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Confirmation of the pomegranate bacterial blight causing pathogen *Xanthomonas citri* pv. *punicae* (*Xcp*) to assess disease severity in pomegranate genotype

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Abstract
Pomegranate is affected by many biotic stresses, among bacterial blight and wilt complex are major. Bacterial blight of pomegranate is caused by the pathogen *Xanthomonas citri* pv. punicae (*Xcp*), a gram-negative bacterium. It is leading to 60-80 % yield loss in the blight infested pomegranate orchard. Farmers are facing great difficulty in managing the disease in effective manner. The disease symptom can be seen on all the parts of pomegranate, except flower. In present study, pathogen was isolated from one of the major pomegranate cultivating belt of Karnataka (India), i.e. Bagalkot. Pathogen confirmation was done through morphological appearance and pathogenicity test. Also, molecular confirmation through gene specific primers and partial sequencing of 16S rRNA. Thus, confirmed pathogen was used for screen the resistance in pomegranate genotypes Bhagwa and IC318735. Bhagwa and IC318735 exhibited 31.5 % and 0.9 % disease severity respectively at 9 day post inoculation of pathogen. This relative tolerant genotype can be used in advanced breeding programs.

Keywords: Pomegranate, Xanthomonas, bacterial blight, pathogen, PCR, 16S rRNA

Introduction
Pomegranate (*Punica granatum* L.) is a tropical fruit crop belongs to the family Lythraceae (Huang and Shi, 2002). It is a deciduous shrub, bearing a non-climacteric fruit (Shulman *et al*., 1984) diploid, with chromosome number 2n = 2x = 16 (Sheidai and Noormohammadi, 2005) [40]. It is believed to be originated from Iran, eventually spread over to the Mediterranean Europe, Asia, as a result it is good adopted to arid or semiarid region (Pal *et al*., 2014) [26]. Its domestication was independently taking over the places, but it was most popular in Iraq (Evreinoff 1949; Zukovskij 1950) [9, 44]. It was spread to the Mediterranean countries at a very early date and now extensively cultivated in Mediterranean region especially in Spain, Morocco, Egypt and Afghanistan. It is also grown in drier parts of Southeast Asia, Burma, China, Japan, USA, West Indies, Russia, Bulgaria, Tropical America, Southern Italy and India. The five major producers of pomegranate are India, Iran, China, USA and Turkey, (da Silva *et al*., 2013; Holland and Bar-Ya'akov, 2014) [5, 13]. India being home to the finest varieties of pomegranate, the fruits have soft seeds with fewer acids. In fact, the fruit quality is much superior to those grown in Spain and Iran in edible quality and attractiveness. In India majorly grown in western Maharashtra, north-western Karnataka, Gujarat, Andhra Pradesh and Tamil Nadu (Benagi *et al*., 2012; Raghuwanshi *et al*., 2013) [4, 32]. Extensive cultivation of the pomegranate in the villages of Solapur, Nasik, Sangli, Ahmednagar, Pune and Satara districts made Maharashtra the country's pomegranate bowl, with 71.21 % of the total area under pomegranate cultivation in the country.

Over a period, its global market is increasing in a higher rate, since the fruit has a wide consumer preference for its attractive, juicy, sweet, acidic and refreshing arils with medicinal value. Pomegranate fruits are good source of carbohydrates and minerals such as calcium, iron and sulphur that possess pharmaceutical and therapeutic properties. It is rich in vitamin C and citric acid is the predominant organic acid in pomegranate (Malhotra *et al*., 1983) [23]. Glucose (5.46%) and fructose (6.14%) are the main sugars with no sucrose in fruits. Sweet varieties are mildly laxative, whereas sour types are good against inflammation of stomach and heartache. Flower buds are very useful in Ayurveda for managing bronchitis, also it is valuable because of the health-promoting traits in edible and non-edible parts, like stem bark and fruit rind is used to treat diarrhea and indigestion.

