IDENTIFICATION AND CHARACTERIZATION OF CAUSAL PATHOGEN OF CITRUS CANKER IN SYLHET REGION

M. K. M. Rahman[1], A. Muqit[2], M. M. Islam[2] and M. U. Salam[3]

[1] PhD Fellow, [2] Professor, Dept. Plant Pathology and Seed Science, Sylhet Agricultural University, Sylhet-3100, [3] Senior Agriculture Sector Development Expert, FAO, UN, FAO Representative, Bangladesh.

*Corresponding author, email: muqit.ppath@sau.ac.bd

ABSTRACT

Rahman, M. K. M., Muqit, A., Islam, M. M. and Salam, M. U. 2021. Identification and Characterization of Causal Pathogen of Citrus Canker in Sylhet Region. Bangladesh J. Plant Pathol. 37(1&2):77-86

Isolation, identification and characterization of citrus canker causing pathogen collected from the orchards of Sreemangal, Juri, Beanibazar, Goainghat and Jaintapur upazila of Sylhet region were conducted during March to June 2019. Several morphological, biochemical and molecular tests conducted on 18 isolates from 11 citrus species confirmed the pathogen as *Xanthomonas axonopodis* pv. *citri.* The isolated bacteria were gram negative in methyl red, indole, nitrate, oxidase, urease, mannitol fermentation tests and gram positive in catalase, KOH, starch hydrolysis and TSI tests. The growth of the isolate was negative at 41 C temperature and in 4% NaCl. The primer set 16s rRNA F-27: AGA GTT TGA TCM TGG CTC AG and R-1492: CGG TTA CCT TGT TAC GAC TT was used for conventional PCR. Five representative PCR products from Mandarin, Malta, Jara lebu, Seedless lebu and Kagzi lebu, after amplification with universal primers for bacteria isolation 16s rRNA gene of F-27 and R-1492 were sequenced and compared with sequences of other *X. axonopodis* pv. *citri* available in NCBI database using Basic Local Alignment Search Tool (BLAST) algorithm. Blast homology showed 98-99% sequence identity with the corresponding nucleotide sequences of ribosomal protein genes of *X. axonopodis* pv. *citri* strains found in USA GenBank (E3CP003778.1, B2CP009013.1, D2DQ490311.1) China GenBank (E2CP011827.2, A2 CP023661.1, C1FJ600360.1), Thailand GenBank (E1HQ875739.1), Brazil GenBank (B3 AE008923.1) and Argentina GenBank (D1KY229747.1). Fresh extract of *Allium sativum* at 70µl showed maximum (15.52±0.22 mm) inhibition zone against the isolated bacteria. The isolated bacterium was highly susceptible (89.5%) to ciprofloxacin.

Key word: Identification, characterization, citrus, canker

INTRODUCTION

Citrus viz. mandarin, malta, pomelo, lemon, lime, etc. is a good source of vitamin-c and are much common and popular fruits in Bangladesh. The internal juicy part of all citrus fruit is edible excepting Jara lemon (*Citrus medica*) in which the edible part is outer rind of the fruit., Area and production of citrus is increasing due to its growing demand. The total citrus production was around 21 thousand metric tons during 2009-2010 (BBS 2010) which boosted up to 142 thousand metric tons during 2019-20. Sylhet region is well known for quality citrus production because of favorable climatic and soil conditions. Around 15% of the total citrus production (20,636 tons) is produced in Sylhet region. Citrus suffers from many diseases of which citrus canker is a major disease prevailing all over citrus fruits produced in Bangladesh (DAE 2014). The disease is caused by *Xanthomonas axonopodis* pv. *citri* in all growth stages of citrus plant with characteristic symptoms on leaves, shoots, twigs and fruits (CABI 2006). In susceptible varieties the disease causes defoliation, dieback, premature fruit drop and blemished fruits which consequently reduce fruit production and market value. Though the pathogen was reported much earlier but its characterization especially molecular detection is yet to be done. The DNA-based methods proved to be reliable and accurate techniques and have been mostly used to

improve the identification and detection of plant pathogens due to their accuracy, specificity and sensitivity (Hartung *et al*. 1993, 1996, Cubero and Graham 2002, Mavrodieva *et al*. 2004, Coletta-Filho *et al*. 2006, Golmohammadi *et al*. 2007). Therefore, the present study was conducted to isolate and identify the citrus canker pathogen from various citrus species and also to make biochemical and molecular characterization of the pathogen.

