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PCR AMPLIFICATION AND SEQUENCING OF *XANTHOMONAS AXONOPODIS* PV.*CITRI* IN CITRUS CANKER AND ITS ANTAGONISTIC CONTROL MEASURES

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Abstract

The present investigation was designed to characterize the *Xanthomonas axonopodis* pv. *citri* (*XAC*) in *Citrus limon* canker through biochemical and molecular approaches and evaluation of its biological control measures. Canker infected pathogen was isolated from *Citrus limon* leaves and characterized by biochemical test, PCR analysis and gene sequencing methods. For biological control, antibiotics, antimicrobial and antagonistic sensitivity were determined by disc diffusion method. The isolated bacteria showed negative results in gram staining test, KOH test, MacConey agar test, Tween 80 hydrolysis test and King's medium B test, while it showed positive results in Catalase test, Simmon citrate, Methyl red and KIA test. In molecular detection, PCR amplified 1400bp DNA fragments of the isolated bacteria. 16S rDNA sequenced results showed approximately 82% similarity with *Xanthomonas citri* strain 3213. Ethanol extract of *Allium sativum* showed highest 14.6±0.4mm diameter of zone of inhibition against the isolated bacteria. Standard Gentamycin showed highest 25.0±0.0mm diameter of zone of inhibition against the isolated bacteria. The soil bacteria *Bacillus subtilis* showed 18.3±0.4mm diameter of zone of inhibition against the isolated *XAC* bacteria. The present techniques would be helpful for biological control of *Citrus limon* canker disease.

Keywords: Citrus canker, *XAC*, Molecular characterization, Biological control

1. Introduction

Citrus limon is one of the major fruit crop all over the world belonging to the family of Rutaceae. *Citrus limon* is widely grown in the tropical, subtropical, and borderline subtropical/temperate areas of the world. Citrus fruits and trees have a tremendous economic, social and cultural impact in our society. It is delectable, juicy and seedless fruit having great

nutritional significance (khan et al., 1992). *Citrus limon* fruits contain a variety of vitamins, minerals, fiber, and phytochemicals such as carotenoids, flavonoids, and limonoids, which appear to have biological activities and health benefits. Citrus fruits have antioxidant and antimutagenic properties and positive associations with bone, cardiovascular, and immune system of health (Codoñer-Franch and Valls-Bellés, 2010). In Bangladesh, about 91% people are suffering from the deficiency of vitamin-C (Haque, 2005). Citrus fruits serve as a potential source of vitamins, especially vitamin-C and minerals (Alam et al., 2003). Different types of citrus species like, *Citrus limon, Citrus aurantifolia* and *Citrus grandis* are commonly cultivated in Bangladesh.

Citrus limon plants are attacked by a number of diseases like citrus canker, gummosis, citrus decline, citrus tristeza virus, greening etc. Citrus canker is caused by the bacterium *XAC* that is probably the worst enemy to citrus plants (Sahi et al., 2007). The bacterium, *Xanthomonas*, causes different symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits (Burning and Gabriel, 2003; Schubert and Sun, 2003; Graham et al., 2004; Zekri et al., 2004). As such, disease severity on susceptible variety results in defoliation, dieback, premature fruit drop and blemished fruit, which consequently decrease fruit production and market value (Zekri et al., 2004). Recently, the uses of synthetic fertilizers and pesticides have resulted in deterioration of soil structure and increase in soil-borne phytopathogens (Bailey and Lazarovits, 2003). Uses of pesticides and copper compounds to control bacterial diseases have a harmful effect on human and the emergence of multidrug resistant pathogens. To cope with this problem biological control have been applied. Biocontrol techniques are involving the use of naturally occurring nonpathogenic microorganisms that are able to reduce the activity of plant pathogens and thereby suppress diseases. Antagonistic microorganisms can compete with the pathogen for nutrients, inhibit pathogen multiplication by secreting antibiotics or toxins, or reduce pathogen population through hyper-parasitisms. Many bio-control agents were isolated by screening of the large number of soil or plant-associated microorganisms for antagonism against phyto-pathogens *in- vitro* or *in* vivo (Berg et al., 2001). Therefore, detection of responsible pathogens and their proper management of this disease are needed to reduce the yield loss as well as economic loss. In Bangladesh, there are few work have done regarding biochemical characterization, molecular identification and biological control of citrus canker of *Citrus limon.*

The present investigation was designed to isolate and characterize the isolated bacteria through different types of biochemical and molecular techniques. *In-vitro* antibiotic sensitivity, antimicrobial screening and antagonistic activities were performed against the isolated bacteria for biological control management.

