Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 2418-2422
Received: 19-07-2017
Accepted: 21-08-2017

Md. Samiul Islam
Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Razia Sultana
Department of Genetic, Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Dr. Md. Khalekuzzaman
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Dr. Biswanath Sikdar
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Dr. Uzzal Kumar Acharjee
Associate Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Md. Faruk Hasan
Assistant Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Dr. Md. Asadul Islam
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Correspondence
Dr. Md. Asadul Islam
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Isolation and characterization of bacterial spot disease of citrus through biochemical approaches and its control measures

Md. Samiul Islam, Razia Sultana, Dr. Md. Khalekuzzaman, Dr. Biswanath Sikdar, Dr. Uzzal Kumar Acharjee, Md. Faruk Hasan and Dr. Md. Asadul Islam

Abstract
Citrus bacterial spot disease is caused by *Xanthomonas campestris* pv. *citrumelo*, which produced unsightly blemishes on leaves and stems of nursery plants and reduce its market value. The present study was done to identify the pathogen from citrus infected leaves, and find out its biological control. Different biochemical tests including gram staining, motility, Simmons citrate, urease, catalase, potassium hydroxide, Triple sugar iron, Kligler iron agar, MacConkey agar, Kovacs oxidase tests showed positive response against the isolated bacteria. All biochemical test results ensured that, the isolated bacteria was gram negative. Chloramphenicol (30 mcg) revealed the highest antibiotic sensitivity with 32±0.5 mm diameter of zone of inhibition against the isolated bacteria. The raw extract of *Allium sativum* showed highest antibacterial activity with 16.0±0.5 mm diameter against the isolated bacteria. The study will be helpful to confirm the efficacy of these antibiotics and plant extracts to make biological control of the disease.

Keywords: Bacterial spot, *Xanthomonas*, Biochemical assays, Sensitivity test, Bio-control

Introduction
Citrus is one of the most edible fruits in worldwide, which belongs to the Rutaceae family. In world, citrus are the second most planting and production fruit plant [1]. It is very effectual secondary metabolites source for nutrition, health, drugs, and other industrial solicitation [2]. Additionally, it reflect various therapeutic qualities. Citrus fruit, like lemons and limes particularly have high concentration of citric acid. Basically, citrus fruit juice used in numerous periodic application for healing blow, scabies and other skin problems [3]. The cooked fruits are used against colds and as perspiration increaser. The citrus juice, which mixed up with water is used against dyspepsia alkaline and fresh juice used against catarrhal angina. The curtail fruit juice is applied to upper stomach vomiting and also applied in chronic obstacle of liver and spleen [4]. The low quality of citrus fruit is frighten to attack for many diseases, such as citrus canker [5], bacterial spot [6], black pit [7], citrus blast [8], citrus greening [9], Citrus variegated chlorosis [10] etc. Among these diseases, bacterial spot disease of citrus has its own discretion, which is occur under nursery condition. This disease is also referred to as bacteriosis or bacterial leaf spot and occurs on leaves, twigs, and fruits. Except Bacillus species, most of the plant pathogenic bacteria are gram-negative [11]. Gram staining test help to distinguish between gram positive and gram negative bacterium. Characterization is an important step to identify the pathogen whose are responsible for causing the disease. After that the control or management systems are easily conducted. Although the research has been conducted on its control measures of this disease, but the need of literature is available on the biochemical characterization of this bacterium. That's why the present investigation was carried out to isolate, characterize and evaluate their antibiotic and antimicrobial sensitivity assay of the bacteria responsible for bacterial spot disease of citrus.

Materials and Methods
Plant materials
Disease infected citrus plant leaves were collecting from Rajshahi University Campus, Rajshahi, Bangladesh and were identifying by a research officer of Bangladesh Fruits Research Institute, Binodpur, Rajshahi. Spot disease infected leaves of citrus plant were used as plant material for this present investigation.

Isolation and purification of causal organism
Spot disease infected leaves were disinfested using a dilute sodium hypochlorite solution (10%) and rinsed thoroughly. Cut the infected area and placed on LB liquid media for allow to growing bacteria and incubated for 16 hours at 37ºC. After the bacteria have grown into LB liquid medium, use a sterile loop to streak the bacteria onto a solid nutrient agar media plates and incubated for 16 hours at 37ºC. One of creamy white colony was pick up by loop and streaked on another media plate for pure culture.

