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Isolation, characterization of phytopathogenic bacterial types in pomegranate crops of Chitradurga district: Biochemical and molecular insights

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ABSTRACT

Bacteria are major pathogens leading to destructive disease inflicting considerable quantitative and qualitative losses in pomegranate. Characterisation of isolates and identification of new strains enables assessment of the evolution of virulence and symptomology. It also helps design better control and management practices. The present study aimed to isolate and characterise biochemical and molecular methods of bacteria from 4 taluks of Chitradurga district, Karnataka, India, with a unique agro-climatic zone. The results of the study report through staining, cultural, and biochemical studies a total of 17 bacterial isolates belonging to Pseudomonas spp., and Erwinia spp. and Xanthomonas species. Isolates of X. axonopodis pv. punicae (Xap) were isolated using selective-medium. Further, staining, cultural, biochemical and molecular variability identified 4 isolates of X. axonopodis pv. punicae (Xap) (Xa-1, Xa-2, Xc-3, Xc-4). The 16sRNA gene analysis indicated the Xc-4 strain was unique, as evidenced by the phylogenetic tree. A strong correlation between in vitro studies and disease severity in the indicator plant assay was observed. In summary, a consortia of bacteria predominantly belonging to Xanthomonas was identified in the Chitradurga area of Pomogranate plants. Evolution of the pathogen in this district could be proposed to be driven by genetic substrates enabling molecular evolution due to its host specificity and other environmental factors. The study has implications for pathogenicity, disease severity and virulence studies. Further, it bears implications in agriculture and the fruit industry with implications for fruit quality and post-harvest technology.

Keywords: *Phytopathogenic bacteria; X. axonopodis pv. Punicae; type-III secretion system (T3SS).*

1.0. Introduction

Phytopathogenic bacteria infect a large spectrum of food-producing plants worldwide (1). Despite its high economic and nutritional value, pomegranate farming is hampered by a variety of phytopathogenic bacteria that reduce fruit quality and output. They induce symptoms such as spots, blights, cankers, and tissue rots when they colonise the plant's surface or tissues, resulting in plant overgrowth, stunning, root branching, and leaf epinasty (2). Thus, they have a deleterious impact on both the qualitative and quantitative characteristics of crops and constitute a significant threat to the global food supply chain. Pomegranate (*Punica granatum* L.) is a member of the Lythraceae family and has long been a popular fruit in tropical and subtropical climates around the world. The fruit's peel, seeds, and pulp have great medicinal and pharmacological value and are a good source of carbohydrates and minerals like calcium, iron, and potassium. Additionally, they are currently marketed as a nutraceutical and functional food source with health-promoting advantages (3). Numerous bioactive substances, as well as significant concentrations of water, sugars, and organic acids like malic, citric, and ascorbic acid, are found in pomegranate fruit. According to 4, it is also abundant in polyphenols, including fatty acids (punicic acid), minerals (potassium and phosphorus), vitamins (C and K), flavonoids (anthocyanins), and tannins (ellagitannins and proanthocyanidins). The bioactive substances support antibacterial, anti-inflammatory, and antioxidant properties, among other health advantages. In addition, help prevent and treat chronic conditions such as diabetes mellitus, obesity, renal disease, cardiovascular disease, and neurodegenerative illnesses (5). Numerous bacterial and fungal diseases cause disease in the crop, affecting both its quality and quantity. Approximately 37 distinct pomegranate diseases have been linked to over 55 pathogens worldwide (6). The main pathogens that cause blasts, wood canker, branch dieback, rots, and wilts are Xanthomonas, *B. cinerea*, *Penicillium* spp., *Al. alternata*, and *C. granati*. Fruit rind and aril deterioration during storage is facilitated by pathogens, which also cause fruit to perish (7). According to 8, bacterial infections are prevalent in areas where pomegranates are grown and cause significant fruit losses during the growing season. The problem of bacterial blight is dangerous and has the potential to spread like wildfire.