2. Materials and Methods

2.1 Plant materials

In the present investigation, canker infected *Citrus limon* plant leaves (Fig. 1A.) were collected from the Rajshahi University Campus, Rajshahi, and were identified by Bangladesh Council of Scientific and Industrial Research (BCSIR), Binodpur, Rajshahi, Bangladesh. Canker disease infected leaves were used as plant material.

2.2 Isolation of causal organism

Disease infected leaves and fruits were disinfected using a dilute sodium hypochlorite solution (10%) and rinsed thoroughly. The infected area was cut and placed on Luria and Bertani (LB) liquid media (Bertani, 1951) and incubated at 37^{o}C for 16 h. After the bacteria have grown into LB liquid medium, bacteria were streak onto a solid nutrient agar plates and incubated at 37^{o}C for 12 h. Single creamy yellow colony was picked by wire loop and streaked on another media plate for pure culture.

2.3 Biochemical test of the isolated bacteria

Several morphological and biochemical tests were conducted to characterize the isolated bacteria. All the morphological, physiological and biochemical including Gram staining test were performed by standard microbiological technique according to Bergey et al. (1935). Potassium hydroxide test (KOH) test was used to differentiate gram negative bacteria. For the KOH solubility test, bacteria were aseptically removed from petridishes with an inoculating wire loop, mixed with 3% KOH solution on a clean slide for 1min and observed for formation of a thread-like mass. KOH test was performed according to Halebian et al. (1981) method. Sulphide-Indole-Motility (SIM) test medium is recommended for the differentiation of gram-negative enteric bacilli on the basis of sulfide production, indole formation and motility. Using a needle, strains were introduced into test tubes containing SIM medium and were incubated at room temperature until the growth was evident according to Kirsop and Doyle (1991). Turbidity away from the line of inoculation was a positive indicator of motility. Indole production from tryptophan was tested using the method of Clarke and Cowan (1952). The catalase production was determined by adding the H_20_2 (3%

v/v) to a bacterial culture and the presence of catalase indicated by bubbles of free oxygen gas (Cappuccino and Sherman, 2001). The citrate test was done according to Simmons (1926) method, to determine whether the isolated bacteria better suited to aerobic or anaerobic environments, using Simmons citrate agar medium. For Kovac oxidase test, a loopful inoculum from pure culture was picked up by sterilized platinum loop. The inoculum was smeared over the area of filter paper containing oxidize reagent to develop deep blue or purple color within ten seconds indicating the oxidation of the reagent (Kovac, 1956). Bacteria were inoculated into the MR broth medium in test tubes for methyl red test. Test tubes were incubated at 37^0C for 16 hours. After incubation 2-3 drops of Methyl red reagent was added. MacConkey agar test was performed for isolation of gram negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria (MacConkey, 1905). MacConkey agar was inoculated with bacteria using streak plate technique and incubated the test tubes at 37 ^{0}C for 16 hours. KIA medium was prepared by using usable amount in 1 liter distilled water and sterilized at 121ºC for 20 minutes (Taylor and Silliker, 1958). The tube cooled in a slanted position to obtain a butt of 1.5-2.0 cm. The 24 hours of old culture of each isolate were stabbing the butt and streaking the surface of the tube. Tubes were incubated aerobically at 37ºC for 16 h. Production of H_2S was observed after 7 day incubation at 28^oC in triple iron salts agar. The composition of King's medium B was added when temperature become lowered and autoclaved. Bacterial culture was streaked on the KB medium and incubated at 30ºC for four days. The plates were then subjected to observe under UV-pro light for fluorescent. Positive control was maintained by inoculating unknown *Pseudomonas* culture on KB medium (King et al., 1954). Tween 80 hydrolysis test was done using purified *XAC* cultures according to Sierra (1957) method. Tween 80 was added to the molten media. The media was poured into petridish. Each isolate of *XAC* was streaked on a medium. The culture was incubated at 27ºC for seven days to observe opaque milky precipitate/milky crystal formation. For biochemical test, different chemicals were collected from Oxide Ltd. Basingstoke, Hampshire, England.

