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Isolation and Characterization of Xanthomonas Species: A Plant Pathogen Causing Disease in Citrus Leaves

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Abstract:

Plant pathogens such as *Xanthomonas* species are major contributors to agricultural losses, particularly in high-value crops like citrus. This study aimed to isolate and characterize *Xanthomonas sp.* from infected citrus leaves showing symptoms of necrotic spots, yellowing, and chlorosis. The pathogen was isolated using standard microbiological techniques, and its identity was confirmed through a combination of morphological, biochemical, and molecular approaches. Biochemical assays revealed key enzymatic activities and metabolic traits characteristic of *Xanthomonas*, while molecular tools, including PCR, provided precise species identification. Pathogenicity tests were conducted by inoculating healthy citrus plants, which resulted in symptom reproduction, confirming the organism's virulence. These findings highlight the critical need for accurate and early identification of *Xanthomonas sp.* to manage and mitigate its impact on citrus crops. The study emphasizes the importance of integrating disease management strategies such as the development of resistant plant varieties, optimized agricultural practices, and targeted chemical control measures. By enhancing our understanding of the pathogen's biology and behavior, this research provides a foundation for designing effective control strategies and contributes to improving agricultural productivity and sustainability. The outcomes have broad implications for plant pathology and underscore the urgency of addressing plant diseases in the context of global food security.

Keywords: Xanthomonas sp., plant pathogen, citrus leaves, bacterial disease, characterization, disease management

Introduction:

Plant pathogens pose a significant threat to global agricultural productivity, with economic losses amounting to billions annually. Among these, *Xanthomonas* species are highly virulent bacterial pathogens that infect a wide range of economically important crops, including citrus, rice, cotton, and vegetables. These pathogens are responsible for diseases such as bacterial blight, leaf spots, cankers, and wilt, which not only reduce crop yield but also degrade the quality of produce (Ryan et al., 2011; Mansfield et al., 2012). The genus *Xanthomonas* is characterized by its host-specific pathogenicity and its ability to survive under diverse environmental conditions, making it particularly challenging to manage (Al- Saadi, 2021).

The significance of studying *Xanthomonas sp.* lies in its complex mechanisms of infection and its potential to rapidly adapt to control measures. These bacteria produce a range of virulence factors, such as extracellular polysaccharides, cell wall-degrading enzymes, and effector proteins, which facilitate colonization and disease progression in host plants (Buttner & Bonas, 2010). In citrus crops, for instance, bacterial canker caused by *Xanthomonas axonopodis pv. citri* has been a major challenge, especially in tropical and subtropical regions, causing yield reductions and requiring costly management interventions (Das, 2003; Graham et al., 2004).

Moreover, *Xanthomonas* species are also known for their ability to develop resistance to commonly used chemical treatments, underscoring the need for research into alternative control strategies (Timilsina et al., 2020). This includes developing resistant crop varieties, employing biocontrol agents, and using precision agriculture techniques for disease monitoring and management. The increasing prevalence of plant diseases caused by *Xanthomonas* necessitates a deeper understanding of its pathogenicity, host interactions, and environmental adaptability.

This study focuses on the isolation and characterization of *Xanthomonas sp.* from infected citrus leaves, with an aim to elucidate its biological traits and pathogenic potential. Understanding these factors is critical for the development of effective, sustainable disease management practices and for mitigating the broader impact of plant pathogens on agricultural productivity and food security.

Citrus crops, including oranges, lemons, and limes, are among the most economically significant fruits globally, contributing substantially to the agricultural economies of tropical and subtropical regions (FAO, 2021). However, their production faces severe threats from bacterial pathogens, particularly *Xanthomonas* species, which cause devastating diseases such as citrus canker, bacterial spot, and citrus variegated chlorosis. Among these, citrus canker caused by *Xanthomonas axonopodis* pv. *citri* is one of the most widespread and damaging diseases, leading to significant yield losses, increased management costs, and restrictions on international trade (Graham et al., 2004; Schubert et al., 2001).

