarose gel in Tris-Acetate-EDTA (TAE) buffer (45 mMTris-acetate, 1 mM EDTA, pH 8.0).

After electrophoresis, the genomic DNA was further used for PCR amplification with universal 16S rRNA primers (F-5'-GAGTTTGATCCTGGCTCA-3' ; R-5' -AGAAAGGAGGTGATCCAG-3'). PCR was performed in a thermal cycler (Eppendorf, Vapo

protect), with 100 ng of genomic DNA, 1 µl of each primer, 10 µl of 2xPCR master mix and sterile distilled water to make a final volume of 20 µl. The thermal cycler was programmed with an initial denaturation at 94°C for four min followed by 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min with the final extension at 72°C for 10 min. The amplified products were purified and directly sequenced from both ends using a commercial facility (Eurofins Scientific India Pvt. Ltd.). Obtained DNA sequences were analysed and compared with the available NCBI database using BLAST analysis.

Quantification of Exopolysaccharide (EPS)

The amount of exopolysaccharide (EPS) produced by the nine Xap isolates was determined according to the method conducted by Suryawanshi *et al*. (2018). A single colony of each Xap isolate was inoculated in 50 ml of nutrient broth (NB) supplemented with one per cent glucose and incubated at 28°C in a shaking incubator at 120 rpm. After 72h, the optical density of the bacterial cultures was measured and adjusted to 1.0 at 600 nm with NB. The bacterial cells were centrifuged at 13,000 rpm for 15 minutes and the culture supernatants were transferred into new 100 ml tubes and mixed with two volumes of absolute ethanol and the tubes were placed at -20°C overnight. The precipitated crude EPS was collected by centrifugation for 30 min at 13,000 rpm. The EPS pellets were dried at 55°C for 12 h and the dry weight of each was measured. The amount of EPS production was represented as mg/100 ml culture.

Results and Discussion

Collection of *Xap* Isolates

The bacterial blight infected leaf samples of pomegranate were collected from different regions of India (Table 1). Infected samples showed a characteristic symptom of small water soaked lesion on leaves. With time increased, the lesion turned into a necrotic spot (Plate 1). Each sample was homogenized with sterile distilled water followed by serial dilution and culturing Xap on nutrient agar media. Similarly, Harshita *et al*. (2018) collected samples from five different localities of Tamil Nadu, showing symptoms like blightening with an oily ring on the leaves. Priyanka *et al*. (2020) collected the Pomegranate leaves showing bacterial blight symptoms from different geographical locations such as Hosdurga, Hiryur (Chitradurga district), Arsikere (Hassan district), Sira, Madhugiri (Tumkur districts) and Chintamani (Kolar district) of Karnataka during the August - September 2016.

Plate 1: Symptoms of bacterial blight on pomegranate leaves and fruits as observed under field conditions

Isolation and Maintenance of *Xanthomonas axonopodis* pv. *punicae*

Nine Xap isolates produced yellow, mucoid, round and smooth colonies on Nutrient agar (NA). The individual colonies of Xap were raised, glistening, circular, mucoid and vibrant yellow pigmented on NA (Plate 2). The single emerging colonies were sub cultured on both NA plates and purified culture of the pathogen was maintained in NA medium as shown in Plate 2. Arora (2016) isolated *X. campestris* pv. *punicae* from leaf, fruit and node and the colonies were mucoid, circular, convex, yellow, rounded, glistening and raised on nutrient agar medium. Ashish and Arora (2014) observed that the colonies produced by Xap on nutrient agar medium were pale yellow, circular, convex, slightly raised, opaque and mucoid.

Pathogenicity Assay

The symptom of minute water soaked lesions were observed on leaves of pomegranate cultivar Bhagwa

Plate 2: Pure culture of *Xanthomonas axonopodis* pv. *punicae*

after seven days of inoculation as minute water soaked lesions. These spots later turned into brown to dark brown surrounded by diffused water soaked margin or yellow halo, with circular to irregular lesions on the leaves. As the disease progressed, the spots gradually enlarged in size and slowly developed necrosis, followed by withering of leaves, which proved the pathogenecity in all isolates tested (Plate 3). Re-isolation was carried out from the inoculated leaves displaying the typical symptoms of bacterial blight by serial dilution technique on NAG medium. The pure culture was similar to that of the mother culture.