2.4 DNA purification

For genomic DNA purification, the isolated bacterial single colony was cultured in LB broth medium at 37ºC for overnight. The culture was centrifuge and the liquid was discarded. The total genomic DNA were isolated from bacterial mass by heat lysis and selective precipitation of cell debris and polysaccharides with CTAB (Cetyltrimethyl

Ammonium-Bromide), and the procedure was maintained as similar as described by Ausbel et al. (1994) with slight modification in the incubation period and amount of chemicals which was used. The DNA was re-suspended in TE buffer and quantified using a spectrophotometer then electrophoresed on 1% agar gel by comparison with DNA samples of known concentration.

2.5 PCR analysis

The amplification of 16S rRNA gene from the isolated DNA was done by PCR reaction in a thermo cycler (Nyx, Technic, Inc., USA), using the primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1391R (5′-GACGGCGGTGTGTRCA-3′). PCR was performed in volumes of 25ml, containing nuclease free ddH_2O 15µL, dNTP mix 1.0µL, forward primer 1.0µL, reverse primer 1.0µL, DNA template 1.5µL, $MgCl_2$ 2.5µL, *Taq* buffer B 2.5µL and *Taq* polymerase (Takara, Japan) 0.5µL. The procedure was as following: initial denaturation at 95ºC for 5min; 35 cycles of denaturation for 40s at 95ºC, annealing for 1min at 65ºC, and extension for 2min at 72ºC; the final extension at 72ºC for 10 min, followed by cooling to 4ºC until the sample was recovered. Gel electrophoresis was used to visualize the PCR products lengths. 0.5x TBE buffer was used in agar gel and visualized under a UV trans-illuminator.

2.6 Sequencing and phylogenetic analysis of *XAC* 16S rRNA

Total genomic DNA was extracted from the *XAC* isolates using CTAB (Cetyltrimethyl Ammonium Bromide) method described by Ausbel et al. (1994). The 16S rRNA gene was amplified by PCR using the primers 27F and 1391R. DNA was purified by agar gel electrophoresis method using AccuPrep® Gel Purification, Bioneer kits. The purified products were sequenced in sequencing service laboratory, National Institute of Biotechnology (NIB), Bangladesh, using *XAC* bacterial gene specific primers 27F(5′-AGAGTTTGATCCTGGCTC-3′) and 1391R (5′-GACGGCGGTGTGTRCA-3′). All sequences were compared with their respective type strains using the BLASTN program in the GenBank nucleotide database (http://www.ncbi. nlm.nih.gov/BLAST). Phylogenetic trees were constructed in GEB, RU, DNA Lab., Bangladesh.

2.7 Screening of antimicrobial activity

In the present investigation, screening of antimicrobial activities was determined by moderate disc diffusion method (Hasan and sikdar, 2016). Six different plants extracts

namely, *Piper betle, Carica papaya, Azadirachta indica, Averrhoa carambola, Allium sativum,* and *Zingiber officinale* were used for antimicrobial screening against the isolated bacteria. The collected plants materials were washed air-dried and grinding a fine powder by a grinding machine. 20g of dried powder was soaked in 200ml ethanol in round bottom flask at room temperature for seven days with occasional shaking. The extracts were filtered by cotton white cloth followed by Whatman No.41 (Whatman, UK) filter paper. The filtrate was evaporated at 45^0C to dryness and the dried substance was kept in sterile bottle under refrigerated condition until use. An inoculum suspension was swabbed uniformly to solidified 20mL LB agar media for bacteria and the inoculum was allowed to dry for 5 minutes. 6mm diameter paperdiscs were used. Aliquot of 10, 20, 40µL from each plant crude extract (500µgmL^{-1}) was added into each disc on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37^oC for 24 h. The resulting inhibition zones were measured in millimeters (mm).