MATERIALS AND METHODS

The experiment was conducted at the Sylhet Agricultural University (SAU), Sylhet. The infected plant parts like leaves, stem, and fruits of citrus were collected during March to June 2019 from the Sylhet Agricultural University campus and also from the villages of Jaintapur, Goainghat, Beanibazar, Juri, and Sreemangal Upazila under Sylhet region (Table 1). The infected plant parts were washed thoroughly with sterile distilled water and ooze tests of leaf, stem and fruits was done to confirm the presence of bacterium. The biochemical tests were conducted in the veterinary central laboratory of Sylhet Agricultural University, Sylhet.

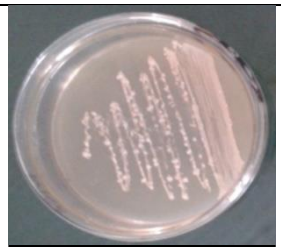
Enumeration viable count: Plant **s**ample (25g) was aseptically macerated in 225 ml of sterile distilled water and homogenized in a sterile blender to make 1:10 dilution. Later on a series of 10 fold dilutions ranging from 10^{-2} to 10^{-9} were prepared according to the recommendation of international standardization (ISO 1995). One liter of nutrient agar was prepared with 3g beef extract (Difco), 5g peptone (Difco) and 15g agar. Similarly, nutrient broth (1L) was prepared with 3g beef extract (Difco) and 5g peptone (Difco) without agar. Then 18g of nutrient agar (NA) medium was taken in a conical flask (1000 ml) and adjusted the volume up to 1000 ml by adding distilled water. One ml of each ten-fold dilution was transferred and spread on NA plate using a sterile pipette for each dilution. The plates were incubated at 28 C for 24 hours and single colonies were counted and averaged by multiplying with dilution factor according to ISO (1995). The count was expressed as the number of colony forming unit per gram (cfu/gm) or per ml (cfu/ml) of sample.

Table 1. Samples of *Xanthomonas axonopodis* pv. *citri (Xac)* collected from different citrus growing areas of greater Sylhet region

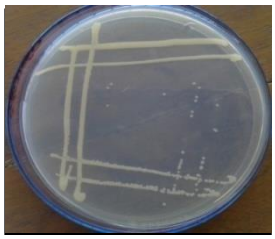
Locations	Name of germplasm	Name of variety	Plant parts used	Sample name
Sreemangal	Malta	BARI Malta-1	Leaf	Xac -01
Sreemangal	Seedless lebu	Local	Leaf	Xac -02
Sreemangal	Jaralebu	Local	Leaf	Xac -03
Sreemangal	Colombo lebu	Local	fruit	Xac -04
Sreemangal	Pomello	Local	fruit	Xac -05
Sreemangal	Kagzilebu	Local	Stem	Xac -06
Juri	Mandarin	Local	Leaf	Xac -07
Beanibazar	Mandarin	Local	Leaf	Xac -08
Gowainghat	Jaralebu	Local	Leaf	Xac -09
Gowainghat	Jaralebu	Local	Leaf	Xac -10
Gowainghat	Ada jamir	Local	Leaf	Xac -11
Gowainghat	Malta	BARI Malta-1	Leaf	Xac -12
Jaintapur	Jaralebu	Local	Leaf	Xac -13
Jaintapur	Seedless	Local	Stem	Xac -14
Jaintapur	Satkora	Local	Stem	Xac -15
Jaintapur	Sorbotilebu	Local	Leaf	Xac -16
Jaintapur	Elachilebu	Local	Leaf	Xac -17
Jaintapur	Kagzilebu	Local	Leaf	Xac -18

Preparation of yeast peptone glucose agar (YPGA) media: Yeast peptone glucose agar medium was prepared with 5g yeast extract, 5g bactopeptone, 10g glucose and 20g agar in one liter of distilled water.