Morphological Test
Gram staining: Gram staining reagents was prepared by taking crystal violet, gram's iodine, decolorizes (alcohol), and stain safranin. Bacterial sample was smears on a slide and carefully fixed by heat. One drop crystal violet was place on smears and holds it 1 minutes and rinse with distilled water. One drop of gram's iodine was place on smears for 1 minutes and rinse with distilled water. Added decolorize reagents on the sample. If it is gram negative, removing the crystal violet. Finally one drop of safranin was added for 30 seconds and rinse with distilled water and placed to air dry for several minutes. The slide was observed under 100X microscope along with one drop of immersion oil to examine shape, size, arrangement and staining reaction of bacterial isolates [12].

Motility test: Soft agar medium was prepare in a test tube for motility test. One isolated colony was pick from the culture and inoculates the medium by stabbing the center of the medium to a depth of 1 inch [13].

Biochemical test
Simmons citrate test: Citrate medium [14] was prepare in deionized water and sterilized at 121ºC for 20 minutes. After that the media was place on a tube and cooled in a slanted position. The isolated colony was pick with a needle and the slant surface was lightly streaked. The incubation was done at 37ºC for 16 hours.

Urease test: Urease medium was prepare to add deionized water. The pH was adjusted to 6.7 and autoclaved at 121ºC for 20 minutes except urea. After autoclaved, cooled the media to 50 to 55ºC, then urea base was add into the media and mixed thoroughly. The tubes were slanted during cooling until solidified. The isolates were inoculating into the slant and incubate at 37ºC for 16 hours [15].

Catalase test: Transferred a small amount of bacterial colony in the clean glass slide by the use of sterile loop. A drop of hydrogen peroxide was place on a glass slide [16].

Potassium hydroxide test (KOH): In KOH test, one or two isolated colonies from the pure culture were placed on a clean slide. After that added one drop of 0.3M KOH on the top of the colonies and waited for 1 minute and observed [17].

Triple sugar iron agar test (TSI): This test was performed to determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium [18], with or without the production of gas, along with the determination of possible hydrogen sulfide (H_2S) production. The TSI agar slant (long butt and short slant) containing three types of sugars (Dextrose, Lactose and Sucrose) was stab, streak with inoculums and incubate at 37^0C for 16 hours.

Kligler iron agar test (KIA): The medium was prepare one liter deionized water and sterilized at 121ºC for 20 minutes [19]. The overnight culture of each isolate were stabbing the butt and streaking the surface of the tube as well as incubated aerobically at 37ºC for 16 hours.

MacConkey agar test: The medium was prepare by petri plates. The pH was adjusted to 7.1 and sterilized at 121ºC for 20 minutes. The medium was pour into the petri dishes and cool to solidify at room temperature in the laminar airflow. The isolated colony were taking with sterile loop and streaking the petri plates. The incubation was done at 37ºC for 16 hours [14].

Kovac's oxidase test: One drop of 1% Kovacs' reagent (1gm Tetramethy-p-phenylenediamine Dihydrochloride in 100 ml distilled water) was placed on the middle of the Whatman filter paper and platinum loop full of bacterial strain was carefully rubbed on the filter paper [20].

Antibiotic Sensitivity Test
Different types of antibiotics like, Amoxicillin, Azithromycin, Carbenicillin, Cefotaxime, Clarithromycin, Chloramphenicol, Gentamycin, Kanamycin, Streptomycin and Tetracycline were used for antibiotic sensitivity test. For this purpose, Kirby-Bauer disk diffusion method has been used [21]. First LB agar medium was prepared for making culture plates. 1-2 ml of isolated bacterial colony were poured in each petri plates and left the airflow cabinet for solidification. Finally each different antibiotics were placing centrally on the respective plates and incubating overnight at 37ºC.

Antibacterial Activity Test against Plant Extracts
Four plants extracts like, *Allium sativum*, *Allium cepa*, *Gingiber officinale*, *Ocimum basilicum* were used for antibacterial activity test. Selected plants were collected, dried, cut in a small piece and pest in mortar pestle. The extracts were stored in a glass bottle. This test was screened by using the disc diffusion method. An inoculum suspension was swabbed uniformly to solidify of LB agar for bacteria and allowed to dry for 5 minutes. Disc of 6 mm in diameter were used. Aliquot of 10, 15, 20µl from each plant raw extract was added into each disc on the seeded medium and allowed to stand on the bench for 30 minutes for proper diffusion and incubated at 37ºC for 16 hours [22].