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Pomegranate bacterial blight has been documented worldwide in a number of nations, including South Africa (9), Iran, Iraq, Pakistan (10), and Turkey (11). All plant parts, with the exception of roots and flowers, exhibit symptoms of bacterial blight; fruits are particularly susceptible. The symptoms include cankers on stems, branches, and trunks, as well as spots on fruits and foliage (12). In India, the oldest recorded blight are (13) . Since then, several researchers from various regions of the nation, including Tamil Nadu, Himachal Pradesh, Haryana, Karnataka, Maharashtra, and Rajasthan, have reported blight and the resulting losses (14,15,16,17,18,19,20).

Bacterial infection in crops starts with adhering to the plant's surface through biofilms, and enters through wounds, stomata, or hydathodes and uses enzymes to break down plant tissues (2). The majority of plant infections introduce effector proteins into plant cells via a type-III secretion system (T3SS), which interferes with host cellular functions and permits bacteria to proliferate and spread illness. To evade plant defence proteins, they also synthesise effectors (ETS) (21). The bacterial blight spreads quickly in altered environmental conditions, leading to significant defoliation and decreased photosynthesis (22). Environmental stress, extensive pesticide usage, and other control methods cause mutation or recombination, which leads to new forms of plant infectious illnesses (23). Additionally, the temporal and spatial dissemination of infectious pathogens is accentuated by human mobility. A high level of genetic variability among the strains of *X. axonopodis pv. punicae* has been reported, which is independent of host cultivar and geographical origins (24). Further, in the last decades, outbreaks are attributed to horizontal transmission of the pathogen aided by genetically homogenous clones of apparently healthy, but latently infected, planting material of elite pomegranate cultivars (25). In order to decipher plant defence mechanisms and create efficient management strategies, it is critical to comprehend disease resistance reactions in pomegranate genotypes against bacterial blight. Epidemiology and laboratory methods enable assessment of pathogen emergence, strain variability and outbreaks of disease symptomology (26). With this background, the present study was carried out to isolate and identify phytopathogenic bacterial types present in pomegranate crops of Chitradurga district using morphological, biochemical, and molecular methods.

2.0. Material and Methods

Field Survey and Sample Collection

A single individual collected the sample, adhering to GLP (Good Laboratory Practices). A systematic field survey was conducted across five taluks in Chitradurga district in the month of May, 2025. From Hiriyur, Challakere, Molakalmuru, Holalkere, and Chitradurga taluk (longitude 76.210329°, latitude 14.361161°) respectively. Pomegranate plants with visible symptoms such as leaf spots, fruit rot, stem blight, and bacterial ooze were selected. A total of 45 symptomatic leaves (n=20), fruits (n=15), and stems (n=10) were collected in individual sterile bags and labelled and transported to the laboratory.

Isolation of Bacterial Strains

Asepsis and microbiology standards were adhered to during isolation of the bacteria. High-grade chemical and growth media were used. Infected issue samples were surface-sterilised using 0.1% mercuric chloride followed by rinsing in sterile distilled water (27).

The tissues were macerated in phosphate-buffered saline (PBS), and aliquots were streaked onto Nutrient Agar (NA) and King's B agar medium plates and incubated at 28°C for 48–72 hours. After incubation, the colonies with characteristic bacterial morphology were sub-cultured to obtain pure isolates (28).

Characterization of Isolates

Morphological Characteristics

Colony morphology such as shape, colour, elevation, margin, and opacity were documented using standard methods (29)

Biochemical Tests

Gram staining, Catalase and oxidase, Gelatin and starch hydrolysis were carried out as described in (30)

Molecular Identification

DNA extraction using CTAB method was completed for all isolates (31). Quality and quantity were estimated by a Spectrophotometer and 1% agarose gel. PCR amplification targeting 16S rRNA gene was carried out using primer (32). 33 PCR primer seqs- 16S rRNA primers (F-5'-GAGTTTGATCCTGGCTCA-3'; R-5'-AGAAAGGAGGTGATCCAG-3') The PCR products were sequenced and the sequence was searched with the Basic Local Alignment Search Tool (BLAST) using the ncbi-blast server to find regions of similarity between sequences in the database.