Isolation and purification of pathogen: Infected plant tissues were macerated in Petri plates containing 0.5 - 1.0 ml of 0.85% NaCl at pH 7. The diseased plant parts were then transferred separately into a few drops of sterile water on a sterilized glass slide under aseptic conditions. The plant parts were left for one minute to allow bacterial ooze to come out in water. An aliquot of the extract was streaked on yeast peptone glucose agar (YPGA) medium. Three Petri plates containing YPGA medium were streaked at a time without recharging the wire loop. The inoculated plates were incubated at 25±3 C for 48 h in inverted position. The colony growth was observed after 3-5 days of incubation and single colony of bacteria was streaked in YPGA media in petri dish and incubated at 25±3 C for 48 h. The single colony was recorded after 3-5 days of incubation as pale yellow colored bacterial colonies (Plate 1). The purified bacterial colonies were streaked on YPGA slants and stored at 5 C in refrigerator for further studies. Pathogenicity test was conducted on two years old Jara lebu (*Citrus medica)* seedling raised in 25 cm earthen pots and inoculated with a syringe without the needle (Plate 2).



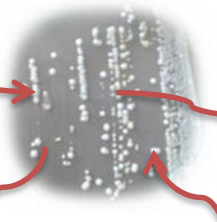
Pure Culture of *Xac*



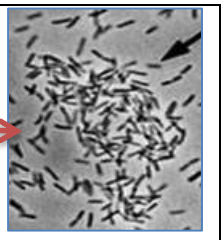
Single colony of *Xac*

Plate 1. Isolation and pure culture of *Xac*

Canker lesion on citrus leaf



Pure culture grown from diseased leaf



Rod shaped bacteria under microscope

Inoculated citrus plant

New lesion on leaf

Plate 2. Steps of inoculation procedure for pathogenicity test

Identification and characterization of pathogen: The colony morphology, gram reaction, growth at different temperatures, growth at 4% NaCl, fluorescence under UV, KOH and starch hydrolysis tests were performed to identify the pathogen. Biochemical tests like triple sugar iron (TSI), indole, methyl red, catalase, nitrate, oxidase, urease, gelatin liquefaction, mannitol fermentation and sucrose fermentation were also conducted. Besides, antibiotic susceptibility, herbal sensitivity and molecular tests were done to characterize the pathogen. The Promega DNA extraction kit was used for molecular identification. The primers set F-27 and R-1492 with sequence (5'3') of AGA GTT TGA TCM TGG CTC AG and CGG TTA CCT TGT TAC GAC TT possessing Amplicon (bp) of 1465 were used for amplification. The DNA sequences were compared with other *Xanthomonas axonopodis* species available in NCBI database using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences. Multiple sequence alignments were performed using aligner tool. The phylogenetic tree was constructed based on 16 srRNA protein gene and compared with previously reported isolates from China, India, Brazil, Argentina, USA and Thailand using Clustal Omega and MEGA (version 5.22).

Antibiotic sensitivity test: Sensitivity of isolates to different antibacterial agents was determined *in-vitro* by modified disk diffusion test of Kirby-Bauer method (Ordax *et al.* 2009). Susceptibility test was done in Mueller-Hinton agar medium and the discs of tetracycline (TE) 30µg, cefotaxime (CTX) 30µg, streptomycin (S) 10µg, gentamycin (GEN) 10µg,

chloramphenicol (C) 30µg, ciprofloxacin (CIP) 5µg, bacitracin (B) 10µg were used for the test.

