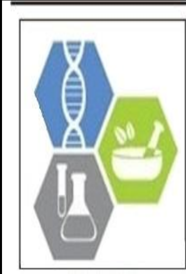

Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(6): 1526-1532
© 2017 JEZS
Received: 23-09-2017
Accepted: 27-10-2017

Md. Roushan Ali
MS Student, 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-6205, Bangladesh

Rizwoana Sharmin Lia,
MS Student, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Afrin Akter
MS Student, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Mst. Sabina Eyesmin Sumi
MS Student, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Md. Firose Hossain
MS Student, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

S. M. Zia Hasan
MS Student, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Dr. Md. Asadul Islam
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Dr. Md. Khalekuzzaman
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Dr. Biswanath Sikdar
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Correspondence
Md. Faruk Hasan
Assistant Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

Isolation and characterization of a canker disease causing pathogen from *Citrus aurantifolia* and evaluation of its biological control measure

Md. Roushan Ali, Md. Faruk Hasan, Rizwoana Sharmin Lia, Afrin Akter, Mst. Sabina Eyesmin Sumi, Md. Firose Hossain, SM Zia Hasan, Dr. Md. Asadul Islam, Dr. Md. Khalekuzzaman and Dr. Biswanath Sikdar

Abstract
The present investigation was designed to isolate and characterize of *Xanthomonas axonopodis* pv. *citri* bacteria in *Citrus aurantifolia* canker disease and evaluation of its antibiotic susceptibility, antibacterial sensitivity and antagonistic activity. Isolated bacterium was characterized by different biochemical test method. Isolated bacterium was found to be gram negative, small size, rod shaped, motile and pink in color. The biochemical tests showed catalase positive, glucose fermenting, lactose non-fermenting and urease test negative. The antibiotic susceptibility, antimicrobial sensitivity and antagonistic activity were determined by disc diffusion method. Gentamycin showed highest 21.0±0.0mm diameter of zone of inhibition against the isolated bacteria. *Allium sativum* extract showed highest 19.9±0.4mm diameter of zone of inhibition against the isolated bacteria. The highest antagonistic activity was found to be 17.4±0.3mm diameter of zone of inhibition by soil borne bacteria X against the isolated bacteria. The present study would be helpful for biological control of canker disease.

Keywords: *Citrus aurantifolia,* bacterial canker disease, characterization, biological control

1. Introduction
Citrus aurantifolia is an important fleshy, juicy and edible fruit plant belonging to the family of Rutaceae. It was thought to be originated in South East Asia and now occurs in more than 30 countries around the world [1]. *Citrus aurantifolia* is rich in vitamin C, various fruit acids and up to 10% fruit sugar [2]. It is also a good source of different electrolytes-folate and potassium [3], phytochemicals [4] that are helpful to overcome the neural tube defects, anemia [3] and Asthma [5].

Canker is a disease affecting citrus species caused by the bacterium *Xanthomonas axonopodis* pv. *citri.* Infection causes lesions on the leaves, stems, and fruit of citrus trees, including lime, oranges, and grapefruits [6]. This devastating disease affects most commercial citrus cultivars, resulting in significant economic losses mainly due to its effect on fruits, which are no longer suitable for commercializing as fresh fruit in national and international markets. Moreover, disease severity on susceptible varieties of *Citrus aurantifolia*, results in defoliation, dieback, premature fruit drop and blemished fruit, which consequently decrease fruit production and market value [7,8]. There are many types of citrus canker disease caused by various pathovars and variants of the bacterium *Xanthomonas axonopodis* [8]. According to symptoms similarity, the separation of these bacterium forms is very difficult based on host range, cultural and physiological characteristics, and bacteriophage sensitivity patterns [9], serology [10], plasmid fingerprints [11] and by various RFLP and polymerase chain reaction (PCR) analyses [12,13]. All cultivars of citrus are susceptible to canker, but grapefruit, Mexican lime and lemon are highly susceptible, whereas sour orange and sweet orange are moderately susceptible. However, Mandarins are moderately resistant and all young above-ground tissues of citrus are susceptible to *X. axonopodis* [1]. Hussain *et al.* (2010) [14] reported that the different aspects of the disease have been potentially addressed and adequately researched in various parts of the world, thereby generating substantial information on the biology and management of the disease. It is demanded of the modern age to substitute the chemical control method with safe and environment friendly management strategies, like biological and genetic control.

This would lead to the alleviation of the dependence on the chemicals that will be eco-friendly for the environment. Moreover, traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Recently, a report has been published on antagonism which is representing one of the most widely distributed forms of natural defense against bacteria and fungi [15].

Therefore, the main objective of this study was to characterize the causal agent of *Citrus aurantifolia* canker disease and establish a suitable biological control method.

2. Materials and methods

2.1 Plant materials

Canker disease infected plant leaves of *Citrus aurantifolia* were collected from Rajshahi University Campus, Rajshahi, Bangladesh and were identified by Md. Ashraful Alam, Scientific Officer, Bangladesh Fruits Research Institute, Binodpur, Rajshahi. Canker disease infected leave of *Citrus aurantifolia* was used as plant material for this present research.

