



## Morpho-Cultural characterization of *Xanthomonas citri* pv. *punicae*, an incitant of bacterial blight of pomegranate

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### Abstract

Bacterial blight, incited by *Xanthomonas citri* pv. *punicae*, is one of the most destructive diseases threatening pomegranate (*Punica granatum* L.) cultivation across Karnataka and other major growing states of India. The causal organism was isolated from naturally infected pomegranate leaves and fruits showing typical bacterial blight symptoms. The pathogen produced characteristic yellow, circular, smooth, convex and mucoid colonies on the nutrient agar medium. Its pathogenicity was confirmed through a detached leaf assay on healthy pomegranate leaves (cv. Bhagwa), wherein typical water-soaked lesions progressed into dark brown to black necrotic spots within 10 to 12 days of inoculation. Morphological characterization revealed the organism to be a Gram-negative, rod-shaped bacterium measuring 1.2-2.0 µm in length and 0.4-0.7 µm in width. Cultural characterization on seven different media showed that nutrient agar supported the maximum bacterial multiplication, while modified Wakimoto agar failed to support any growth. Colony diameter varied considerably across media, ranging from 1.12 mm on nutrient agar to 4.51 mm on yeast glucose calcium carbonate agar, although the colonies retained their characteristic round, smooth-margined and mucoid appearance irrespective of the medium used. Despite variation in growth intensity across media, the morphological and cultural features recorded were consistent with the known characters of the pathogen and the confirmed pathogenicity further strengthens its identity as *X. citri* pv. *punicae*.

**Keywords:** Pomegranate, bacterial blight, *Xanthomonas citri* pv. *punicae*, morphological characterization, cultural characterization

### Introduction

Pomegranate (*Punica granatum* L.) is one of the most economically important fruit crop of the semi-arid tropics, valued for its nutritional richness, antioxidant properties and high export potential. India, particularly the state of Maharashtra and Karnataka, occupies a prominent position in global pomegranate production. However, the sustainability of this high value crop is seriously threatened by bacterial blight, caused by *Xanthomonas citri* pv. *punicae* (formerly *X. axonopodis* pv. *punicae*), a disease that can bring an yield losses ranging from 60 to 80 per cent under epidemic conditions (Sharma *et al.*, 2010)<sup>[9]</sup>.

The pathogen incites symptoms on leaves, stems and fruits in the form of small, irregular, water-soaked lesions that later turn necrotic, coalesce and cause defoliation, twig dieback and fruit cracking, rendering the produce unmarketable (Sharma *et al.*, 2017)<sup>[8]</sup>. The pathogen disseminates through infected planting material, wind-borne inoculum, rain splash and contaminated pruning tools (Yenjerappa *et al.*, 2011)<sup>[11]</sup>. Precise identification and characterization of the causal organism therefore constitute a prerequisite for the development of reliable diagnostic and management strategies.

Although the disease has been studied in different pomegranate growing regions of India, regional variability in the pathogen's morphological and cultural characteristics has often been reported, underscoring the need for continuous characterization of isolates from newly affected areas. With this background, the present investigation was undertaken to isolate the causal organism from naturally infected pomegranate tissue, confirm its pathogenicity and

characterize it morphologically and culturally on a range of nutrient media.

### Materials and Methods

#### 1. Isolation of the causal organism

Infected pomegranate leaves and fruits showing typical bacterial blight symptoms were collected from an affected orchard. Small pieces of symptomatic tissues were excised from the margin of the advancing lesions, surface sterilized and macerated in sterile distilled water to release bacterial ooze. A loopful of this suspension was streaked onto Nutrient agar (NA) by the standard streak plate method and incubated at 28 ± 1 °C for 48 to 72 h.

#### 2. Pathogenicity

The pathogenicity of the isolate was confirmed using a detached leaf assay. Healthy leaves of pomegranate cv. Bhagwa were collected, thoroughly washed under running tap water, surface sterilized and placed on moist sterile filter paper in Petri plates to maintain humidity. A 48 hour old bacterial suspension of the isolate was inoculated onto the healthy leaves by pin-prick method and the inoculated leaves were incubated at 30 °C. Sterile distilled water served as a control. The leaves were observed daily for the appearance of symptoms.

#### 3. Morphological characterization

##### Gram staining

Gram staining was performed using a 24 hour old bacterial culture to determine the Gram reaction. A smear was prepared on a clean glass slide, air dried, heat fixed and flooded with crystal violet for 1 min, followed by washing.

Gram's iodine was applied for 1 min, washed and the smear was decolorized with ethyl alcohol (95 %) for 1 min. It was then counterstained with safranin for 30 sec, washed, air dried and examined under a compound microscope.

