The Bioscan 5 (3) : 373-376, 2010
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES

ISOLATION AND CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* FROM *CITRUS AURANTIFOLIA CHRISTM* (SWINGLE.)

B.SUJATHA* AND D.V.R. SAI GOPAL
Microbiology Division, Department of Virology,
Sri Venkateswara University, Tirupati - 517 502, A.P., INDIA
E-mail: sujatha72_bachu@yahoo.com

KEY WORDS
Canker
Xanthomonas
Proteins
Plasmid
Pathogenicity

Received on :
02.05.2010
Accepted on :
17.08.2010

***Corresponding author**

ABSTRACT

Citrus Canker was caused by *Xanthomonas axonopodis*. It causes serious damage to Citrus industry. The pathogen was isolated from the infected fruits of citrus orchards in the present study. It was characterized by preliminary studies and identified as gram negative rod shaped organism. Analysis of the protein profiles by SDS-PAGE revealed differences. A change was observed in total proteins, soluble and insoluble proteins. Total proteins showed difference between 116 KDa and 18.4 KDa with reference to the marker. Plasmid DNA analysis also showed differences. These differences in the protein and plasmid profile quote the pathogenicity of the bacteria towards the citrus crop.

INTRODUCTION

Citrus Canker is widespread in all the citrus growing areas of the world. The disease has originated from India, Java or some other parts of Asia. In India, Citrus canker was reported from Punjab by Luthra and Sattar in 1940. Citrus Industry is receiving serious losses because of Canker disease. This is caused by the bacterium *Xanthomonas axonopodis*. Species and pathovars of bacteria belonging to the genus *Xanthomonas* cause diseases to several plant species of major economic value. Sweet oranges or Sathgudi were very much affected by heavy incidence (88%) of Fruit Canker (C. I. P. Annual Report, 1982 and 1985). Citrus canker affects all above ground plant parts, with raised initial symptoms on leaves appearing as tiny, slightly raised blister like lesions with time foliar lesions turn grey, then tan/brown, a water soaked margin appears and the entire lesion is usually surrounded by a chlorotic margin. The centre of the lesion becomes characteristically raised and spongy or corky. The lesions are visible from both sides of the leaves. Wind driven rain is the main natural dispersal agent and the wind mainly aid in the penetration of the bacterium through the stomatal pores or wounds made by thorns. The bacteria ooze out form the lesions and start new growth when there is free moisture (Gottwald *et al.,* 2002). The genus *Xanthomonas* composed of strains that exhibit a high level of host specificity. The pathogen consists of two plasmids that are earlier reported pXAC33 and pXAC64 (Da Silver *et al.,* 2002).The plasmid profile diversity states that the virulent genes are responsible to cause full virulence in Citrus plants (Tondo *et al.,* 2010). The present paper deals with the isolation of the pathogen, its identification, biochemical characterization and molecular analysis.

MATERIALS AND METHODS

Isolation of pathogen

A few typical lesions from the cankerous fruits were excised using a sterile razor in the laminar flow bench. The excised lesions are surfaced sterilized using 70% alcohol followed by serial washings with distilled water. Later the lesions were tweezed by using a sterile forceps or needle using aseptic conditions and left aside for 10min, for release of the bacteria. A loopful of tweezed bacteria was taken and streaked on the different selective media like *Xanthomonas* growth media (XGM) (Galactose-20g, Yeast extract-10g, Calcium carbonate-20g, Agar Agar-20g, Distilled water-1000mL),Glucose Yeast extract Peptone(GYP media: Peptone-0.5g, Yeast extract-0.5g, Agar agar-1.5g, Glucose-1g, Distilled water-100mL).

Identification of the pathogen

The bacteria were identified by the standard Gram's staining technique.

Identification of the pathogen by biochemical tests

The pathogen was identified biochemically by performing standard procedures explained in the laboratory manuals by Aneja (2003), Gunasekaran (2002). IMVIC tests, fermentation of Carbohydrates, Catalase test, Hydrolysis of Gelatin, Casein Hydrolysis were used to identify the pathogen.

Isolation of bacterial plasmid

The bacterial plasmid was isolated by following Sambrook *et*

al. (1989) from the fruit isolate.

