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Isolation, characterization and control of canker disease causing bacteria of selected citrus plants in Rajshahi, Bangladesh

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Abstract
Xanthomonas axonopodis pv. *citri* is responsible for the overwhelming important disease of citrus known as citrus canker. Isolated bacteria were characterized by different physical, biochemical, carbohydrate utilization and vitamin C tolerance test. The result of vitamin C tolerance revealed that all three isolates had the ability to tolerate higher level of vitamin C as compared with that of *Rhizobium* indicating that vitamin C is less toxic to canker bacteria. Optimum temperature and pH for growth of isolated bacteria were 28 ^{0}C and pH 7. The isolates showed similar morphological and biochemical characteristic that confirm the identity of isolates as belonging to those of *Xanthomonas axonopodis* pv. citri. Isolated bacteria were gram negative, rod shaped, motile and highly sensitive to Ciprofloxacin (29.0±0.00mm diameter of zone of inhibition). However, isolated bacteria X1, X2 and X3 were respectively highly sensitive to extract of *Spondia mombin* (23.0mm/30µl) , *Lawsonia inermis* (20.0mm/100µl) and *Candle bush* (25.0mm/30µl) respectively. The highest antagonistic activity was found by soil borne bacterial isolates D, A & B and A against the isolated bacteria X1 (10.0±0.0mm zone of inhibition), X2 (14.0±0.0mm zone of inhibition) and X3 (13.0±0.0mm zone of inhibition) respectively. The results reveal that the isolated *X. axonopodis* contain unique characteristics which are vital for better understanding about local type of citrus canker disease as well as for designing locally effective control measures of this notorious disease.

Keywords: *Xanthomonas axonopodis*, citrus canker, plant extract, vitamin C

1. Introduction
Citrus is well known as the third most important fruit crop of the world [1]. Therefore, they have an incredible economic, social and cultural impact in our society [2]. Several varieties and species of citrus plants are susceptible to different diseases caused by fungi, bacteria, viruses and phytoplasma. *Xanthomonas axonopodis* pv. *citri* (syn. *Xanthomonas citri* subsp. *citri*) is responsible for the citrus canker and it is one of the most critical disease of citrus throughout the world including Africa, Asia, Australia, South America and USA [3]. Due to canker disease lesions found on the leaves, stems, and fruit of citrus trees, including lime, oranges, and grapefruits [4]. This pestilential disease affects mainly commercial citrus cultivars, resulting in significant economic losses mainly due to its effect on fruits, which are no longer suitable for commercializing as fresh fruits in international markets. For controlling citrus canker need eradication of infected plants and construction of windbreaks that greatly increase production costs [5]. Multiple types of citrus canker disease caused by various pathovars and variations of the bacterium *Xanthomonas axonopodis* [5].

The separation of this bacterium according to the similarity of symptoms, cultural and physiological characteristics, and bacteriophage sensitivity patterns [6], serology [7], plasmid fingerprints [8] and by various RFLP and polymerase chain reaction (PCR) analyses [9] is very challenging task. All cultivars of citrus are susceptible to canker, but highly susceptible are grapefruit, Mexican lime and lemon, whereas moderately susceptible are sour orange and sweet orange. However, Mandarins are moderately resistant and all young above-ground tissues of citrus are susceptible to *X. axonopodis* [10]. It was 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 [10]. This would lead to the mitigation of the necessity on the chemicals that will be eco-friendly for the environment. Moreover, traditional antibiotics can selectively kill or stop the growth of bacteria. Recently, a report has been published on antagonism that represent the most widely distributed forms of natural defense against bacteria and fungi [11]. Therefore, the main objective of this study was to characterize the *Xanthomonas axonopodis* responsible for canker disease with an intention to provide supportive data for establishment of a proper biological control method.

Materials and Methods
Plant materials
Canker disease containing plant leaves of lemon, pomelo and malta were collected from Fruit Research Institute, Rajshahi, Bangladesh. Canker disease infected leaves were used as plant material for this present research.

Isolation and culture of bacteria

Surface-sterilization of the leaves containing canker disease was done by 10% bleach for 5 min, followed by seven rinses in sterile distilled water. Infected portion of the leaves were excised with a sterile scalpel and placed onto sterile mortar then crushed with pestle by adding filter water. The crushed portion of the leaves were placed in Luria-Bertani (LB) liquid medium and incubated at 37°C for overnight. After overnight culture bacteria have grown in LB liquid medium then a sterile loop was used to streak the bacteria onto YDC medium which is specific for the canker bacteria and incubated at 37°C for 18 hours for observing the growth of the colony. The colonies of bacteria in YDC media were yellow, mucoid and convex. Colonies of canker bacteria grown on LB agar plate were white.

Characterization of bacterial isolates

Gram staining: Gram staining test of isolated bacteria was done as previously described [12].

SIM-medium test (Sulphide-Indole-Motility medium): The main purpose of SIM test is done to know the hydrogen sulfide production, indole, and motility of the organism. First of all 50 ml distilled water containing fresh conical flax was taken and 1.5 gm ready-made media was added. Then pouring the medium into three test tubes (7 ml of media), cotton plug is used to cover the mouth of the test tubes and autoclaved at 121°C for 20 min. After cooling, a single bacterial colony was taken in the test tubes and inoculated. Finally, all of the test tubes were kept for incubation at 37°C for 48 hrs. After 48 hours Kovac's reagent is added top of the test tubes.

