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Identification and Characterization of the Pathogen Causing Canker Disease in Acid Lime (*Citrus aurantiifolia*) cv. Petlur Acid Lime

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In this study, the pathogen responsible for canker disease in Acid Lime Cv. Petlur Acid Lime was identified through a comprehensive analysis involving both morphological and molecular characterization techniques. Cultures of the *Xanthomonas campestris pv. citri (Xcc)* bacterial isolate, grown on artificial media, exhibited distinct characteristics: small, round, and mucoid colonies with a gram-negative nature, as revealed by Gram staining. Molecular identification was performed using universal primers 27F and 1492R, resulting in a specific DNA band of 424 bp, confirming the presence of the pathogen in the samples.

Keywords: Citrus canker; xanthomonas campestris pv. citri; acid lime; morphological characterization; molecular identification.

1. INTRODUCTION

Acid lime (*Citrus aurantifolia*), a small citrus fruit from the Rutaceae family, has gained global popularity for its tangy flavor, versatile use in cooking, and medicinal properties. Thriving in various soil and climate conditions, it has become a fundamental crop in tropical and subtropical regions worldwide. India, recognized as the leading producer of acid lime globally, cultivates this fruit over 248 thousand hectares, with key cultivation regions in states such as Andhra Pradesh, Telangana, Maharashtra, Assam, Chhattisgarh, Karnataka, Bihar, and Gujarat. Following India, the USA, Spain, and Israel also contribute significantly to global acid lime production. Notably, Andhra Pradesh stands out with a vast cultivation area of 45.8 million hectares, yielding 686.6 metric tons, owing to its favorable climate conditions. This widespread cultivation underscores the economic significance of acid lime and its vital role in agriculture.

Several factors limit the productivity of this crop, and one of the major constraints is the lack of knowledge about disease management. One of the significant diseases affecting acid lime is canker, caused by *Xanthomonas campestris pv. citri.* The symptoms of citrus canker include pustules developing into necrotic lesions, characterized by erumpent corky tissues surrounded by oily or water-soaked margins and a yellow halo [1]. The disease severity leads to defoliation, dieback, premature fruit drop, and blemished fruit. Fruit lesions are especially economically detrimental, making the fruit unacceptable for the fresh market and lowering its market value significantly [2]. Early, rapid, and specific detection of the canker-associated bacterium is crucial for effective plant disease management. Without accurate diagnosis, timely control measures cannot be implemented. Traditional methods for detecting and identifying bacterial pathogens involve isolating the pathogen on selective media and conducting identification through molecular analysis [3,4]. However, these methods have limitations. PCR-based molecular markers offer a more specific and reliable alternative [5]. With these considerations in mind, our experiment was meticulously planned and conducted at Citrus Research Station, Petlur, between 2021-23. The primary objective was to identify the pathogen responsible for causing canker disease in citrus.

2. MATERIALS AND METHODS

Young citrus plants displaying symptoms of canker infection on their leaves were gathered from the experimental area of Citrus Research Station, Petlur. These samples were then processed to isolate the causal bacterium in the laboratory of the Department of Plant Pathology at Citrus Research Station, Petlur. The isolation of the bacterium was carried out using a specialized medium called Nutrient Agar, following the method outlined by Khan and Chohan [6]. The isolated organism was purified and multiplied before undergoing identification at both morphological and molecular levels. Koch's postulates were rigorously applied to confirm the pathogen's identity. Bacterial DNA was extracted using the modified CTAB method as described by Murray and Thompson [7]. Subsequently, the quantity and quality of the bacterial DNA were assessed using Nanodrop for concentration and Agarose gel for purity, respectively. Molecular identification was conducted using universal primers, namely 27F and 1492R.

3. RESULTS AND DISCUSSION

3.1 Morphological Identification of Pathogen Symptomology

Citrus canker manifests as pustules evolving into necrotic lesions, characterized by erumpent corky tissues surrounded by oily or water-soaked margins and a yellow halo. The disease's severity leads to defoliation, dieback, premature fruit drop, and the development of blemished fruit.

Similar observations were documented by Shehzadi and Naz [8] and Das [2] in citrus plants infested with *Xanthomonas*. They reported that citrus canker disease appears as necrotic raised lesions on fruits, leaves, and stems. Severe infestations can cause fruit drop, and defoliation, and may even lead to tree decline.

The presence of the pathogen causing citrus canker was first reported in the USA in 1910 [9]. Subsequently, citrus canker has been observed in citrus-producing countries worldwide [2].