2.8 Antibiotic sensitivity test

Antibiotic sensitivity test was done against isolated bacteria according to Bauer et al. (1966). The isolated bacterial strain was inoculated in nutrient broths medium and incubated at 37^0C for overnight with continuous shaking at 150 rpm. LB agar medium was prepared in sterile conical flasks, cooled down to 40-50^0C and placed in 90mm petridish. 20mL of liquid medium was poured in each petridish and left the airflow cabinet for solidification. Using a loop the colony was streaked on LB agar culture plates. Commercially available and frequently prescribed standard antibiotics namely, Gentamycin, Doxycycline, Erythromycin, Tetracycline, Clarithromycin, Chloramphenicol, Penicillin, Amoxycillin, Neomycin, Cefotaxime, Kanamycin and Azithromyin were used to test antibiotic sensitivity of the isolated bacteria. Antibiotic discs were placed centrally on the respective plates and incubate at 37^0C for overnight. After incubation, the zone of inhibition was observed and measured manually with the help of mm scale**.**

2.9 Antagonistic activity of *Bacillus subtilis* against *XAC*

Agar well diffusion method (Vaglas, 2007) was used to evaluate the antagonistic activity of *Bacillus subtilis* against *XAC* bacteria. Previously isolated and identified *Bacillus subtilis* was collected as antagonistic microorganism from Professor Joarder DNA and Chromosome Research Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh. A hole with a diameter of 6mm was punched aseptically

with a sterile cork borer or a tip. The agar plate surface was inoculated by spreading a volume of *XAC* strain (optical density at 620 nm) grown for 16 h and re-suspended in sterile water with four different doses *viz*., 10µl, 20µl, 40µl and 50 µl/well of *Bacillus subtilis* bacteria solution against the isolated plant pathogen *XAC*. Then, agar plates were incubated at 37ºC for 16 h. After incubation, the zone of inhibition was observed and measured manually with the help of mm scale.

3.0 Statistical analysis

All experiments were performed at least three times. Data represent the means and standard errors (Mean±SE) from at least three replicates of a representative experiment. The data were calculated using Microsoft Excel 2010 software.

3. Results

3.1 Isolation of bacteria

From liquid culture, subculture was done by streaking onto the LB agar medium in 90mm petridish. Visual observation was identified the colony morphology of the bacteria. The isolated colonies of citrus canker infected leaves were found to be yellow in color and small to medium in size; smooth, convex and mucoid (**Fig. 1B**).

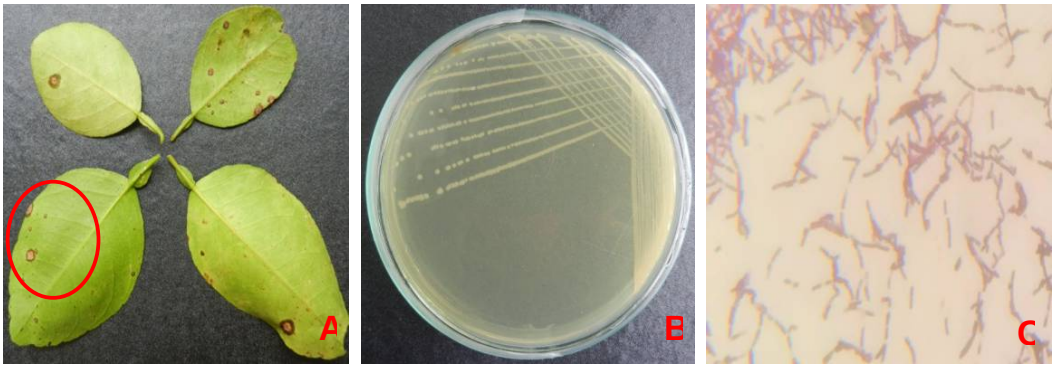

Fig.1- Showing the plant sample, causal organism isolation and gram staining (A) Citrus canker disease, (B) Isolated bacterial colonies, (C) Gram negative bacteria

3.2 Physiological and biochemical characteristic of *XAC* bacterium

In gram staining reaction, the isolated bacteria were found to be pinkish in color, small and rod shaped (**Fig. 1C**). In different biochemical test, the isolates were thread like viscous appearance in KOH solubility test. Motility of the isolated bacteria was found in SIM medium where no H_2S and indole ring was formed after inoculation of isolated bacteria. Bubbles were produced in catalase test and it indicated that isolated bacteria can breakdown

H_2O_2 and catalase positive. Deep blue color was formed in citrate test and no purple color was formed in oxidase test. A red ring was formed after inoculation of bacteria in methyl red test. Isolated bacteria grown well on MacConkey agar and produced pink colony and it was lactose fermenting. No H_2S was produced in Kligler Iron agar test, both the butt and slant turned into yellow color. No fluorescent color was found during fluorescent pigment test. Milky white precipitate was appeared in Tween 80 Hydrolysis test. The results of morphological, physiological and biochemical test of isolated bacteria are presented in Table 1.

