www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(6): 101-105

www.thepharmajournal.com
Received: 01-03-2023
Accepted: 08-04-2023

Ganesuni Lakshmi Prasanna
Ph.D., Scholar, Department of Plant Pathology, Dr. YSRHU-College of Horticulture Anantharajupeta, Andhra Pradesh, India

K Gopal
Associate Dean, Department of Plant Pathology Dr. YSRHU-College of Horticulture Anantharajupeta, Andhra Pradesh, India

CH Ruth
Professor and Head, Department of Plant Pathology, Dr. YSRHU- College of Horticulture Anantharajupeta, Andhra Pradesh, India

Y. Sireesha
Assistant Professor, Department of Plant Pathology, Dr. YSRHU- College of Horticulture Anantharajupeta, Andhra Pradesh, India

Syed Sadarunnisa
Professor and Head, Department of Vegetable Science, Dr. YSRHU- College of Horticulture Anantharajupeta, Andhra Pradesh, India

VV Padmaja
Associate Professor, Department of Plant Physiology, Dr. YSRHU- College of Horticulture Anantharajupeta, Andhra Pradesh, India

Corresponding Author:
Ganesuni Lakshmi Prasanna
Ph.D., Scholar, Department of Plant Pathology Dr. YSRHU-College of Horticulture Anantharajupeta, Andhra Pradesh, India

Colonial and morphological variability among the isolates of *Xanthomonas axonopodis* pv. *citri* Causing acid lime canker

Ganesuni Lakshmi Prasanna, K Gopal, CH Ruth, Y Sireesha, Syed Sadarunnisa and VV Padmaja

Abstract
The objective of the present investigation was to distinguish between different *Xanthomonas axonopodis* pv. *citri* (Xac) isolates, the causative agent of canker in acid lime. 32 different isolates were gathered from acid lime canker lesions (leaves and fruits) in Andhra Pradesh's potential growing regions. In the present study, morphological characteristics and biochemical technique *i.e* Grams staining were used to identify different Xac isolates. All 32 isolates grown on nutrient agar media produced small, yellow to orange yellow colonies with a variety of margins, according to the morphological studies. Grams staining test were performed on these isolates and all the isolates were found negative to Grams staining test.

Keywords: Colonial, Gram staining, Morphological, Nutrient agar and *Xanthomonas axonopodis* pv. *citri*

1. Introduction
One of the five economically significant citrus species grown as a commercial crop in India is acid lime (*Citrus aurantifolia* Swingle). In India, an area of 3.17 lakh ha was used for acid lime cultivation, yielding 37.17 lakh MT (NHB, 2021-22) [12] with a productivity of 10.3 MT/hectare, it accounts for 12.5% of production and 14.9% of the total fruit area. It is grown in almost all of India's states, with Andhra Pradesh being one of the most significant producers of acid limes in the nation with an annual production of 731 lakh MT (equivalent to 80% of production in India) and a productivity of 15 MT.

The production of acid lime fruits is hampered by a number of diseases brought on by bacterial, fungal, viral, and nematode pathogens. Among these ailments, bacterial canker impairs plant development and fruit quality in India and all other countries that grow acid lime (Hameed *et al*., 2020) [4]. The citrus canker, one of the major farming challenges, was first noted in Punjab, India (Luthra and Sattar, 1942) [8]. Canker occurrence was further recorded in Tamil Nadu, Andhra Pradesh, Karnataka, Rajasthan, Madhya Pradesh, Assam and Uttar Pradesh. The bacterium *Xanthomonas axonopodis* pv. *citri* causes bacterial canker, a devastating disease that affects many commercial cultivars of citrus and closely related species worldwide when warm temperatures and rainfall are present at the time of shoot emergence and fruit development (Shivankar and Ghosh, 2015) [15]. Citrus canker symptoms range from pustules to necrotic lesions with erupted corky tissues encircled by oily or wet margins and a yellow halo. Defoliation, dieback, premature fruit drop, and imperfect fruit are all symptoms of the disease severity. Fruit with lesions is most economically significant damaged and has very low value because it cannot be sold fresh (Shehzadi and Naz, 2019) [14].