2.2 Isolation and culture of bacteria

Canker disease infected leaves were surface-sterilized with 10% bleach for 5 min, followed by seven rinses in sterile distilled water. Infected portion of the leaves were aseptically excised with a sterile scalpel and placed onto sterile mortar and crushed with pestle. The crushed tissue was placed in Luria-Bertani (LB) liquid medium and incubated at 37°C for overnight. After the bacteria have grown in LB liquid medium, a sterile loop was used to streak the bacteria onto a solid agar medium and incubated at 37°C for 16 hours to examine periodically for colony growth.

2.3 Morphological and biochemical characterization

The isolated white color bacteria was characterized by some morphological and biochemical test.

Gram staining: Gram staining test was used to differentiate bacterial species into gram-positive and gram-negative, based on the physical properties of their cell walls. Gram staining was carried out according to Chaudhry and Rashid (2011) [16] method. Crystal violet, ethanol, iodine, and safranin were used. At first, the isolated bacterial culture was heat fixed onto a glass slide. Then crystal violet was added to the bacterial sample and incubated for 1 min. After washing the slide, iodine was added in the medium. Then safranin was used to counterstaining. After all these steps the slide was used to observe under the light microscope at 100X using oil immersion.

SIM-medium test (Sulphide-Indole-Motility medium): SIM medium test was performed to test for hydrogen sulfide, indole, and motility of the organism. First of all in conical flask 50 ml distilled water and 1.5 gm ready-made media was taken. Then the medium, two test tube, conical flask, cotton were autoclaved at 121°C for 20 min. In the laminar air flow, 25 ml media was taken in two test tubes. After cooling, a single bacterial colony was taken and inoculated. Finally, test tubes were kept for incubation at 37°C for 24 hrs. It will react with the sodium thiosulfate in the medium and the indicator, ferric ammonium citrate, to produce ferrous sulfide which falls out of solution as a blackish precipitate. The indole portion of the test was performed by adding Kovac's reagent to the inoculated medium. The Kovac's reagent reacts with the indole to produce a pinkish-red or reddish-purple ring around the top of the test tube.

Simmon's citrate test: Simmon's citrate test was performed using Simmon's citrate agar medium to detect the bacterial isolate for the ability to utilize citrate as its carbon and energy source [17]. The citrate test is often a part of the battery of tests used to identify gram-negative pathogens and environmental isolate. In the laminar air flow, medium was poured in two test tubes and kept in slant condition. Next, a single pure isolated colony was picked with a needle and the slant surface was lightly streaked and incubated at 37°C for 48 hrs.

Catalase test: Catalase test was used to identify organisms that produce the enzyme catalase. A single colony was taken on a clean slide and hydrogen per oxide was added, smeared carefully. The catalase production was determined by adding the H_2O_2 (3% v/v) to a bacterial culture and the presence of catalase indicated by bubbles of free oxygen gas [18].

MacConkey agar test: This test was used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria [19]. At first, appropriate amount of MacConkey agar kits were taken in conical flask and pH was adjusted to 7.0 and boiled to dissolve agar and sterilized at 15 lbs and 121°C for 20 min. MacConkey agar was inoculated with bacteria using streak plate technique and was incubated the plate in incubator at 37°C for 24 hrs.

Kligler Iron Agar (KIA) test: KIA test was used to aid in the differentiation of enteric gram-negative bacilli on the basis of carbohydrate fermentation and H_2S production. For KIA test, 55gm of the medium was suspended in 1 liter deionized water, mixed well and sterilized at 121°C for 20 min. The tubes were cooled in a slanted position to obtain a butt of 1.5 - 2.0 cm depth. Then the medium was inoculated with the colony in study by stabbing the butt and streaking the surface of the tube. After that, the tubes were incubated at 37°C for 24 hrs and the results were recorded.

Urease test: Urease test was used to determine the ability of an organism to split urea through the production of the enzyme urease [20]. Media were autoclaved at 121°C for 20 min. Then autoclaved medium was poured into two test tubes and kept in a rack in slant condition. The surface of a urea agar slant was streaked with a portion of a well-isolated colony and the tube was incubated at 37°C for 48 hrs.

Kovac's oxidase test: This test is used to determine bacteria that produce cytochrome c oxidase enzyme of electron transport chain. For Kovac's oxidase test, a well isolated single colony was picked by a sterilized platinum loop. Then the inoculum was smeared over the center of filter paper containing a single drop of 1% kovac's oxidase reagent. The change of color was obtained within 10 seconds (Kovac 1956) [21] Mubeen *et al*. (2015a) [27].

Triple Sugar Iron (TSI) Agar test: Triple Sugar Iron (TSI) Agar is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It was used to differentiate enteric based on the ability to reduce sulfur and ferment carbohydrates. In a conical flask, appropriate amount of readymade TSI medium kits were taken in 50 ml distilled water. Two test tubes and the medium was autoclaved, then

taken in laminar air flow. Then autoclaved medium was poured into two test tubes and kept in a rack in slant condition. After cooling, a sterilized inoculating loop was used for the streaking of a pure bacterial colony the on surface of the slant and incubated at 37°C for 24 hrs.