In-vitro Pathogenicity Evaluation-Host Plant Inoculation (Detached Leaf Assay)

Bacterial cultures were grown in nutrient broth for 24 hours. Suspension adjusted to 10^8 CFU/mL using spectrophotometric OD values at 600 nm. Healthy, surface-sterilized middle aged pomegranate leaves were inoculated with bacterial suspensions using the prick and drop method using a sterilised needle at several sites on the leaf. Control leaves were treated with sterile distilled water. Incubation was done in moist chambers at 28°C and leaves observed over 7 days for symptoms (32).

Scoring was based on the following criteria:

- Lesion diameter
- Color change
- Water-soaked appearance
- Presence of bacterial ooze

Re-Isolation and confirmation of pathogenicity by Koch's Postulates

Pathogens were re-isolated from infected tissues using standard methods and compared with original isolates using staining and biochemical methods to confirm the causality.

3.0. Results

A total of 17 distinct bacterial isolates were isolated and preserved on slants for further studies. The Gram staining results indicated majority of the bacteria were Gram-negative rods. Bacteria were positive for the catalase and oxidase tests. The Gelatin hydrolysis and starch hydrolysis indicated variable results, aiding in differentiation. Most isolates exhibited yellow to cream-colored mucoid colonies suggestive of *Xanthomonas* species. The biochemical tests of the Xanthomonas are summarised in the table-1.

Successful isolation of DNA was carried out from all bacteria and the quality and quantity. The 16S rRNA gene sequence was used as a primer for amplification of the DNA from all isolates. Figure-3 depicts the PCR products of the blight strains amplified using 16sRNA primer, electrophoresed on 2% agarose gel.

Studies on cultural, morphological, physiological, biochemical and genetic features of the pathogen are of immense use in understanding the nature of the pathogen. BlastP results of the strain Xa-1 indicated 100% alignment to 140 blast sequences in databases. Major hits (128 were to Xanthomonas (g-proteobacteria). Major alignments were to *Xanthomonas axonopodis* (23), *Xanthomonas perforans*(12), *Xanthomonas citri pv. Fuscans*(15), *Xanthomonas euvesicatoria*(24) causative agents of blight in fruits and vegetables. The Xc-4 blast indicated 100% alignment to 148 blast sequences in databases. Major hits (122) were to Xanthomonas(g-proteobacteria) and one uncultured bacterium. Major alignment was to *Xanthomonas axonopodis* (21), *Xanthomonas perforans*(10), *Xanthomonas euvesicatoria* (74), *Xanthomonas citri pv. Fuscans* (9). The data is summarised as a phylogenetic tree in figure-4A and B and respectively.

The *In-vitro* Pathogenicity Evaluation-Host Plant Inoculation (Detached Leaf Assay)

Symptomology was scored visually and recorded

Re-Isolation and confirmation of pathogenicity by Koch's Postulates.

The results were Gram staining positive and similar biochemical tests as seen in the isolated bacteria confirming the pathogen its pathogenicity.

4.0. Discussion

India is the world's largest pomegranate grower with a cultivable area of 2.3 lakh ha and an annual production of 28 lakh tonnes. Maharashtra covers the largest area (1.4 lakh ha) and is the India's largest pomegranate producer, followed by Gujarat, Karnataka and Andhra Pradesh (34). Bacterial blight caused by Xanthomonas axonopodis pv. punicae (Xap) is one of the most devastating diseases of pomegranate, resulting in yield loss of 70-90 per cent (35 and 36). Figure-5 is a representative image of the infection. Fruit bacterial infection variability is a complicated, multifaceted phenomenon that is impacted by maturation stage, environmental factors, particular fruit species and postharvest handling (37,38,39). In recent times, Xap has spread to different geographical regions, indicating the wide adaptability of the pathogen. This variation impacts not only the kind of bacterial populations that are present (composition), but also their density and pathogenicity, which can range from minor tissue damage to serious contamination (40). Bacterial variability is determined by a number of variables, including the fact that isolates of bacteria exhibit notable variations in the course of the disease, with certain strains such as the jackfruit-bronzing disease strain JEN-14, being significantly more aggressive than others (41). b. The main pathogen in crops, such as kiwifruit, can change depending on the season. For instance, *Pectobacterium carotovorum* subsp. Actinidiae causes greater harm in the summer and *Pseudomonas syringae* pv. actinidiae in the winter and spring. c. Same species variation as demonstrated by the different, unique genotypic groups of *Pseudomonas syringae* pv. Syringae discovered on stone fruit (42). 43,44 and other researchers used morphological and DNA-based techniques to report similar diversity in *Xanthomonas axonopodis* pv *punicae*.