#### Negative staining

Negative staining was carried out using nigrosin to study cell shape and size. A loopful of fresh culture was mixed with a drop of nigrosin near one end of a clean slide and the suspension was spread into a thin film using the edge of another slide held at ~45°. The smear was air dried without heat fixation and examined under the microscope.

#### 4. Cultural characterization

The cultural characteristics of the pathogen was studied on seven different culture media, viz., Nutrient Agar (NA), Nutrient Glucose Agar (NGA), Yeast Extract Nutrient Agar (YNA), Yeast Glucose Calcium Carbonate Agar (YGCA), Yeast Dextrose Calcium Carbonate Agar (YDCA), Nutrient Sucrose Agar (NSA) and Modified Wakimoto Agar (MWA). Sterilized media (autoclaved at 1.10 kg/cm<sup>2</sup> for 15 min) were poured into sterile Petri plates and allowed to solidify aseptically. A 72 hour old bacterial suspension, serially diluted in sterile distilled water, was spread (100 µl of 10<sup>-7</sup> dilution) onto the plates using a sterile spreader and incubated at 28 ± 1 °C for 48 h after which the colony characters were recorded.

### Results and Discussion

#### 1. Isolation of the pathogen

Bacterial growth was observed on NA plates within 48 to 72 h of incubation. A single, well-isolated colony exhibiting yellow, circular, smooth, convex and mucoid appearance, characteristic of the pathogen, was selected and purified through repeated sub-culturing on fresh NA medium until a pure culture was obtained. The purified isolate was maintained on NA slants for further studies.

#### 2. Pathogenicity

The pathogenicity of the isolate was confirmed through a detached leaf assay on healthy pomegranate leaves (cv. Bhagwa) maintained under humid conditions. Characteristic bacterial blight symptoms appeared 10 to 12 days after inoculation, beginning as minute water-soaked lesions at the point of inoculation which gradually enlarged into dark brown to black necrotic lesions resembling those observed under natural field infection. Re-isolation of the pathogen from the inoculated leaves yielded colonies identical in morphology to the original isolate, thereby fulfilling Koch's postulates and confirming the pathogenic nature of isolate. These results corroborate the findings of Ramdas and Patil (2020) [7], who confirmed the pathogenicity of the pathogen on pomegranate cv. Ganesh through the pin-prick inoculation method, fulfilling Koch's postulates by re-isolating the pathogen from infected tissue. Similarly, Manjula and Khan (2003) [6] and Chavan *et al.* (2017) [1] demonstrated the pathogenicity of the organism through the reproduction of typical symptoms and subsequent recovery of the pathogen from inoculated tissue.

#### 3. Morphological characterization

**Gram staining:** The bacterial cells appeared pink following Gram staining, confirming that the organism did not retain the primary stain and was therefore Gram-negative in nature.

**Negative staining:** Microscopic examination of nigrosin-stained cells revealed rod-shaped bacteria measuring approximately 1.2 - 2.0 µm in length and 0.4 - 0.7 µm in width.

The Gram-negative reaction and rod-shaped morphology recorded in the present study are characteristic features of *X. citri* pv. *punicae*. Similar observations were made by Gamangatti and Patil (2014) [3], who described *X. axonopodis* pv. *punicae* as Gram-negative, rod-shaped, non-spore-forming bacteria occurring singly or occasionally in pairs. Comparable findings were reported by Kale *et al.* (2012) [5], wherein the pathogen exhibited a Gram-negative reaction, rod-shaped morphology, slightly raised and pale yellow colonies. Similarly, Kabade *et al.* (2020) [4] reported a consistent Gram-negative reaction among the isolates of the pathogen collected from different pomegranate growing regions, confirming the stability of this diagnostic character.

#### 4. Cultural characterization

The cultural characteristics of the pathogen was evaluated on seven differential media, namely NA, NGA, YNA, YGCA, YDCA, NSA and MWA (Table 1). All the media supported growth of the pathogen except Modified Wakimoto Agar, on which no growth was observed. Considerable variation in colony number, size and pigmentation was recorded among the different media tested. The colony count expressed as cfu per 100 µl was maximum on Nutrient agar (143 × 10<sup>7</sup>), followed by Yeast extract nutrient agar (111 × 10<sup>7</sup>) and Nutrient glucose agar (61 × 10<sup>7</sup>), whereas comparatively fewer colonies developed on Yeast dextrose calcium carbonate agar (42 × 10<sup>7</sup>), Nutrient sucrose agar (38 × 10<sup>7</sup>) and Yeast glucose calcium carbonate agar (8 × 10<sup>7</sup>).