Table 1-Effect of the isolated bacteria in morphological and different biochemical test media

Test Name	Reaction	Appearance	Remarks
Gram staining	-(ve)	Rod shaped, small, pink color	Pink color confirming gram -(ve) bacteria
KOH test	+(ve)	Thread like viscous appearance	Thread like appearance confirmed KOH +(ve) bacteria
SIM test	+(ve)	No H_2S was appeared, Motility was observed	Isolated bacteria was motile
Catalase test	+(ve)	Bubbles were produced	Bacteria contained catalase enzyme
Oxidase test	-(ve)	No purple color was produced	Produce no purple color
MR test	+(ve)	Red ring was formed	Produced red ring
MacConkey Agar test	+(ve)	Bacteria grew well and colony retained pink color	Gram negative was lactose fermenting
KIA test	+(ve)	No H_2S produced, yellow butt and slant	Isolated bacteria fermented sugars
Fluorescent Pigment	-(ve)	No fluorescent of any color was produced	Produced no fluorescent color
Tween 80 Hydrolysis	+(ve)	White precipitate was appeared	Hydrolyzed the tween 80

Notes: KOH=Potassium Hydroxide Test, SIM=Sulphide-Indole-Motility, MR= Methyl Red, KIA= Kligler Iron Agar, + (ve)= Positive, - (ve)= Negative

3.3 Molecular characterization of *XAC*

The pathogen was detected by PCR in a naturally infected citrus plant. As expected, the pathogen was detected by PCR assay from infected citrus leaves. The purified genomic DNA was quantified using a spectrophotometer and electrophoresed on 1% agar gel which show clear band in lane 1, 2 and 3 comparisons with DNA samples of known concentration (**Fig. 2A**). In PCR analysis, the DNA amplified PCR products showed a 1400bp length clear band in lane 2 and 3 while lane 1 did not show any band in agar gel electrophoresis (**Fig. 2B**).

For phylogenetic analysis, partial 16S rRNA gene sequences were PCR amplified from *XAC* strain, which was classified in the same class as the *Xanthomonas citri* strain 3213 (**Fig. 2C**). The sequence identity of the 16S rRNA sequences from the citrus canker bacteria *XAC* strain was approximately 82% similarity with *Xanthomonas citri* strain 3213.