The pathogen exhibits considerable variability when cultured on different artificial media. Growth on Phyto-Xynocamp Agar Base Media enabled the characterisation of Xanthomonas. Its utility has been successfully demonstrated by its adoption by the 45. Earlier efforts to characterise the pathogen include growth and colony morphology on different media like SX, SM, BSCAA, MXP, MD-5 and Tween (46).

Biochemical test methods are employed to identify and differentiate bacteria based on their specific metabolic activities, enzyme production, and chemical utilisation. Xanthomonas spp., Pseudomonas spp., and Erwinia spp. were the main species identified in the present study based on colony, culture and biochemical tests. These tests detect phenotypic differences, such as carbohydrate fermentation or protein metabolism, by analysing changes in pH, color, or gas production, crucial for species identification. The Biochemical test of the four isolates confirmed they are Gram-positive, aerobic and have other features unique to Xanthomonas. Previous studies by researchers on bacteria on pomogranates include Pant and Shashidhar report Acinetobacter, Micrococcus, Pantoea, Microbacterium, Strenotrophomonas, Bacillus, Staphylococcus and Exiguobacterium,*Gluconobacter oxydans* (47), Xanthomonas (48). Host Plant Inoculation data suggested 11 of the isolated bacteria demonstrated pathogenicity with visible symptoms on leaf tissue. Three isolates of the Xanthomonas showed strong virulence, causing necrotic lesions within 48 hours in the Host Plant inoculation assay. The data complement the observation from *in vitro* studies that Xanthomonas are dominant and highly virulent. A correlation was observed between colony morphology and disease severity. It is reported pathogen causes water-soaked, angular, and dark brown/black necrotic lesions on leaves, stems, and fruits including necrotic spots, within 2–3 days (48-72 hours) of inoculation (49). It is reported that high humidity (>60%), warm temperatures (25–33°C), and rain splash accelerate the development of these necrotizing lesions and these symptoms are indicative of the Virulence (50,51). Xanthomonas use effectors (ETS) and Extracellular polysaccharides (EPS) to overcome plant defense proteins (52). Extracellular polysaccharides (EPS), clog the xylem vessels and cause wilting. The type III secretion systems (T3SS) supply virulence factors, which decrease host immune responses and promote the spread of disease (22, 53). Through the type III secretion system (T3SS), Xap secretes the effector proteins XopN and XopL to promote pathogenesis and inhibit host immune responses. The TTSS-effector inhibits plant immune responses, such as programmed cell death (PCD), and supports the proliferation and pathogenicity of XopL for a significant amount of time throughout the development of blight disease (54). Particularly in poor weather conditions, the blight can spread quickly, resulting in severe defoliation and decreased photosynthesis. Studies of the Indian strain of *Xanthomonas axonopodis* implicate six non-TAL or Xop effectors (55) also, highlight XopL and XopN are major contributors to virulence and (54). 56 report using null mutants virulence, *in planta* bacterial growth, and reactive oxygen species (ROS) production studies and suggest multiple non-TAL (Xop) T3SS effectors have roles in suppression of plant immune responses. (33) report Virulence diversity in Xap for Xanthomonas axonopodis pv. Punicae isolates on Pomegranate Variety cv. Bhagwa. The results implicate infection of different isolates is directly correlated with the EPS production. The Phylogentic distance tree grouped the Xa-1 strain along with other Xanthomonas strains with *X. oryzae* as the outgroup. The Xc-4 strain had two nodes with *Xanthomonas campestris* pv. campestris strain sequence as the outgroup. The blast sequence was not grouped in the first cluster along with other Xanthomonas strains suggesting its unique sequence. Xa-1 strain has higher similarity with *Xanthomonas axonopodis*, where Xc-4 strain *Xanthomonas euvesicatoria* suggesting the different evolutionary trajectories.