(Hamayun, 2003; Mahboob *et al*.; Sidhu *et al*., 2007, 2018) [10, 22, 42] which contains number of alkaloids belonging to pyridine group. The bark is also used in tanning industry. These can be used for a wide range of human diseases including cancers, diabetics, obesity, hypertension (Basu and Penugonda, 2009) [3] and Alzheimer's disease. Metabolome analysis revealed that pomegranate aril, seed, rind, flower, bark and root contain a wide range of phytochemicals, including gallotannins, ellagic acid, flavonoids, antioxidants, terpenoids and alkaloids (Prakash and Prakash, 2011) [28]. Despite all these beneficiary nature, phytosanitary rules by the many countries are now major concern for export of the pomegranate with good quality.

Cultivation of Pomegranate is well suited to dry tropics and sub-tropics and performs well even in soils of low fertility status and saline conditions. The management of pomegranate orchard and maintenance of fruit quality is very difficult due to number of pest and diseases. Major pests are thrips, anar butterfly, white flies, mite, mealy bugs, scale insects, fruit sucking moth, bark eating caterpillar, stem borer, shot hole borer, root knot nematodes & aphids, these pests are not only cause heavy losses, but also responsible for the spread of major diseases such as bacterial, fungal and viral pathogens causing various infectious diseases like leaf spots and rots, fruit spots, fruit rot, wilt (root rot and stem canker) and bacterial blight or oily spot (Pal *et al*., 2014) [26]. Among biotic stresses, yield and quality of pomegranate is severely affected by bacterial blight caused by *Xanthomonas citri* pv. *punicae* (*Xcp*) (Hingorani and Singh, 1960) [13]. This can render up to 60-80 per cent of yield losses followed by wilt complex (Mondal and Mani, 2009) [25]. As a result, cultivation of pomegranate is decreasing in peninsular India (Kale *et al*., 2012; Awasthi, 2015) [2, 18].

The oily spot/bacterial blight of pomegranate was first time observed in the India at Delhi (Hingorani and Mehta, 1952) [12]. Later, it was reported from Karnataka (Hingorani and Singh, 1960; Ramesh and Ram, 1991) [13, 33]. Himachal Pradesh (Sohi *et al*., 1964), Haryana (Kanwar, 1976) [20] and Maharashtra (Dhandar *et al*., 2004; Jadhav and Sharma, 2009; Sharma *et al*., 2017; Sharma *et al*., 2011; Sharma *et al*., 2010) [7, 17, 35-37]. Apart from India, it is also been reported in Pakistan (Akhtar and Bhatti, 1992) [1], Western Cape and the Limpopo provinces of South Africa, (Petersen *et al*., 2010) [27] and recently in Turkey (Icoz *et al*., 2014) [16].

The disease symptoms in the field, initially starts on stem, slowly infect to leaves and then to fruits (Deshpande *et al*., 2014; Ramesh and Ram, 1991) [6, 33]. On stem, initially observed brown to black spot around the nodes. In advance stages of nodal infection girdling and cracking of nodes lead to break down of branches, called as stem blight. On leaves, symptom appears as small, irregular, water-soaked spots with 2 to 5 mm in size necrotic center of pin head size in the beginning (3-10 days after infection). These oily spots are translucent against light. Later, these spots turn light to dark brown and are surrounded by prominent irregular yellow margin. These spots may coalesce to form bigger patches looks like blight/burnt appearance. On severe infection leaves may drop off. On fruits, initially start with oily spot, turn to brown to black spots, surrounded by oily tinge on pericarp. Later the spots start cracking as a result splitting of the fruit takes place. The disease spreads as the bacterium survives on the tree as well as in the litters. The high temperature and high relative humidity favour the disease. The disease spreads to healthy plants through wind splashed rains and in new area through infected cuttings (Deshpande *et al*., 2014) [6]. It is been proven that the pathogen can be virulent in the field till 7-8 months and pathogen can be isolated from the infected field debris up to 1 years in laboratory condition on nutrient glucose agar media (Rani and Verma, 2002) [34]. Complete field sanitization and maintaining the hygiene is very important when starting with new orchard.

