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CHARACTERIZATION OF CITRUS BACTERIAL SPOT BACTERIA THROUGH BIOCHEMICAL APPROACHES AND ITS CONTROL MEASURES

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Abstract

Citrus species are among the most important fruit crops in the world. Citrus bacterial spot disease is caused by the bacteria *Xanthomona axonopodis* pv. *citrumelo* which may cause huge damage of citrus production. The pathogen was isolated and cultured on LB liquid media at pH 7.5 and bacterial growth developed within 48 to 72 hours. Colonies on the nutrient agar media were straw yellow and creamy in color. Isolated bacteria identified by Gram staining, Endospore staining, KOH and MacConey agar test and the result showed that isolated bacteria were gram negative. It also showed positive result in case of Catalase, Simmons citrate agar, TSI, Oxidase and KIA test. In case of carbohydrate test, highest OD (0.26) was observed in Maltose containing medium of the isolate. The isolate was tested against ten plant extracts and fifteen antibiotics. The isolate was highly sensitive against cefotaxime, neomycin, streptomycin, gentamicin, chloramphenicol, azithromycin antibiotics and the susceptibility zones were 28±0.5, 26±0.5, 23±0.5, 17±0.5, 20±0.5, 17±0.5 mm respectively. Antimicrobial activity of ten plant extracts showed that the isolateis susceptible to *Momordica charantia* extract, revealed a wide antibacterial spectrum against the bacterial strain.

Keywords: Citrus bacterial spot, infectious disease, *Xanthomonas axonopodis*, gram negative bacteria, antimicrobial activity.

Introduction

Citrus is one of the most cultivated fruits in the world. *Citrus* is a genus of flowering trees and shrubs in the rue family, Rutaceae. Plants in the genus produce *citrus* fruits, including important crops like oranges, lemons, grapefruit and limes. The most recent research indicates an origin of *citrus* in Australia, New Caledonia and New Guinea (Liu *et al*., 2012). The generic name originated from Latin, where it referred to either the plant known as Citron (*C. medica*) or a conifer tree (*Thuja*). It is somehow related to the ancient Greek word for cedar. This may be due to perceived similarities in the smell of citrus leaves and fruit with that of cedar (Spiegel-Roy and Goldschmidt, 1996). *Xanthomona axonopodis* pv. *citrumelo* causes citrus bacterial spot (CBS). Citrus bacterial spot is known to occur only under nursery conditions. In citrus nurseries, *X. axonopodis* pv. *citrumelo* most commonly infects the rootstocks citrumelo (*C. paradisi* × *Poncirus trifoliata*), especially cv. Swingle, and also citrange (*C. sinensis* × *P. trifoliata*) and *P. trifoliate* itself. In artificial inoculation experiments, leaves of oranges, grapefruits (*C. paradisi*) and other *Citrus* spp. were less affected (Gottwald *et al*., 1993). Fruits of citrumelo were more susceptible to infection by an aggressive strain of *X. axonopodis* pv. *citrumelo* than grapefruits, while other citrus fruits were even less infected (Graham *et al*., 1992). The pathogen was first recognized as distinct in 1987, after analysis of a first outbreak of citrus bacterial spot at a citrus nursery in central Florida (USA) (Schoulties *et al*., 1987). The disease particularly affects young citrus plants (in nurseries), rather than established trees (in citrus groves). It causes lesions on leaves, fruits and stems of citrus, like *X. axonopodis* pv. *citri*, but these are sunken and not raised. Some strains of "*X. axonopodis* pv. *citrumelo*" are less aggressive (Lawson *et al*., 1989; Graham and Gottwald, 1990), not causing any water-soaking of tissues. These lesions also expand more slowly. Indeed, the most aggressive strains associated with the initial outbreaks were never found on mature commercial citrus, and have not been found again in nature since 1987 (Stall and Civerolo, 1991). So, the strains which can now be isolated as *X. axonopodis* pv. *citrumelo* are relatively harmless. It is thought that the aggressive strains of *X. axonopodis* pv. *citrumelo* which caused the original

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bacterial spot outbreaks in Florida were eliminated by eradicative action mainly targeting pv. *citri*. In any case, the disease can be controlled by sanitation in nurseries, since its spread is relatively slow. The less aggressive strains which now occur do not require any particular control measures. In the USA, it has been decided to make no further attempt to eradicate *X. axonopodis* pv. *citrumelo*. Graham and Gottwald (1991) reviewed the status of canker and bacterial spot in Florida (USA) and the possibilities for their eradication.