Proper detection and management techniques are greatly complicated by the presence of various bacterial forms. Rangaswami and Soumini (1957) [16] have reported the occurrence of pathogen strains (pathotypes) in India. For the management of plant diseases, early, rapid, and precise detection of canker-associated bacteria is crucial (Mansfield *et al*., 2012) [9]. Without a diagnosis, the disease cannot be controlled at a specific time (Mccartney *et al.*, 2003) [10]. Traditional methods for identifying and detecting bacterial pathogens involved isolating the pathogen on specific media and identifying it through biochemical tests (Gottwald *et al.*, 2002) [3]. The different types of bacterial canker causal agent can be distinguished using a variety of methods, including morphological, biochemical (Jadhav *et al*., 2018; Mehmood *et al*., 2019) [7, 11] etc. Understanding the morphology and biochemical basis of pathogens may be crucial for adopting and helping to create efficient disease management strategies (Isokar *et al.*, 2020) [6].

Therefore, the current study concentrated on an accurate detection and variability among the *Xanthomonas axonopodis* pv. *citri* isolates in order to determine the management practices of a specific disease.

2. Material and Methods

2.1 Isolation, purification and maintenance of *Xanthomonas axonopodis* pv. *citri* isolates

To isolate the pathogen from infectious acid lime leaves and fruits, the canker lesion along with 2mm of the tissue surrounding the lesion was removed and isolation of *Xanthomonas axonopodis* pv. *citri* was done by the method described by Janse (2005) [20]. As a basal medium, a nutrient agar (NA) medium containing 0.3% beef extract, 0.2% yeast extract, 0.5% peptone, 0.5% sodium chloride, and 1.5% agar agar was used. The canker-infected leave and fruits were thoroughly washed with tap water and surface sterilized for 2-3 minutes with 0.1% sodium hypochlorite solution, followed by three consecutive washings with sterilized distilled water to remove any excess or traces of sodium hypochlorite solution in the samples. On sterilized tissue paper, the leaves were allowed to dry. Infected leaves with cankerous spots were aseptically excised with a sterile scalpel, placed on a sterile slide, and macerated with the help of a glass rod by adding a small amount of sterile distilled water. Using a sterile inoculation loop, the macerate was transferred onto a Petri-plate containing nutrient agar medium. The plates were incubated for 1-2 days at 28±2 ℃.

Following isolation, typical yellow colonies were subjected to purification. *Xanthomonas axonopodis* pv. *citri* single colonies were isolated from NA plates using a sterile inoculation loop and incubated at 28±2 ℃. After 24 hours, the inoculated plates were observed, and the purified cultures were maintained on NA media. A single colony with a yellow-orange colony was selected from the plates by the inoculation loop and streaked onto another plate containing pure culture media. After purification on NA plates, bacteria were stored in test tubes on NA slants in the refrigerator at 4 ℃ and used as mother or stock culture. The isolates were labeled as Xac-1 to 32 and sub cultured at regular intervals.

2.2 Identification of the pathogen

The identification of isolates was confirmed using standard microbiological procedures such as cultural/morphological, and biochemical characterization of the pathogen.

2.2.1 Cultural/morphological characterization

The colony shape, elevations, margin, pigmentation, and cell shape of various test isolates were studied using nutrient agar according to the standard procedures described by Islam *et al*. (2014) [5].

2.2.2 Gram staining

Gram staining is used to distinguish between Gram positive and Gram negative bacteria. Gerhardt's (1981) [2] protocol was followed for the Gram staining procedure. Bacteria were initially heat fixed on a glass slide and treated with 0.5% crystal violet for 30 seconds before being washed with a slow flow of tap water. The slide was then treated with iodine solution for 1 minute, washed with tap water again, and decolorized with 95% ethanol for 30 seconds before being washed again and treated with safranin counter stain for 1 minute. The slide was washed in a gentle and indirect stream of tap water until no colour was visible in the effluent, and then blotted dry with absorbent tissue paper. The results of the Gram staining procedure were observed using a bright field microscope while immersed in oil.

