International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Int. J. of Life Sciences, 2021; 9 (2):175-180
ISSN:2320-7817(p) | 2320-964X(o)

Research Article Open Access

Isolation and study of bacteriophage of citrus canker causing bacterial pathogen

Gokule Kajal[1], Ahiwale Sangita[2] and Walhe Rajan[1]*

[1]MES' AbasahebGarware College, Pune. India
[2] Rayat Shikshan Sanstha's Mahatma Phule Mahavidyalay, Pimpri, Pune. India
*Corresponding author: E-mail: rawalhe@rediffmail.com

Manuscript details:
Received: 07.05.2020
Accepted: 22.05.2021
Published: 31.05.2021

Cite this article as:
Gokule Kajal, Ahiwale Sangita and Walhe Rajan (2021) Isolation and study of bacteriophage of citrus canker causing bacterial pathogen, *Int. J. of Life Sciences*, 9 (2): 175-180.

Available online on http://www.ijlsci.in
ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

ABSTRACT

India is at second position in the world in agricultural yield of fruits and vegetables. Citrus fruit is one of the most widely produced fruit crop globally. The five groups of cultivated citrus include sweet orange, mandarins, grapefruits, pommel and the oft-grouped lemons and limes. Limes are of vast economic value and find its place in Asian cuisines. Many species of citrus plants are susceptible to the infections and disease caused by bacteria, fungi, viruses, etc. Citrus canker is a frightening bacterial disease of all citrus crops, caused by *Xanthomonasaxonopodis*. Plant diseases could be controlled by variety of ways including chemical, biological, resistance breeding, etc. Phages were discovered against plant pathogens almost a century ago but only recently been assessed for their use as biological control agents. Bacteriophages of citrus canker bacteria were first isolated and evaluated for its host specific properties in India. In the present work, two citrus canker causing bacterial cultures were isolated from citrus canker infected lime leaves. From water samples, bacteriophages were isolated which lysed the citrus canker causing bacteria tested with double agar layer plaque assay technique.

Keywords: Plant pathogen, Xanthomonas sp., Bacteriophage, Citrus cancer.

INTRODUCTION

Agriculture yield in Indian field is the main source of food stuff of fruits and vegetables in that India is the second country after China in the world in its production (Neeraj et.al.2017). Citrus fruit is the most widely produced fruit crop globally. The Citrus species are diploids and domesticated in Southeast Asia before thousands of years and then spread all over the world (Ollitrault and Navarro, 2012). Citrus is world's leading fruit crop with annual growth of approximate 60 million megatons. These include five groups of cultivated citrus: sweet oranges, mandarins, grapefruits, pommel and the oft-grouped lemons and limes (Donkersley *et al.* 2018). Limes are of great economic significance and way to Asian

cuisines and are used for their juice, though zest and leaves are also used in cooking in South America. Limes are cultivated almost all tropical and subtropical regions of the world. Though on recommendations of World Health Organization, its demand is growing, pests and pathogens have abridged its productivity all over the world. Citrus is well identified for its abundance of vitamin C as well as many micro and macronutrients.

Many species and varieties of citrus plants are vulnerable to range of diseases caused by fungi, bacteria, viruses, and phytoplasma (Chaity *et al.* 2019). Several kinds of citrus canker diseases are caused by different pathovars and variations of the bacterium Xanthomonas*axonopodis*. Citrus canker is one of the serious diseases of citrus all over the world. The lesions appeared on leaves, fruits, stems of citrus plant that influences the commercial value of citrus in the market.

Citrus canker is one of the terrifying diseases of citrus that has influenced all types of important citrus crops (Das, 2003). It causes widespread harm to citrus and its severity of the infection ranges with the species, the varieties it is affecting and also the existing climatic conditions. The disease is endemic in India, Japan and rest of the South- East Asian countries and from here it reached to almost all citrus producing continents.

Plant diseases could be managed by variety of ways including chemical control, resistance breeding, integration of cultural practices, etc. As a chemical preventive measure, copper-based bactericides are used universally for citrus canker caused by *Xanthomonasaxonopodis*pv. *Citri* (Xac) (Graham *et al.* 2006). Moreover emergence of antibiotics and copper resistant bacterial strains in the field delayed the efficacy of chemical control measures (Tewfike and Desoky, 2015). Also, many alternative preventive measures were tried recently, including, biological control of disease with nonpathogenic Pseudomonas strains and use of bacterial viruses to control bacterial diseases. Bacteriophages of citrus canker bacteria were first isolated and evaluated for its host specific properties in India by Uppal (Naseem *et al.* 2017). Mastumoto and Okabe described the phage isolated from diseased leaves and soil beneath diseased citrus plant in Formosa (Wakimoto, 1967).