The present research work has been undertaken to characterize the bacteria of citrus bacterial spot disease through biochemical approaches and to analyze its control measures by different antibiotics and plant extracts. The goal of this present study was to provide useful data to citrus breeders and to characterize the isolated bacteria using some biochemical tests and to study the effect of some antibiotics and plant extracts against isolated bacteria.

Materials and Methods

Collection and processing of infected leaves

The present investigation was conducted during the period of November 2015 at Professor Joarder DNA and Chromosome Research Laboratory in the Department of Genetic Engineering and Biotechnology, University of Rajshahi. Leaves from different sections of the same citrus plant were collected from Rajshahi University area and tested for the presence of *X. axonopodis* pv. *citrumelo*as described below by different morphological and biochemical tests. Infected plant leaves were surface disinfested using a dilute sodium hypochlorite solution (10%) and rinsed thoroughly. Surface-disinfested tissue was then placed in a LB liquid medium and allowed to grow bacteria into LB liquid medium by overnight. After that, a sterile loop was used to streak the bacteria onto a solid agar medium. The bacteria were allowed to grow for at least 48 hours at room temperature and examined periodically for colony growth. Both solidified and liquid media were used for the present study. Plating is essential to obtain single colony from bacterial culture. In order to identify bacteria, it is necessary to obtain a pure culture. This is done by using the streak-plate method. Isolation, plating, streaking and subculture were done manually. At each time of plating and streaking, precaution was taken to minimize cross contamination of samples.

Biochemical characterization of the isolated bacteria

Isolated bacteria were characterized by some morphological and biochemical tests. Colony morphology of the isolated bacteria on the agar plate was recorded after 24 h of growth on LB agar plate at 37°C. It was yellow and creamy white in color. Gram negative test was done on the basis of their physiological and chemical properties of cell wall of the test pathogen. In order to characterize bacteria a series of biochemical tests were conducted as described by Bergey's Manual of Systematic Bacteriology (Bergey *et al*., 1994).

Gram (+/-) staining test, Endospore (+/-) staining test, SIM test, Methyl red test (MR), Simmons citrate utilization test, MacConkey test, Catalase test, Triple Sugar Iron test (TSI), KOH test, Klinger's Iron Agar test (KIA test), Oxidase test, Urease test, King's medium B test and Carbohydrate utilization test were carried out using isolated bacterial colonies or broth cultures.

In MacConkey test, MacConkey agar was used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacteria (Macconkey, 1905). In case of KOH test, bacteria were aseptically removed from the agar medium with a toothpick placed on a glass slide into a drop of 3 % KOH, and stirred for 10 seconds using a quick circular motion (Suslow *et al*.,1982).

Antimicrobial and antibacterial activity screening test using different plant extracts and antibiotic against isolated bacteria

Antibiotic susceptibility test and antimicrobial activity test were usually carried out to determine which antibiotic or plant extract will be most successful to treat this test pathogen. Antimicrobial activity test was done by using ten different plant extracts (*Phyllanthus emblica, Azadirachta indica, Terminalia arjuna, Justicia adhatoda, Momordica charantia, Ocimum tenuiflorum, Aloe vera* etc. plant leaf extracts and *Allium sativum, Zingiber officinale, Allium cepa* etc. plant raw materials). Agar disk diffusion method was used for the test (Alzoreky and Nakahar, 2003). Materials of ten plant species were harvested in November 2016 from different parts of Rajshahi University campus. The isolated bacterial strains were grown overnight in nutrient broths that were placed in the shaker at 37°C

temperature and 150 rpm for the antimicrobial activity test. The antimicrobial activity of the plant extracts was evaluated and the resulting inhibition zones were measured in millimeter (mm) scale.