3.2 Collection, Isolation, and Purification of the Pathogen

Young citrus plants exhibiting canker infections on their leaves were gathered from the experimental area of Citrus Research Station, Petlur. Precautions were taken to isolate the pathogen, which was identified in these samples. The pathogen was isolated on Nutrient Agar media following the method outlined by Khan and Chohan [6]. Diseased samples, along with healthy portions, were cut into small pieces (0.5 to 1 cm) and disinfected with a 1.00% sodium hypochlorite solution. After two washes in autoclaved distilled water, the samples were dried on sterilized blotter paper. These disinfected bits were then placed in sterilized petri plates containing NA (nutrient agar) media and incubated at a temperature of 27 ± 1°C for 48 hours. Single purified colonies of the bacterium were streaked onto NA (nutrient agar) and YDC (Yeast Dextrose Calcium Carbonate) media to obtain a purified bacterial culture [10]. The inoculated plates were incubated at 28 ± 1°C for 48 to 72 hours, and observations of the growth pattern, colony color, shape, and appearance were recorded.

3.3 Identification of the Pathogen

Following the pathogen's purification via microscopic investigations, a comprehensive analysis of various characteristics was conducted to identify the causative agent of citrus canker disease, which was determined to be *Xanthomonas campestris pv. citri*.

3.4 Microscopic Observations

Microscopic examination revealed that the colonies of the *Xcc* bacterial isolate were small, round, mucoid, and displayed a yellow coloration due to the accumulation of xanthomandian. The distinctive yellow hue was a result of xanthomonadin pigmentation, a substance produced by *Xanthomonas.* The colonies exhibited mucoid characteristics due to the production of extracellular polysaccharide slime, facilitated by the addition of glucose in the culture medium. The colonies were observed to be small to medium-sized, convex, and singularly arranged in terms of size, shape, and arrangement.

These findings were consistent with previous studies conducted by Shehzadi and Naz [8] and Das [2] in citrus, validating the consistency of our observations.

3.5 Pathogenicity Test

The isolate underwent a standardized pathogenicity test following established procedures and was designated as the Dr. YSRHU - CRS, Petluru isolate. After 21 days, the pathogen was successfully isolated and cultured, confirming adherence to Koch’s postulates. These results corroborated the earlier findings by Shehzadi and Naz [8] and Das [2], further establishing the pathogenic nature of *Xcc* in acid lime (Fig 1).

3.6 Molecular Identification of Pathogen

3.6.1 DNA isolation

The DNA was extracted from the isolate using the CTAB method of DNA isolation. The quality of the acquired DNA was assessed by running it through agarose gel electrophoresis. A distinct band was observed, indicating the presence of DNA. The quantity was also verified, and DNA of good quality, ranging between 1.8 to 2.0, was used for subsequent studies.

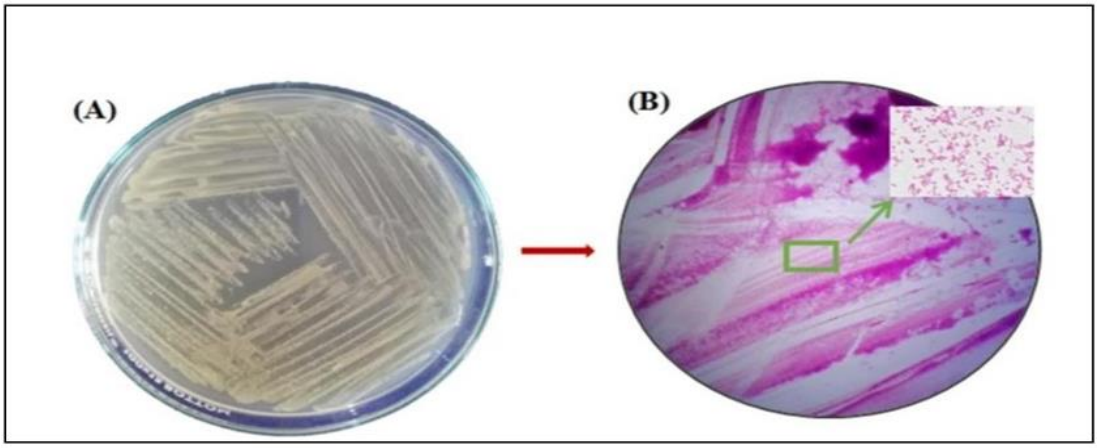

Fig. 1. Yellow to dark yellow colonies on nutrient agar medium

Picture 1. PCR amplification using ISSR primers
**Lane M: 100 bp DNA ladder*

3.6.2 PCR amplification using ISSR primers

The isolated DNA was subjected to PCR amplification using universal primers 27F and 1492R to identify the pathogen. The PCR product showed a band width size of 424 bp, as evident in Plate. The obtained banding pattern confirmed the isolated pathogen as *Xanthomonas campestris pv. citri.*

These results align with the findings reported by Shehzadi and Naz [8] in acid lime.

4. CONCLUSION

Early, rapid, and specific detection of canker-associated bacteria is crucial for effective plant disease management. In this study, the pathogen was identified based on both morphological and molecular characterization. On artificial media, colonies of the *Xcc* bacterial isolate were observed to be small, round, and mucoid, with a gram- negative nature confirmed by gram staining. The bandwidth size of 424 bp further validated the presence of the pathogen.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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