Catalase test: Catalase test was used to know that the organisms can produce catalase enzyme or not. It is done by taking single colony on a clean slide and hydrogen per oxide was added and smeared carefully. The presence of catalase was indicated by bubbles of free oxygen gas.

MacConkey agar test: This test was used for the isolation of gram-negative bacteria and the ifferentiate either the bacteria can ferment the lactose if fermentation occur it will turn into pink in color. At first, suitable amount of MacConkey agar kits were taken in fresh conical flask and pH was adjusted to 7.0 and sterilized at 121°C for 20 min. Then the media containing flax pour into sterile Petri disk after becoming cool MacConkey agar containing plate one colony was taken and streaking was done. Finally incubated the plate in incubator at 37°C for 24 hrs.

Kligler Iron Agar (KIA) test: KIA test was done to identify gram-negative bacteria on the basis of fermentation of carbohydrate and production of H_2S. To do KIA test, 55gm of the medium was dissolved in 1 liter deionized water, mixed well and sterilized at 121°C for 20 min. After strilazion media containg flax pour in the test tubes. The tubes were cooled in a slanted position for obtaining a butt around1.5 - 2.0 cm depth. Then the colony was inoculate in the medium with the by stabbing the butt and streaking the surface of the tube. Finally, the tubes were incubated at 37°C for 24 hrs.

Urease test: Urease test was done to know the ability of the organisms to split urea through the production of the enzyme urease [20]. After preparing the media it was pour into test tubes, cover the tubes with cotton plug and autoclaved at 121°C for 20 min. After autoclaving the medium test tubes are kept in a rack for creating slant condition. Then well- isolated colony was incubated in the surface of the slant and streaking was done. Then it was incubated at 37°C for 48 hrs for observation.

Kovac's oxidase test: It is one kind of test that determine bacteria that produce cytochrome c oxidase enzyme of electron transport chain. For doing this test, isolated single colony was picked by a loop which is sterilized and put into the center of filter paper followed by adding a 1% kovac's oxidase reagent.

Triple Sugar Iron (TSI) Agar test: Triple Sugar Iron (TSI) Agar test is done to know either the organism can ferment glucose (dextrose), sucrose and lactose or not and phenol red is present as a pH indicator. In a conical flask, taken in laminar air flow. It was used to differentiate enteric based on the ability to reduce sulfur and ferment carbohydrates. In a conical flask, exact amount of readymade TSI medium kits were taken in 50 ml distilled water after dissolving it was pour into test tubes and cover the mouth of the test tubes by cotton plug. Then the medium containing test tubes were kept into the autoclaved, then taken in laminar air flow. Then autoclaved test tubes were kept in a rack in slant condition. After cooling, a single colony is picked by sterilized loop and done the streaking of a pure bacterial colony the on surface of the slant finally incubated at 37°C for 24 hrs.

Methyl Red (MR) test: MR test is done to determine that either organism produce stable acids by mechanisms of mixed acid fermentation of glucose. After inoculating colony in MR-VP incubate at 37°C for 24-28 hours. Following 28 hours add about 5 drops of the methyl red indicator solution to the tube.

Voges-Proskauer test: Voges-Proskauer test is done to know that the organism can produce acetylmethyl by fermenting glucose. After preparing the media colony of the bacteria is incubated at 37°C for 24-48 hours. After incubation 3 ml of 5% alcoholic α-naphthol solution was added to each tube followed by 1 ml of potassium hydroxide creatine solution. Then the tubes were then shaken vigorously for 1-2 minutes.

KOH test: KOH test is done to know either bacteria is gram positive or negative like gram staining test. If bacteria is gram negative the cell wall of bacteria will easily broke because of the expose of the dilute alkali solution while the tough thick peptidoglycan wall of gram positive bacteria will not lysis. For doing this test one drop of KOH (3%) solution taken on the slide and mixed well by taking a colony from a sterile loop in the slide. After homogenized immersed the loop slowly.

Simmons Citrate Test: The test is done to know the isolated bacteria have the ability to utilize the citrate as its carbon and its energy source. To do this test 1.92 gram of simmons citrate kit pour into the 80 ml of distill water and mixed it gently. After mixing the media is transfer to the test tubes and close the tubes by cotton plug then Sterilized it at

121°C under 15 psi pressure for 15 minutes. Then the medium was cooled in slanted position (long slant, shallow butt) to give a forest green color due to the pH of the sample and the bromothymol blue. After cooling colony of the bacteria is inoculate. Finally incubated at 35°C for 48 hours.

Tween 80 hydrolysis test: Tween 80 hydrolysis test is one of the lipase hydrolysis tests it is done to know that bacteria can hydrolysis the lipid or not. To do this test 10 gram peptone, 5 gram of yeast extract, 10 gram sodium chloride, 15 gram agar and make the mixture up to 1000 ml with distilled water. After properly mixing poured the medium into the test tubes and close the mouth with cotton plug. Then sterilized it at 121°C under 15 psi pressure for 15 minutes. Then the medium kept in autoclave and let it to be cooled in slanted condition. After cooling colony of the bacteria is inoculate and incubated at 35°C for 48 hours for observation.