During the year 2007, the total production of pomegranate in India was down by 60 per cent (Raghavan, 2007) [31]. Many growers have found no options to mitigate their disease effectively and uprooted their crop due to unbearable losses. Even after following several cultural practices beginning with healthy cuttings, avoiding rainy season, giving rest period, farmers have faced difficulty in managing the disease either through chemical control or biological control measures (Awasthi, 2015) [2]. The disease has been observed as serious threat to the cultivation of recommended varieties of pomegranate. Of the several disease management strategies, varietal resistance is considered to be as best alternative. Even some of the wild accessions (Nana and IC318735) are reported as a moderately resistance to bacterial blight. Here confirmed pathogen was challenge inoculated to susceptible cultivar Bhagwa and wild type IC318735 to check disease severity.

Methodology

Isolation of bacterial blight causing pathogen and pathogenicity test

Bacterial blight infected pomegranate leaves were collected from the infected field. Blighted leaves with fresh oily spots were chopped and immersed in 0.01 % mercuric chloride for 30 sec. Treated leaf pieces were then washed in sterile distilled water for three to four times to remove residual $HgCl_2$. Then samples were crushed on a glass slide using a scalpel and forceps. Finely smashed macerate was streaked on the nutrient glucose agar [NGA media composition: Beef extract (3 g/L), Peptone (5g/L), Dextrose (10g/L), Agar-Agar type-I (20g/L), adjust. pH ~ 7, make up the volume 1 L and autoclaved] plate and incubated at 28 °C for 48 h. After the colony growth, *Xcp* colonies were identified based on morphological characters and pure culture was streaked on the NGA plates. Streaked plates were incubated at 28 °C for 48 h for the multiplication of isolated bacteria.

Pathogenicity testing of isolated bacteria was done. For that, a single colony from the streaked plates was inoculated into NG broth and incubated at 28 °C for 48 h on an incubator shaker set at 120 rpm. The 48-h old bacterial suspension was diluted to a spectrophotometric optical density of OD=0.6 measured at 600 nm. Parallelly, six months old plants of the bacterial blight susceptible pomegranate variety 'Bhagwa' were covered with moist, transparent plastic bags, 24 h prior to inoculation to maintain optimum relative humidity. The diluted bacterial suspension was sprayed on the entire plant using a high-pressure vacuum pump and covered again with moist polythene bags for another 24 h. Observations were recorded for the appearance of blight symptoms on the leaves. Re-isolated bacteria from the infected leaves were re-cultured on NGA plates. Newly cultured bacteria were maintained as 50 % glycerol stock and stored at -80 ^{0}C.

Genomic DNA isolation of *Xcp*

A single colony of *Xcp* cultured on NGA plates was inoculated to NG broth and incubated overnight on an incubator shaker set at 28 °C with 120 rpm rotation. Then 1.5 mL of overnight grown *Xcp* culture (OD_{600nm}=0.6) was transferred into a 2 mL micro centrifuge tube and centrifuged for 5 minutes at 10000 rpm to pellet the bacterial cells and supernatant was discarded. The bacterial pellet was re-suspended in 400 µl of TE (10 mM of Tris HCl and 1 mM of EDTA, pH 8) buffer, added with 50 µl of 10 % SDS, 50 µl of Proteinase K and incubated at 37 °C for 1 h. 500 µl of Chloroform: Isoamyl alcohol (24:1) was added to the bacterial suspension, mixed by inverting the tubes and centrifuged at 12000 rpm for 10 minutes. The supernatant was transferred to a fresh micro centrifuge tube and then an equal volume (~ 500 µl) of isopropanol and 100 µl of 3 M sodium acetate was added and incubated overnight at -20 °C. The overnight incubated mixture was centrifuged at 13000 rpm for 15 minutes. The supernatant was discarded without disturbing the pellet and the pellet was washed twice with 70 % alcohol by centrifuging at 5000 rpm for 5 min. Alcohol was decanted without disturbing the pellet. The pellet was air-dried at room temperature to remove the traces of alcohol and re-suspended in 50 µl of sterile water. Bacterial DNA was stored at -20 °C for further use.