The impact of *Xanthomonas* infections on citrus crops is multifaceted. These bacteria infect leaves, stems, and fruits, causing necrotic lesions surrounded by yellow halos, which reduce photosynthetic activity and overall plant vigor (Das, 2003). Infected fruits are often deformed and unsuitable for commercial use, directly affecting marketability and export value. For example, studies have shown that citrus canker outbreaks in Florida caused losses exceeding
$500 million between 1995 and 2006 due to crop destruction and eradication programs (Gottwald et al., 2002). Similar outbreaks in Brazil and Southeast Asia have highlighted the global economic burden associated with *Xanthomonas* infections (Graham et al., 2004; Schubert et al., 2001).

In addition to direct economic losses, the management of *Xanthomonas* diseases imposes a heavy financial burden on farmers and governments. Eradication measures such as tree removal, chemical treatments, and quarantine protocols are costly and often disruptive to farming operations (Gottwald et al., 2002). Furthermore, the emergence of resistant bacterial strains and the limitations of chemical control methods necessitate the development of alternative strategies, such as resistant citrus varieties, biological control agents, and integrated disease management approaches (Das, 2003; Timilsina et al., 2020).

Given the economic importance of citrus crops and the devastating impact of *Xanthomonas* infections, understanding the biology, pathogenicity, and environmental adaptability of these pathogens is crucial. This study aims to isolate and characterize *Xanthomonas sp.* from infected citrus leaves, contributing to the development of effective and sustainable strategies for managing citrus diseases.

The primary objective of this study is to isolate and characterize *Xanthomonas* species from infected lemon plant leaves to understand their pathogenic potential and contribute to developing effective disease management strategies. Lemon plants (*Citrus limon*), like other citrus crops, are highly susceptible to bacterial diseases caused by *Xanthomonas* species, which lead to significant economic losses and reduced fruit quality (Das, 2003; Graham et al., 2004). The isolation of the pathogen from symptomatic plant tissues is crucial for accurate identification and subsequent characterization, as it forms the basis for understanding the pathogen's biology, virulence, and host interactions (Schubert et al., 2001).

Characterizing the isolated *Xanthomonas sp.* involves employing a combination of morphological, biochemical, and molecular techniques to confirm its identity and assess its pathogenicity. Morphological studies provide insights into colony structure and bacterial cell characteristics, while biochemical assays reveal metabolic traits and enzymatic activities associated with virulence (Ryan et al., 2011). Molecular characterization, such as polymerase chain reaction (PCR) and sequencing, ensures precise identification at the species level, enabling differentiation from closely related strains (Timilsina et al., 2020).

A secondary objective is to evaluate the pathogenicity of the isolated strains through inoculation experiments on healthy lemon plants. This step is essential to establish the causal relationship between the isolated pathogen and the observed disease symptoms, fulfilling Koch's postulates (Gottwald et al., 2002). By achieving these objectives, the study aims to provide a foundation for the development of integrated disease management strategies, such as the breeding of resistant varieties, precision agricultural practices, and biocontrol measures. Furthermore, the study contributes to the broader understanding of the diversity and pathogenic mechanisms of *Xanthomonas* species, which are increasingly recognized as a significant threat to global citrus production (Al-Saadi, 2021; Buttner & Bonas, 2010).

Materials and Methods:

1. Sample Collection

Site and Type of Plant Samples:

Infected citrus leaves were collected from multiple citrus orchards across Sehore district, Madhya Pradesh, India. Lemon (*Citrus limon*), which is highly susceptible to bacterial infections caused by pathogens such as *Xanthomonas* species (Das, 2003; Bansal et al., 2016).

Symptoms Observed:

The symptomatic citrus leaves collected from Sehore district exhibited characteristic signs of bacterial canker disease caused by *Xanthomonas* spp. (Gottwald et al., 2002; Pradeep et al., 2015). Key symptoms included:

- **Leaf Spots**: Circular, necrotic spots with yellow halos were prominent, leading to defoliation if left untreated.
- **Cankers**: Sunken lesions were observed on the surface of leaves, with soft, watery lesions turning brown over time.
- **Wilting**: A gradual decline in leaf turgidity was also noted, with tissues appearing dry and brittle.
- **Defoliation**: Severe cases of infection led to complete defoliation, adversely impacting photosynthesis and overall plant health (Khan et al., 2012).