Methyl Red (MR) test: For Methyl Red (MR) test, 2-3 drops of MR reagent was added. Bacteria were inoculated into the MR broth medium in the test tubes and were incubated at 37°C for 18 hrs. The suspension was poured 1/3 into a clean non sterile tube then run the MR test in the tube with 2/3 medium.
For biochemical test, different chemicals were collected from Oxido Ltd. Basingstoke, Hampshire, England.

2.4 Antibiotic susceptibility assay
Antibiotic susceptibility was determined by moderate disc diffusion method [22]. Commercially available fifteen different antibiotics namely, Amoxicillin, Erythromycin, Gentamycin, Chloramphenicol, Clarithromycin, Ampicillin, Tetracycline, Carbenicillin, Neomycin, Streptomycin, Azithromycin, Kanamycin, Doxycycline, Cefotaxime and Penicillin (Sigma, UNI-Chemical Company, China and S.R.L, India.) were used to determine the antibiotic susceptibility against the isolated bacteria. Briefly, 20ml quantities of nutrient agar were plated in petridish with 0.1ml of a 10^{-2} dilution of isolated bacterial culture. Antibiotics discs (6mm in diameter) were impregnated on isolated organism-seeded plates. The activity was determined after 16 hrs of incubation at 37°C. The diameters of zone of inhibition produced by the antibiotics were then measured in millimeters (mm) scale. All the tests were performed manually and enough care was taken for plating, streaking and handling of the isolated pathogen.

2.5 Antibacterial activity screening
Antimicrobial activity test was performed by moderate agar disc diffusion method [23]. Six different plants extracts, namely, *Aliium cepa, Allium sativum, Adhatoda vasica,Terminalia arjuna, Ocimum sanctum,* and *Azadirachta indica* were used against the isolated bacteria. Different parts of four plant species were harvested from Rajshahi university campus, Rajshahi, Bangladesh. Collected parts of the plants were cut, air-dried powdered in a grinding machine and stored in an airtight polybag. Powdered dried plants were extracted by distilled water in flat bottom conical flask, through occasional shaking and stirring for 10 days. The content was pressed through the markin cloth to get the maximum amount of extracts. The whole mixture was then filtered by Whatman No. 41(Whatman, UK) filter paper and the remaining filtrate were dried *in vacuo* to afford a blackish mass. The output extracts and fraction were collected in glass vials and preserved in a refrigerator at 4°C. Briefly, 20ml quantities of nutrient agar were placed in a petridish with 0.1ml of a 10^{-2} dilution of isolated bacterial culture. Discs (6mm in diameter) were impregnated on isolated organism seeded plates. 10µl, 20µl and 30µl/disc of each plant extracts were taken with the help of micropipette and incubation at 37°C for 16 hrs. The diameters of zone of inhibition produced by the plant extracts were then measured in mm scale.

2.6 Antagonistic effect of soil bacteria
Antagonistic effect of soil bacteria was performed by moderate disc diffusion method [24] against the isolated bacteria. Two soil borne bacterial strains were isolated from soil, near the roots of a flat bean tree. For the isolation of soil borne bacteria, 2gm soil was taken to an empty tube and dissolve in 10ml distilled water. The tube was incubated in the water bath for 12 min at 80°C followed by 37°C for 18 hrs in an incubator. Bacterial inoculums were cultured on a LB nutrient agar plate using an inoculating loop and incubated at 37°C for 18 hrs. From the mixed culture of soil bacteria, two types of single colonies were observed and cultured separately. For the betterment of study, they were indicated as soil borne bacteria X and Y. The discs (6mm diameters) were made by punching the Whatman No. 41 (Whatman, UK) filter paper with the help of punch machine. Briefly, 20ml quantities of nutrient agar were placed in a petridish with 0.1ml of a 10^{-2} dilution of isolated bacterial culture. Discs (6mm in diameter) were impregnated on isolated organism seeded plates. 10µl, 20µl and 30µl/disc of each soil bacterial culture was taken with the help of micropipette and were incubated at 37°C for 18 hrs. The antagonistic activities of the two soil bacteria against the isolated bacteria were determined by measuring the diameter zone of inhibition using mm scale.

2.7 Statistical Analysis
All the above experiments of the present study were conducted in triplicate for consistency of results and statistical purpose. The data were expressed as mean and standard error (Mean±SE) and analyzed by one way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. The data were calculated using Microsoft Excel 2010 software.

3. Results
3.1 Isolation and purification of bacteria
The infected leaf samples **(Fig. 1 a**) placed on LB liquid media showed the bacterial colonies after of incubation at 37ºC. The turbid condition in the media indicates the bacteria were grown. By streaking method, single colonies were found and partially identified based on colony morphology. The colonies were creamy white in color. The size and shape of colonies were found to be small to medium, convex, and mucoid (**Fig. 1b**).