Extraction of plasmid

A single bacterial colony was inoculated into 100mL of nutrient broth and kept in rotary shaker for vigorous shaking and continued for about 16-18hr. 1.5mL of bacterial culture was transferred to eppendorf tube and centrifuged at 6000rpm fir 5 min at 4°C. Supernatant was discarded and pellet was collected. To this 300μL of P_1 buffer (Tris 50mM-6.055g, EDTA10mM-3.722 g, distilled water 1000mL adjust the pH to 8.0) was added and vortexed. Again 300 μL of P_2 (NaOH-8.09g dissolved in 950mL 20%SDS-50mL mix thoroughly and stored at room temperature) was added followed by 300 μL of P_3 buffer (Potassium acetate-147.2g dissolved in 400mL and adjusted the pH to 5.5 with GAA and made up to 500mL). They were kept at -20°C for 5 min and centrifuged at 14,000rpm for 2min. Supernatant was taken and to this 600 μL of isopropanol was added. Later the tubes were spinned at 14,000 rpm for 30 min at 25°C. Finally the pellet was washed twice with 70% ethanol, dried and dissolved in TE buffer. The plasmid DNA was analyzed by Agarose gel electrophoresis (0.7%). The gel was stained with ethidium bromide and photographed. ECORI and HINDIII double digested DNA marker was used.

Isolation of protein from the bacteria

The total bacterial protein, soluble, and insoluble were isolated by using Sodium dodecyl polyacrylamide gel electrophoresis (resolving gel-12%, stacking gel-4%; Laemmli, 1970; Sambrook *et al.,* 1989).

Isolation of total, soluble and insoluble bacterial protein

1.5mL of bacterial culture was taken into eppendorf tube and centrifuged at 6000rpm for 2 min. Supernatant was discarded and to the pellet 120 μL of sample buffer (0.5M Tris Hcl-1mL, Glycerol-0.8mL, 10%SDS-1.6mL, 2-Mercaptoehtanol-0.4mL 0.05%Bromo phenol blue-0.2mL, and Distilled water-4 mL) was added. Later the tubes were heated at 95°C for 5min and cooled at 4°C for 5min. After cooling the tubes were centrifuged at 10,000rpm for 5min and supernatant was taken as sample for total proteins. For preparation of samples for soluble and insoluble Proteins, large quantity (25mL) of culture was taken and pelleted down (6000rpm for 5min). The pellet was subjected to sonication (5cycles with a break for 2-3 sec), suspended in Tris buffer pH8.8 (Tris base-18.5g/100mL), sample buffer was added, heated and cooled. It was taken as insoluble protein sample for running the electrophoresis process. The supernatant was suspended in Tris buffer pH 8.8, sample buffer was added, heated and cooled. It was taken as soluble protein sample for running the electrophoresis process.

RESULTS AND DISUSSION

The following are the results indicating the Morphological (Table 1) and biochemical characterization (Table 2) of *Xanthomonas axonopodis*. On Luria and Nutrient agar media, after 48hr of incubation small, round mucous yellow to orange colonies were observed (Fig. 1). On *Xanthomonas* selective media and Glucose yeast extract peptone media, yellow colored appear against white background of the media. The

Table 1: Morphological identification

Test	Organism
Configuration	Smalland round
Margins	Smooth
Surface	Mucous
Pigmentation	Yellow
Opacity	Opaque
Gram's reaction	-ve rods with slight bulged ends
Arrangement	Singles
Optimal temperature	28°C-37°C
pH	Neutral

Table 2: Biochemical characterization

Indole test	-ve
Methyl Red Test	+ve
Voges-Proskauer test	+ve
Citrate utilization test	+ve
Gelatin utilization test	+ve
Catalase test	+ve
Sugar fermentation	
Glucose	+ve for acid and –ve for gas production
Galactose	+ve for acid and –ve for gas production
Casein hydrolysis	-ve

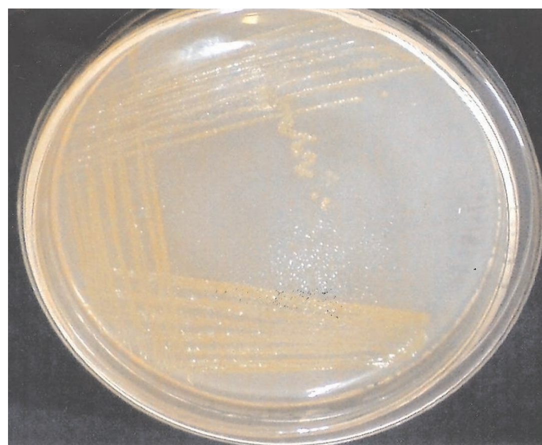

Figure 1: *Xanthomonas axonopodis* on nutriet Agar medium

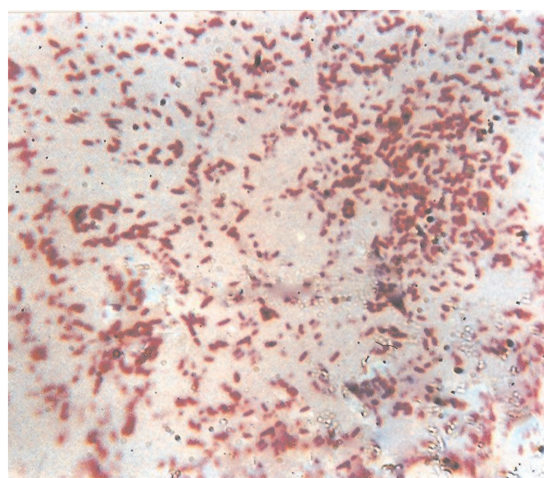

Figure 2: Gram staining picture of *Xanthomonas axonopodis*

bacterial cells retained pink color after staining, so it was identified as gram negative bacteria (Fig. 2).