Molecular confirmation of *Xcp* identity

For further confirmation of isolated bacteria as *Xcp*, isolated genomic DNA was subjected to PCR amplification using previously reported *Xcp* specific primers (Table 1). PCR was carried out in a 10 µl PCR mixture containing 50 ng of genomic DNA, 0.5 µM each of forward and reverse primer, 5 µl of 2X master mix (Ampliqon, Denmark). Thermal cycler was programmed with an initial denaturation for 3 min at 94 ^{0}C followed by 30 cycles each of 94 ^{0}C for 30 sec, annealing at 55 ^{0}C for 40 sec and extension at 72 ^{0}C for 30 sec, and with a final extension for 5 min at 72 ^{0}C.

Further genomic DNA confirmed with partial gene sequencing of 16S rRNA gene (JQ067629.1). Primers were designed (Table 2.) to 16S rRNA gene in order to amplify 1200-1400 bp and partial sequencing of the gene was carried out. The gene was PCR amplified using high-fidelity DNA polymerase (Phusion^{TM}F531L). PCR was carried out with 10 µl of 5X Phusion^{TM} HF buffer, 1 µl of 10 mM dNTPs, 2.5 µM each of forward and reverse primer, 100 ng of genomic DNA, 1.5 µl of DMSO, 0.5 µl Phusion^{TM} high-fidelity DNA polymerase and the total volume was made up to 50 µl. PCR product was purified using GSure® PCR DNA purification kit and both forward and reverse strands were sequenced by Sanger sequencing chemistry ABI3730. Raw reads were trimmed for low-quality bases (Phred Score < 20) and assembled into a single sequence using Codon Code Aligner. The sequence was deposited at the NCBI database (MN971672.1). The obtained sequence was BLAST searched against the NCBI nr database and top 20 hits were used for multiple sequence alignment and phylogenetic tree was constructed. After confirmation, *Xcp* bacterial culture was deposited at the National Centre for Microbial Resources, Pune, Maharashtra.

Phenotypic evaluation of pomegranate genotypes for Bacterial blight disease severity analysis

Bacterial blight disease severity analysis was done in pomegranate genotype IC318735 and Bhagwa at 3 time point; 0 h (before pathogen inoculation), 3day post inoculation (dpi) and 9 dpi. Randomly, 50 leaves per plant were selected for recording the observations on disease incidence and disease severity. Number of leaves infected and disease grade on each leaf (in scale of 1-5, Table 2.) were recorded. Percent disease severity (DS) were determined using the formula:

$$\% \text{ severity} = \frac{\text{Number of infected leaves X Grade X 100}}{\text{Total Number of leaves X Max. Grade}}$$

Result

Pathogen confirmation by morphology and pathogenicity test

Pathogen (Bacteria) isolated from the bacterial blight infected pomegranate leaves were produced slightly raised yellowish creamy, mucoid colonies on the nutrient glucose agar (NGA) at 3 days post-incubation. (Fig.1a). Thus, isolated bacteria were checked for bacterial blight pathogenicity on susceptible pomegranate genotype 'Bhagwa' through challenge inoculation (Fig. 1c). Leaves of the challenge inoculated plants exhibited slight discolouration on the upper surface at 3 dpi (Fig. 1d). By 9 dpi, prominent oily spots surrounding pale brown spot (typical symptoms of bacterial blight) started appearing on the lower surfaces of the leaves (Fig. 1e). Oily spots were turned into dark brown spots, surrounded by yellow oily hallow at 12 dpi (Fig. 1f), later coalesced to form blight like appearance. The appearance of previously reported bacterial blight symptoms on par with the susceptible plant challenge inoculated with isolated bacteria. That confirmed the pathogenicity of the new isolate and newly isolated bacteria were named as Strain Bagalkot isolate.