3.2 Morphological and biochemical characteristics of isolated bacteria
Gram-negative bacteria have a thinner layer (10% of cell envelope), and are stained pink with safranin. Here, the isolated bacteria found to gram negative, motile and rod shaped under light microscope at 100X magnification (**Fig. 1c**). SIM medium was recommended for the differentiation of gram negative enteric bacilli on the basis of sulfide production, indole formation and motility. After adding the kovac's reagent, bacteria did not produce red/pink color band on the top of the tube and H_2S was not produced as no black precipitation was formed. In citrate test, the media inoculated with the strains changed from green to the royal blue color as the bacteria metabolized citrate while, the control medium had the deep forest green color. The catalase test was performed to identify organisms that produce the enzyme. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The presence of bubbles resulting from production of oxygen gas clearly indicates a catalase positive result in this study. In MacConkey agar test, isolated bacteria were grown on MacConkey agar medium. So, they were gram-negative enteric bacteria. The lactose fermenting capability of the strain was also detected by the MacConkey agar test. The isolated bacteria produce pink color around the colony so it was lactose fermenting. The

isolated bacteria yielded a yellow slant and cracks, splits, or bubbles in the medium indicate gas production. In Urease test, isolated bacteria did not hydrolyze urea. In Kovac's test, medium containing filter paper and oxidizing agent reagent did not produce any color. The isolated bacteria were grown well on TSI agar medium and were considered as gram-negative enteric bacteria. In carbohydrates test, isolated bacteria did not produce hydrogen sulfide but they were lactose, glucose or sucrose fermenting. In the MR test, the test bacteria were grown in a broth medium containing glucose and the color of methyl red changed from yellow to red. Bacteria showed positive results against Methyl Red test. The results of the morphological and biochemical test are represented in **Table 1.**

Table 1: Responses of the isolated bacteria in different biochemical test media

Name of the Test	Reaction	Appearance	Remarks
Gram staining	-ve	Small, rod shaped, pink in color	Gram staining showed gram negative bacteria
Sulphide –Indole-Motility(SIM)	+ve, (-ve)	Motile, no H_2S and Indole production	Bacteria showed motility but no indole and H_2S production
Simmon's citrate agar	+ve	Color changed from green to the royal blue	Citrate metabolizing gram negative bacteria
Catalase	+ve	Presence of bubbles	Bacteria formed bubbles from production of O_2 gas
MacConkey agar	+ve	No pink color around the colony	Bacteria showed no pink color, lactose non-fermenting
Kligler Iron Agar(KIA)	+ve	Yields yellow slants	Bacteria yields yellow slants confirming lactose fermenting
Urease	-ve	Slant remains yellow	Bacteria did not hydrolyze urea
Kovac's oxidase	-ve	No color formation	Bacteria did not produce any characteristic color
Triple Sugar Iron(TSI)	+ve	Color changed from red to yellow	Bacteria didn't produce H_2S and confirming lactose fermenting
Methyl Red	+ve	Color changed from yellow to Red ring	Bacteria had the ability to utilize glucose

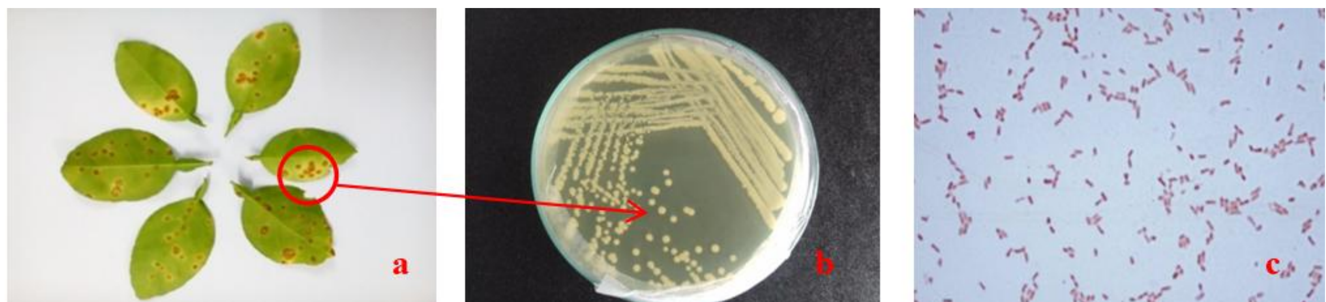

Fig. 1: Showing the plant sample, causal organisms isolation and gram staining (a) Bacterial Canker disease of Citrus, (b) Bacterial colonies (creamy) of isolated *Xanthomonas axonopodis* pv. *citri*, (c) Gram negative bacteria

3.3 Antibiotic susceptibility assay

The antibiotic susceptibility assay was examined using fifteen standard antibiotics against isolated bacteria. The standard Gentamycin revealed highest antibiotic activity with 21.0±0.0mm diameter of zone of inhibition at 10µg/disc concentration followed by Chloramphenicol and Tetracycline with 20.6±0.2mm diameter of zone of inhibition at 30µg/disc concentration. On the left hands, the standard Ampicillin showed lowest activity with 6.0±0.0mm diameter of zone of inhibition at 10µg/disc concentration against isolated bacteria (Fig. 2 a-c). The results of antibiotic sensitivity tests are represented in Table 2.