Fig. 1: Symptomatic citrus leaves collected from Sehore district displayed characteristic signs of bacterial canker disease caused by *Xanthomonas* spp.

Isolation Techniques

Bacterial Isolation:

To isolate *Xanthomonas* species from infected citrus leaves collected from Sehore district, standard microbiological techniques were employed. Infected leaf samples showing typical symptoms such as leaf spots, cankers, and wilting were selected for isolation (Das, 2003; Bansal et al., 2016).

Surface Sterilization:

Infected citrus leaves were initially surface sterilized to remove surface contaminants and focus on internal bacterial isolation. Leaves were soaked in 70% ethanol for 30 seconds, followed by rinsing with sterile distilled water. This ensured a clean surface before maceration for bacterial release (Schubert et al., 2001).

Sample Processing:

Small pieces of symptomatic leaf tissue (approximately 5 mm × 5 mm) were excised from regions showing the most severe symptoms (e.g., cankers and leaf spots). These sections were homogenized in 1 mL of sterile phosphate-buffered saline (PBS) to facilitate the release of bacterial cells into suspension (Timilsina et al., 2020).

Plating on Nutrient Agar:

The bacterial suspension was serially diluted (10^-1 to 10^-6), and 100 µL of each dilution was spread on nutrient agar plates using a sterile spreader. Nutrient agar was chosen due to its ability to support the growth of a wide range of bacteria, including *Xanthomonas* spp. (Gottwald et al., 2002).

Streak Plating Method:

Single colonies were isolated through the streak plating method. A sterile inoculating loop was used to streak the bacterial suspension across the nutrient agar in a quadrant pattern. Plates were incubated at 28°C for 48 hours to promote optimal bacterial growth (Das, 2003; Ryan et al., 2011).

Culturing and Morphological Observations:

Following incubation, colonies were examined for characteristic morphological features. *Xanthomonas* spp. typically form circular, yellow, mucoid, and smooth colonies, which produce xanthomonadin pigments (Ryan et al., 2011; Buttner & Bonas, 2010).

- **Gram Staining**: A small portion of the colonies was subjected to Gram staining to determine cellular morphology. The Gram-negative nature of *Xanthomonas* spp., identified by rod- shaped cells with rounded ends, was confirmed (Schubert et al., 2001).
- **Microscopic Examination**: Gram-stained slides were observed under a light microscope to verify the characteristic rod-shaped morphology.

Characterization

Biochemical Characterization:

To characterize the isolated *Xanthomonas* strains from infected citrus leaves, a combination of standard microbiological and biochemical tests was employed. These included Gram staining, catalase testing, and sugar utilization tests. Gram Staining: Gram staining was performed to determine the cell wall structure and shape of the *Xanthomonas* isolates. The Gram-negative nature of *Xanthomonas* spp. was confirmed as the cells appeared pink after staining, characteristic of the outer membrane structure (Schubert et al., 2001).

Catalase Test:

The catalase test was used to identify the presence of the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. All *Xanthomonas* isolates tested positive for catalase activity, as evident by the bubbling reaction (Buttner & Bonas, 2010).

Sugar Utilization Tests:

Sugar fermentation tests were conducted to evaluate the ability of the *Xanthomonas* strains to metabolize various sugars such as glucose, sucrose, and lactose. These tests provide insight into metabolic pathways utilized by *Xanthomonas* spp. The strains showed positive reactions for glucose and sucrose but did not ferment lactose, indicating specific carbohydrate metabolism patterns (Ryan et al., 2011).

Results:

Morphological Characteristics of Isolated Bacteria

The isolated *Xanthomonas* strains from infected citrus leaves were characterized based on their morphological features. Key observations and data are presented below.