Molecular confirmation of the *Xcp* genomic DNA by PCR and partial 16S rRNA sequencing

Isolated bacteria were further confirmed by amplification of *Xcp* specific PCR amplicons using DNA isolated from the bacteria as a template and previously reported primers. Observed amplicon sizes were 190 bp for *XopQ* primer (Fig. 2a), 491 bp for KKM 5&6 primer [Fig 2b & 2e(1)], 128 bp for *XopL* primer (Fig.2c), 145 bp for *XopN* primers (Fig. 2d) and 230 bp for GAPDH primers [Plate 2e (2)]. The observed PCR amplicons were identical to the previously reported PCR amplicons in *Xcp*, further confirming the pathogen's taxonomy.

Further, the relatedness of *Xcp* strain Bagalkot was confirmed by partial sequencing of 16S rRNA gene (Fig. 2f). Both forward and reverse sequencing reads were trimmed for low-quality reads (Q<30) and aligned into a single consensus sequence of 1173 bp. The 16S rRNA gene sequence of *Xcp* Strain Bagalkot was submitted to the NCBI GenBank with the accession MN971672.1. BLAST analysis of the 16S RNA against the NCBI database showed 100 % identity with *Xanthomonas citri pv. Punicae* BDP1, B0022 and other 7 strains of *Xcp* with a 100% coverage (Fig. 3). With this confirmation, pathogen culture was deposited at National Centre for Microbial Resources (NCMR) along with the partial 16S rRNA gene sequence data. And it has been allotted with the accession number of MCC 4627.

Disease severity analysis of pomegranate genotype

Disease incidence was started early (3 dpi) in the Bhagw. Till 8 dpi disease incidence was not reported in IC318735, whereas it started at 9 dpi. In Bhagwa disease severity at 3 dpi was 2.1 %, at 6 dpi 14.5 % and at 9 dpi 31.5 %. With respect

to disease severity there was significant difference between susceptible cultivar Bhagwa and relatively tolerant genotype IC318735 (Table 3.).

Discussion

Freshly isolated *Xanthomonas citri* pv. *punicae* from the bacterial blight-infected leaves have exhibited similar colony characters on the NGA media as previously reported. Slightly raised; yellowish creamy, mucoid colonies developed after 3 days post incubation on nutrient glucose agar (NGA) (Doddaraju *et al*., 2019; Manjula, 2002; Ramesh and Ram, 1991; Sharma and Chandra, 2013) [33, 37]. However, delayed colony growth up to 5-6 days after incubation was observed when the pathogen was isolated and incubated during winter (Nov-Feb) and summer (March-May). Rapid raising of colonies was observed in the rainy season (June-Sept) even though culture had been maintained in the controlled condition. Serial dilution of infected leaf macerate minimum of 10×10^{-3} led to the isolation of pure colonies avoiding contamination with many other bacteria and fungus.

Challenge inoculation was made to susceptible cultivars to prove Koch's postulate and it has been proven when, water-soaked lesions started appearing on the lower surface of the leaves (at sixth day in winter). Water-soaked lesions appeared on the lower surface of the leaves at 3 to 9 days of post inoculation, depending on the season of inoculation. The appearance of water-soaked lesions could be varied from 4 to 19 days depending on temperature and humidity conditions. Varying incubation periods reported for blight development ranged from 9-12 days (HINGORANI and JIT, 1959) [11]; 4-12 days (Kanwar, 1976) [20]; 17-40 days (Ramesh and Ram, 1991) [33] and 7-15 days. It was observed that, younger leaves are less infected compared to the older ones. Once the challenge inoculated leaves wither, new leaves were free from the disease, indicating no systemic spread of the disease (Upasana *et al*., 2001).