Mannitol salt agar or **MSA** is a commonly used selective and differential growth medium. The medium is usually prepared using at a concentration of 11.1 g in every 100 ml distilled water (concentration may vary depending on manufacturer). MSA helps to demonstrate the ability of a bacterium to grow in a 7.5% salt environment incorporation of 7.5% sodium chloride in the medium helps to select only those bacteria which can tolerate high salt concentrations.

Carbohydrate Utilization test: For doing this test suspended the ingredient containing peptone, NaCl and phenol red into distilled water and mixed well. Distributed 9 mL of media in fermentation tubes .Sterilized by autoclaving at 15 lbs pressure (121ºC) for 30 minutes. Aseptically added filter sterilized 1mL carbohydrate solution to sterile basal medium. Incubated the tube at 37ºC for 24-36 hours. Glucose, Fructose, Sucrose, Cellulose, Maltose, Galactose, Lactose are used in carbohydrate test.

Antibiotic vulnerability assay

The pattern either the bacteria is sensitivity or resistance was determined *in vitro* employing standard disc diffusion method as described previously [13, 14]. Different antibiotics namely Rifampicin, Amoxicillin, Erythromycin, Gentamycin, Cephatexin, Ampicillin, Tetracycline, Ciprofloxacin, Neomycin, Cefradine and Doxycycline (Sigma, UNI-Chemical Company, China and S.R.L, India.) were used to determine the susceptibility against the isolated bacteria. Briefly, 300µl, of fresh broth culture of isolated bacteria was pour uniformly on a nutrient agar plate and spread with a sterile glass spreader. Antibiotics discs were placed on isolated organism-seeded plates. The activity was determined after 14 hrs of incubation at 28°C. The diameters of zone due to antibiotics were then measured in millimeters scale. All the tests (plating, streaking and handling of the isolated bacteria) were performed manually and carefully.

Antibacterial activity of plan extracts

Antimicrobial activity test was done by using agar disc diffusion method [13]. For evaluation twelve different plants extracts are used namely, *Aliium cepa, Allium sativum, Lawsonia inermis, Azardirachta indica, Zingier officinal, Mintha viridis, Hibiscus rosa-sinesis, Psidium guajava, Ficus carica, Coccinia grandis, Spondias mombin* and *Cassia alata* were used against the isolated bacteria. The parts of the plants were cut, dried making powder form by using a grinding machine. Powdered dried plants were extracted by methanol in conical flask, through occasional shaking and stirring for 14 days. To obtain the maximum amount of extracts the content was pressed through the markin cloth and the whole mixture was then filtered by Whatman filter paper after that the remaining filtrate were dried *in vacuo* to afford a blackish mass. Then output extracts and fraction were collected in vials and preserved in a refrigerator at 4°C. Briefly, 250µl, of fresh broth culture containing isolated bacteria was pour carefully on a nutrient agar plate and spread with a sterile glass spreader. Discs were saturated on isolated organism seeded plates. 20µl, 30µl and 40µl/disc of each plant extracts were taken with the help of micropipette and incubate at 37°C for 14 hrs. Finally diameters of zone of inhibition produced due to the plant extracts were then measured in mm scale.

Antagonistic effect of soil bacteria

Antagonistic effect of soil bacteria was done by disc diffusion method against the bacteria which was isolated. Five soil borne bacterial strains were isolated from soil those are *Pseudomonas* (A), *Acetobacter* (B), *Brevibacillus* (C), *Rhizobium* (D) and *Bacillus* (E). Briefly, 300µl, of fresh broth culture containing isolated bacteria was pour homogenously on a nutrient agar plate and spread with a sterile spreader. The discs (6mm in diameter) which were made by punching the Whatman filter paper No.1 filter paper by punch machine, these discs were then autoclave at 121°C for 20 minutes for sterilization and that discs were saturated on isolated organism seeded plates. 5oµl disc of each soil bacteria was taken by the help of micropipette and incubate at 37°C for 14 hrs. Finally diameters of zone of inhibition produced due to soil bacteria were then measured using mm scale.

Determination of optimum growth conditions

Optimum growth condition was measured as previously described [15]. To determine the optimum pH of bacterial growth, culture medium was adjusted to pH 6, 7 and 8. For determination of optimum temperatures, inoculated media were incubated at 28^0C, 37^0C and 42 ^{0}C. The growths of bacteria at different condition were determined at different time intervals by measuring optical density at 660 nm with photoelectric colorimeter.

Effect of vitamin C on growth of bacterial Isolates

Pure culture of the Isolate X1, X2 and X3 were prepared in 100mL nutrient broth media. On next day, 2g of vitamin-C was dissolved in 100mL distilled water and sterilized by membrane filtration technique. Then, 2mL, 3mL, 4mL, 5mL, 6mL and 7mL of sterilized vitamin-C solution was added to autoclaved and cooled nutrient broth to prepare 400ppm, 600ppm, 800ppm, 1000ppm, 1200ppm and 1400ppm solution of vitamin-C. Autoclaved distilled water without vitamin-C was added in control nutrient broth. Then 1mL of bacterial solution was added in each flask of nutrient broth and incubated at 37^0C. OD of each sample was measured at 4 hours interval up to stationary phase.