3. Results and Discussion

3.1 Isolation and identification of the pathogen

The pathogen was isolated on nutrient agar medium as a basal medium and a total of thirty-two strains were isolated from infected leaf and fruit samples. All the thirty-two isolates showing typical characters of *Xanthomonas axonopodis* pv. *citri* with varied colonies were identified on the basis of cultural characteristics and staining methods. The results of gram staining showed that the pathogen was Gram negative bacterium when counter discoloured with safranin, produced red colour bacterial cells. The bacterium isolated during the study, on the other hand, formed small, shiny yellow to orange yellow colonies with varied margins on nutrient agar media, a *Xanthomonas* species characteristic attributed to the production of the xanthomonadin pigment by members of the species (Das, 2003) [1].

3.1.1 Cultural characterization

The colonies were found to have creamy white- creamy white with pale yellow tinge- yellow- yellow orange colour, which were obtained by streaking the bacterial suspension on nutrient agar containing plates. Single colonies from these plates were picked and further streaked on another nutrient agar plates to get the pure cultures of thirty-two isolates. The cultural characters of the pathogen isolates were also recorded and have been presented in Table 1 and Plate 1. It is clear from the table 1 and Plate 1 that isolates Xac-1, 4, 7, 9, 10, 11, 15, 17, 18, 19, 20, 22, 27, 29 and 31 were yellow in colour, isolates Xac-2, 3, 13, 23 and 32 were yellow orange in colour, isolates Xac-5, 8, 14, 21, 24, 25 and 28 were creamy white in colour and rest of the isolates Xac-6, 12, 16, 26 and 30 were creamy white with pale yellow tinge in colour. As far as the colony characters *viz*., elevation, size and texture were concerned, colonies of isolates Xac- 1, 3, 5, 8, 9, 10, 14, 15, 16, 18, 19, 20, 22, 23, 24, 25, 27, 29, 31 and 32 were mucoid, convex with regular margins, isolates Xac-2, 11, 12 and 21 were mucoid, slightly raised with regular margins, isolates Xac-6 and 13 were mucoid, flattened with regular margins and isolates Xac-4, 17, 26 and 30 were Smooth, flattened with regular margins while, all other isolates Xac-7 and 28 were Smooth, slightly raised with regular margined colonies.

The pathogen isolates were identified as *Xanthomonas axonopodis* pv. *citri* based on cultural, morphological, and biochemical observations. Gram staining revealed that colonies of various isolates on nutrient agar medium were small, yellow, mucoid, and gramme negative, which is consistent with the findings of Shehzadi and Naz (2019) [14]. The work of Dulaimi *et al*. (2018) [21], Mehmood *et al*. (2019) [11], and Isokar *et al.* (2020) [6] on the variation in colour and shape of colonies of *Xanthomonas axonopodis* pv. *citri* on nutrient agar media supported the findings of present study.

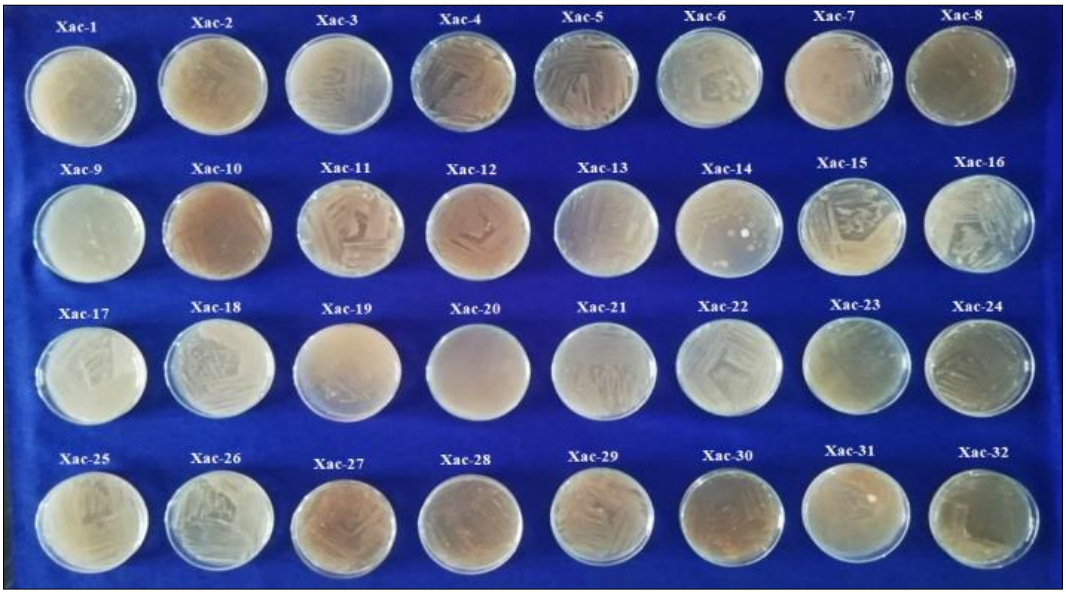
Table 1: The cultural, morphological and biochemical characterization of *Xanthomonas axonopodis* pv. *citri* isolates