Herbal sensitivity test: The diseased free leaves of neem and mahogany plants were collected from local villages and garlic, zinger and onion were collected from local market. The bulb of onion (*Allium cepa*) and garlic (*Allium sativum*), rhizom of ginger (*Zingiber officinale*), leaf of neem (*Azadirachta indica*) and seed of mahogany (*Swietenia macrophylla*) were used for herbal test. Antibacterial activity of aqueous extracts was evaluated against *Xanthomnas axonopodis* pv. *citri* through agar well diffusion method (Irobi and Banso 1994). The plant parts were disinfected with 1% sodium hypochlorite and three washings with distilled water and then dried under shade for 24 h at room temperature. A total of 15g dried plant parts were grinded in 100 ml of distilled water and the homogenate was filtered through muslin cloth and then centrifuged at room temperature for 15 min at 8000 rpm and finally 40µl, 50µl, 70µl of plant extracts were made. Suspensions of freshly cultured *Xac* with 40µl, 50µl, 70µl ml of diluted inoculum were swabbed on the nutrient agar plates. Wells of 5 mm diameter were punched into the agar plates and 40µl, 50µl, 70µl of plant extracts were separately poured into the wells. Herbal extracts inhibitory response was recorded after 24 h of incubation at 28 C. Normal growth was recorded as resistant to extracts and clear zone was recorded as sensitive one and the inhibition zone was measured in millimeter.

RESULTS AND DISCUSSION

Symptoms on leaves: Pinpoint spots surrounded the tissue with a convex surface eventually became white or grayish and finally ruptured as pustules (Plate 3). The spots size ranged from 3 to 11 mm diameter (B) based mainly on the age of host tissue and cultivar. Lesions appear on the underside of leaves at 7 to 10 days after infection and then on upper surface. The young lesions were water soaked, raised or 'pustule' on both surfaces of leaf particularly on the lower leaf which eventually become corky and crater form with a raised margin, sunken center and yellow halo surrounding the lesions (D) and also water-soaked margin around the necrotic tissue (C).

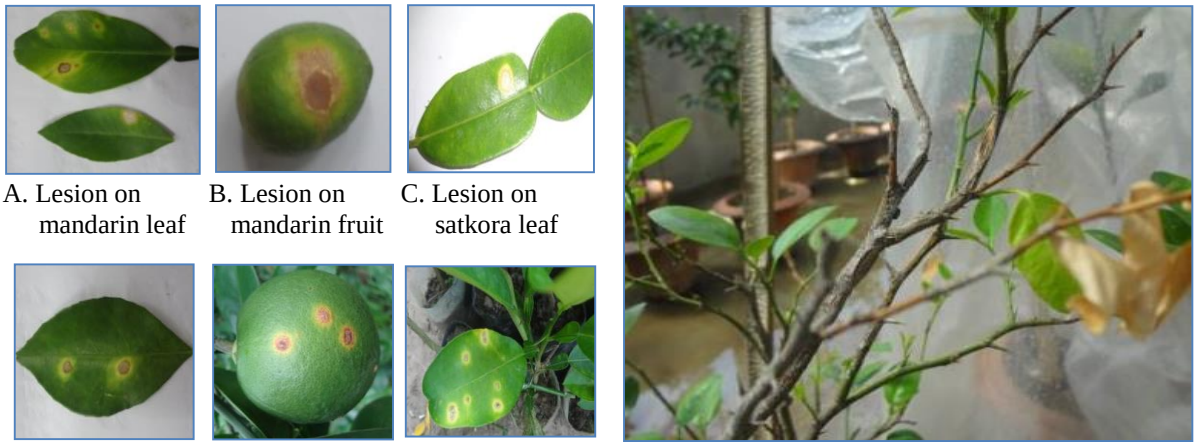
Symptoms on fruit and stem: Citrus canker lesions on fruit (Plate 3. B, E) and stems varied in size and the rind was more susceptible causing premature fruit drop, rind-blemishing fruit and occasionally the lesions penetrated the rind deeply to expose secondary infection. The center of lesions on leaf, stem and twig appeared raised and corky. Lesions on stems remain viable for several seasons and supported long-term survival of the bacteria.

Total Viable count: The highest value of total viable count (TVC) of *Xac* was $288X10^6$ in pomelo leaf collected from Sreemangal while the lowest value ($109X10^3$) was recorded in pomelo leaf sample collected from Jaintapur (Table 2).