Statistical tools for analysis
Above study were conducted in repeated triplicate for accurate results and statistical analysis. All the data were revealed as mean and standard error using 2013 Microsoft Excel sheet version. The actual values with $P<0.5$ was calculated statistically significant.

Results
Isolation and Purification
The infected leaves samples placed on LB liquid media showed the bacterial colonies after 16 hours of incubation at 37ºC. The turbid condition in the media indicates the bacteria were grown. By streaking method, single colonies were found. The colonies were creamy white in color. The size and shape of colonies were found to be small, medium, convex and mucoid.

Morphological characterization of isolated bacteria

Gram Staining and motility test

In gram staining test, bacterial colonies slide were observed under the light microscope at 100X using oil immersion. The isolates showed to be gram negative, rod shaped and pinkish in color when stained with counter-stained by the safranin which was the indication for gram negative bacterium. After incubated the medium at 37ºC for 16 hours, a positive motility test was observed. The growth area was extending away from the line of inoculation which indicates the isolated bacteria were motile.

Biochemical test

In Simmons citrate test, the isolated bacteria showed positive result against Simmons citrate agar, because the medium turns the blue color, which indicates the isolated bacteria were able to utilizing citrate. The isolated bacteria were negative to urease test, because no pink color was formed in the media, which indicates the isolated bacteria were unable to hydrolyzing urea to ammonia. After added hydrogen peroxide on the clean slide, which contains a small amount of bacterial colonies, the oxygen bubbles were clearly indicates the isolated bacteria were catalase positive. In KOH test, the isolated bacteria was gram negative, because it showed viscous strings and formed thread like slime. Color changed of the TSI medium showed the positive result, that's mean slant yellow and butt yellow color indicates the isolated bacteria were glucose and lactose fermenting. But no H_2S was formed in the medium. In KIA test, yellow color in the medium confirmed the positive result, which indicates the isolated bacteria were glucose and lactose fermenting, but no gas was formed in the medium. Characterize the isolated bacteria as MacConkey agar positive, because the medium turns the pink color after overnight incubation at 37ºC, which indicates the isolated bacteria were able to ferment lactose. Finally in Kovac's oxidase test, the isolated bacteria was gram negative because that gave no purple color after 60 seconds. All characterization results have been presented in a Table 1.

Table 1: Summary of morphological and biochemical test of isolated bacteria

Test	Results	Optimization	Remarks
Gram staining	-ve	Small, rod shaped, Pink color colony	Isolated bacteria was gram negative
Motility	+ve	Growth area extend away from the inoculation line	Isolated bacteria was motile
Simmons citrate	+ve	Blue color	Isolated bacteria was capable to utilized citrate
Urease	-ve	No color	Isolated bacteria were not able to hydrolyzing urea to ammonia
Catalase	+ve	Oxygen bubbles	Isolated bacteria was able to produce catalase enzyme
KOH	+ve	Viscous and thread like slime	Isolated bacteria was gram negative,
TSI	+ve	Yellow color	Isolated bacteria were glucose and lactose fermenting, but no gas and H2S was formed
KIA	+ve	Yellow color	Isolated bacteria were glucose and lactose fermenting, but no gas was formed
MacConkey agar	+ve	Pink color	Isolated bacteria were lactose fermenting
Kovac's oxidase	-ve	Colorless	Isolated bacteria gave no purple color after 60 seconds.

Antibiotic Sensitivity Test

In antibiotic test, ten different types of antibiotic discs were used against isolated bacteria. Chloramphenicol revealed the highest significant antibiotic activity with inhibition zone of 32.0±0.5mm, while tetracycline showed the lowest 13.0±0.5mm inhibition zone against the isolated bacteria. Most of the antibiotic showed a moderate antibiotic spectrum against the isolated bacteria. The results of antibiotic sensitivity test have been presented in a Table 2.