In the present study, bacterial agent of citrus canker was isolated from leaves, characterized for biochemical and other properties; specific phage of isolate was isolated and studied.

MATERIALS AND METHODS

Isolation of bacterial pathogen:
Collection and processing of plant disease causing bacterial sample was done as per Jadhav et.al. 2018, with slight changes. A diseased lime plant sample was collected from the farm located in a Chinchoshigaon in RajgurunagarTahsil, Maharashtra, India. The collected leaves were washed with distilled water and infected part of leaves were cut aseptically into small pieces. The cut part of leaf were surface sterilized with 0.1% Mercuric Chloride for 30-40 sec. To remove traces of HgCl2, the leaf part was washed with sterilize distilled water. Small piece of infected leaf was kept on a clean slide and 1-2 drop of sterile distilled water were placed on it and teased well with sterile scalpel. Slide was observed under microscope for the presence of ooze. Samples giving cloudy discharge were treated as positive sample of the bacterial ooze. Water drop from positive slide was taken with a inoculation loop and streaked on sterile nutrient agar plate. Inoculated plates were kept for incubation at 37°C for 24-48 hrs. After incubation cultural characteristics of bacterial isolates were studied and noted. The isolates were morphotyped.

Biochemical characteristics of bacterial isolate:
Different tests such as Catalase, Oxidase, KOH test, sugar utilization and tests, etc. were performed with the fresh growth of isolates (Ali et a, 2017; Cruickshank et.al.1975., Chaity *et al.* 2019; Jadhav *et al.* 2018).

Bacterial growth curve:
Freshloopfulgrowth from the isolated culture plates was streaked on the nutrient agar plate and Incubated at 37°C for about 24 hours. Single colony was picked up and inoculated it into a sterile 50 ml of nutrient broth and incubated overnight at 37°C. From this flask, 2.5 ml was broth culture was inoculated to 250 ml broth; OD at zero hour was noted and incubated. At the intervals of 30 min. 1 ml aliquot was removed and OD recorded at 600 nm till static readings.

Antibiotic susceptibility test:
Susceptibility of isolates to different antibacterial agents was determined in-vitro by employing a

modified disk diffusion test of the Kirby-Bauer method as per Islam et al., 2014 with some modifications. Isolate was inoculated in sterile nutrient broth and incubated to obtain fresh growth for the test.To maintain the bacterial density similar during testing, OD 600 was measured by colorimeter and 1.0 ml of culture growth from fresh broth culture was spread on the surface of Mueller-Hinton agar (HiMedia). Then the antibiotic poly-discs (HiMedia) were applied on the inoculated plates with the help of sterile needle and incubated at 37°C for 24-48 hours in an inverted position. The zone of inhibition was measured and results were recorded as sensitive, intermediate or resistant to the individual antibiotic as per zone of inhibition chart given by the manufacturer.

Isolation of bacteriophage and phage lysate:
The isolation of bacteriophage from water sample was performed by double agar layer plaque assay technique described by Ahmad *et al.* 2014 a; Harshitha *et al.* 2018, 1994; Ranjani *et al.* 2018; Weiss *et al.* 1994 with some modifications. Water samples were collected from various sites.,vizKhed, Ambegaon canal and Mula River Ahmadnagar (Maharashtra, India). Water sample was filter through sterile membrane filter assembly (filter having pore size 0.20 um). Fresh growth of host bacterial culture (isolated form diseased leaves) suspension grown in phage broth (0.5 ml) and 0.2 ml filter water (source of phages) was added in 3ml sterile soft nutrient agar (nutrient broth with 0.6% agar), mixed and poured on sterile nutrient agar plates. These plates were incubated at 37°C and observed for plaque formation after 24hrs. For preparation of phage filtrate (phase lysates), single plaque was picked up with sterile corkborer and suspended it in 200 ml sterile phage broth. Log phase hostculture (0.1 ml) was inoculated in the phage broth. Flask was incubated at 37°C for 24hrs. After incubation, the content in the flask was centrifuge at 10,000 rpm for 20 minutes; supernatant was collected and passed it through membrane filter.

Plaque characteristics:
The nature of plaque was studied on nutrient agar. An aliquot (1 ml) of respective phage lysate was mixed with 1 ml of the mid-log phase culture of respective host. An aliquot of 0.1 ml of each mixture was spread onto the surface of the respective medium. Plates were incubated at 37 °C and observed for its morphology after 24 h for the development ofplaques (Gasic *et al.* 2011).