Morphological and physiological characterization: The collected samples from the plants showing symptoms of citrus canker were grown in nutrient agar (NA) and then incubated at 28 C for overnight. The *X. axonopodis* colonies grown on NA were pale yellow and mucoid. The colony morphology on the media was round, convex and smooth-edged, mucoid and creamy yellow. After 3-5 days of incubation at 28 C, *Xac* showed gram reaction, KOH and starch hydrolysis tests as negative, positive, and positive reaction, respectively (Table 3).

Biochemical characterization: A series of biochemical tests were done for biochemical characterization of *Xac* isolates (Table-4). All the isolates were positive to TSI test, catalase, gelatin liquefaction and sucrose fermentation test and negative to indole, methyl red nitrate, oxidase, urease and mannitor fermentation tests. The growth of all *Xac* isolates was ceased at 41C and no isolate grew at 4% NaCl except the isolate *Xac2*.

Pathogenicity test: The initial disease symptom was characterized as the development of small water soaked lesions, which later on transformed into a necrotic corky lesion on leaves (Table 5). The symptom developed by isolate 10 within 5 days whereas 9 days was required by the isolate 4, 9, 11, 14, and 18. The spot size was increased up to 13-16 days of inoculation and turned brown yellow to dark coffee brown in color which later on developed into dry necrotic, corky patches on leaves. The re-isolated culture resembled the original mother culture and hence the pathogenicity test was confirmed.



A. Lesion on mandarin leaf B. Lesion on mandarin fruit C. Lesion on satkora leaf

D. Lesion on malta leaf E. Lesion on malta fruit F. Lesion on kagzi leaf G. Lesion on kagzi twig

Plate 3. Canker infected citrus plant showing symptoms on leaf, fruit and twig.

Molecular detection of *Xanthomonas axonopodis* pv. *citri*:

Polymerase chain reaction with primer 16s rRNA was performed on sample extracts obtained from field infected citrus trees at different target locations. The primer set 16s rRNA, F-27: AGA GTT TGA TCM TGG CTC AG and R-1492: CGG TTA CCT TGT TAC GAC was chosen for conventional PCR. The amplified DNA after electrophoresis on 1.2% agarose gel showed a band of about 1465 bp by the extracts from malta plants infected with *Xac* strains of Gowainghat upazila (Figure 1). Five representative PCR products of mandarin, jara lebu, and kagzi lebu, after amplification with universal primers for bacteria isolation 16s rRNA gene of F-27 and R-1492 were sequenced and compared with sequences of other *Xanthomonas axonopodis* pv. *citri* available in the NCBI database using Basic Local Alignment Search Tool (BLAST) algorithm. Blast homology showed 98-99% sequence identity with the corresponding nucleotide sequences of ribosomal protein genes of *Xanthomonas axonopodis* pv. *citri* strains found in USA GenBank (E3CP003778.1, B2CP009013.1, D2DQ490311.1) China GenBank (E2CP011827.2, A2CP023661.1, C1FJ600360.1), Thailand GenBank (E1HQ875739.1), Brazil GenBank (B3AE008923.1) and Argentina GenBank (D1KY229747.1).

Herbal sensitivity test: Significant antibacterial activity was observed in onion (*A. sativum)* and ginger (*Z. officinale)* against the tested pathogens (Table 6). No inhibition zone was observed at 40µl. The *A. sativum* 70µl and *Z. officinale* 70µl showed higher inhibition zone of 18 mm and 9 mm, respectively. The highest inhibition zone was produced by *Allium sativum* 70µl.

Antibiotic sensitivity test: Comparing the zone created by the isolates with the standard zone of inhibition, all isolates were found 100% resistant to bacitracin. The ciprofloxacin was found as the most effective among the tested antibiotics (Table 7 and Plate 4). The *Xac* isolate was susceptible (S) to the antibiotics streptomycin, tetracyclin, chloramphenicol, gentamycin, ciprofloxasin; intermediate (I) to streptomycin, chloramphenicol, cefotaxime, gentamycin, ciprofloxasin and resistant (R) to tetracyclin, bacitracin, cefotaxime (Table 7).