Results

Isolation of the bacteria form leaves of citrus plant

By streaking method in YDC media, single colonies were found and partially identified based on colony morphology. The colonies were mucoid, convex and yellow creamy in color. Isolated bacteria also grown in LB Agar media. The colour of colonies were creamy white and the size, shape of colonies were found to be small mucoid and convex in medium.

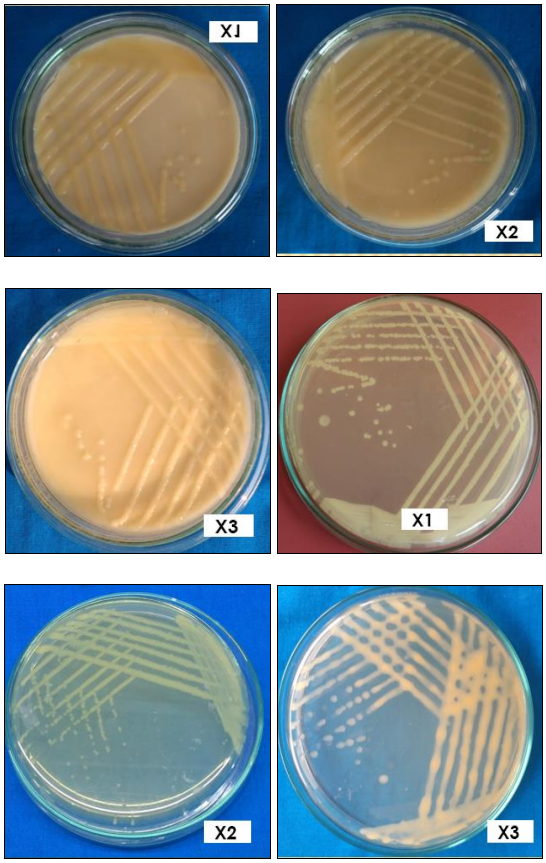
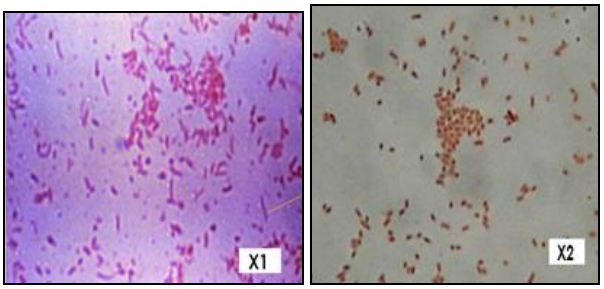


Fig 1: Isolated bacterial strains (X1, X2, X3, respectively) in different media and pure cultures.

Characterization of bacterial isolates

Gram staining

Gram staining test of isolated bacteria was done as previously described (12).



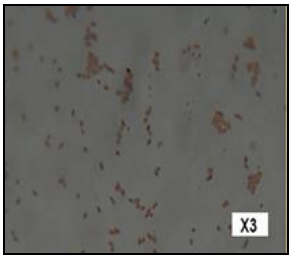


Fig. 2: Gram staining of the isolates X1, X2, X3 respectively.

Morphological and biochemical characteristics of the isolated bacteria

In this study, the isolated bacteria were gram negative, motile and rod shaped under light microscope at 100X magnification. SIM medium was recommended for the differentiation of gram negative on the basis of sulfide production, indole formation and motility. After adding the kovac's reagent, bacteria failed to produce red/pink color band on the top of the tube and H2S was not produced as a result black precipitation was formed. In citrate test, bacteria inoculate in the media strains changed from green to the royal blue color as the bacteria metabolized citrate while, the control medium had green color. The catalase test was performed to identify either the organisms produce the enzyme if the enzyme is produced it detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The presence of bubbles indicated the production of oxygen gas and it showed catalase positive. In MacConkey agar test, isolated bacteria grew on MacConkey agar medium and produced pink color around the colony so it was lactose fermenting and they were gram-negative bacteria. In Urease test, isolated bacteria hydrolyze urea. In Kovac's test, medium containing sterilized filter paper and oxidizing agent reagent did not produce any color. The isolated bacteria were grown on TSI agar medium and were considered as gram-negative enteric bacteria and it also produced gas in X1 and X3 except X2. In carbohydrates test, isolated bacteria ferment lactose, glucose, fructose, galactose, sucrose, maltose except cellulose. Mannitol fermentation test isolated bacteria fermented mannitol. In the MR test, the isolated bacteria were grown in a broth medium containing glucose and the color of methyl red changed from yellow to red that showed positive result. All isolated bacteria showed positive result to VP because color converted yellow to red. In KOH test, the results showed that the bacterial suspension becomes viscous and form thread like slime when picked up with an inoculation loop indicating that all of three isolates were Gram negative bacteria. In Kligler Iron Agar (KIA) test, it was found that slant color was converted from red to yellow for all three isolates indicating that these isolates can ferment dextrose and lactose. However, gas was produced by isolate X1 and X2, but gas was produced by isolate X3. In tween 80 hydrolysis test the crystal formation indicated the positive result of lipase activity test.