Molecular confirmation of *Xcp* DNA by PCR amplification has shown amplicon size of 491 bp, 190 bp, 230 bp and 128bp to KKM, Xopq, GAPDH and XopL genes primer of *Xcp*-strain Baglkot respectively and they were identical to the previously reported isolates of *Xcp* (Doddaraju *et al*., 2019; Kalyan *et al*., 2012; Kumar *et al*., 2021; Sharma *et al*., 2022) [8, 19, 21, 35]. The 16S rRNA sequence of *Xcp* strain Baglkot also exhibited 100 % identity with the BDP1, B0022 and seven other strains of *Xcp* isolated from different agro-climatic regions, which were grouped into a single clade in the molecular phylogeny. The closest neighbouring clade of *Xcp* strain Baglkot consisted of *Xanthomonas citri* pv. *glycines*, *Xanthomonas citri* pv. *Malvacearum*, *Xanthomonas phaseoli* pv. *phaseoli*. The partial sequencing of 16S rRNA gene could able to differentiate other *Xanthomonas citri* strains and confirmed the genomic DNA (Radhika *et al*., 2021) [30].

Isolated pathogen was virulent and could able to develop early symptoms in the susceptible cultivar Bhagwa. Relatively tolerant genotype IC318735 has exhibited tolerance till 8 dpi. Earlier 4.91 % disease severity was reported in IC318735 (Kumar *et al*., 2021) [21]. The re-evaluated them under the greenhouse conditions exhibited higher tolerance compared to previously reported studies (Priya *et al*., 2016) [29]

Table 1: List of primers used for pathogen *Xanthomonas citri*pv, *punicae* confirmation

Sl. No	Gene	Sequence (5'-3')	Annealing Temp.
1	KKM 5-Forward	AGAGTTTGATCCTGGCTAG	55
	KKM 6-Reverse	AGGAGGTGATCCAGCCGCA	55
2	XopN-Forward	GGATCAGGCTGCGTAGTTT	55
	XopN-Reverse	GGATCAGGCTGCGTAGTTT	55
3	XopL-Forward	CCCAGAATCGAATGACGAGAG	55
	XopL-Reverse	CTGGCTTGCTTCGTGATAAAC	55
4	XopQ-Forward	GCGAGGAACTTGGAATGCTC	55
	XopQ-Reverse	AGGTCGAAGGCTTTTTGCG	55
5	Primer16S1-Forward	GTGAGGAATACATCGGAATCTAC	55
	Primer 16S1-Reverse	GGTTAAGCTACCTGCTTCTG	55
6	Primer 16S2-Forward	CGTAGGGAAACTTACGCTAATA	55
	Primer 16S2-Reverse	GCTACCTTGTTACGACTTCA	55