Determination of phage titer:
Phage titer determination was doneas per Bhoyar *et al.* 2017 with some changes.In short it was done by setting up 6 dilution tubes numbered -1 to -6 and a controltube. To each eachtube 9ml sterile water was added. One ml phage lysate was added in first tube, then diluted itserially. Then, 1 ml mid-log host culture and 1 ml diluted lysate from each tube (one tube at a time) was added in soft agar,mixed well and double agar overlay plaque assay was performed as earlier. For a positive control, 1.0 ml bacterialsuspension was added in soft agar and poured it on sterile nutrient agar. Poured plates were incubated at 37°C for 24hrs. Plaque count was noted and phage titer was estimated.

One step growth experiment (curve):
One-step growth experiment was performed as described by as described by Ahmad *et al.* 2014 and Gasic *et al.* 2011 with some modifications.The mid-log phase host culture (9.0 ml) was mixed with phage lysate (1.0 ml). Phages were allowed to adsorb for 10 min at about 37 °C. The mixture was then centrifuged (10,000× g, 20 min), the pellet formed was resuspendedin 10 ml fresh phage broth medium. Aliquots of the suspension (0.1ml each) were withdrawn at 5 min intervals over a period of about 1 h and titer determined by double agar overlay plaque assay described above. The plaque forming units (PFU) in each tube were determined and noted.

RESULTS AND DISCUSSION

We attempted to isolate causative agent for citrus canker from infected leaves from canker affected plantthat werefound to be aerobic organisms. Two bacterial isolate obtained (isolate A and isolate B) were selected on the basis of distinct cultural and morphologicalcharacteristics. Both the isolates were found to be Gram negative rods (results not mentioned here) with typical cultural features of the genus Xantho-monas.The typical yellow colour colonies were seen on the medium after incubation, the yellow colour may be due to the production of Xanthin produced by the isolate; the characteristic of the genus Xnthomonas (Haider et al, 2020). The colonies were medium (1-2 mm), convex, opaque and yellowish in colour.

Biochemical characteristics of both isolates were studied in order to check similarity of biochemical

features with genus *Xanthomonas* by subjecting tovarious biochemical tests as shown in Table: 1.

One bacterial isolate i.e. isolate A was selected for growth curve experiment to find out the mid-log phase. The Isolate requires approximately 2.5 h to complete its lag phase; the mid-log phase of isolate was achieved after 6 h. Soares et al, 2010 also have obtained similar time log phase with *Xanthomonascitri* subsp. *Citri* in nutrient broth; we got little earlier mid log phase.

The isolate was found to be sensitive to the antibiotics tested except Cefadroxi, Ciprofloxime and Cefotaxime which indicating the organism carries less hazard of horizontal gene transfers (Table 2).

Table 1: Biochemical tests

Sr. No.	Test	Results	
		Isolate A	**Isolate B**
1	Catalase test	Positive	Positive
2	Oxidase test	Negative	Negative
3	KOH test	Positive	Positive
4	H_2S production test	Negative	Negative
5	Sugar utilization Manos Galactose Fructose	 Positive Positive Positive	 Positive Positive Positive

Table 2: Antibiotic susceptibility test:

Antibiotics tested	Result
Amikacin	Sensitive
Netilmicin	Sensitive
Cefadroxil	Resistant
Sparfloxacin	Sensitive
Ceftriaxone	Sensitive
Ciprofloxacin	Resistant
Gentamicin	Sensitive
Cefotaxime	Resistant
Cefoperazone	Sensitive
Lomefloxacin	Sensitive
Ceftazidime	Resistant

Figure 1: Plaques from sewage water sample

Table 3: Plaque characteristics.

Sample site	Size of Plaque in mm	Shape of Plaque	Appearance of Plaque
Mula river(Ahmadnagar)	2	Circular	Clear
Canal(Khed-Ambegaon)	Plaques were not isolated		