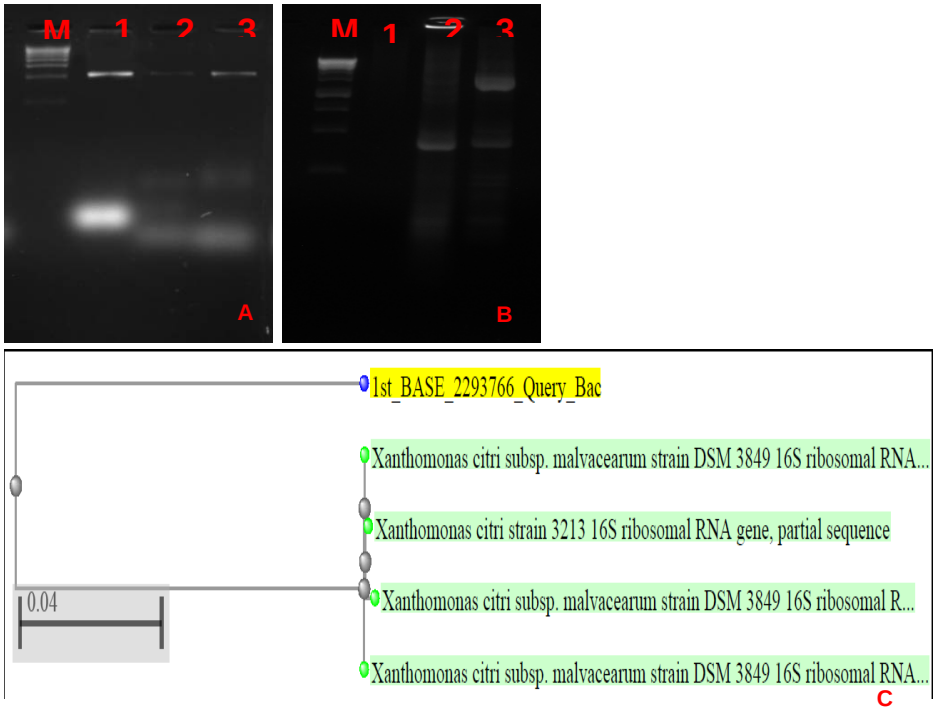

Fig. 2-Genomic DNA, PCR analysis and sequencing of *Xanthomonas axonopodis* by agarose gel electrophoresis (A) Total genomic DNA, (B) PCR amplification, (C) Phylogenetic tree of *Xanthomonas* species based on 16S rRNA gene sequences, M= size marker 1 kb DNA ladder, 1,2,3=clones of *XAC*

3.4 Screening of antimicrobial activity

For antibacterial activity determination, six different medicinal plant extracts were used against the isolated bacteria. The highest antibacterial activity with 14.6±0.4mm diameter of zone of inhibition was showed by *Allium sativum* ethanol extract (**Fig. 3B**) at 20mg/disc concentration followed by 12.5±0.4mm diameter of zone of inhibition by same extract at10mg/disc concentration against the isolated bacteria. On the left hand, ethanol extract of *Piper betle, Carica papaya,* and *Azadiracta indica* showed the lowest 6.0±0.0mm diameter of zone of inhibition at the concentration of 5mg/disc against the isolated bacteria. The data are represented in **Table 2**.

Table 2-Antimicrobial activity of some plant extracts against the isolated bacteria

Name of the plants	DOZOI (in mm) in different doses (in mg), (M±SE)			Sensitivity pattern
	5mg/disc	10mg/disc	20mg/disc	
Piper betle	6.0±0.0	7.0±0.0	8.3±0.6	Resistant
Carica papaya	6.0±0.0	7.3±0.4	8.0±0.0	Resistant
Azadirachta indica	6.0±0.0	7.3±0.4	8.6±0.4	Resistant
Averrhoa carambola	6.6±0.4	7.6±0.4	7.0±0.9	Resistant
Allium sativum	10.3±0.4	12.5±0.4	14.6±0.4	Intermediate
Zingiber officinale	7.0±0.6	7.0±0.0	7.5±0.5	Resistant

Note: Resistant =<10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm; DOZOI=Diameter of zone of inhibition, M±SE= mean and standard error

3.5 Antibiotic susceptibility assay

In antibiotic susceptibility assay, different types of standard antibiotic discs were used against the isolated bacteria. The highest antibiotic activity with 25.0±0.0mm diameter of zone of inhibition was showed by Gentamycin (**Fig. 3A**) at 10µg/disc concentration following by Cefotaxime with 22.3±0.4mm diameter of zone of inhibition at 30µg/disc concentration against isolated bacteria. On the other hands, penicillin and Azithromycin showed the lowest 10.0±0.0mm diameter of zone of inhibition at 10µg/disc and 15µg/disc concentration respectively. The sensitivity patterns of antibiotics are represented in **Table 3**.

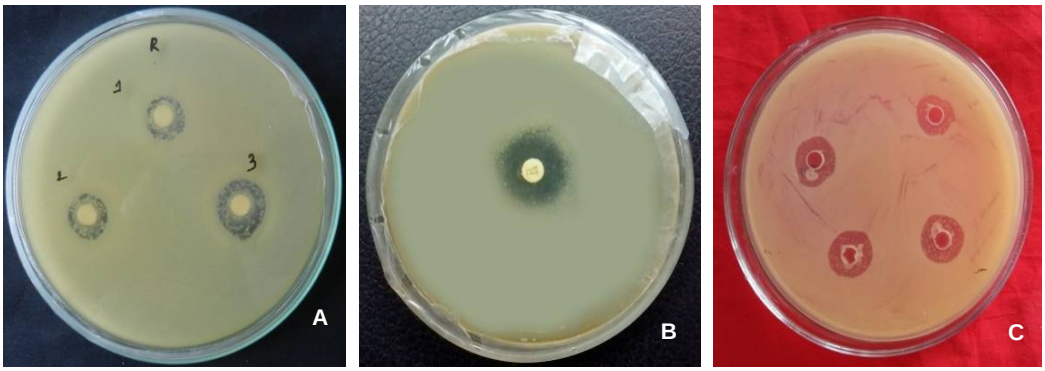

Fig. 3-Showing the highest antibiotic, antibacterial and antagonistic activities against *Xanthomonas axonopodis* pv. *citri (*A) *Allium sativum* extract, (B) Gentamycin, (C) *Bacillus subtilis*