Mondal *et al.* (2012) observed the initial symptoms of Xap on foliage as irregular, water-soaked spots (2 to 5 mm diameter), turned to necrotic with a brown centre and finally dark brown Similarly, Raghuwanshi *et al*. (2013) inoculated the broth cultures to prove the pathogenecity of *X. a*. pv. *punicae,* in healthy susceptible pomegranate seedlings for which they obtained symptoms similar to our experiment. Bora and Katki (2014), while reporting the occurrence of bacterial blight of pomegranate in Assam, also observed water soaked oily spots and proved the pathogenecity of *X.a*. pv. *punicae* on the inoculated seedlings of pomegranate. Icoz *et al.* (2014) observed blight disease of pomegranate from Turkey and

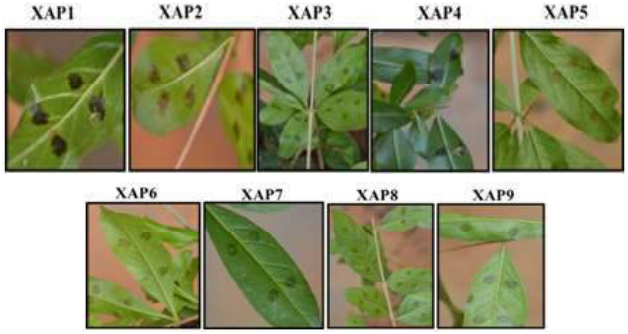

Plate 3: Symptoms on leaves proving pathogenicity test in all Xap isolates

reported dark brown, angular to irregular shaped spots on leaves and fruits, canker on stems, branches and trunks.

Confirmation of the Pathogen through 16S rRNA Sequencing

PCR yielded an amplicon of expected 1500 bp size in all the isolates (Plate 5). The 16S rRNA sequences of the nine isolates were sequenced and compared with the previously published 16s rRNA gene sequences of Xap. All the nine isolates showed 98.5 to 100 per cent similarity with previously identified *X. a*. pv. *punicae* isolates present in the NCBI database.

Plate 4 : Agarose gel confirmation of DNA of nine isolates

The 16S rRNA loci is a highly conserved region in prokaryotes and is widely used in bacteria's molecular identification by using universal 16S rRNA primers. Similarly, Priyanka *et al*. (2020) isolated *X. axonopodis* pv. *punicae* and through PCR using 16S rRNA universal primers and the amplified product was of 1450 bp size of 1450 bp.

Further, the unpurified PCR product was sequenced and confirmed that all the isolates were *X. axonopodis* pv. *punicae* using BLAST and comparing the aligned sequence with the available database in NCBI. Xap1, Xap2, Xap3, Xap4, Xap5, Xap6, Xap7, Xap8 and Xap9 were showing more than 98 to 99 per cent identity to *Xanthomonas axonopodis* pv. *punicae* strains existing in the NCBI database.

Exopolysaccharide (EPS) Production and Disease Severity of *Xap* Isolates

There is a significant difference in the production of EPS among the isolates. *Xap* 1 produced a high amount of EPS (106 mg), whereas *Xap* 9 produced the least amount of EPS (20 mg) (Table 2).

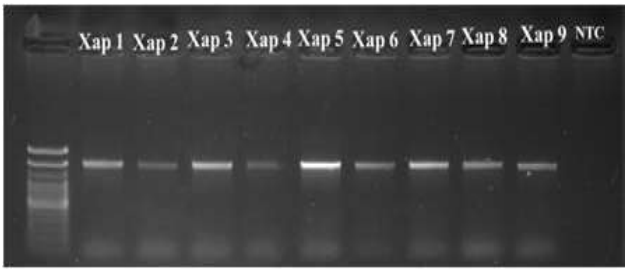

Plate 5: Gel electrophoresis image showing 16S rRNA amplification in the nine isolates of Xap (1500bp). Lane M, Marker; lanes Xap1–Xap9, isolates and lane NTC (no template control).

Extracellular polysaccharides (EPS) are abundantly produced by several plant pathogenic bacteria, especially those inhabiting the phyllosphere. Besides protecting the bacteria from hostile environmental conditions, they play a diverse role in bacterial aggregation and virulence. Singh and Walid (2019) quantified EPS from twenty seven isolates of *Xanthomonas axonopodis* pv. *punicae* and correlated with aggressiveness of the pathogen. The EPS production ranged 2.64 mg/100 ml in *Xap* 125 to 5.19 mg/100 ml in *Xap*102. A high pearson correlation coefficient (0.88) was observed during 0-10 days of inoculation, suggesting the possible role of EPS in facilitating the bacteria to establish itself during the early stages of infection.