Colony diameter ranged from 1.12 mm on Nutrient agar to 4.51 mm on Yeast glucose calcium carbonate agar, followed by Nutrient sucrose agar (3.60 mm) and Yeast dextrose calcium carbonate agar (2.93 mm). Colony colour varied from light yellow to dark yellow, indicating slight variation in the intensity of pigmentation produced by the pathogen among the different media tested.

Despite the observed variability, the pathogen consistently exhibited the characteristic circular, smooth and mucoid colonies across the media, indicating that the basic cultural identity of the pathogen remains stable even as nutrient availability modulates its growth intensity. Comparable observations were reported by Kabade *et al.* (2020) [4] and Chormale *et al.* (2021) [2], who observed that *X. axonopodis* pv. *punicae* produced circular, convex and mucoid colonies with variation in colony size, colony number and pigmentation across the different culture media.

The pathogen did not grow on modified Wakimoto agar, although the same inoculum grew well on all the other media tested. This medium is chemically defined, with cellobiose as the sole carbon source and ammonium chloride as the sole nitrogen source, and it has no peptone, yeast extract or other complex supplement. As it has been reported to support the growth of *X. oryzae* pv. *oryzae* (Tulasi *et al.*, 2026) [10], the failure of *X. citri* pv. *punicae* to grow on it probably reflects the nutritional requirements of the pathogen. The result suggests that media based on peptone, beef extract or yeast extract are better suited than minimal formulations for its isolation and cultural characterization.

**Table 1:** Cultural characterization of *Xanthomonas citri* pv. *punicae* on differential media

| Sl No. | Media                                 | Colony number (10 <sup>-7</sup> dilution)* | Colony size (mm)* | Colony colour | Colony shape | Colony margin | Mucoidness |
|--------|---------------------------------------|--------------------------------------------|-------------------|---------------|--------------|---------------|------------|
| 1      | Nutrient agar                         | 143                                        | 1.12              | Yellow        | Round        | Smooth        | Mucoid     |
| 2      | Nutrient glucose agar                 | 61                                         | 1.26              | Yellow        | Round        | Smooth        | Mucoid     |
| 3      | Yeast extract nutrient agar           | 111                                        | 1.68              | Dark yellow   | Round        | Smooth        | Mucoid     |
| 4      | Yeast glucose calcium carbonate agar  | 8                                          | 4.51              | Dark yellow   | Round        | Smooth        | Mucoid     |
| 5      | Yeast dextrose calcium carbonate agar | 42                                         | 2.93              | Yellow        | Round        | Smooth        | Mucoid     |
| 6      | Nutrient sucrose agar                 | 38                                         | 3.60              | Light yellow  | Round        | Smooth        | Mucoid     |
| 7      | Modified Wakimoto agar                | 0                                          | -                 | -             | -            | -             | -          |
|        | S. Em. ±                              | 0.55                                       | -                 | -             | -            | -             | -          |
|        | C.D. @ 1%                             | 1.68                                       | -                 | -             | -            | -             | -          |