Table 1: Different morphological and biochemical tests

Name of Test		Results		
		Isolate X1	Isolate X2	Isolate X3
Oxidase		+	+	+
Catalase test		+	+	+
KOH Test		+	+	+
Triple Sugar Iron (TSI)	Gas	+	+	+

	H_2S	+	+	+
	Sugar Fermentation	+	+	+
Sulfide indole motility test	Sulfide	+	+	+
	Indole	+	+	+
	Motility	+	+	+
Citrate utilization test		+	+	+
Name of Test		Results		
		Isolate X1	Isolate X2	Isolate X3
Growth on MacConkey Agar		+	+	+
Mannitol Fermentation Test		+	+	+
Kligler Iron Agar		+	+	+
Tween 80 hydrolysis test		+	+	+
Methyl Red Test		-	-	-
Voges-Proskauer reaction		-	-	-
Urease Test		-	-	-

Table 2. Carbohydrate utilization test

Test	Isolate X1	Isolate X2	Isolate X3
Glucose	+	+	+
Sucrose	+	+	+
Lactose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Maltose	+	+	+
Cellusoe	-	-	-

Antibiotic vulnerability assay

The antibiotic sensitivity assay was done using eleven standard antibiotics against isolated bacteria. The Ciprofloxacin revealed highest antibiotic activity with 29.0±0.0 mm diameter of zone of inhibition in X1, 30.0±0.0 mm in X2 and 35.0±0.0 mm in X3 at 5µg/disc concentration (Table 2). On the other hand hands, the standard Erythromycin (15µg/disc) showed lowest activity with 5.0±0.0mm diameter of zone of inhibition at 10µg/disc concentration against isolate X1 bacteria (Table 2). Ampicillin (5µg/disc), Cephalexin (30µg/disc) and Cefradine (30µg/disc) show no zone of inhibition against isolate X2 while only Cephalexin (30µg/disc) showed no zone of inhibition against isolate X3 (Table 2).

Table 3. Antibiotic sensitivity tests

Antibiotic discs	Disc conc. (µg)	Zone of inhibition (mm)		
		X1	X2	X3
Rifampicin	5	15 (I)	15 (I)	11 (I)
Tetracycline	30	16 (S)	16 (S)	17 (S)
Erythromycin	15	4 (R)	7 (R)	5 (S)
Ampicillin	25	6 (R)	0 (R)	5 (R)
Cephatexin	30	6 (R)	0 (R)	0 (R)
Neomycin	30	7 (R)	8 (R)	7 (R)
Ciprofloxacin	5	29 (S)	30 (S)	35 (S)
Cefradine	25	11(R)	0 (R)	7 (R)
Amoxycillin	30	5 (R)	4 (R)	4 (R)
Doxycycline	30	22 (S)	12 (S)	20 (S)
Gentamycine	30	21 (S)	20 (S)	16 (S)

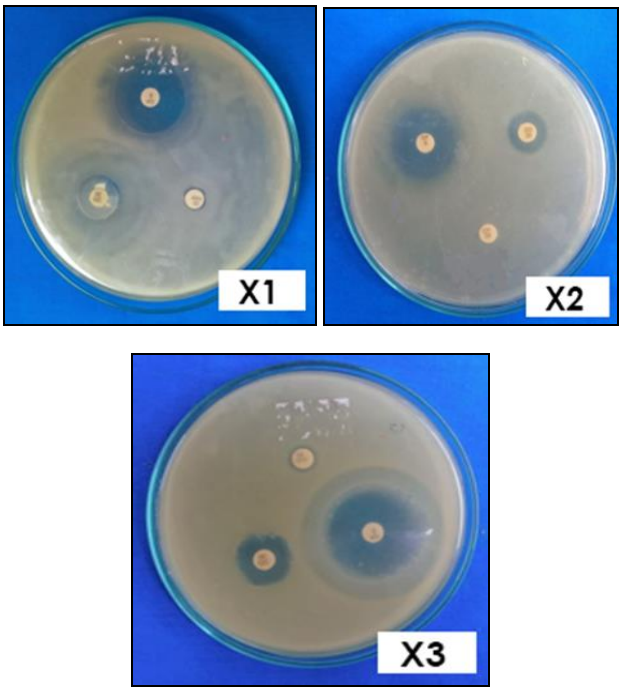


Fig 3: Antibiotic effects of isolates X1, X2, X3 respectively.

Antibacterial activity screening

Antibacterial activities of twelve different plant extracts were determined against the isolated bacteria. The extract of *Spondia mombin* showed highest antibacterial activity with 23.0±0.0 mm diameter of zone of inhibition at 30µl/disc concentrations against Isolate X1 (Fig. 1). On the contrary, the extract of *Coccinia grandis*, *Zingier officinal* and *Mentha viridis* failed to control the growth of isolated bacteria (Fig. 1). But, the extract of *Lawsonia inermis* showed highest antibacterial activity against Isolate X2 while extract of *Spondia mombin*, *Cassia alata*, *Fiscus carica*, *Psidium guajava*, *Coccinia grandis* and *Mentha officinal* showed no response against Isolate X2. *Cassia alata* showed highest antibacterial activity with 25.0±0.0 mm of zone of inhibition against Isolate X3 (Fig. 1).