The isolated bacteria from infected citrus of different upazila of Sylhet region showed positive reactions to KOH, starch hydrolysis, triple sugar iron agar, catalase, gelatin liquefy action, sucrose fermentation tests and negative reaction to indole, methyl red, urease, nitrate production, oxidase, and mannitol fermentation. These results were partially agreed with the findings of Ali *et al.* (2017), Islam *et al.* (2014), Abubaker *et al*. (2016), Arshiya *et al.* (2014), Hussain *et al*. (2010), Haider *et al.* (2020), Mubeen *et al.* (2015), who used several biochemical tests to identify and characterize different strains of citrus canker causing bacteria.

In genome sequencing 1st_BASE_3512640_3M_ 27F, 1st_BASE_ 3587168 _ 6_ 27_ F and 1st_ BASE_ 3696378 _ 3_27_F showed better homology and clustered in two clades. In Clade-I, 1st_BASE_ 3512640_3M_27F and 1st_ BASE_ 3587168_ 6_27_F clustered with the reference strains from China and Thailand with a bootstrap value of 99% while another strain 1st_BASE_3696378_3_27_F clustered with USA, Argentina and China strains with a bootstrap value of 71%. All the out-group strains

CP003778.1, CP011827.2, CPAE008923.1, CP009013.1 and CP023661.1 clustered together in clade III with 100% bootstrap value.

Table 2. Total viable colony (TVC) count of different *Xac* samples

Name of fruit tree	*Xac* sample	Total viable count (cfu/gm)
Malta	Xac-01	$208X10^5$
Seedless lebu	Xac -02	$209X10^4$
Colombo lebu	Xac -03	$53X10^5$
Pomello	Xac -04	$288X10^6$
Kagzi lebu	Xac -05	285X106
Mandarin	Xac -06	$97X10^4$
Jara lebu	Xac -09	$107X10^4$

Table 3. Gram reaction KOH and starch hydrolysis tests of *Xac* isolates

Isolates	Gram's reaction	KOH test	Starch hydrolysis
Xac1	-ve	+ve	+ve
Xac2	-ve	+ve	+ve
Xac3	-ve	+ve	+ve
Xac4	-ve	+ve	+ve
Xac5	-ve	+ve	+ve
Xac6	-ve	+ve	+ve
Xac7	-ve	+ve	+ve
Xac8	-ve	+ve	+ve
Xac9	-ve	+ve	+ve
Xac10	-ve	+ve	+ve
Xac11	-ve	+ve	+ve
Xac12	-ve	+ve	+ve
Xac13	-ve	+ve	+ve
Xac14	-ve	+ve	+ve
Xac15	-ve	+ve	+ve
Xac16	-ve	+ve	+ve
Xac17	-ve	+ve	+ve
Xac18	-ve	+ve	+ve

The standard ceprofloxacin showed highest antibiotic activity with 22±0.27 mm zone of inhibition while basitracin showed lowest 4.9±.51 mm inhibition zone against *Xanthomonas axonopodis* pv. *citri.* Mubeen *et al.* (2015) found 1.4cm, 1.6cm, 1.8cm and 2.2cm inhibition zones produced by sinobionic, benzyl penicillin sodium, streptomycin sulphate and kanamycin sulphate, respectively, but no inhibition zone was produced by ampicillin sodium and chloramphenicol sodium against *Xanthomonas axonopodis*. The *Allium sativum* extract showed highest antibacterial activity i.e. 15.52±0.22 mm inhibition zone at 70µl/disc and 11.29±0.29 mm inhibition zone at 50µl/disc. The *Z. officinale* extracts showed the 7.58±0.24 mm inhibition zone at 70µl/disc and 5.41±0.14 mm at 50µl/disc concentration. Besides, *A. cepa, S. macrophylla,* and *A. indica* extract produced no inhibition zone against the tested bacteria. Praba and Kumaresan (2014) found significant antibiotic activity of *A. sativum* extract against different bacterial species at 50% concentration. Hussain *et al.* (2011) reported that guava leaf, beleric myrobalan fruit, pomegranate fruit peel, nut gall fruit and myrobalan wood fruit had a pronounced inhibitory effect against citrus canker bacteria. Neem cake suspension was found very effective in controlling the *Xanthomonas axonopodis* bacteria against citrus canker disease (Das and Singh 2000). Ahmad and Beg (2001) reported similar antimicrobial effect of different plant extract against *Xanthomonas axonopodis* bacteria isolated from citrus canker.