Table 3 -Effect of some standard antibiotics against isolated bacteria

Name of antibiotic	Disc potency (µg/disc)	DOZOI (in mm, M±SE)	Sensitivity pattern
Tetracycline	30	19.2±1.2	Susceptible
Doxycycline	30	21.0±0.4	Susceptible
Erythromycin	15	17.5±0.4	Susceptible
Gentamycin	10	25.0±0.0	Susceptible
Clarithromycin	15	20.5 ±0.4	Susceptible
Chloramphenicol	30	18.9±0.6	Susceptible
Penicillin	10	10.0±0.0	Resistant
Amoxicillin	10	10.6±0.4	Intermediate
Neomycin	30	19.3±0.0	Susceptible
Kanamycin	30	19.2±1.2	Susceptible
Cefotaxime	30	22.3±0.4	Susceptible
Azithromycin	15	10.0±0.0	Resistant

Note: Resistant =<10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm, DOZOI=Diameter of zone of inhibition, M±SE= mean and standard error

3.6 *Bacillus subtilis* exhibited antagonistic activity against the pathogen of citrus canker

In the present study, the antagonistic activity of soil bacteria *Bacillus subtilis* was determined against the isolated *XAC* bacteria. *Bacillus subtilis* (**Fig. 3C**) showed highest 18.3±0.4mm zone of inhibition at 50µl/disc concentration followed by 16.9±0.6mm at 40µl/disc concentration. On the other hands, the lowest diameter of zone of inhibition was found to be 13.0±0.0mm at 10µl/disc concentration. The effects of soil borne bacteria are given in **Table 4**.

Table 4 -Antagonistic effect of *Bacillus subtilis* against the *XAC* bacteria

Name of soil bacteria	Diameter of zone of inhibition (in mm), in different doses (in µl), (Mean±SE)			
	10µl/disc	20µl/disc	40µl/disc	50µl/disc
Bacillus subtilis	13.0±0.0	14.6±0.4	16.9±0.6	18.3±0.4

Note: M±SE= Mean and standard error

4. Discussion

There are several types of citrus canker disease caused by various pathovars and variants of the bacterium *Xanthomonas* axonopodis (Graham et al., 2004). According to the symptoms similarity, the separation of these bacterium forms are very difficult depend on host range, cultural, physiological characteristics and bacteriophage sensitivity (Civerolo, 1984). In the present study, bacteria isolated from citrus canker disease are gram negative, small and rod shaped. The similar results were reported by Gottwald et al. (2002). Das (2003) reported that *Xanthomonas axonopodis* isolated from citrus canker infected leaves are rod shaped measuring 1.5-2.0 x 0.5-0.75µm and gram negative. In KOH test thread like slime was found. Our work confirmed the work of Halebian et al. (1981) where all isolates responded positively to loop test by forming a thread when uplifted gently. Moreover, Suslow et al. (1982) performed KOH test to accurately characterized gram negative bacteria of wheat which supports our present findings. Indole ring was formed in SIM medium test and these results confirmed the result of Abhang et al. (2015) who also recorded indole formation for seven isolates of *Xanthomonas axonopodis* from citrus canker infected leaves. The isolated bacteria found catalase positive result. Das (2003) reported that the bacterial cells of *Xanthomonas citri* are positive for catalase test. Different biochemical test such as Oxidase test, Methyl Red test, Kligler Iron agar test, Fluorescent Pigment test and Tween 80 Hydrolysis test characterized the *Xac* as gram negative bacteria. Our results confirmed the work of Verniere et al. (1998) who used several biochemical tests to identify and differentiate different pathotypes of citrus canker bacteria. Similarly, Mohan and Schaad (1987) observed non-fluorescent pigmentation occurred in gram negative bacteria on KB media as compared to *Pseudomomas syringae*. For detection of phytopathogenic bacteria, modern molecular method involving specific amplification of target DNA fragments was used. Based on PCR amplification technique a number of molecular methods have been developed for the identification of *Xanthomonas axonopodis* pv.*citri*. As expected, PCR products showed a 1400bp length clear band in agar gel electrophoresis which is very similar to the original length of *XAC* specific gene. Design of specific primers and DNA probes for identification and detection has been reported for a number of plant pathogenic bacteria (Hartung et al, 1993; Leite et al, 1994) including xanthomonads. Moreover, consistent results of amplification of a 561bp fragment from *Xanthomonas axonopodis* pv. *citri* by XACF and XACR were also obtained by Parka et al. (2006). In sequencing and phylogenetic analysis, the sequence identity of the 16S rRNA sequences from the citrus canker bacteria *XAC* strain