Table 2: Effect of some standard antibiotics against the isolated bacteria

Name of Antibiotics	Disc potency (µg/disc)	Diameter of zone of inhibition (in mm), (Mean±SE)	ANOVA
Amoxicillin	10	9.4 ±0.23	P<0.05
Erythromycin	10	14.0 ±0.0	P<0.05
Gentamycin	10	21.0 ±0.0	P<0.05
Chloramphenicol	30	20.6 ±0.2	P<0.05
Clarithromycin	15	16.0 ±0.0	P<0.05
Ampicillin	10	06.0 ±0.0	P<0.05
Tetracycline	30	20.6 ±0.2	P<0.05
Carbenicillin	100	14.7 ±1.0	P<0.05
Neomycin	30	20.3 ±0.4	P<0.05
Streptomycin	10	19.0 ±0.0	P<0.05
Azithromycin	15	15.9 ±0.8	P<0.05
Kanamycin	30	15.0 ±0.0	P<0.05
Doxycycline	30	14.9 ±1.2	P<0.05
Cefotaxime	30	12.6 ±0.6	P<0.05
Penicillin	10	07.0 ±0.0	P<0.05

Legend: Mean±SE=mean and standard error, ANOVA=analysis of variance, Resistant (R) =<10 mm; Intermediate (I) =11-15 mm; Susceptible (S) =>15 mm

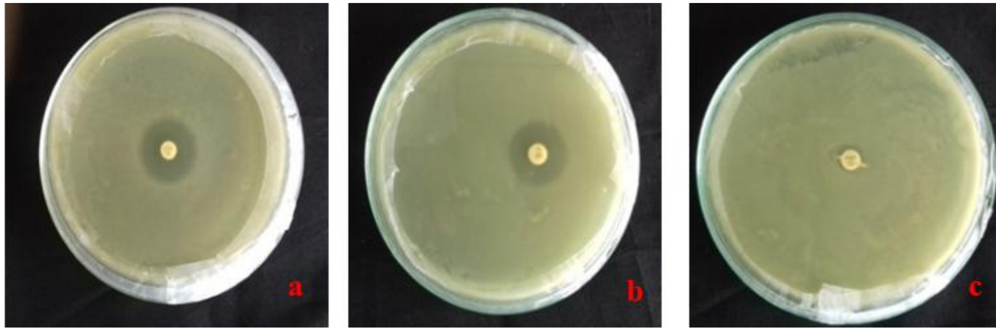

Fig. 2: Showing the antibiotic activities against *Xanthomonas axonopodis* pv. *citri* (**a**) Gentamycin (**b**)Neomycin (**c**) Ampicillin

3.4 Antibacterial activity screening

Antibacterial activities of six different plant extracts were determined against the isolated bacteria. The extract of *Allium sativum* showed highest antibacterial activity with 19.9±0.4mm diameter of zone of inhibition at 30µl/disc concentrations followed by 17.6±0.5mm diameter of zone of inhibition at 20µl/disc concentration of the same plant extract. On the left hand, the extract of *Adhatoda vasica* showed no inhibition zone against the isolated bacteria (**Fig. 3 a, b**). The results are given in Table 3.

Table 3: Antimicrobial activity screening of some plant extracts against the isolated bacteria

Name of plant extract	Dose of plant extract (in µl) and zone of inhibition (in mm), (Mean±SE)			ANOVA
	10µl/disc	20µl/disc	30µl/disc	
Allium cepa	7.9±0.5	8.0±0.0	9.0±0.0	P<0.05
Allium sativum	10.0±0.0	17.6±0.5	19.9±0.4	P<0.05
Adhatoda vasica	0.0±0.0	0.0±0.0	0.0±0.0	P<0.05
Terminalia arjuna	9.3±0.3	11.6±0.3	12.5±0.4	P<0.05
Ocimum sanctum	0.0±0.0	0.0±0.0	7.0±0.0	P<0.05
Azadirachta indica	0.0±0.0	7.0±0.0	7.0±0.0	P<0.05

Legend: Mean±SE=mean and standard error, ANOVA=analysis of variance, Resistant(R) =<10 mm; Intermediate (I) =11-15 mm; Susceptible(S) =>15 mm

3.5 Antagonistic effect of soil bacteria

In the present investigation, two soil bacteria were used to determine the antagonistic effect against the isolated bacteria. The soil borne bacteria X showed the highest antagonistic activity with 17.4±0.3mm diameter of zone of inhibition at 30µl/disc concentrations followed by 11.5±0.2mm diameter of zone of inhibition by the same bacteria at 20µl/disc concentration against the isolated bacteria (**Fig. 3c**). On the other hand, the soil borne bacteria Y showed no zone of inhibition at 10µl/disc concentration against the isolated bacteria of canker disease. The results are represented in Table 4.