ACKNOWLEDGEMENT

This work was funded by the Bangladesh Bureau of Educational Information and Statistics (BANBEIS) under the project entitled “Citrus canker disease in Sylhet region: epidemiology, quality degradation and management approaches” was duly acknowledged

Table 4. Biochemical test on different chemical properties of *X. axonopodis* pv. *citri* (*Xac*)

Isolates of *Xac*	Growth at 41^{0}C	Growth NaCl	FL under UV	TSI		Indole test	Methyl Red test	Catalase	Nitrate test	Oxidase test	Urease test	Gel	Ma	Su
				Gas	H_2S									
Xac1	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 2	-	+	-	+	+	-	-	+	-	-	-	+	-	+
Xac 3	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 4	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 5	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 6	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 7	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 8	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 9	-	-	-	+	+	-	-	+	+	-	-	+	-	+
Xac 10	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 11	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 12	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 13	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 14	-	-	-	+	+	-	-	+	+	-	-	+	-	+
Xac 15	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 16	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 17	-	-	-	+	+	-	-	+	-	-	-	+	-	+
Xac 18	-	-	-	+	+	-	-	+	-	-	-	+	-	+

Fl= fluorescent, TSI = triple sugar iron, Gel= gelatin liquefaction, Ma= mannitol fermentation, Su= sucrose fermentation.

Table 5. Pathogenicity of *Xanthomon asaxonopodis* pv. *citri* isolates at different days after inoculation

Isolates	Growth of *Xac* at different days after inoculation					Days to symptom development
	5	6	7	8	9	
Xac-01		+				15
Xac-02		+				12
Xac-03			+			14
Xac-04					+	15
Xac-05			+			13
Xac-06				+		15
Xac-07		+				14
Xac-08				+		16
Xac-09					+	15
Xac-10	+					16
Xac-11					+	15
Xac-12				+		16
Xac-13				+		13
Xac-14					+	15
Xac-15		+				13
Xac-16				+		16
Xac-17			+			13
Xac-18					+	16