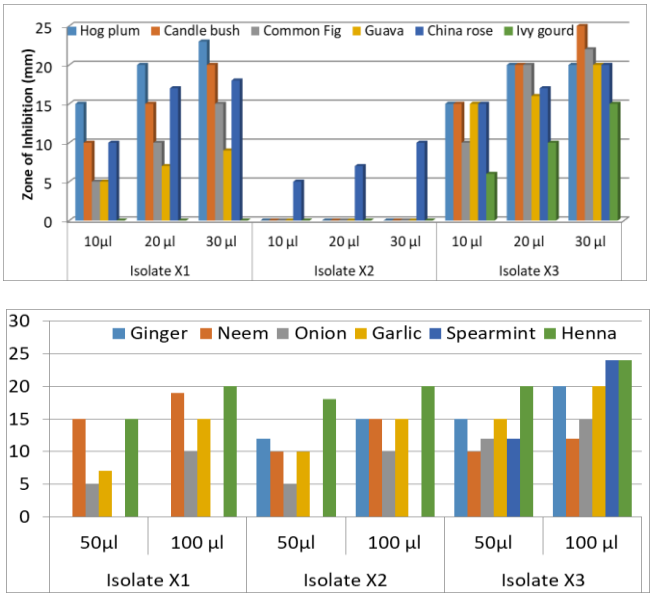


Fig 1: Antibacterial effect of plants extract against bacterial isolates

Antagonistic effect of isolated soil bacteria

Five soil bacteria were used to determine the antagonistic effect against the isolated bacteria. The soil borne bacteria D showed the highest antagonistic activity with 10±0.0 mm diameter of zone of inhibition at 30µl/disc concentrations against Isolate X1 and X2 (Fig 2). Similarly, soil bacteria A & B showed highest antagonistic activity with 14±0.0 mm diameter of zone of inhibition at 30µl/disc concentrations against Isolate X2 and soil bacteria A showed the highest antagonistic activity with 13±0.0 mm diameter of zone of inhibition at 30µl/disc concentrations against Isolate X3 (Fig. 2).

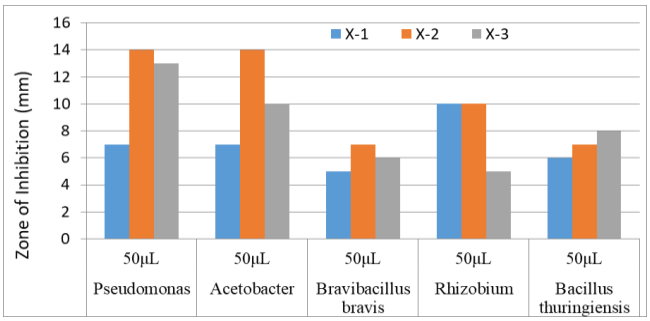
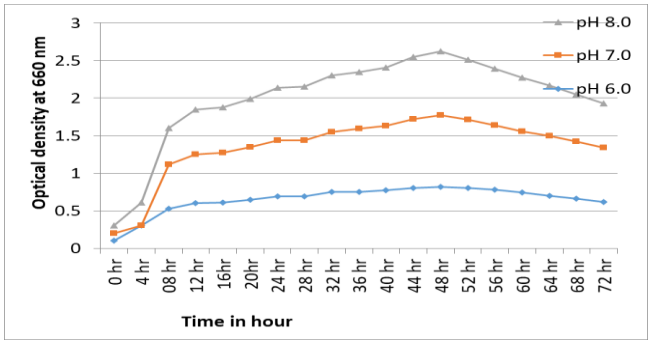
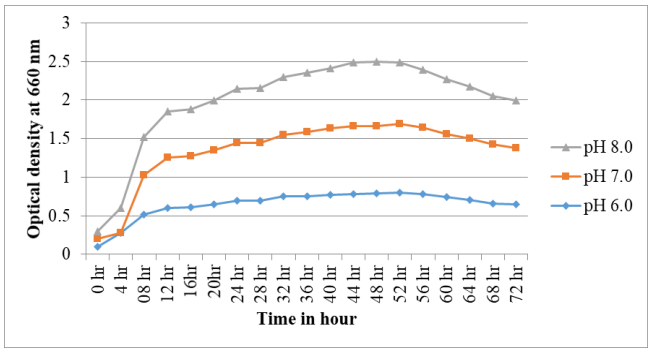


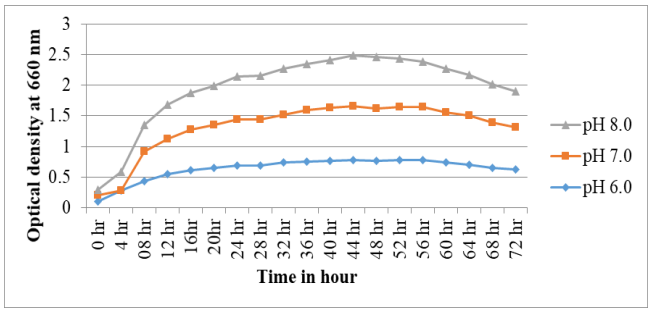
Fig 2: Antagonistic efficacies of different soil bacteria against isolated bacteria

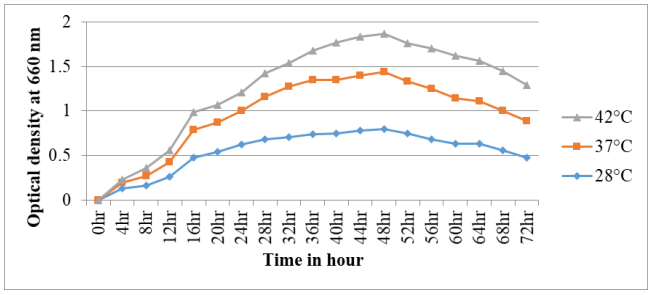
Effect of pH and temperature on Growth of Bacterial Isolates