Antibacterial activity test of fifteen antibiotics (Gentamicin, Erythromycin, Doxycycline, Tetracycline, Penicillin, Amoxycillin, Chloramphenicol, Clarithromycin, Cefotaxime, Neomycin, Kanamycin, Azithromyin, Ampicillin, Stretomycin and Carbenicillin) against isolated bacteria was also done carefully by serial dilution technique. Antibiotic disks were placed centrally on the respective plates and incubate overnight at 37°C. After overnight incubation zones were observed on the plate and measured with the help of millimeter (mm) scale. The entire test was performed manually and enough care was taken for plating, streaking and handling of the test pathogen.

Results

Isolation and purification of bacteria

The leaves samples were collected and bacterial liquid culture obtained after overnight incubation at 37°C by following standard protocol. From the liquid culture, mixed culture was obtained which contain yellow and white colonies. From the mixed culture, pure culture was isolated and the bacteria were partially identified based on colony morphology (Fig. 1). The size and shape of colonies were found to be small to medium, convex and mucoid. It was yellow and creamy white in color. Purification was done by streaking method.

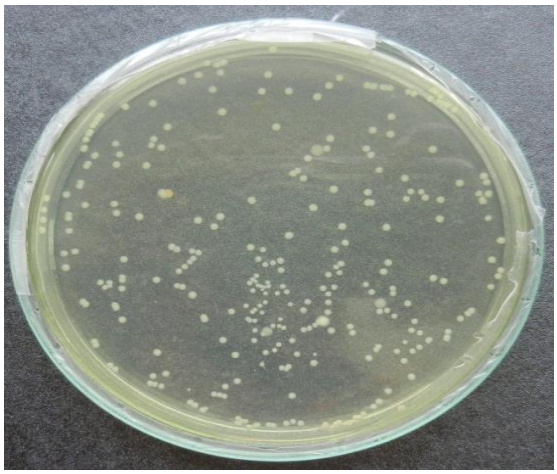

Fig. 1. Nature of the colony of isolated bacteria

Biochemical characterization of isolated bacteria

Various biochemical tests were carried out to characterize the isolated bacteria. The results of the biochemical tests are summarized in Table 1.

Gram staining test

The isolated bacteria were gram-negative which showed the pink color in gram staining test.

Endospore staining test

The isolated bacteria did not contain any endospore in endospore staining test.

SIM-medium test (Sulphide-Indole-Motility medium)

After adding kovac's reagent, bacteria did not produce red/pink color band on the top of tube and H_2S was not produced as no black precipitation formed and after incubation of 48 hours isolated bacteria showed motility in SIM medium.

Table 1. Morphological and biochemical tests of isolated bacteria

Name of the test	Results
Gram staining	Gram negative and rod shaped
Endospore staining	Negative
SIM	Motile, H_2S and Indole production negative
Simmons citrate	Positive
Catalase	Positive
MacConkey Agar	Gram negative and lactose fermenting
Triple Sugar Iron (TSI)	Carbohydrates fermenting bacteria
Methyl Red (MR)	Negative
Klinger Iron Agar (KIA)	Positive
KOH	Gram negative
Oxidase	Positive
Urease	Negative
King's medium B	Negative

Methyl Red test

Methyl red positive isolates showed red coloration as a result of high acid production and negative isolates showed yellow coloration as a result of less acid production.

Simmons citrate agar test

The uninoculated medium had the deep forest green color. The test tubes containing media inoculated with the strains changed from green to the royal blue color as the bacteria metabolized citrate.

Catalase test

This test was used to identify organisms that produced the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicated a catalase positive result.

MacConkey agar test

The sample (isolate) and control strain were grown on MacConkey agar plates for the identification of gram-negative enteric bacteria. As the sample grown well on the MacConkey agar, they were gram-negative enteric bacteria. The lactose fermenting capability of the strain was also detected from the MacConkey agar test. The isolated strain was grown on the media and it was gram negative. The isolate produced pink color around the colony so it was lactose fermenting.