Table 2: Effects of antibiotics against isolated bacteria

Name of Antibiotic	Symbol	Disc potency (µg)	Zone of inhibition(mm) (M±SE)	Sensitivity pattern
Amoxicillin	*AML*	10	11.0±0.5	Intermediate
Azithromycin	*AZM*	15	24.0±0.5	Susceptible
Carbenicillin	*CB*	100	19.0±0.5	Susceptible
Cefotaxime	*CTX*	30	29.0±0.5	Susceptible
Clarithromycin	*CLR*	15	25.0±0.5	Susceptible
Chloramphenicol	*C*	30	32.0±0.5	Susceptible
Gentamycin	*GEN*	10	30.0±0.5	Susceptible
Kanamycin	*K*	30	22.0±0.5	Susceptible
Streptomycin	*S*	10	26.0±0.5	Susceptible
Tetracycline	*TE*	30	13.0±0.5	Intermediate

Note: Resistant =<10 mm; Intermediate =<10-15 mm; Susceptible =>15 mm [21]

Antibacterial Activity Test against Plant Extracts

In antibacterial susceptibility test, raw extracts of four different plants were used for evaluate the control measures of this disease. Among these, the raw extracts of *Allium sativum* revealed the highest antibacterial activity with16.0±0.5mm diameter of zone of inhibition in20µl/disc concentration followed by 14.0±0.5mm diameter zone of inhibition for same extract in 15µl/disc concentration against the isolated bacteria. The raw extract of *Gingiber officinale* showed the lowest antibacterial activity with 5.0±0.5mm diameter of zone of inhibition against the isolated bacteria. Likewise, the other raw extract of plant showed moderate antibacterial activity. The test results have been presented in a Table 3.

Table 3: Effects of some plant extracts against isolated bacteria

Name of plant extract	Zone of inhibition in different doses (mm) (M±SE)			Sensitivity pattern
	10µl	15 µl	20µl	
Aliium sativum	11.0±0.5	14.0±0.5	16.0±0.5	Intermediate /susceptible
Allium cepa	7.0±0.5	8.0±0.5	9.0±0.5	Resistant
Gingiber officinale	5.0±0.5	7.5.0±0.5	8.0±0.5	Resistant
Ocimum basilicum	6.0±0.5	7.5.0±0.5	8.0±0.5	Resistant

Note: Resistant =<10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm

Discussion

In this study, bacterial spot disease affected leaves were collected from the citrus plant and the *Xanthomonas campestris* pv *citrumelo* was isolated and purified. Several biochemical tests such as Gram staining, Motility, Simmons citrate, Urease, Catalase, KOH, TSI, KIA, MacConkey agar, Kovac's oxidase test was done to characterize the isolated bacteria as gram negative bacteria. The isolated bacteria was gram negative, which shows pink color and rod shaped size in staining procedure [23].The motility test which was accurately confirmed the isolated bacteria was motile [13]. The isolated bacteria was able to utilized citrate and the media color change it to blue [14]. The isolated bacteria showed negative result against the urease test which confirmed the bacteria was not able to hydrolyze urea [15]. The isolated formed bubbles after added hydrogen peroxide on the top of the bacterial colonies, which was confirmed our bacteria was catalase positive [16]. In addition, Suslow *et al.* (1982) [17] performed KOH test to finally confirmed characterized gram negative bacteria of wheat, so our isolated bacteria was clearly indicates as gram negative. In TSI test, the gram negative bacteria observed positive results against these two test [14]. The slant and butt yellow color indicates the isolated bacteria was glucose and lactose fermenting. No H_2S was formed, because no black precipitation was found in the medium. The KIA test confirmed the isolated bacteria was glucose and lactose fermenting but no gas was formed in the medium. Taylor and Silliker, (1958) [19] performed the KIA test in *Salmonellae* food samples which was clearly characterized between gram positive and gram negative bacteria. The isolated bacteria turns the MacConkey agar media pink color after overnight incubation, which indicates the isolated bacteria was able to ferment lactose [14]. Kovac's reagent gives no purple color after 60 seconds, which indicates the isolated bacteria was gram negative bacteria [20]. In antibiotic test, Chloramphenicol showed highest 32.0±0.5mm diameter zone of inhibition, while tetracycline showed the lowest 13.0±0.5mm inhibition zone against isolated bacteria. Antibiotic sensitivity test was helpful to find out the control measures of this disease. The zone of inhibition on a plate clearly identified the sensitivity pattern of the isolated bacteria against the different types of antibiotics [21]. In antibacterial assay, *Allium sativum* extract revealed the highest 16.0±0.5mm diameter of zone of inhibition and *Gingiber officinale* extract showed the lowest 5.0±0.5mm diameter of zone of inhibition against isolated bacteria. Hindi and Chabuck, (2013) [22] performed antibacterial activity against some lemon extracts which evaluates the biological control of this disease. So, current investigation indicates that, the antibacterial activity depends on the species of the plants.