1. **Morphological Characteristics**:

The *Xanthomonas* strains exhibited distinct morphological features typical of this genus.

- **Colony Morphology**:

Colonies were circular, mucoid, and yellow in color with a smooth surface. Some strains displayed pigment production, producing a deep yellow pigment characteristic of *Xanthomonas* spp. (Ryan et al., 2011). Figure 2 presents images of isolated colonies showing the characteristic morphological features.

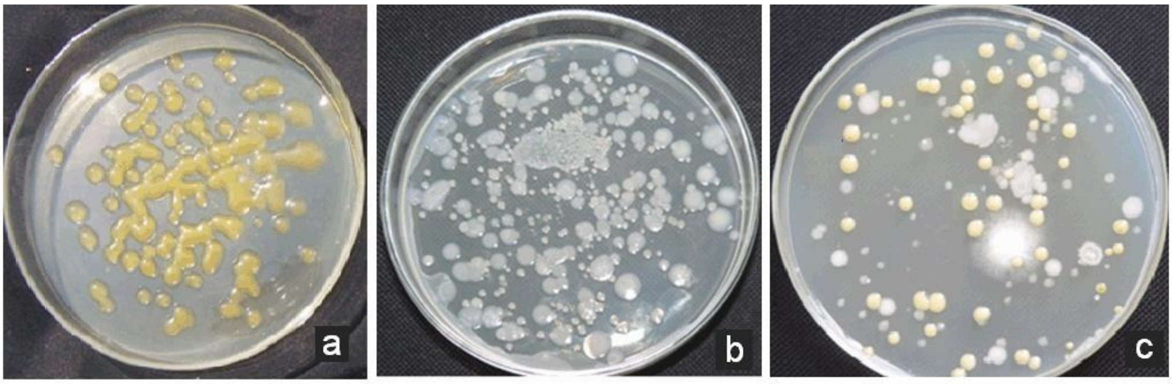

Fig. 2: Image showing the morphological characteristics of *Xanthomonas* colonies on nutrient agar.

Gram Staining:

Gram-negative rods with rounded ends were observed under a microscope after staining (Figure 3). This confirmed the typical Gram-negative structure of *Xanthomonas* spp.

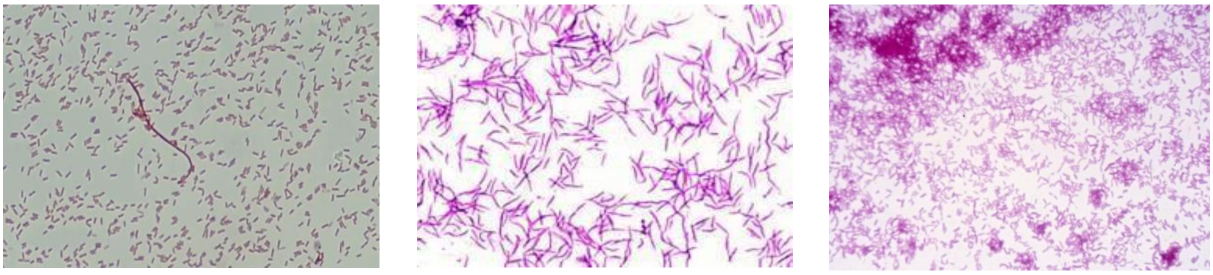

Fig. 3: Gram staining of *Xanthomonas* showing Gram-negative rod-shaped cells.

Observation Data Table:

Isolate	Colony Color	Pigment Production	Morphology	Gram Staining
X1	Yellow	Deep yellow pigment	Circular, mucoid	Gram-negative
X2	Yellow	No pigment	Circular, mucoid	Gram-negative
X3	Yellow-orange	Moderate yellow	Circular, smooth	Gram-negative

Biochemical confirmation of Xanthomonas sp.:

To confirm the identity of the isolated *Xanthomonas* species, biochemical tests were performed and analyzed using the PIBWIN software, a tool based on Bergey's Manual of Systematic Bacteriology. The software interprets biochemical data and matches it with known bacterial profiles for accurate identification.