Sr. No.	Area of collection	Isolate	Cultural/Morphological characteristics		Gram staining
			Pigmentation	Colony character	
1.	Kuppayapalem	XAC-1	Yellow	Mucoid, convex with regular margins	-ve
2.	Dakkili	XAC-2	Yellow orange	Mucoid, slightly raised with regular margins	-ve
3.	Devulapalle	XAC-3	Yellow orange	Mucoid, convex with regular margins	-ve
4.	Petluru	XAC-4	Yellow	Smooth, flattened with regular margins	-ve
5.	Peruru	XAC-5	Creamy white	Mucoid, convex with regular margins	-ve
6.	Tumaya	XAC-6	Creamy white with pale yellow tinge	Mucoid, flattened with regular margins	-ve
7.	MPPS Nayanipalle	XAC-7	Yellow	Smooth, slightly raised with regular margins	-ve
8.	Gudur	XAC-8	Creamy white	Mucoid, convex with regular margins	-ve
9.	Kambalapadu	XAC-9	Yellow	Mucoid, convex with regular margins	-ve
10.	Chinnarikatla	XAC-10	Yellow	Mucoid, convex with regular margins	-ve
11.	Pedarikatla	XAC-11	Yellow	Mucoid, slightly raised with regular margins	-ve
12.	Neredupalli	XAC-12	Creamy white with pale yellow tinge	Mucoid, slightly raised with regular margins	-ve
13.	Peddhairlapadu	XAC-13	Yellow orange	Mucoid, flattened with regular margins	-ve
14.	Pillolapalli	XAC-14	Creamy white	Mucoid, convex with regular margins	-ve
15.	Mallavaram	XAC-15	Yellow	Mucoid, convex with regular margins	-ve
16.	Vellampalli	XAC-16	Creamy white with pale yellow tinge	Mucoid, convex with regular margins	-ve
17.	Rachavaripalem	XAC-17	Yellow	Smooth, flattened with irregular margins	-ve
18.	Kesavabotlavari-palem	XAC-18	Yellow	Mucoid, convex with regular margins	-ve
19.	Jafalapuram	XAC-19	Yellow	Mucoid, convex with regular margins	-ve
20.	Kothasangatipalli	XAC-20	Yellow	Mucoid, convex with regular margins	-ve
21.	Pendlimarri	XAC-21	Creamy white	Mucoid, slightly raised with regular margins	-ve
22.	Kodur	XAC-22	Yellow	Mucoid, convex with regular margins	-ve
23.	Maravaripalli	XAC-23	Yellow orange	Mucoid, convex with regular margins	-ve
24.	Anantharajupeta	XAC-24	Creamy white	Mucoid, convex with regular margins	-ve
25.	Obulavaripalli	XAC-25	Creamy white	Mucoid, convex with regular margins	-ve
26.	Yerraguntakota (Y.Kota)	XAC-26	Creamy white with pale yellow tinge	Smooth, flattened with regular margins	-ve
27.	Chennarajupodu	XAC-27	Yellow	Mucoid, convex with regular margins	-ve
28.	Kasturivaripalli	XAC-28	Creamy white	Smooth, slightly raised with regular margins	-ve
29.	K. Kandulavaripalli	XAC-29	Yellow	Mucoid, convex with regular margins	-ve
30.	Proddatur	XAC-30	Creamy white with pale yellow tinge	Smooth, flattened with regular margins	-ve
31.	Tirupati	XAC-31	Yellow	Mucoid, convex with regular margins	-ve
32.	Venkataramanna-gudem	XAC-32	Yellow orange	Mucoid, convex with regular margins	-ve