Table 2: Scoring of disease symptoms for disease severity analysis

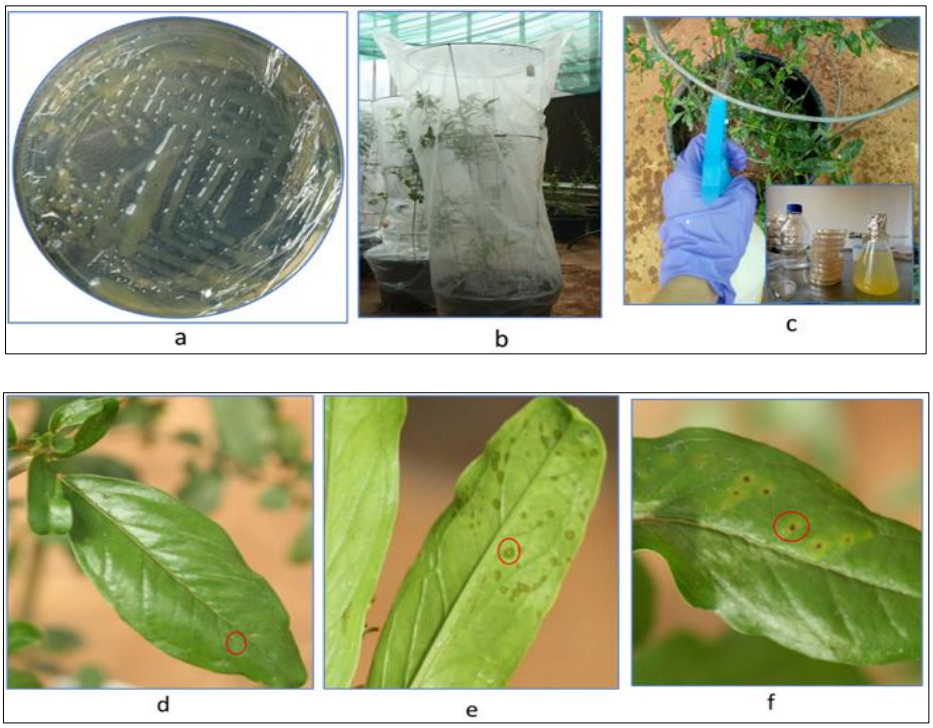
Severity Grade	% Severity	Symptoms on Leaves and fruits
0	0	Disease not seen
1	01-10	Disease not easily visible, very few units/plant
2	11-25	Disease visible easily in each direction, but most (75%) of units look healthy
3	26-50	Both disease and healthy units are equally observed
4	51-75	Disease seen very easily, with only some healthy units
5	76-100	Lesions covered all over the leaves

Grade1: 1-2 spots, Grade2: 5-10 spots, Grade 3: 25%, Grade 4: 50%, grade 5: 75-100%

Table 3: Disease severity analysis in pomegranate genotype

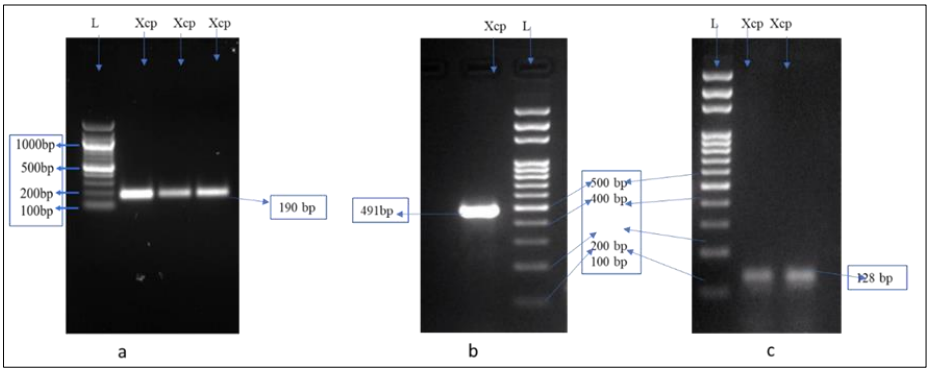
Observation	Bhagwa	IC318735	Mean
0 h	0	0	0
3 dpi	2.1	0	1.05
6 dpi	14.5	0	7.25
9 dpi	31.5	0.9	16.2
	Days	Genotype	Interaction
S.E.M	0.280624	0.093541	0.396863
CD @5%	1.159149	0.386383	1.639285