Triple Sugar Iron Agar test

The sample (isolate) and control strain were grown on TSI agar plates for the identification of gram-negative enteric bacteria. As the sample grown well on the TSI agar, they were considered as gram-negative enteric bacteria. Lactose or sucrose or glucose fermentation has occurred. Since these substances were present in higher concentrations, they serve as substrates for continued fermentative activities with maintenance of an acid reaction in both the slant and the butt. The isolated bacteria did not produce Hydrogen sulfide and were lactose, glucose or sucrose fermenting.

Kligler Iron Agar test

This is a complex medium that contains a large amount of lactose and a very small amount of glucose; a pH indicator (yellow in acid and red in base); and iron, which is precipitated as a black sulfide if H_2S is produced. Lactose (+) organisms yielded a yellow slant and lactose (-) organisms yield a red slant. Cracks, splits, or bubbles in the medium indicated gas production. The isolated strains were grown on the media and it was lactose fermenting because it yielded yellow slants.

KOH test

The KOH test is a confirmation test of the Gram stain, especially with difficult bacterial species. Gram+ cells with their tough thick cell walls of peptidoglycan do not lyse whereas gram- cells with their thinner more porous cell walls lyse and DNA comes out of the cells. As a result, the bacterial smear becomes a viscous, stringy, sticky mess,

when picked up with a toothpick during KOH test which was absent in gram positive bacteria. The isolated bacteria were KOH test positive.

Oxidase test

The oxidase test was used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome coxidase oxidizes the reagent (tetramethyl-p-phenylenediamine**)** to **(**indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and was colorless. The isolated bacteria were oxidase test positive.

Urease test

This test was used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. If organisms produce urease, the color of the slant changes from yellow to pink. If organisms do not produce urease, the agar slant and butt remain yellow. The isolated bacteria could not hydrolyze urea. So, it was urease test negative.

King's medium B test

Kings Medium B Base was recommended for non-selective isolation, cultivation and pigment production of *Pseudomonas* species. As the isolated gram negative bacteria didn't produce fluorescent of any color which indicated that the isolates were not *Pseudomonas* species. The isolated bacteria were King's medium B test negative.

Carbohydrate utilization test

To find out the utilization of carbohydrates by isolate, five different carbohydrates were used. Utilization observed in nutrient broth with respective sugars, at 35°C for 72 hours. Isolated bacteria revealed positive result in carbohydrate utilization. The overall results of carbohydrate utilization test of the screened bacterial strain are summarized in the Table 2.

Table 2. Carbohydrate utilization test of isolated bacteria

Carbohydrates	Optimum density
Maltose	0.26
Fructose	0.15
Lactose	0.25
Sucrose	0.10
Glucose	0.13

Results of antibacterial activity screening test using different plant extracts against isolated bacteria

Antimicrobial activity of ten plant extracts was evaluated *in vitro* against isolated bacteria. The obtained results showed that the bacteria was resistant to all plant extracts except *M. charantia* extract which showed susceptibility against the isolated bacteria (Fig. 2). The result of antimicrobial activity test is summarized in the Table 3.

Antibacterial activity of some antibiotics against isolated bacteria

Antibacterial activity was tested using of fifteen antibiotics and was evaluated *in vitro* against isolated bacterial species. Most of the tested antibiotic showed high level of antibacterial activity. Isolated bacteria was highly sensitive against cefotaxime, neomycin, streptomycin, gentamicin, chloramphenicol, azithromycin antibiotics and the susceptibility zones were 28±0.5, 26±0.5, 23±0.5, 17±0.5, 20±0.5, 17±0.5 mm, respectively. The obtained result showed that, cefotaxime revealed highest antibacterial activity with 28±0.5 mm zone of inhibition against isolated bacteria (Fig. 3). The result of antibiotic sensitivity test is summarized in the Table 4.