3.1.2 Gram staining

Microscopic examination of Grams stained *X. axonopodis* pv. *citri* mount elucidated that the test bacterium did not retained violet colour of the primary stain i.e. crystal violet but bacterial cells appeared pink colour due to counter staining with the stain safari (Table 1 and Plate 2). Hence the test bacterium was gram negative, rod shaped, which is the characteristic feature of the plant pathogenic bacteria. All the 32 isolates were negative to Gram staining.

The present studies on biochemical characterization of *Xanthomonas axonopodis* pv. *citri* showed their negative reactions for Gram's staining by Suryawanshi *et al*. (2011) [19], Ali *et al*. (2017) [18], Shehzadi and Naz (2019) [14] and Iqbal *et al.* (2021) [17].



A

B

Plate 1: Pure cutures of 32 isolates of *Xanthomonas axonopodis* pv. *citri* from the potential acid lime growing areas of Andhra Pradesh (A) petri-plates and (B) test tubes

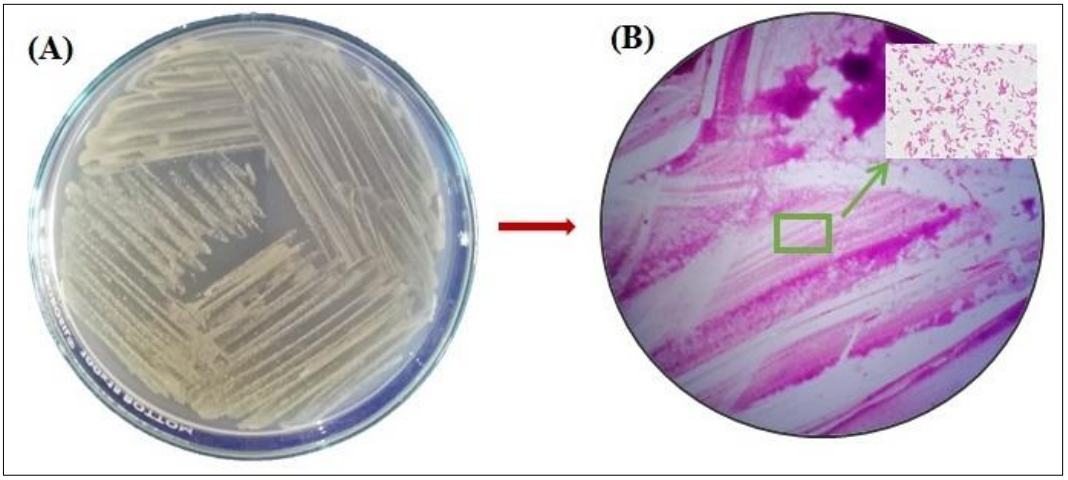


Plate 2: (A) Pure culture plate of *Xanthomonas axonopodis* pv. *citri* and (B) Gram staining proved that the isolated bacteria as Gram –ve bacteria

Conclusion

We came to the conclusion that *Xanthomonas axonopodis* pv. *citri* isolates differ greatly from one another. Designing effective management strategies may benefit from having an understanding of the morphological and biochemical characterization of the isolates. This information was not only important for understanding of pathogen distribution and diversity, but also could be used to identify novel pathotypes of the pathogen and to select the best suitable management practices against *Xanthomonas axonopodis* pv. *citri*.

Acknowledgements

This is a part of a Ph.D. thesis of the first author which has been completed under supervision of the second author. This work was supported by Dr. YSR Horticultural University for which the authors are grateful.