Acknowledgements

The authors wish to thanks Ministry of Education, People's Republic of Bangladesh, Project No. 58, 2015-2016 for providing all the financial support to carry the whole research work. Also thanks to Professor Joarder DNA and Chromosome Research Laboratory, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi for providing all the lab facilities and guidance.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Spiegel-Roy P, Goldschmidt EE. Biology of Citrus, Edn. Cambridge University Press. 1996; 136(1):369-374.
2. Khan MM, Khan MA, Inam-ulHaq M, Ahmad R, Aziz I. Incidence of citrus canker caused by X. campestris pv. citri orchard in Faisalabad District. Proceed. 1st Inter. sem. citriculture in Pakistan. Dec. 2-5. University of Agriculture Faisalabad, 1992, 311-314.
3. Ram L, Singh S. Medicinal importance of citrus products and by-products-a review. Agricultural Reviews. 2006; 27(3):170-180.
4. Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, *et al*. Flavonoids in food and their health benefits. Plant foods for human nutrition. 2004; 59(3):113-122.
5. Graham JH, Gottwald TR, Cubero J, Achor DS. *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. Molecular plant pathology. 2004; 5(1):1-15.
6. Jalan N, Aritua V, Kumar D, Yu F, Jones JB, Graham JH, *et al*. Comparative genomic analysis of *Xanthomonas axonopodis* pv. *citrumelo* F1, which causes citrus bacterial spot disease, and related strains provides insights into virulence and host specificity. Journal of bacteriology. 2011; 193(22):6342-6357.
7. Knorr LC. Citrus diseases a bibliography. PANS Pest Articles & News Summaries. 1973; 19(3):441-477.
8. Mirik M, Baloglu S, Aysan Y, Cetinkaya-Yildiz R, Kusek M, Sahin F. First outbreak and occurrence of citrus blast disease, caused by *Pseudomonas syringae* pv. syringae, on orange and mandarin trees in Turkey. Plant pathology. 2005; 54(2):238-238.
9. Graca JD. Citrus greening disease. Annual review of phytopathology. 1991; 29(1):109-136.
10. Van Sluys MA, De Oliveira MC, Monteiro-Vitorello CB, Miyaki CY, Furlan LR, Camargo LEA, *et al*. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. Journal of Bacteriology. 2003; 185(3):1018-1026.
11. Schaad NW, Jones JB, Chun W. Laboratory guide for the identification of plant pathogenic bacteria. Edn 3, American Phytopathological Society press, America, 2011, 275-278.
12. Bradbury JF. Isolation and preliminary study of bacteria from plants. PANS Pest Articles & News Summaries. 1970; 16(4):632-637.
13. Adler J, Dahl MM. A method for measuring the motility

of bacteria and for comparing random and non-random motility. Microbiology. 1967; 46(2):161-173.
14. MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. Edn 3, Williams and Wilkins, Philadelphia, PA, 2000, 309-314.
15. Cheney JE, Collins CH. Formaldehyde disinfection in laboratories: limitations and hazards. British journal of biomedical science. 1995; 52(3):195-201.
16. Vashist H, Sharma D, Gupta A. A review on commonly used biochemical test for bacteria. Innovare Journal of Life Science. 2013; 1(1):1-7.
17. Suslow TV, Schroth MN, Isaka M. Application of rapid method for gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology. 1982; 72(1):917-918.
18. Mandelbaum RT, Allan DL, Wackett LP. Isolation and characterization of a Pseudomonas sp. that mineralizes the s-triazine herbicide atrazine. Applied and Environmental Microbiology. 1995; 61(4): 1451-1457.
19. Taylor WI, Silliker JH. Isolation of Salmonellae from Food Samples: III. Dulcitol Lactose Iron Agar, a New Differential Tube Medium for Confirmation of Microorganisms of the Genus *Salmonella*. Applied microbiology. 1958; 6(5): 335-338.
20. Kovacs N. Identification of *Pseudomonas pyocyanea* by oxidase reaction. Nature. 1956; 178(4535): 703-703.
21. Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. In Manual of Clinical Microbiology. Edn 11, American Society of Microbiology, 2015, 1253-1273.
22. Hindi NKK, Chabuck ZAG. Antimicrobial activity of different aqueous lemon extracts. Journal of Applied Pharmaceutical Science. 2013; 3:74-78.
23. Mustansar M, Hafiz MI, Arshad, Yasir I, Muhammad IU, Iram B. Biochemical characterization of *Xanthomonas axopondis* pv *citri*: A gram negative bacterium causing citrus canker. International Journal of Science and Nature. 2015; 6(1): 151-154.