Table 3. Antibacterial activity of some plant extracts against isolated bacteria

Name of plant extract	Dose of plant extract (zone in mm)			Sensitivity pattern
	10 µl	20 µl	30 µl	
Phyllanthus emblica (amalaki)	12 mm	11 mm	12.5 mm	Resistant
Azadirachta indica (neem)	7 mm	7.5 mm	8 mm	Resistant
Terminalia arjuna (arjun)	9 mm	8 mm	8.5 mm	Resistant
Justicia adhatoda (vasaka)	6 mm	6.5 mm	6 mm	Resistant
Momordica charantia (bitter guard)	12 mm	15 mm	20 mm	Susceptible
Ocimum tenuiflorum (tulsi)	7 mm	8 mm	10 mm	Resistant
Aloe vera (Aloe vera)	7 mm	16 mm	10 mm	Resistant
Allium sativum (garlic)	10 mm	8 mm	7 mm	Resistant
Zingiber officinale (zinger)	8 mm	7 mm	7 mm	Resistant
Allium cepa (onion)	10 mm	6 mm	7 mm	Resistant

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

Table 4. Antibiotic sensitivity test of isolated bacteria

Name of antibiotic	Disc concentration	Zone diameter (mm)	Sensitivity pattern
Tetracycline	30 µg	16±0.5 mm	Susceptible
Doxycycline	30 µg	8±0.5 mm	Resistant
Erythromycin	15 µg	8±0.5 mm	Resistant
Gentamicin	10 µg	17±0.5 mm	Susceptible
Clarithromycin	15 µg	12±0.5 mm	Intermediate
Chloramphenicol	30 µg	20±0.5 mm	Susceptible
Penicillin	10 µg	10±0.5 mm	Resistant
Amoxycillin	10 µg	15±0.5 mm	Intermediate
Neomycin	30 µg	26±0.5 mm	Susceptible
Kanamycin	30 µg	19±0.5 mm	Susceptible
Cefotaxime	30 µg	28±0.5 mm	Susceptible
Azithromycin	15 µg	17±0.5 mm	Susceptible
Carbenicillin	100 µg	15±0.5 mm	Intermediate
Streptomycin	10 µg	23±0.5 mm	Susceptible
Ampicillin	10 µg	16±0.5 mm	Susceptible

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

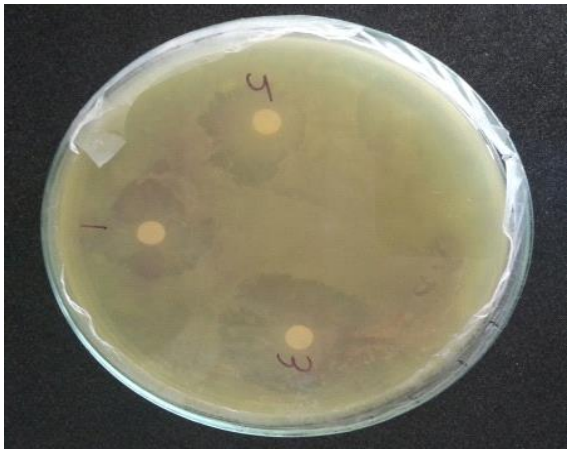

Fig. 2. Antimicrobial activity of *M. charantia* (bitter guard) against isolate

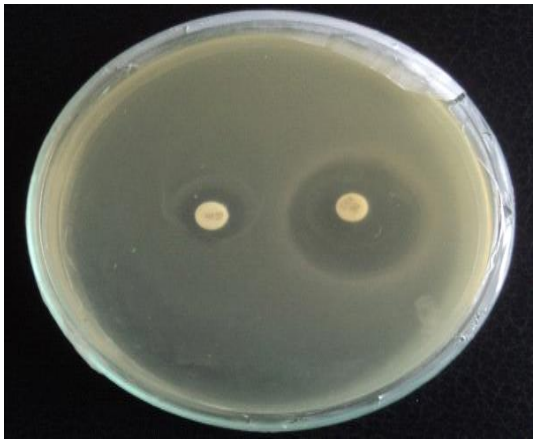

Fig. 3. Antibacterial activity of Erythromycin and Cefotaxime (8±0.5 mm, 28±0.5 mm) against isolate