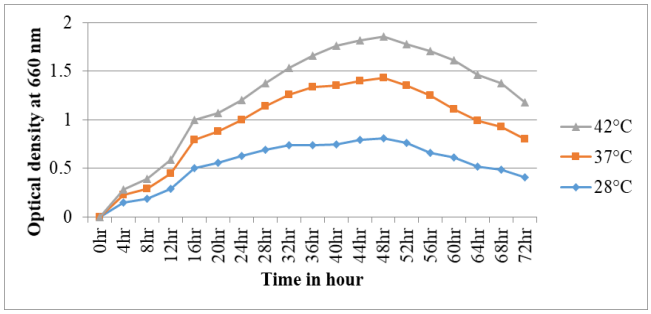
The bacterial growth depends on temperature and pH. Optimum pH for growth of isolate X1, X2 and X3 was determined at 28 ^{0}C, 37 ^{0}C and 42 ^{0}C and pH 6, 7 and 8 in liquid broth medium. The result showed that all the studied isolates (X1 X2 and X3) exhibited maximum growth at pH 7 and temperature 28 ^{0}C.











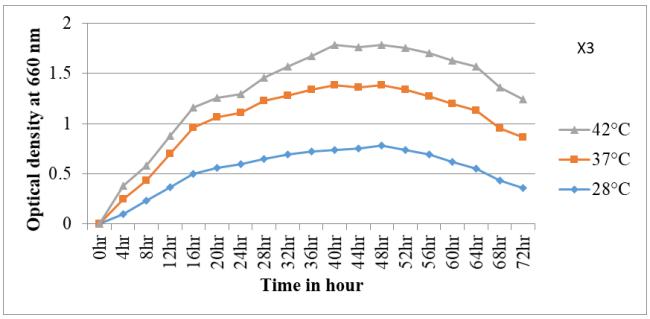


Fig 3: Effect of pH and temperature on growth of bacterial isolates

Effect of vitamin C on growth of bacterial Isolates

The result showed that the growth rate of three Isolates (X1, X2 and X3) gradually decreased with the increase of vitamin-C concentration (Fig. 3, 4 and 5). The highest growth rate of Isolates was observed in control broth followed by lowest concentration of vitamin-C (400 ppm) indicating that vitamin-C resist the bacterial growth in broth (Fig. 3, 4, 5 and 6). However, the Isolate X1, X2 and X3 showed some level of resistance to higher concentration of vitamin-C (1200ppm and 1400ppm) resulting in better growth of those bacteria as compared with that of *Rhizobium* (Fig. 3, 4, 5 and 6).