The Biochemical tests like IMVIC, Catalase test, Fermentation of Carbohydrates, Gelatinase and Casein hydrolysis, were performed by using both negative and positive controls.

Plasmid isolation

The gel was observed under the UV-transillumination. Two forms of plasmids were observed with reference to the marker from the fruit isolate (Fig. 3).The Molecular weight of the first and second form were 45,000bp and 25,000bp (approx) respectively.

Protein

The bacterial pellet was taken as total protein sample and resolved using SDS-PAGE. It showed a series of several protein bands ranging between 116 to 18.4 KDa of the Marker (Fig. 4 and 5). Difference was observed, nearing from second to fourth band of the marker. Both soluble and insoluble protein profiles were checked, they also showed difference in their pattern (Fig. 6).

Hasse (1915) was the first person to identify and classify the causative organism of canker. The pathogen *Xanthomonas* was isolated from the infected lesions of the fruit collected form the Citrus orchards. The bacteria was isolated and grown on Luria or Nutrient Agar medium. After that its require a selective medium *Xanthomonas* Galactose medium.

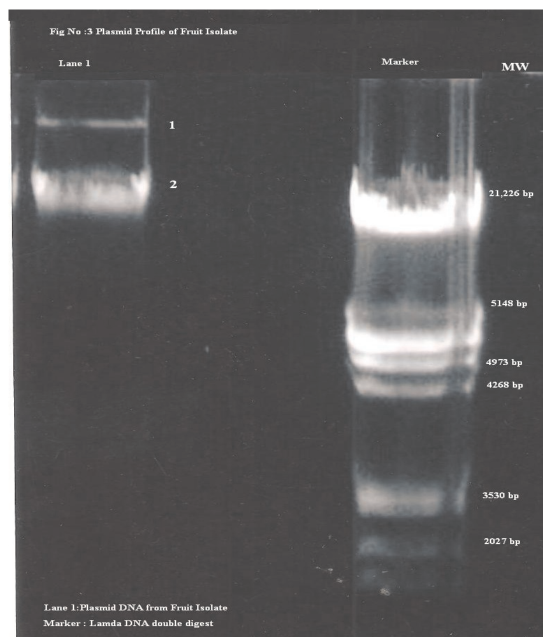

Figure 3: Plasmid profile of fruit isolate

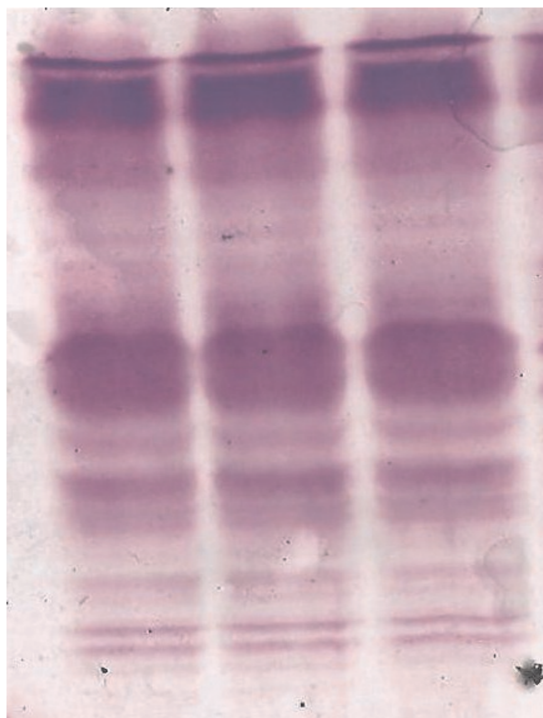

Figure 4: Total protein profile of fruit isolate

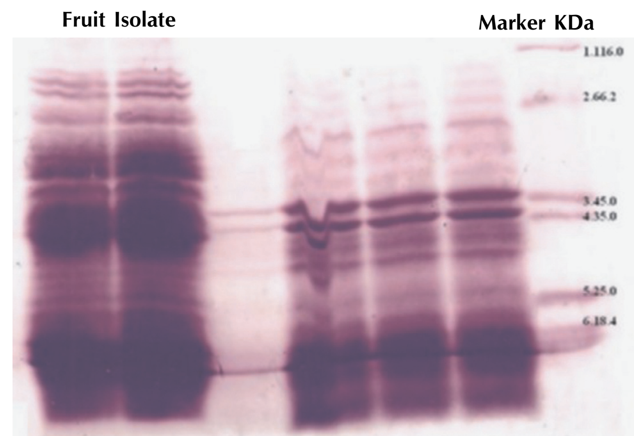

Figure 5: Total protein profile of fruit isolate with marker

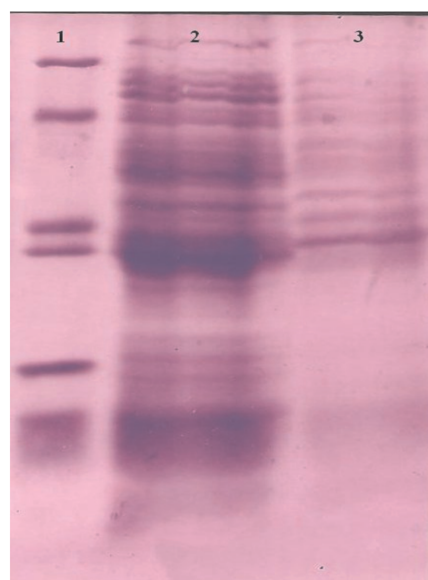

Figure 6: SDS Page of soluble and insoluble proteins;1 = Marker; 2 = Soluble proteins; 3 = Insoluble proteins

Previous studies of plasmid DNA profile analysis showed that bacterial strains were different from each other in terms of plasmid number and molecular weight (Mohammadin *et al.,* 2001). Several virulence and avirulence factors are present with plasmid. The presence of plasmid containing (Pathogen A gene) indicates their importance in causing virulence. Primers can be used for Citrus Canker diagnosis which is based on plasmid profile (Cubero and Graham, 2001).

The characterization of plasmids and the proteins helps to reveal the pathogenicity of unknown strains. The total, soluble and insoluble protein profile aid to identify the role played by the organisms in playing pathogenic role in the host plant. Plants like bacterial natriuretic peptides (PNPs) are a class of extracellular systemically mobile molecules that can enable a plant pathogen to modify host responses to create conditions favourable to its own survival (Natalia *et al.,* 2008).