Table 4: Antagonistic effect of soil bacteria against the isolated bacteria

Name of soil bacteria	Dose of soil bacteria (in µl) and zone of inhibition (in mm), (Mean±SE)			ANOVA
	10µl/disc	20µl/disc	30µl/disc	
Soil borne bacteria X	7.0±0.0	11.5±0.2	17.4±0.3	P<0.05
Soil borne bacteria Y	0.0±0.0	7.5±0.2	9.5±0.5	P<0.05

Legend: Mean±SE=mean and standard error, ANOVA=analysis of variance

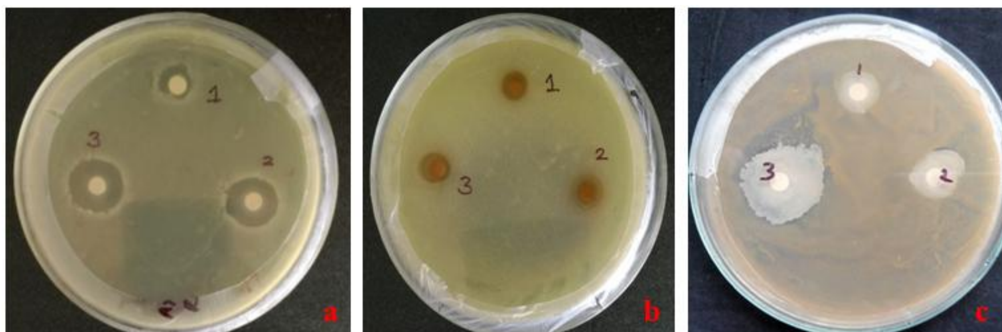

Fig. 3: Showing the antimicrobial and antagonistic activities against *Xanthomonas axonopodis* pv. *citri* **(a)** Inhibition zone of *Allium sativum* extract **(b)** Inhibition zone of *Terminalia arjuna* extract **(c)** Inhibition zone of Soil borne bacteria X

4. Discussion

Citrus canker is an economically important disease that is found all over the world. The in efficiency of currently applied methods to control the citrus canker disease along with the need to develop sustainable methods of disease management has started the hunt for a suitable alternative. The biological control of plant diseases with bacterial antagonism is a potential alternative of chemical control. The present study was carried out to evaluate proper biological control of *Citrus aurantifolia* canker disease by the different antagonistic agent. In gram staining, the isolated bacteria showed gram negative as well assmall size, rod shaped and

pink color. Bacteria isolated from *Citrus aurantifolia* by Abubaker *et al*. (2016) [25] also showed similar results by different biochemical test. Arshiya *et al.* (2014) [26] also found that the different strains of *Xanthomonas axonopodis* pv. *citri* bacteria isolated from citrus canker were gram-negative, obligate aerobes and non-spore forming rod yellow giving convex round and mucoid colonies on YDC (Yeast, Dextrose, Calcium carbonate) agar medium. Our results confirmed the work of Mubeen *et al.* (2015a) [27] who used gram reaction tests to identify and differentiate different pathotypes of citrus canker causing bacteria. In biochemical test, the isolated bacteria showed positive results to Simmon's citrate, Catalase, MacConkey agar, Kligler Iron Agar test, Triple Sugar Iron Agar, Methyl Red test and negative results to Urease and Kovac's oxidase test. Arshiya *et al.* (2014) [26] found that the different strains of *Xanthomonas axonopodis* pv. *citri* bacteria isolated from citrus canker were positive for starch hydrolysis, gelatin liquefaction, aesculin hydrolysis and tween 80 lipolysis, H_2S production, urease production, while all strains showed oxidase test negative. Our results confirmed the work of Mubeen *et al.* (2015a) [27], Islam *et al.* (2014) [29] and Hussain *et al*. (2010) [14] who used several biochemical tests to identify and characterize different strains of citrus canker causing bacteria. In antibiotic assay, Gentamycin showed highest 21.0±0.0mm diameter of zone of inhibition and Ampicillin showed lowest 6.0±0.0mm diameter of zone of inhibition against *Xanthomonas axonopodis* pv. *citri.* Mubeen *et al.* (2015b) [28] reported 1.4cm, 1.6cm, 1.8cm and 2.2cm inhibition zones by Sinobionic, Benzylpenicillin sodium, Streptomycin sulphate and Kanamycin sulphate respectively, while Ampicillin sodium and Chloramphenicol sodium did not show any inhibition zone against the *Xanthomonas axonopodis* bacteria. These results support our present findings. Different types of antibiotics, such as Tetracycline and Penicillin, were injected into infected citrus trees to temporarily relieve HLB symptoms and decrease Las bacterial titers [30]. Islam *et al.*(2014) [29] found the similar results for different commercial antibiotics against *Xanthomonas axonopodis* bacteria isolated from citrus canker which appreciate our research findings. In the antibacterial activity assay, *Allium sativum* extract showed highest antibacterial activity with 19.9±0.4mm diameter of zone of inhibition while *Adhatoda vasica* extract showed no inhibition zone against the isolated bacteria. Our result is quite similar to Praba and Kumaresan (2014) [31] and they worked on *Allium sativum* extract against different bacterial species at 50% concentration and they found significant results. Hussain *et al.*(2010) [14] reported that guava leaf, belericmyrobalan fruit, pomegranate fruit peel, nut gall fruit and myrobalan wood fruit had a pronounced effect on the inhibition of citrus canker bacteria, on culture media. Das and Singh (2000) [32] reported that neem cake suspension was found very effective in controlling the *Xanthomonas axonopodis* bacteria in citrus canker disease. Ahmad and Beg (2001) [33] reported similar results for antimicrobial of different plant extract against *Xanthomonas axonopodis* bacteria isolated from citrus canker which support our findings. In antagonistic test, the soil borne bacteria X showed the highest 17.4±0.3mm diameter of zone of inhibition against *Xanthomonas axonopodis* pv. *citri*. Some strains of bacteria *viz.*, *Pseudomonas syringae*, *Erwinia herbicola*, *Bacillus subtilis* and *Pseudomonas fluorescence* isolated from citrus phylloplane were reported to be antagonistic in vitro to the canker pathogen [34]. Moore *et al.* (2013) [35] reported the similar results for newly isolated soil borne *Bacillus* strains against *Salmonella, Shigella* and *Staphylococcus* strains of food bacteria. Similar result was obtained by Huang *et al.* (2012) [36] in citrus bacterial canker. Interactions between *Xanthomonas citri* and antagonistic bacteria including *Bacillus subtilis* have been reported by Pabitra *et al.* (1996) [37] *in vitro* and *in vivo* of citrus plant.