References

1. Das AK. Citrus canker-A review. Journal of Applied Horticulture. 2003;5(1):52-60.
2. Gerhardt P. Manual of methods of General Bacteriology. American Society of Microbiology Washington, D.C; c1981.
3. Gottwald TR, Graham JH, Schubert TS. Citrus canker: The pathogen and its impact. Plant Health Progress. 2002;3(1):15.
4. Hameed A, Atiq M, Rajput NA, Alsamadany H, Alzahrani Y, Sahi ST, *et al*. Role of mineral nutrients in confronting citrus canker caused by Xanthomonas axonopodis pv. citri. International Journal of Biosciences. 2020;17:113-9.
5. Islam MA, Mazumdar RM, Islam S, Alam MJ, Urmee SA. Isolation, Identification and *In Vitro* Antibiotic Sensitivity Pattern of Citrus Canker Causing Organism Xanthomonas axonopodis. Advancements in Life Sciences. 2014 Aug 25;1(4):215-22.
6. Isokar SS, Bramhankar SB, Das AK, Thakur KD, Pillai T, Dinkwar GT, *et al*. Morphological, biochemical and molecular characterization of *Xanthomonas citri* subsp. *citri* isolates from different agroclimatic zones of Maharashtra. International Journal of Chemical Studies. 2020;8(2):62-70.
7. Jadhav RR, More AS, Apet KT, Dandnaik BP. Cultural and bio-chemical characterization of *Xanthomonas axonopodis* pv. *citri*: causing citrus canker. International Journal of Current Microbiology and Applied Sciences.

2018;6:1682-87.
8. Luthra JC, Sattar A. Citrus canker and its control in Punjab. Punjab Fruit J. 1942;6(1):179-82.
9. Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, *et al*. Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular plant pathology. 2012 Aug;13(6):614-29.
10. McCartney HA, Foster SJ, Fraaije BA, Ward E. Molecular diagnostics for fungal plant pathogens. Pest Management Science: formerly Pesticide Science. 2003 Feb;59(2):129-42.
11. Mehmood B, Tariq-Khan M, Matee-ur-Rehman H, Younas MT. Surveillance of citrus canker in peshawar division and its management through host resistance. Pakistan Journal of Phytopathology. 2019 Jun 30;31(1):67-74.
12. NHB. Horticultural Statistics at a glance; c2020-2021.
13. Raghavendra BT, Singh D, Yadava DK, Mondal KK, Sharma P. Virulence analysis and genetic diversity of *Xanthomonas campestris* pv. campestris causing black rot of crucifers. Archives of Phytopathology and Plant Protection. 2013 Jan 1;46(2):227-42.
14. Shehzadi I, Naz S. Morphological, biochemical and genetic characterization of citrus canker pathogen (*Xanthomonas axonopodis*) from citrus cultivars of Punjab, Pakistan. The Journal of Animal and Plant Sciences. 2019;29(1):117-124.
15. Shivankar VJ, Ghosh DK. Tropical and subtropical citrus health management. Satish serial publishing house; c2015.
16. Rangaswami G, Soumini RC. Disease of citrus canker in Madras State. Indian Hort. 1957;5:50-7.
17. Iqbal W, Tang YM, Chau KY, Irfan M, Mohsin M. Nexus between air pollution and NCOV-2019 in China: application of negative binomial regression analysis. Process Safety and Environmental Protection. 2021 Jun 1;150:557-65.
18. Ali Q, Daud MK, Haider MZ, Ali S, Rizwan M, Aslam N, *et al*. Seed priming by sodium nitroprusside improves salt tolerance in wheat (*Triticum aestivum* L.) by enhancing physiological and biochemical parameters. Plant physiology and biochemistry. 2017 Oct 1;119:50-8.
19. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PB, Sehrawat S, Sharma S, Rouse BT. Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. The Journal of Immunology. 2011 Aug 15;187(4):1919-30.
20. Jansen JJ, Van Den Bosch FA, Volberda HW. Managing potential and realized absorptive capacity: how do organizational antecedents matter?. Academy of management journal. 2005 Dec 1;48(6):999-1015.
21. Al-Dulaimi KA, Banks J, Chandran V, Tomeo-Reyes I, Nguyen Thanh K. Classification of white blood cell types from microscope images: Techniques and challenges. Microscopy science: Last approaches on educational programs and applied research (Microscopy Book Series, 8). 2018:17-25.