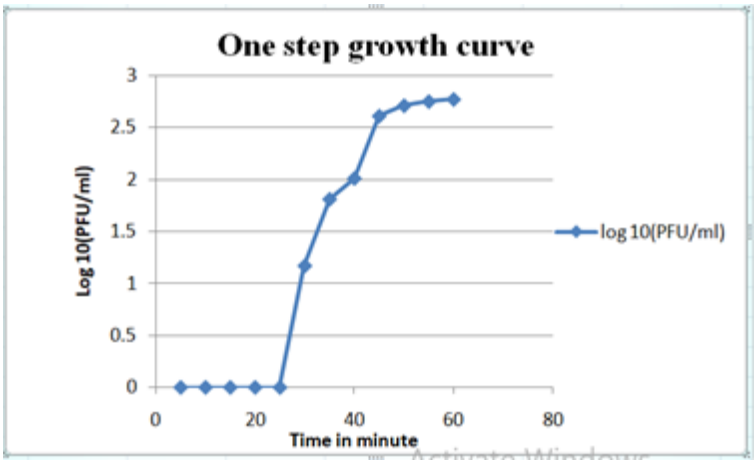

Figure 2: One step growth of the phage isolated

Copper-based bactericides used to control citrus canker after long-term are reported to pose problem of resistance to copper in Citrus causing bacterial pathogens (Graham et al, 2006). Balogh et al, 2008 have showed around 60% reduction in disease severity of citrus canker by phage treatment in a set of five greenhouse experiments, thus claimed reliable control. Bio-control alternatives were tried by many researchers. With the same context, phages specific to bacterial isolate-A were isolated from Ahmadnagar water sample by double agar plaque assay technique (Figure 1). Two lyticphages were isolated; one from those was selected for further studies here. The phagelysate was prepared using the host. Plaque characteristics were studied and results noted as shown in table 3. Plaques were differentiated on the basis of their morphology (size , clarity, turbidity, etc) (Chae et al, 2014; Gasic et al, 2011).

The plaques were counted on different dilution plates and recorded. From those the plaque forming unit was calculated as, phage titer (PFU/ml) = number of plaques/d × v ;Whereas; v = volume of diluted sample used for plating d = dilution factor. The phage titer was found to be 2.52 $\times 10^8$pfu/ml.

One step growth curve was performed to understand the adsorption pattern of the selected phage of the bacterial isolate.(Figure 2). Eclipsed period may be determined in conjunction with the latent period, since bacteriophage adsorbed to its host rapidly. Latent eclipse, and rise period can determined from the changes in the free and total bacteriophage. The latent period of around 20 minutes was found that goes similar with that of reports of Gasic et al, 2011 who have reported to be of 20 minutes with phages of *Xanthomonaseuvesicatoria*KFB 189.

CONCLUSIONS

The phage therapy is the new way to control disease. Instead of chemicals,it is important to use a biological treatment to control or treat disease. The presented work provides the information on potential of phages as a biocontrol agent to reduce the bacterial canker disease of citrus plants with comparatively safe and an easy-to-use tool. Phage isolation and its use as a biological treatment against citrus canker canbe done for the prevention of the economic loss of farmers in India. This approach received increasing attention as alternative treatment of chemotherapeutic approach. Litrature survey indicated that no much work is done about phage as a biocontrol agent against bacteria causing citrus canker in our country; so the climate,atmosphere, environmental factors are different from different geographical locations. Hence with further detailed characterization of phages based on its growth kinetics and host range, electron microscopy studies, biocontrol potential of phages could be considered to combat the citrus canker disease in India.

Acknowledgment: Authors express sincere thanks to Principal of MES' Abasaheb a College Dr. P.B. Buchade, for the facilities for the work.

Conflicts of interest: The authors declare no conflict of interest.

REFERENCES

Ahmad AA, Askora A, Kawasaki T, Fujie M, and Yamada T(2014) The filamentous phage XacF1 causes loss of virulence in *Xanthomonasaxonopodis*pv. *citri*, the causative agent of citrus canker disease. Frontiers in Microbiology, 5 (321): 1-11.

Ahmad AA, Ogawa M, Kawasaki T, Fujie M, and Yamada T (2014) Characterization of Bacteriophages Cp1 and Cp2, the Strain-Typing. Applied and Environmental Microbiology, 80 (1): 77-85.

Ali MR, Hasan MF, Lia SR, Akter A, Sumi MSE, Hossain F, Hasan SM, Islam MA, Khalekuzzaman M and Sidkar B (2017) Isolation and characterization of a canker disease causing pathogen from *Citrus aurantifolia*and evaluation of its biological control measure. Journal of Entomology and Zoology Studies, 5(6): 1526-1532.

Balogh B, Canteros B, Stall R and Jones J (2008) Control of Citrus Canker and Citrus Bacterial Spot with Bacteriophages. Plant Disease, 92 (7): 1048-52.

Bhoyar MS, Singh UB, Sahu U, Nagrale D and Sahu P (2017) Characterization of lytic bacteriophage xcc9sh3 infecting *Xanthomonascampestris*pv. *campestris*. Journal of Plant Pathology, 99 (1): 233-238.