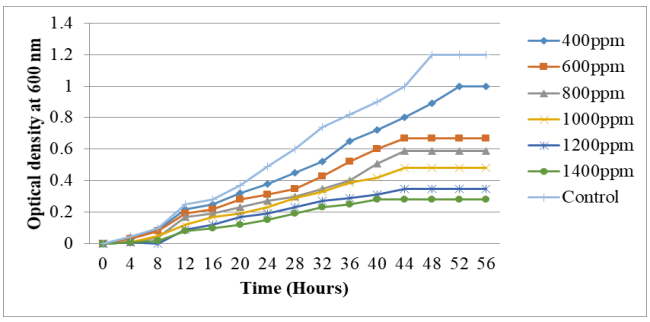


Fig 4: Effect of vitamin C on growth of Canker bacterial isolate X1

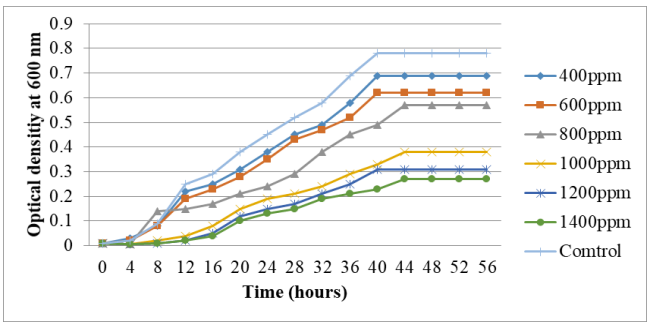


Fig 5: Effect of vitamin C on growth of canker bacterial isolate X2

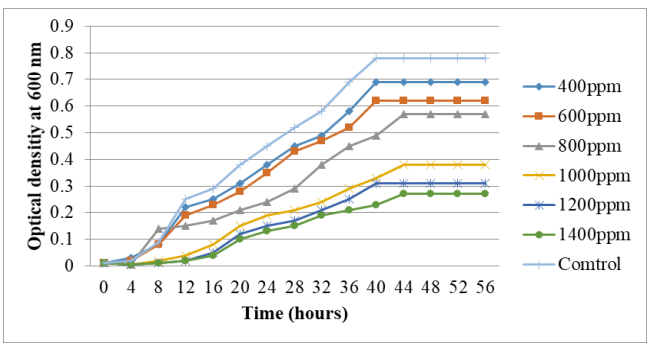


Fig 6: Effect of vitamin C on growth of canker bacterial isolate X3

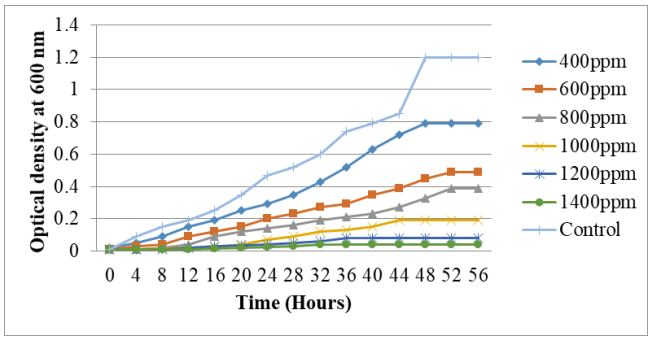


Fig 7: Effect of vitamin C on growth of soil bacteria, *Rhizobium*

Discussion

In this study, the isolate X1, X2 and X3 were rod shaped, gram negative and motile bacteria as shown by others researcher as common characteristics of citrus canker causing bacteria [10, 16, 17]. Colonies on culture media were yellow due to xanthomonadin pigment production. When glucose or other sugars are added to the culture medium, colonies become mucoid due to the production of extracellular polysaccharide slime [18, 19]. The colony of the pure culture of three isolates were cream color which agree with results of other researchers in this field [16]. Biochemical tests indicated that isolated bacteria were positive to Catalase, KIA, Urease test which was supported by result of another study [17]. All three isolates were positive to Potassium hydroxide and Tween 80 hydrolysis test which was also reflected by other studies [3, 17]. The TSI test showed yellow slant which indicates all isolated were lactose fermenting. In SIM test all the isolates showed negative result for Hydrogen sulphide and Indole. Similarly, biochemical tests showed that all isolated bacteria were positive to Methyl red, Urease, and Simmone citrate test which were also agreed by the results of other studies [7, 17]. In case of Macconkey test, all isolates were grown in Macconkey agar plate. It indicated that all isolates were gram negative. Pink color colonies indicated that isolates were lactose fermenting. In case of amylase activity test a clearing zone around the bacterial growth indicated the positive result of amylase activity test. So, it indicated that the organism had hydrolyzed starch [16]. Carbohydrate utilization test showed positive to glucose, fructose, lactose, maltose, galactose, sucrose except cellulose. It was found that the isolates can ferment all the tested carbohydrates except cellulose. Similar studies on canker bacteria showed comparable results [16, 17]. Isolated bacteria from infected plants of citrus which developed susceptibility to different antibiotics [5]. The result showed that all of the three isolates were susceptible to Ciprofloxacin while mixed pattern of sensitivity was observed to other studied antibiotics for different isolates. In the present study, the antagonistic activities of five soil bacteria were tested against the three isolates. Variation in inhibitory efficacy was observed for different soil bacteria against different isolates. Similar findings was reported in some papers [20].

Antibacterial effect of plant extracts collected from 12 different plants viz. Onion, Garlic, Ginger, Henna, Spearmint, Neem, China rose, Guava, Common fig, Ivy gourd, Hog plum and Candle bush were evaluated against three different Canker bacteria. It was found that antibacterial efficacy of plant extract vary with type of bacterial isolates. It was shown in a study that the plant extract of *Spondias mombin* produced the highest zone of

inhibition while no zone of inhibition produced by *Coccinia grandis, Zingier officinal* and *Mentha viridis* for isolate X1. Similarly, extract of *Lawsonia intermis* was effective against Isolate X2 while extract of *Spondia mombin, Cassia alata, Fiscus carica, Psidium guajava, Coccinia grandis, Mentha viridis* showed no inhibitory action against isolate X2. Similar inhibitory action of plant extract against plant pathogenic bacteria are reported by others [21].

Canker disease causing bacteria are adapted to citrus fruits which are very rich in vitamin C. The results of this study on vitamin C tolerance level showed that all three isolated bacteria can tolerate higher level of vitamin C concentration as compared with vitamin C tolerance level of soil bacteria, *Rhizobium.* It was reported that high doses of vitamin C inhibit the growth of *H. pylori* in vitro as well as *in vivo* [22]. It was also reported that Vitamin C can inhibit growth of *S. aureus* [23].

Conclusion

In this study, morphological and biochemical characteristics of the three isolates were similar to the characteristics of *Xanthomonas axonopodies*. The result of vitamin C tolerance revealed that three isolated bacteria had the ability to tolerate higher level of vitamin C which indicated that these bacteria might possess a vitamin C resistant gene. Some particular antibiotics, antagonistic soil bacteria and plant extracts were effective against a particular Isolate for controlling its growth. Taken together, it can be concluded that all three isolated bacteria were belonging to *X. axonopodis* which possessed some fine variation in their ability to resist the toxic effect of antibiotics, plant extracts, soil bacteria and vitamin C. Hence, better understanding of isolate-specific variation is vital for designing locally effective control measures of canker disease.

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Author Contributions

ASC, MEKC, MFH, MAI and BS designed the experiments, developed the methodology and prepared the manuscript. CS, MEKC, MFH, and BS collected the data and carried out analysis. MEKH, MFH, MAI and MFH assisted with manuscript preparation.

Conflict of interest

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

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