5. Conclusion

Canker is one of the most economically devastating bacterial disease of *Citrus aurantifolia* plant caused by *Xanthomonas axonopodis* pv. *citri.* In the present research work, we performed isolation, biochemical characterization and biological control measurement against the isolated bacteria. We found significant result in both antibiotic and antibacterial sensitivity test as well as in antagonistic test against the isolated soil bacteria. Therefore, the isolation, identification and characterization of pathogen responsible for canker disease were detected. Finally, the evaluation of its antibacterial and antagonistic potentiality could useful for bio-control agent in lieu of synthetic antibiotics. It would be helpful for future detection and control of this depletive disease.

6. Acknowledgement

The authors wish to thank Ministry of Science and Technology and Ministry of Education (MoST: 2015-2016 & MoE: 2016-2017) People's Republic of Bangladesh, for financial supports during the whole research work.

6.1 Conflict of interest

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

6.2 Author's contribution

MRA, MFH and BS designed the experiments, developed the methodology and prepare the manuscript. MRA, MFH, MAI, MKZ and BS collected the data and carried out analysis. RSL, AA, MSES, MFH and SMZH assisted with data analysis and manuscript preparation.

7. References

1. Gottwald TR, Graham JH, Schubert TS. Citrus canker: the pathogen and its impact. Plant health progress. 2002; 10:32.
2. Ward RW, Kilmer RL. The citrus industry: a domestic and international economic perspective. Iowa State University Press, 1989.
3. Whitney EN, Rolfes SR. Table of food composition. Understanding nutrition. Belmont: ITP, cap. H (appendix), 1999, 1-89.
4. Katz SH, Weaver W. Encyclopedia of food and culture. New York. Scribner, 2003.
5. Hatch GE. Asthma, inhaled oxidants, and dietary antioxidants. American Journal of Clinical Nutrition. 1995; 61(3):625S-630S.
6. Lakshmi TN, Gopi V, Gouri Sankar T, Sarada G, Lakshmi LM, Ramana KTV, *et al*. Status of diseases in sweet orange and acid lime orchards in Andhra Pradesh, India. International Journal of Current Microbiology and Applied Sciences. 2014; 3(5):513-518.
7. Zekri M, Chamberlain H, Timmer P, Roberts P, Muchove R. Field identification of citrus canker symptoms and decontamination procedures. University of Florida, 2005.
8. 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.
9. Civerolo EL. Bacterial canker disease of citrus. Journal of the Rio Grande Valley Horticultural Society. 1984; 37:127-146.
10. Alvarez AM, Benedict AA, Mizumoto CY, Pollard LW, Civerola EL. Analysis of *Xanthomonas campestris* pv. *citri and X.c. citrumelo* with monoclonal antibodies. Journal of Phytopathology. 1991; 81(8):857-865.
11. Pruvost O, Hartung JH, Civerolo EL, Dubois C, Perrier X. Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus bacterial canker disease. Journal of Phytopathology. 1992; 82:485-490.
12. Cubero J, Graham JH. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. Applied and Environmental Microbiology. 2002; 68:1257-1264.
13. Hartung JS. Plasmid-based hybridization probes for detection and identification of *Xanthomonos campestris* pv. *citri.* Journal of Plant Disease. 1992; 76:889-893.
14. Hussain K, Nawaz K, Majeed A, Haq IU, Lin F, *et al.* Molecular and biochemical characterization of *Xanthomonas axonopodis* pv. *citri* pathotypes. African Journal of Biotechnology. 2010; 9:9092-9095.
15. Kuzina LV, Miller TA, Cooksey DA. *In vitro* activities of antibiotics and antimicrobial peptides against the plant pathogenic bacterium *Xylella fastidiosa*. The Society for Applied Microbiology. 2006; 42(5):514-20.