Chae J, Hung N, Yu SM, Lee HK, and Lee YH (2014) Diversity of Bacteriophages Infecting Xanthomonasoryzaepv. Oryzae in Paddy Fields and Its Potential to Control Bacterial Leaf Blight of Rice. J. Microbiol. Biotechnol., 24(6): 740–747.

Chaity AS, ChowdhuryKhan EM, HasanF, Islam MA, Haque MF, and Sikdar B (2019) Isolation, characterization and control of canker disease causing bacteria of selected citrus plants in Rajshahi, Bangladesh. European Journal of Biotechnology and Bioscience, 7 (3): 9-17.

Cruikshank R, Duguid J, Marmion B, Swain R (1975) Medical microbiology, Churchill Livingstone Edinburgh London and New York Longman group Limited. 5(2) 87-88, 180-18.

Das AK (2003) Citrus canker- A review. Journal of Applied Horticulture, 5 (1): 52-60.

Donkersley P, Silva F, Carvalho C, Al-Sadi AM, and Elliot S (2018) Biological, environmental and socioeconomic threats to citrus limeproduction. Journal of Plant Diseases and Protection, 125:339–356

Gasic K, Ivanovic M, Ignjatov M, Calic A, and Obradovic A (2011) Isolation and characterization of *Xanthomonaseuvesicatoria*bacteriophages. Journal of Plant Pathology ,93(2): 415-423.

Graham J, Gottwald T, and Leite RP Jr. (2006) Prospects for control of Citrus canker with novel chemical compounds. Proc. Fla. State Hort. Soc., 119:82-88.

Haider MF, Ahmad Z, Shakeel Q, Shafiq S, Chand SA, Saddiq WM, Ahsan MA, Kharal M (2020) Biochemical characterization of Xanthomonasaxonopodispv. citri; A major impediment to Citrus orchards. International Journal of Biosciences,V16 (3): 512-517.

Harshitha KN, Manoranjitham SK, Somasundaram E, Rajendran , and Karthikeyan G (2018) Characterization of *Xanthomonasaxonopodis*pv. punicae (Hingorani and Singh) Vauterin*et al*. and Isolation of Xap Specific Bacteriophage. Madras Agric. J., 105 (4-6): 210-214.

Islam MA, Mazumdar RM, Islam S, Alam MJ, and Urmee SA (2014) Isolation, identification and *in-vitro* antibiotic sensitivity pattern of citrus canker causing organism *Xanthomonasaxonopodis*. Advancemts in Life Sciences, 1 (4): 215-22.

Jadhav RR, Apet KT, and Dandnaik BP (2018) Cultural and Bio-Chemical Characterization of *Xanthomonasa-xonopodis*pv. *citri*: Causing Citrus Canker. Int.J.Curr.Microbiol.App.Sci.,6: 1682-1687.

NaseemSaadia Shah HA, and Ali Z (2017) First Report on Characterization of Citrus Disease Causing Bacteria and Related Phages Isolated in Pakistan. International Journal of Agriculture and Biology, 19 (4): 857-64.

Neeraj, Chittora A, Bisht V and Johar V (2017) Marketing and Production of Fruits and Vegetables in India. Int. J. Curr. Microbiol. App. Sci., 6 (9):2896-2907.

Ollitrault P and Navarro L (2012) ML Badenes and DH. Byrne (eds.), Fruit Breeding, Handbook of Plant Breeding 8, 623-62.

Ranjani P, Gowthami Y, Samuel, Gnanamanickam S, and Palani P (2018) Bacteriophages: A New Weapon for the Control of Bacterial Blight Disease in Rice Caused by *Xanthomonasoryzae*. Microbiol. Biotechnol. Letters, 46 (4): 346-59.

Soares MR, Facincani AP, Ferreira RM, Moreira LM, Oliveira J, Ferro J, Ferro M, Meneghini R and GozzoF(2010) Proteome of the phytopathogenXanthomonascitri subsp. citri: a global expression profile. Proteome Science, 8(55): 1-11.

Tewfike TA and Shimaa M D (2015) Biocontrol of *Xanthomonasaxonopodis*causing bacterial spot by application of formulated phage**.** Annals of Agric. Sci. Moshtohor, 53 (4): 615-624.

Wakimoto S (1967) Some characteristics of Citrus Canker Bacteria, *Xanthomonascitri*(Hasse) Dowson, and the Related Phages Isolated from Japan. Ann. Phytopath. Soc. Japan, 33: 301-310.

Weiss BD, Capage MA, Kessel M, and Benson SA (1994) Isolation and Characterization of a Generalized Transducing Phage for Xanthomonascampestrispv. Campestris. Journal of Bacteriology, 176 (11): 3354-3359.