Discussion

Citrus is a detectable and juicy fruit having great nutritional significance (Khan *et al*., 1992). The present status of citrus is threatened by a number of diseases. In this study bacteria was isolated from Citrus Bacterial Spot (CBS) infected leaves and characterized by different biochemical tests. The characteristic symptoms of CBS on leaves were detected by lesions on leaves, fruits and stems of citrus, like *X. axonopodis* pv. *citri*, but these were sunken and not raised. Bacteria were isolated and purified from the infected portion on leaves. The results of the different biochemical tests were carried out on the pathogenic isolates indicated that the isolate was likely *Xanthomonas* spp. and a similar result also found by Abdul-Rahim and Adam (1990). Biochemical and physiological test results of the isolated bacteria were observed.

From the different biochemical test of isolated bacteria, negative results were found in Gram staining, Endospore staining, Macconkey test, Methyl Red test, Urease test, and King's medium B tests. Positive results were recorded in Catalase test, KOH test, Simmons citrate test, and Klinger iron agar tests. TSI test indicated that the bacteria were carbohydrate fermenting. Gram staining result showed they were gram negative bacteria. Farah-Naqvi *et al*. (2013) found positive results in tests for KOH string assay, H_2S, Catalase and negative results in gram staining, Oxidase test and Nitrate reduction of *X. campestris* isolates. Different biochemical tests such as, Starch hydrolysis, Tween 80 hydrolysis, Kovacs' oxidase, Gelatin liquefaction, Fluorescent pigmentation and KOH characterized the *Xac*as gram negative bacteria (Mustansar *et al*., 2015). The results we have found were also similar with the work of Verniere *et al*. (1991) who studied about the biochemical and physiological characteristics of various strains of *X. campestris* pv. *citri*. Similarly, Mohan and Schaad (1987) observed non fluorescent pigmentation occurred in gram negative bacteria on KB media as compared to *Pseudomomas syringae* pv. *syringae*. In KOH test thread like slime was found. All isolates responded positively to loop test by forming a thread when uplifted gently. The loop formation was indicated that the isolated strain was gram negative and similar results were also observed by Halebian *et al*. (1981). Moreover Suslow *et al*. (1982) performed KOH test to accurately characterized gram negative bacteria of wheat.

A total of fifteen antibiotics were used to perform the antibacterial activity against *X. citrumelo*using disc diffusion antibiotic sensitivity test. The results we found were also similar with the work of Dayakar and Gnanamanickam (1996) who reported that kanamycin, tetracycline and chloramphenicol is susceptible to *Xanthomonas campestries.*

Antimicrobial activity of ten medicinal plants was evaluated *in vitro* against isolated *X. citrumelo*.Most of the tested plant extracts showed some levels of antibacterial activity. For gram negative bacteria the zones of inhibition were all above 15 mm for three extracts of *Callistemon viminalis* (Ahmad *et al*., 1998). The resistancy of Gram-negative bacteria towards antibacterial substances was related to lipopolysaccharides in their outer membrane (Gao *et al*., 1999).

Conclusion

Biochemical and physiological test results of the isolated bacteria were observed. In the present research, it was found that the citrus bacterial spot bacteria were gram negative and endospore negative from the morphological and biochemical tests. Citrus bacterial spot bacteria was highly sensitive against streptomycin, gentamicin, chloramphenicol, neomycin, cefotaxime, azithromycin antibiotics and the susceptibility zones were 23±0.5, 17±0.5, 20±0.5, 26±0.5, 28±0.5, 17±0.5 mm, respectively. Antimicrobial activity of ten plant extracts showed that the isolate was susceptible to *M. charantia* extract, revealed a wide antibacterial spectrum against bacterial strains. This study showed that antibiotics and some plant extracts have some activity against the pathogen and may have application in protecting plants from developing CBS. Thus, this study confirms the efficacy of some plant extracts as natural antimicrobials and suggests the possibility of employing them in drugs for treatment of infectious diseases caused by the test pathogens which may contribute in our agricultural sector.

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