was approximately 82% similarity comparison with *Xanthomonas citri* strain 3213.Though we detect the *XAC* gene by PCR and sequencing but still we don't know how this gene is involved in canker disease production.

In screening of antibacterial assay, the highest antibacterial activity was found to be 14.6±0.4mm diameter of zone of inhibition by *Allium sativum* ethanol extract at 20mg/disc concentration, while ethanol extract of *Piper betle*, *Carica papaya*, and *Azadiracta indica* showed lowest 6.0±0.0mm diameter of zone of inhibition at the concentration of 5mg/disc against the isolated *XAC* bacteria. The antibacterial activity of different plant extracts were increased depended on their concentrations. Though *Allium sativum* showed highest 14.6±0.4mm zone of inhibition against the isolated bacteria but it was intermediate resistant to *XAC* bacteria. Praba and Kumaresan (2014) found minimum zone of inhibition by *Allium sativum* extract against *Pseudomonas aeruginosa* at 50% concentration. This findings also confirmed by Whitemore and Naidu (2000) who found inhibitory action of garlic against gram positive and gram negative bacteria. These results support our present findings. In contrast, Hussain et al. (2010) reported that extracted powdered leaf and pod material of *Caesalpinia coriaria* (Jacq) Wild with water and successively amid different solvents such as petroleum ether, benzene, chloroform, methanol and ethanol suggested as a potential plant for the management of phytopathogenic *Xanthomonas* pathovars of tomato, French bean and cotton. In antibiotic susceptibility assay, highest antibiotic activity was 25.0±0.0mm diameter of zone of inhibition by Gentamycin against the *XAC* bacteria. Abhang et al. (2015) recorded 11.0mm, 35.0mm and 37.33mm zone of inhibition for *Xanthomonas axonopodis* pv. *citri* isolated from canker disease infected citrus plant which confirmed our present findings. Moreover, Islam et al. (2014) found 22.2%, 77.7% and 0.0% susceptibility for Gentamycin, Chloramphenicol and Cefotaxime respectively against all the isolates of *Xanthomonas axonopodi*s pv. *citri*, causal organism of citrus canker. These results also support our present findings. In the present study, the antagonistic activity of soil bacteria *Bacillus subtilis* was determined against the isolated *XAC* bacteria. *Bacillus subtilis* showed highest 18.3±0.4mm zone of inhibition at 50µl/disc concentration while the lowest diameter of zone of inhibition was found to be 13.0±0.0mm at 10µl/disc concentration. So, the antagonistic effect of *Bacillus subtilis* was depended on their doses. Huang et al. (2012) found antagonistic activity of *Bacillus sp.* against citrus bacterial canker. Moore et al. (2013) reported the similar results for newly isolated soil borne *Bacillus* strains against the *Salmonella*, *Shigella* and

Staphylococcus strains of food bacteria. Our works confirmed this work regarding the antagonistic activity.

5. Conclusion

From our findings, it can be concluded that bacteria isolated from citrus canker disease is gram negative and responsed positive and negative reaction to different biochemical tests. 16S rDNA sequenced results of isolated bacteria showed approximately 82% similarity with *Xanthomonas citri* strain 3213. Ethanol extract of *Allium sativum* and soil bacteria *Bacillus subtilis* showed highest antagonism against isolated bacteria. Therefore our findings regarding antagonism of ethanol extract of *Allium sativum* and soil bacteria *Bacillus subtilis* against isolated bacteria will be helpful for biological control of citrus canker disease.

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Authors' contribution: DS, MFH, SMZH, ZFZ, MFH, MAI and BS designed the experiments, developed the methodology and prepared the manuscript. DS, MFH, SMZH, ZFZ, MAI and BS collected the data and carried out analysis. MRA assisted with data analysis and manuscript preparation. DS, MFH, SMZH and have equal contribution for this research article.

Conflict of interest

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

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