CD-Critical difference dpi- day post inoculation, SEM-Mean squared error

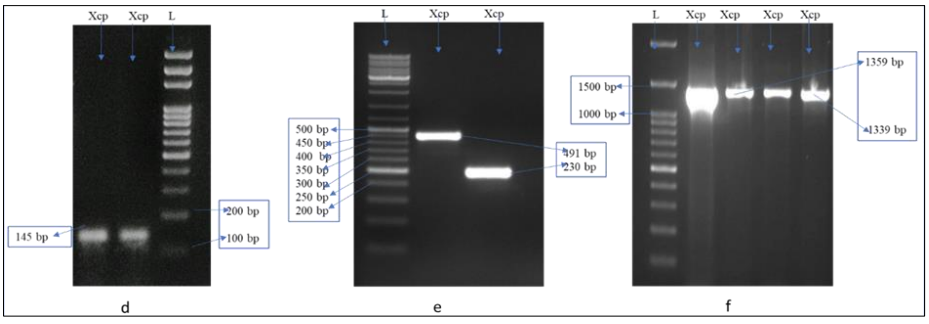


A. Pure culture of *Xanthomonas citri* pv. *Punicae* Strain Bagalkot on NGA.
B. Polythene covering of plant one before pathogen spray.
C. Inoculum preparation and Challenge inoculation.
D. Bacterial blight symptoms on pomegranate leaves 3 day post inoculation (dpi).
E. Bacterial blight symptoms on pomegranate leaves 9 dpi.
F. Bacterial blight symptoms on pomegranate leaves 12 dpi.

Fig 1: Pomegranate bacterial blight causing pathogen *Xanthomonas citri* pv. *punicae* instead of *Xanthomonas citri* pv. *Punicae*



a. XopQ primer, b. KKM5&6 primer c. XopL primer



d. XopN primer, e. (1) KKM5&6 primer, (2) GAPDH primer f. 16S (1) & 16S (2) RNA primer. L: Ladder Xcp: *Xanthomonas citri pv. Punicae* DNA sample

Fig 2: Molecular confirmation pathogen *Xanthomonas citri* pv, *punicae* through pathogen specific primer PCR

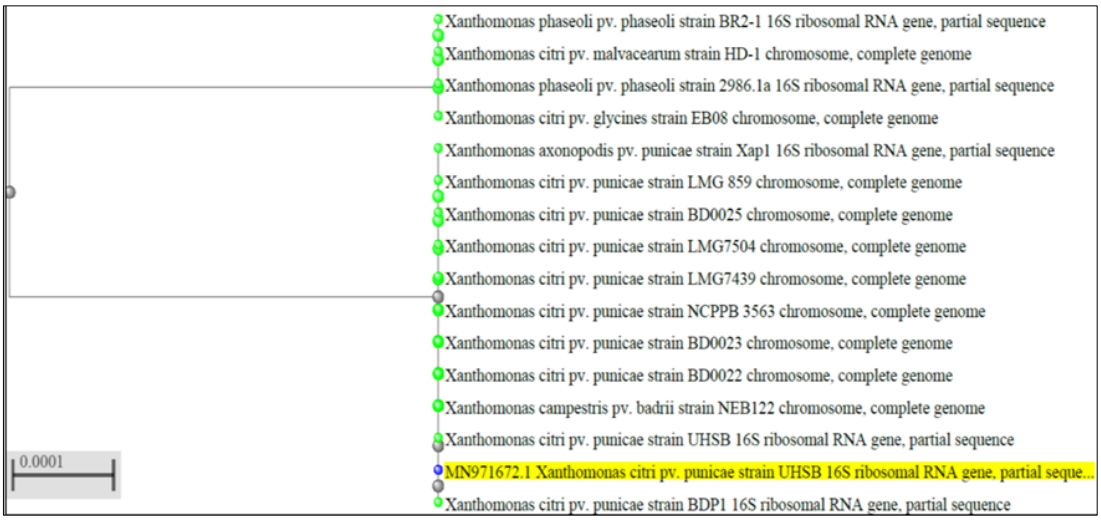


Fig 3: Molecular confirmation of *Xanthomonas citri* pv. *punicae* Strain Bagalkot genomic DNA by phylogenetic analysis of partial sequenced 16S RNA gene of *Xanthomonas citri* pv. *punicae* in NCBI-BLAST

Conclusion

Xcp is a out breaking in many arid and tropical countries. Development of resistant cultivar is first and foremost need of the farmers. In this aspect identification of the tolerant source is the very important. The reconfirmed relatively tolerant pomegranate genotype is carrying lot of undesirable characteristics, which made unfit for conventional breeding. The study of pathogenicity and host tolerance is the initial step to undertake molecular level study of the host pathogen interaction to identify candidate genes.

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