Numerous clusters and subgroups of the pathogen have been found by molecular investigations employing molecular markers, suggesting a high level of genetic diversity in the Indian isolates. 56 report an multilocus sequence analysis MLSA study of 24 isolates collected from major Indian states during disease epidemics document ing phenotypic traits, pathogenicity and cultural characteristics. Sequence-based characterization at nine genetic loci, revealed that all isolates showed more than 99% similarity with *X. axonopodis* pv. *punicae* isolates for the 16S and the IGS rDNA gene sequence confirming their identity as *X. axonopodis* pv. *punicae*. The study proposes clonal evolution and lateral transmission of bacterial blight of pomegranate as a consequence of pathogen migration with the expansion of pomegranate cultivation.

Research insights into the pathogenicity indicate that *Xanthomonas axonopodis* pv. punicae is highly specific to pomegranate, and there are no alternate hosts to this pathogen. Resistance to the pathogen in tolerant lines of pomegranates results from the expression of high transcript levels of defence response genes (57). Few epidemiology studies demonstrate that this variation is location-dependent (58). Also, few authors opine that outcrossing behaviour of pomegranate crop and the acquired antibiotic resistance due to overexposure to antimicrobial agents employed for the control of the disease have contributed to pathogen variability (59,60). Evidence of population shift and local adaptation is reported from Taiwan (61). Horizontal Gene Transfer (HGT), genome rearrangements, emergence of new virulent strains with modified or lost effectors are the mechanisms adopted by the pathogen as part of its virulence arsenal (62,63,64). These research findings implicate the pathogen evolves using various mechanisms to overcome resistance and differentiate at the morphological and genetic level to aid spread or overcome environmental and preventive practices.

Among the different districts surveyed by 65, maximum fruit infection of 38.29 per cent was recorded in Chitradurga district Koppal (32.40%) and Bellary (32.21%) districts. Correspondingly, average severity of the disease on fruits was observed as maximum in Bijapur district (28.95 PDI) and minimum severity of 17.86 PDI was recorded in Bellary district. 65 reported Xa_{13} strain isolated from Hiriyur district. Further, the dendrogram analysis showed two major clusters A and B with cluster-A composed of all other isolates belonging to different isolates of Bagalkot, Bellary, Bijapur, Chitradurga Koppal of Karnataka. In India blight severity increases during June and July and reaches a maximum in September and October, attributed to the seasonal south-west monsoon and then gradually declines (12). Altitude, climate significantly influences the epiphytic bacterial community. Warm weather and high humidity are reported to increase the susceptibility of fruits to bacterial diseases. It could be hypothesised that in the present study, these could be the triggers for the strain variability. The study has few limitations such as only single season sampling, few biochemical assays and genotyping of a single locus to test strain variability.

Table 1: Summary of Staining and biochemical characterization of Xanthomonas bacteria from the pomegranate

Sl No	Isolates	Potassium hydroxide test	Catalase test	Kovac's oxidase test	Production of Fluorescent pigment	Oxidation and fermentation of Glucose	Gram staining
1	*Xa-1*	+	+	+	+	+	-
2	*Xa-2*	+	+	+	+	+	-
3	*Xc-3*	+	+	+	+	+	-
4	*Xc-4*	+	+	+	+	+	-