\*Mean of three replications

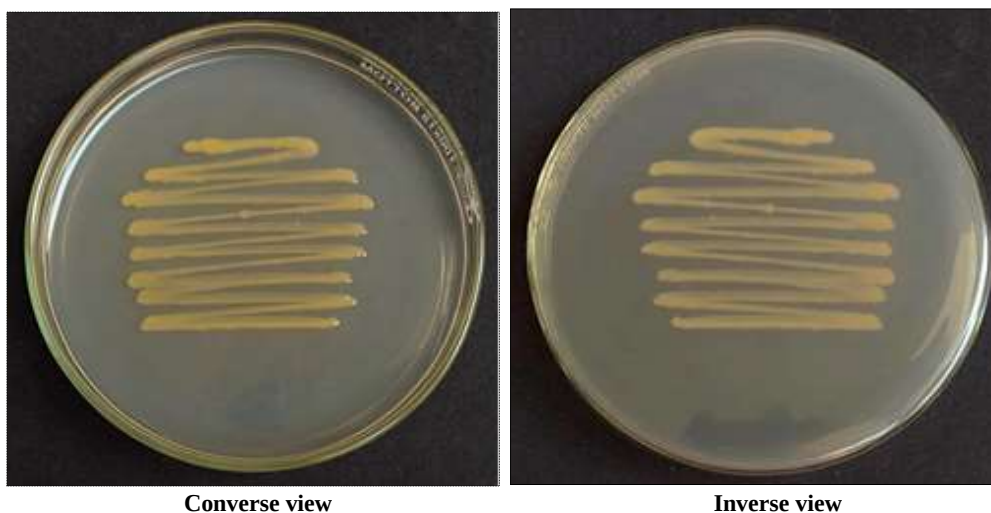


**Fig 1:** Converse and inverse view of *Xanthomonas citri* pv. *punicae* on nutrient agar medium

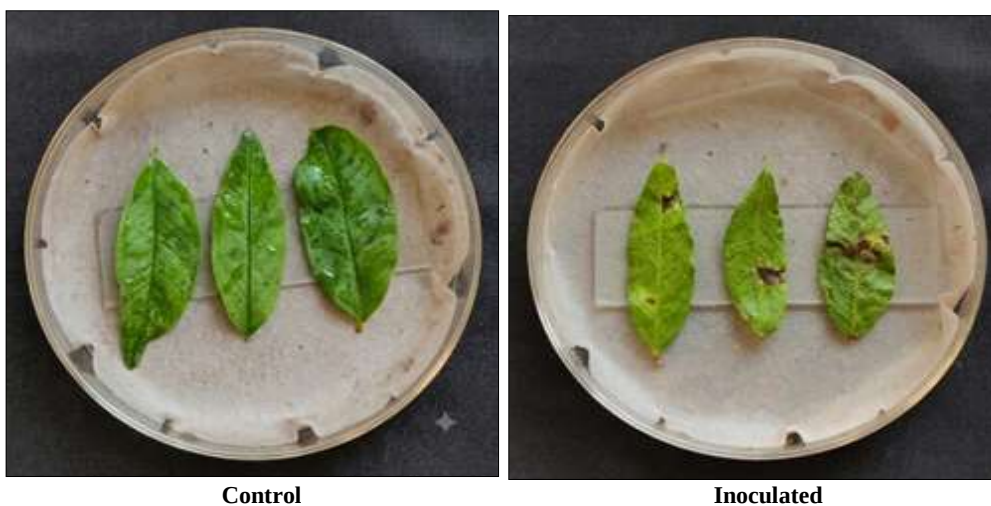
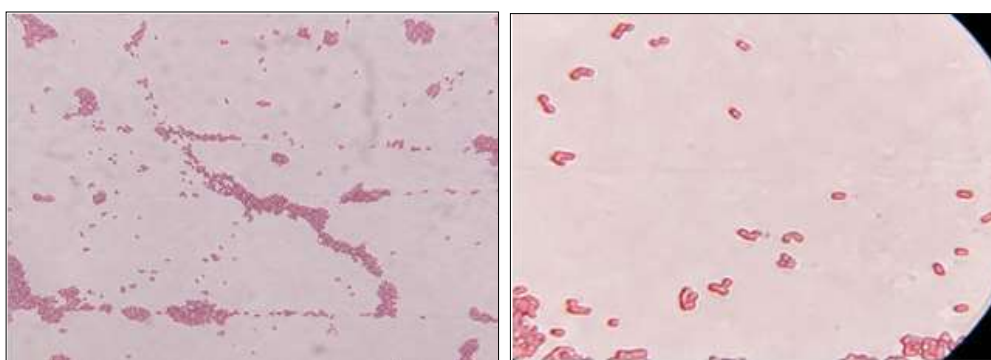
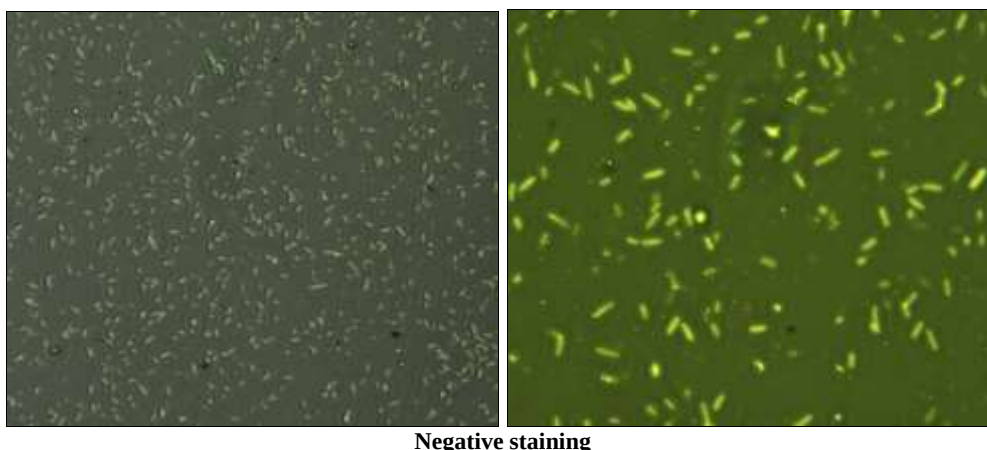