16. Chaudhry Z, Rashid H. Isolation and characterization of *Ralsonia solanacearum* from infected tomato plants of soan skesar valley of Punjab. Pakistan Journal of Botany. 2011; 43(6):2979-2985.
17. Macfaddin JF. Biochemical tests for identification of medical bacteria (3rd edition), Lippencott, Williams and Wilkins. Philadelphia. 2000, 205-220.
18. MacConkey A. Lactose-fermenting bacteria in faeces. Journal of Hygiene. 1905; 5(3):333-379.
19. Cappuccino JG, Sherman N. Microbiology: A Laboratory Manual. Benjamin Cummings, San Francisco. 2001, 185.
20. Cox FR, Cox ME, Martin JR. Rapid urease test for mycobacteria: preliminary observations. Journal of Clinical Microbiology. 1977; 5:656-657.
21. Kovacs N. Identification of *Psedudomonas pyocyanae* by oxidase reaction. Nature. 1956; 178:703.
22. Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966; 45(4):493-496.
23. Hasan MF, Sikdar B. Screening of antimicrobial, cytotoxic and pesticidal activities of *Coccinia grandis* (L.) Voigt. Journal of Microbiology, Biotechnology and Food Sciences. 2016; 5(6):584-588.
24. Ghai S, Sood SS, Jain RK. Antagonistic and antimicrobial activities of some bacterial isolates collected from soil samples. Indian Journal of Microbiology. 2007; l47:77-80.
25. Abubaker MYA, Abu Dibar OAB, Elhassan SM, Yousif NME. First report of citrus bacterial canker disease in lime (*Citrus aurantifolia* Swingle) in Gadaref State-Eastern Sudan. Agriculture and Biology Journal of North America. 2016; 7(5):254-265.
26. Arshiya M, Suryawanshi A, More D, Baig MMV. Repetitive PCR based detection of Genetic Diversity in *Xanthomonas axonopodi s*pv. *citri* Strains. Journal of Applied Biology & Biotechnology. 2014; 2(01):017-022.
27. Mubeen M, Arshad HMI, Iftikhar Y, IrfanUllah M, Bilqees I. Bio-chemical characterization of *Xanthomonas axonopodis* pv. *citri:* a gram negative bacterium causing citrus canker. International Journal of Science and Nature. 2015a; 6(2):151-154.
28. Mubeen M, Arshad HM, Iftikhar Y, Bilqees I, Arooj S, *et al*. *In-vitro* efficacy of antibiotics against *Xanthomonas axonopodis* pv. *citri* through inhabitation zone techniques. International Journal of Agriculture and Applied Science. 2015b; 7(1):67-71.
29. 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.* Journal of Advanced Life Sciences. 2014; 1(4):215-222.
30. Su HJ, Chang SC. Electron microscopically study on the heat and tetracycline response and ultra-structure of the pathogen complex causing citrus likubin disease. (In: Proc. 8th International Congress). Electron Microscopy. 1974; 2:628-629.
31. Praba SK, Kumaresan R. Efficacy of antimicribial activity of aqueous garlic (*Allium sativum*) extract against different bacterial species. Journal of Chemical and Pharmaceutical Research. 2014; 6(10):677-679.
32. Das AK, Singh S. Management of Acid lime canker by using chemicals with compatible cultural practices (Hi-tech Citrus Management–Proc. International Symposium Citric). Nov. 23-27, 1999, Nagpur, Maharashtra, 2000, 1054-1056.
33. Ahmad I, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethno pharmacology. 2001; 74(2):113-123.
34. Kalita P, Bora LC, Bhagabati KN. *Phylloplane microflora* of citrus and their role in management of citrus canker. Indian Phytopathology. 1996; 49:234-237.
35. Moore T, Globa L, Barbare J, Vodyanoy V, Sorokulova I. Antagonistic Activity of Bacillus Bacteria against Food-Borne Pathogens. Journal of Probiotics and Health. 2013; 1(3):1-6.
36. Huang TP, Tzeng DDS, Wong ACL, Chen CH, Lu KM, *et al*. DNA Polymorphisms and Bio-control of *Bacillus* Antagonistic to Citrus Bacterial Canker with Indication of the Interference of Phyllosphere Biofilms. PLoS one. 2012; 7(7):e42124.
37. Pabitra K, Bora LC, Bhagabati KN. *Phylloplane microflora* of citrus and their role in management of citrus canker. Indian Phytopathology. 1996; 49(3):234-237.