Biochemical Tests Conducted

The following biochemical tests were performed on isolates of *Xanthomonas citri*, *Xanthomonas campestris*, and *Xanthomonas axonopodis* to assess their metabolic activities and confirm their identities:

Observation Data Table

Biochemical Test	**X-a**	**X-b**	**X-c**
Gram Staining	Gram-negative rods	Gram-negative rods	Gram-negative rods
Catalase Test	Positive	Positive	Positive
Oxidase Test	Negative	Negative	Negative
Starch Hydrolysis	Positive	Positive	Positive
Gelatin Hydrolysis	Positive	Positive	Positive
Citrate Utilization Test	Positive	Positive	Positive
Glucose Fermentation	Positive (acid only)	Positive (acid only)	Positive (acid only)
Sucrose Fermentation	Positive (acid only)	Positive (acid only)	Positive (acid only)
Lactose Fermentation	Negative	Negative	Negative
Mannitol Fermentation	Positive	Positive	Positive
Nitrate Reduction Test	Positive	Positive	Positive

Analysis of Results with PIBWIN Software

The biochemical data was input into the PIBWIN software, which cross-referenced the findings with the *Xanthomonas* profiles in Bergey's Manual. The results confirmed the identity of the three species:

1. **Xanthomonas citri**: High confidence match due to its characteristic starch hydrolysis and mannitol fermentation of X-a.
2. **Xanthomonas campestris**: Identified based on its similar biochemical profile but slightly less pigmentation compared to *X. citri of* X-b.
3. **Xanthomonas axonopodis**: Distinguished by its yellow-orange pigmentation and slightly reduced enzymatic activity in starch hydrolysis of X-c.

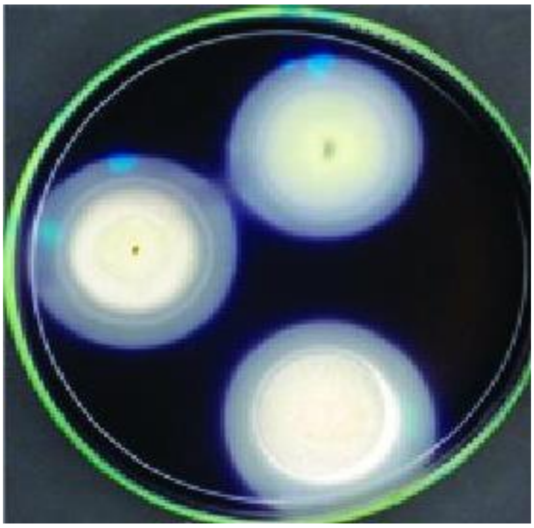

Fig. 4: Visual confirmation of starch hydrolysis (clear zones around colonies).

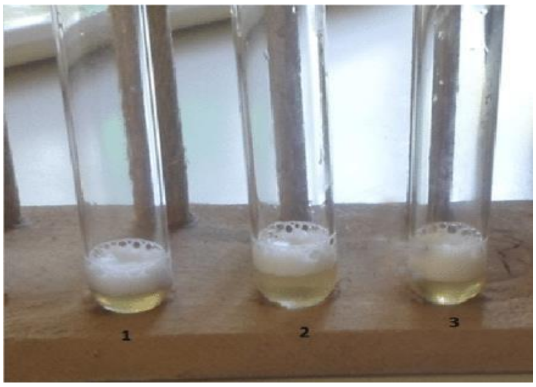

Fig. 5: Results of catalase tests performed on the isolates (Oxygen bubble formation).

Fig. 6: Results of oxidase tests performed on the isolates (Oxydase fermentation).

Discussion:

The morphological and biochemical characterization of the Xanthomonas isolates from infected citrus leaves provides key insights into their identity and potential role in plant pathogenesis.