**Fig 2:** Pathogenicity of *Xanthomonas citri* pv. *punicae* on detached leaves of pomegranate cv. Bhagwa



**Gram staining**



Negative staining

Fig 3: Morphological characteristics of *Xanthomonas citri* pv. *Punicae*

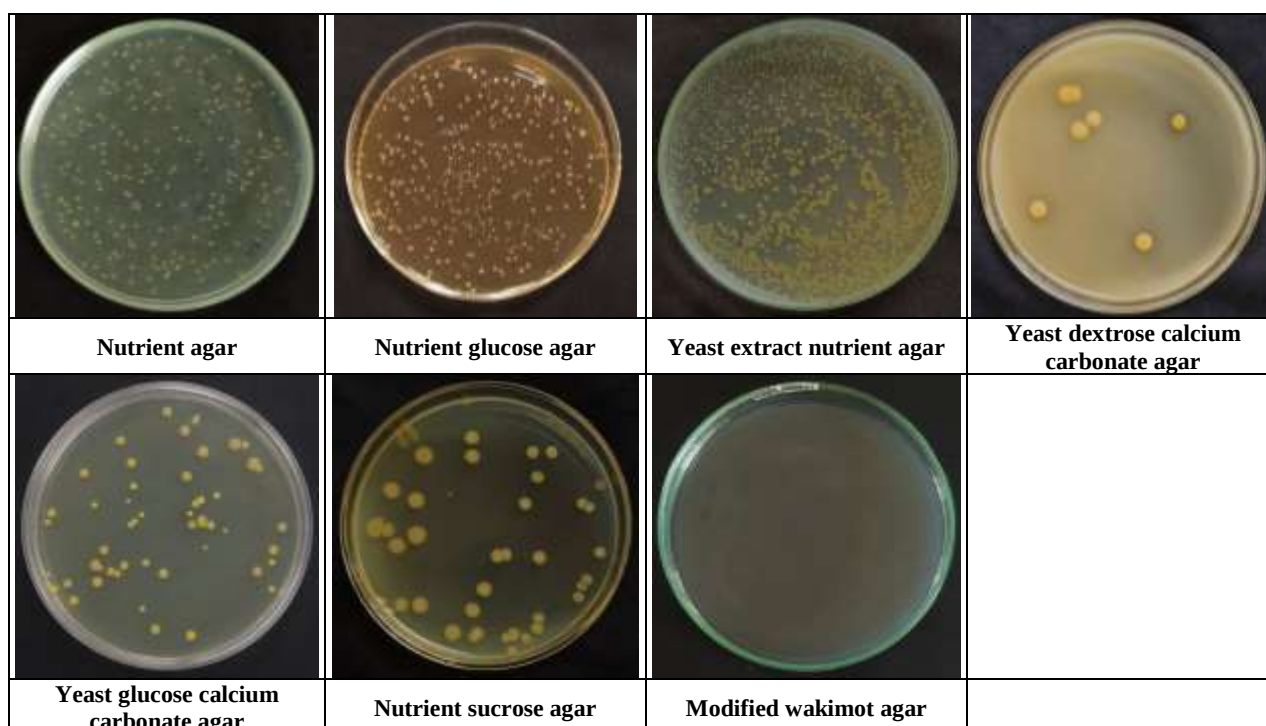


Fig 4: Cultural characteristics of *Xanthomonas citri* pv. *punicae* on differential media

## Conclusion

The bacterial blight pathogen, *Xanthomonas citri* pv. *punicae*, was successfully isolated from naturally infected pomegranate tissues using the streak plate technique on Nutrient agar medium. The pathogenicity was unambiguously confirmed through a detached leaf assay, wherein typical water-soaked and necrotic lesions developed within 10 to 12 days of inoculation and the pathogen was successfully re-isolated, thereby fulfilling Koch's postulates. Morphological characterization confirmed the organism to be a Gram-negative, rod-shaped bacterium, while cultural characterization across seven media revealed Nutrient agar to be the most suitable medium for supporting vigorous bacterial growth, with Modified Wakimoto agar being unsuitable. Although colony size and colour intensity varied with the nutrient composition of the medium, the pathogen consistently retained its characteristic round, smooth, mucoid colony morphology, underscoring the reliability of these features for its routine identification.

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