Further sequencing methods and PCR based primer diagnosis are to be employed to reveal the complete role of the plasmids in confining the bacteria as potent pathogen at the gene level.

REFERENCES

Aneja K. R. 2003. Experiments in Microbiology, Plant pathology and Biotechnology, fourth Edition. pp. 102-106,190,246-275, New Age International (P) Limited, Publishers.

Citrus Improvement Project (APAU), Annual Report for 1982. Tirupathi, Andhra Pradesh, India. p. 56.

Citrus Improvement Project (APAU), Annual Report for 1985. Tirupathi, Andhra Pradesh, India. p. 49.

Cubero, J., Graham, J. H. and Gottwald, T.R. 2001. Quantitative PCR method for diagnosis of citrus bacterial canker. *Applied and Environmental Microbiology.* ***67*****:** 2849-2852.

Da Silver, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S. and Farlan, L. R. 2002. Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. *Nature.* **417:** 459-463.

Gottwald, T. R., Graham, J. H. and Schubert, T. S. 2002. Citrus canker: the pathogen and its impact. Plant Health progress doi: 10. 1094/PHP-2002-0812-01-RY. http://WWW.plant management network.org/php/

Gunasekaran, P. 2002. Laboratory Manual in Microbiology. pp. 53-64, New Age International (P) Limited.

Hasse, C.1915. The cause of Citrus Canker. *J. Agricultural Research.* **4:** 97.

Laemmli, U. K. 1970. Cleavage of Structural proteins during the assembly of the bacteriophage T4. *Nature.* **297:** 680-685.

Luthra, J. C. and Sattar, A. 1940. A note on Citrus canker (Phytomonas citri) in Punjab. *Proc. 27th Indian Sci. Congr.* **111:** 228-229.

Mohammadin, M., Mirazee, M. R. and Rahimian, H. 2001. Physiological and Biochemical Characteristics of Iranian strains of Xanthomonas Pv. Citri, the causal agent of citrus canker disease. *J. Phytopathology.* **149(2):** 65-75.

Natalia, G., Betiana, S., Garavaglia, Lucas D., Daurelio, Alex Valentine, Chris Gehring, Elena G. Orellano and Jorgelina Ottado October 10. 2008.

Sambrook, J., Fristsch, E. F. and Maniatis, T. 1989. Plasmid vectors, In: Molecular cloaning, a Laboratory manual, second edition. pp. 1.1 to 1.110.

Tondo, M. L., Petrocelli, S., Ottado, J., Orellano, E.G. 2010. The Monofunctional Catalase KatE of *Xanthomonas Axonopodis Pv. Citri* Is Required for Full Virulence in Citrus Plants. PLos ONE. **5(5):** e: 10803 doi:1371/journal.pone.0010803.