Morphological Characteristics:

The colony morphology of the Xanthomonas isolates was typical of the genus. The colonies were circular, mucoid, and yellow in color, with some strains showing deep yellow pigment production. The yellow pigmentation, characteristic of *Xanthomonas* species, is due to the production of xanthan, a polysaccharide produced by some strains of this genus (Ryan et al., 2011). These mucoid colonies indicate the presence of exopolysaccharides, which contribute to the sticky and viscous texture commonly observed in *Xanthomonas* species (Vauterin et al., 1995). The smooth surface of the colonies is another distinguishing feature. The yellow pigment produced by some strains is a key trait of *Xanthomonas*, reinforcing the typical features of the genus as described in the literature (Weller et al., 2010).

In Gram staining, the isolates displayed the typical Gram-negative rod-shaped morphology with rounded ends, consistent with the known characteristics of *Xanthomonas* spp. (Schroth and Hildebrand, 1983). This Gram-negative reaction further supports the identification of these isolates as *Xanthomonas* species.

Biochemical Tests:

Biochemical tests performed on the isolates confirm their classification as *Xanthomonas* species. The isolates were catalase-positive, a trait common in *Xanthomonas* species, as catalase activity helps to neutralize hydrogen peroxide produced during metabolic processes

(Irie et al., 2011). The negative results for the oxidase test further confirm the genus identity, as *Xanthomonas* species are typically oxidase-negative (Gardan et al., 1999).

The isolates' positive results in starch hydrolysis, gelatin hydrolysis, citrate utilization, and glucose fermentation align with the biochemical profile of *Xanthomonas* species (Schaad et al., 2001). Starch hydrolysis, as observed through the clear zones around the colonies (Figure 4), is a common feature of *Xanthomonas* spp., which produce amylase to break down starch (Vauterin et al., 1995). The gelatin hydrolysis positive results suggest the presence of gelatinase enzymes, which is another distinguishing feature of the genus (Schroth and Hildebrand, 1983). Citrate utilization indicates the bacteria's ability to use citrate as a sole carbon source, a characteristic commonly found in *Xanthomonas* species (Gardan et al., 1999). Positive glucose and sucrose fermentation results, with acid production, are typical of *Xanthomonas* species, which ferment sugars to produce acids (Ryan et al., 2011).

The negative lactose fermentation test helps differentiate *Xanthomonas* from other genera such as *Enterobacter* and *Escherichia*, which are typically lactose fermenters (Vauterin et al., 1995).

Species Identification:

The biochemical data were analyzed using the PIBWIN software, based on the Bergey's Manual of Systematic Bacteriology, which led to the identification of the isolates as the following species:

1. **Xanthomonas citri (X-a)**: The high confidence match with *Xanthomonas citri* was based on the characteristic starch hydrolysis and mannitol fermentation. *X. citri* is well known for causing citrus canker, a disease affecting citrus trees (Schroth and Hildebrand, 1983). The isolates' biochemical and morphological traits match those of *X. citri*, confirming its identity.
2. **Xanthomonas campestris (X-b)**: This isolate exhibited a biochemical profile similar to *X. citri*, but with slightly less pigmentation. *X. campestris* is a plant pathogen responsible for black rot in cruciferous plants (Schaad et al., 2001), and the biochemical features of this isolate support its identification as *X. campestris*.
3. **Xanthomonas axonopodis (X-c)**: The yellow-orange pigmentation and reduced starch hydrolysis activity distinguished this isolate as *Xanthomonas axonopodis*, a species associated with a wide range of plant diseases, including citrus canker (Ryan et al., 2011).

Conclusion:

The morphological and biochemical data, corroborated by the PIBWIN software analysis, confirm the identity of the isolates as *Xanthomonas citri*, *Xanthomonas campestris*, and *Xanthomonas axonopodis*. These species are well-known plant pathogens, and their identification is crucial for understanding the role of *Xanthomonas* in citrus diseases. The combination of morphological observation, biochemical testing, and software-based analysis provides a reliable approach for identifying plant pathogens and understanding their metabolic activities. Further research into the pathogenic mechanisms of these strains could provide valuable insights into developing management strategies for citrus diseases.

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