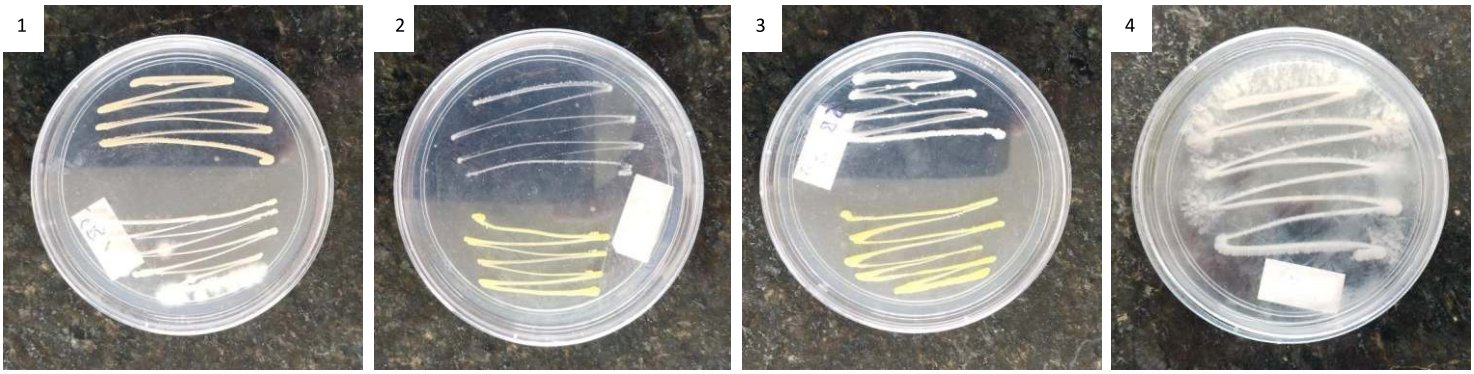

Figure 1: 1,2,3,4 NA plates with bacterial isolates from pomegranate growth

Figure 2: Growth on Phyto-Xynocamp Agar Base Media(media) for the characterization of bacterial isolates

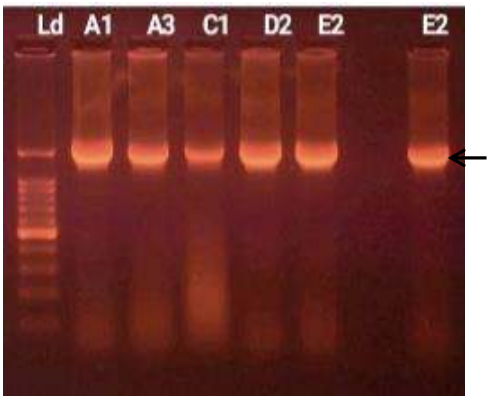

Figure 3: Representative PCR image of 16sRNA amplified PCR products from the various bacterial strains.(Ld,-Marker ladder, A1-E2 –PCR products,arrow-PCR product)

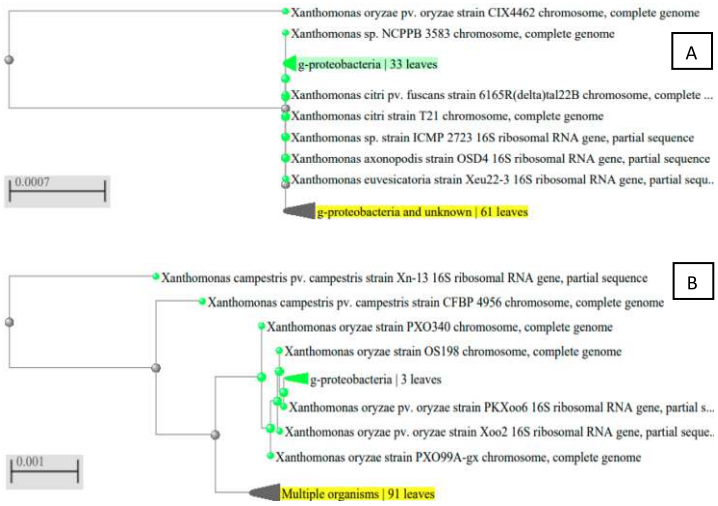

Figure 4: A and B. Phylogeentic trees constructed using NCBI url sequences of the BlastN alignment of 16SRNA sequence. (A-Xa1B-Xc-4)(Yellow include 16S sequence of the strain)

Figure 5: Symptoms of bacterial blight on pomegranate leaves and fruits as observed under field conditions

Conclusion

The current study identified a consortium of epiphytic bacteria from the pomogranante. Specifically 4 strains of Xanthomonas were identified with high Virulence. Whole genome sequencing of the strain will provide further insights into the genetic correlates of the pathogen's virulence. The results will help better management of blight in pomegranate by designing better anti-microbials and thus reduce fruit loss. Thus the study of plant-pathogen and their interaction, molecular pathways and evolution of strain variability in response to various biotic and abiotic factors assumes significance.

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