To assess the disease severity of different isolates, the susceptible cultivar Bhagwa was challenge inoculated with *Xap* isolates. All the nine isolates produced water-soaked lesions at 8 and 15 dpi (Plate 6, Table 3 and Fig. 1). The virulence among the Xap isolates was estimated by measuring lesion length of the necrotic area. *Xap*1 isolate showed no symptom up to 5th dpi, at 8th and 15th dpi lesion length was 0.9 cm and 1.4 cm, respectively, whereas *Xap*9 showed lesion size of 0.9 cm of *Xap*9 at 15 dpi as shown in Table 3. This shows that lesion length increased with an increase in days after post inoculation, indicating that a positive correlation exists between lesion length and dpi.

TABLE 2
Variation in the production of exopolysaccharide (EPS) by Xap isolates

Isolates	Dry weight of EPS (mg)
Xap 1	106.00
Xap 2	70.00
Xap 3	27.00
Xap 4	54.00
Xap 5	36.00
Xap 6	80.00
Xap7	40.00
Xap 8	40.00
Xap 9	20.00
SEm±	0.418
CD at 1%	1.683
CV %	1.526

TABLE 3
Lesion length of Xap isolate at 8 and 15days after post inoculation

Isolates	8 dpi (cm)	15 dpi (cm)
Xap 1	0.967	1.467
Xap 2	0.833	1.200
Xap 3	0.567	0.967
Xap 4	0.600	1.167
Xap 5	0.567	1.000
Xap 6	0.867	1.233
Xap 7	0.500	1.167
Xap 8	0.600	1.000
Xap 9	0.533	0.933
Comparing of means	S.Em±	CD at 1%
Isolates (A)	0.038	0.114
Days after inoculation (B)	0.013	0.054
A × B	0.003	0.161

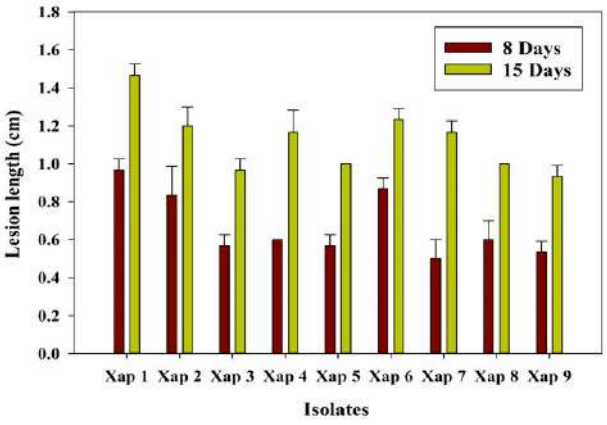

Fig. 1: Lesion length of Xap isolate were recorded at 8 and 15 days after post inoculation

Plate 6 : Lesion length on leaves at 8 and 15 dpi in all Xap isolates

Patil *et al.* (2017) reported the progression of lesion in the *Xap*-4 isolate had more (4.3 mm) after 18 d of inoculation followed by *Xap*-3 (4.1 mm after 18d) and *Xap*-13.6 mm after 18 d of inoculation). Ashish and Arora (2014) observed that small, discoloured and water-soaked spots were initially noticed on the leaves. On the upper surface of leaves, the diffused water-soaked zone was seen around the spot. Later on, these spots increased in size (2.0 - 5.0 mm in diameter), coalesced and extended upto midrib within a week covering almost entire leaf lamina.

The lesion size and the production of EPS of the *Xap* 1 was more at 8 and 15 dpi and while isolate *Xap* 9 was less virulent in accordance with EPS production. EPS such as xanthan and lipopolysaccharides (LPS) produced by *Xanthomonas* are reported to be involved in disease development as virulency factor (Wang *et al*., 2017). Overall *Xap* 1 proved more virulent based on high EPS production. These findings are in concurrence with the earlier works (Thein and Prathuangwong, 2010).

The ability of *Xap* to colonise the plant increases as days progress and there by there is enhanced symptoms on pomegranate. The pathogenicity assay revealed a diverse virulence activity among the *Xap* isolates, with *Xap*1 being highly virulent by producing the highest lesion length and EPS production. This study provides insight into whether EPS has a real role in enhancing the virulence ability of *Xap*. Further validation through molecular characterisation of the EPS genes is required for better disease management.

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