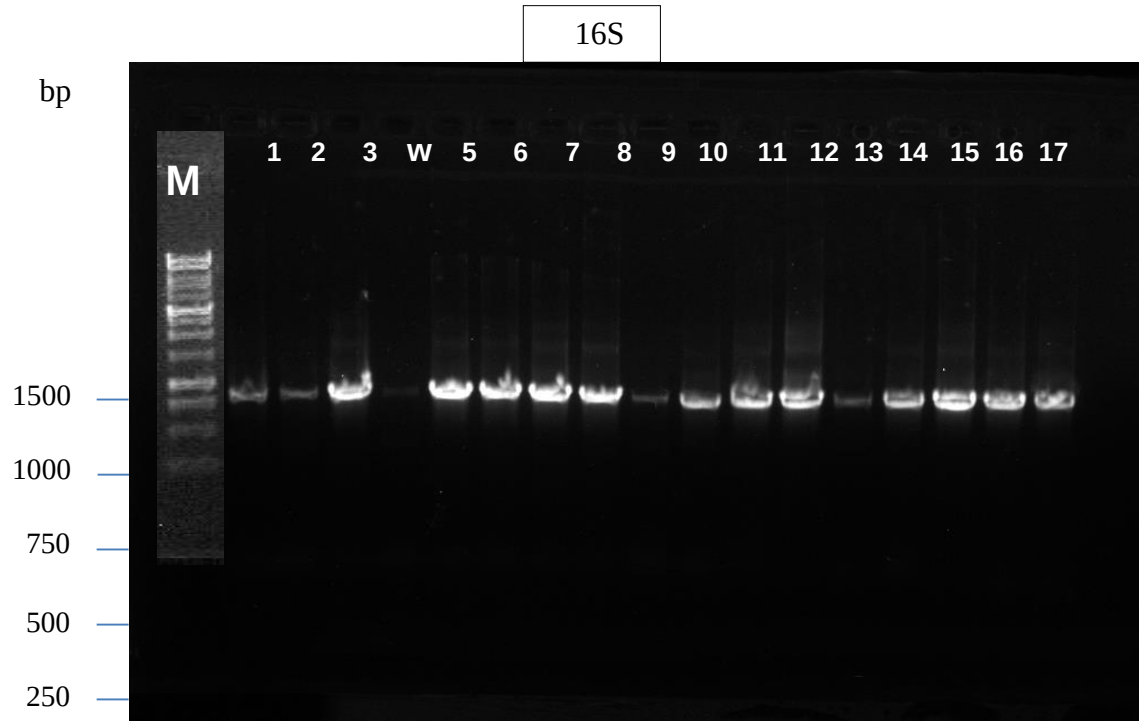


Figure 1. Gel electrophoresis of PCR products: Agarose gel electrophoresis of symptomatic leaf DNA amplified with 16s rRNA universal primer pair F-27 and R-1492 for *Xanthomonas axonopodis* pv. *citri*

Table 6. Antimicrobial sensitivity of plant extract against *X. axonopodis* pv. *citri* isolates

Plant extracts with volume	Inhibition zone (mm) developed by plant extracts against *Xac* isolates (C1-C18)																
	C1	C2	C3	C4	C5	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
A. sativum 70µl	16	15	17	15	16	16	15	15	15	16	16	15	15	15	14	15	18
A. sativum 50 µl	10	11	10	12	12	11	13	12	12	13	11	10	10	9	11	13	12
A. cepa 70µl	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
A. cepa 50µl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Z. officinale 70µl	9	6	8	7	8	7	7	7	7	9	6	8	9	8	7	9	7
Z. officinale 50µl	6	5	5	6	5	5	6	5	6	5	5	5	6	7	5	5	5
S. macrophylla 70µl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. macrophylla 5oµl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. indica 70µl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. indica 50µl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7. Antimicrobial sensitivity test of antibiotics against *X. axonopodis* pv. *citri*

Name of Antibiotics	Disc conc.	No. of isolate	Sensitivity pattern of *Xac.*		
			S (%)	I (%)	R (%)
Streptomycin	10 µg	18	66.6	33.4	-
Tetracyclin	30 µg	18	77.7	-	22.2
Bacitracin	10 µg	18	-	-	100
Chloramphenicol	30 µg	18	66.66	33.33	-
Cefotaxime	30 µg	18	-	16.66	83.33
Gentamycin	10 µg	18	72.22	27.77	-
Ciprofloxasin	5 µg	18	89.5	11.11	-

S= Susceptible, I= Intermediate, R= Resistant

Bacitracin Ciprofloxasin Streptomycin Gentamycin Chloramphenicol Cefotaxime Tetracyclin

Plate 4. Inhibition zone produced by antibiotic disc in *X. axonopodis* pv. *citri* inoculated plates

LITERATURE CITED

Abubaker, M.Y.A., Abu Dibar, O.A.B., Elhassan, S.M. and Yousif, N.M.E. 2016. First report of citrus bacterial canker disease in lime (*Citrus aurantifolia* Swingle) in Gadaref State-Eastern Sudan. Agriculture and Biology J. North America. 7(5):254-265.

Ahmad, I. and Beg, A.Z. 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J. Ethno pharmacology. 74(2):113-123.

Ali, M.R., Hasan, M.F., Hossain, M.F. and Hasan, S.M.Z. 2017. Isolation and characterization of a canker disease causing pathogen from Citrus aurantifolia and evaluation of its biological control measure. J. Entom. and Zoology Studies. 5(6): 1526-1532

Arshiya, M., Suryawanshi, A., More, D. and Baig, M.M.V. 2014. Repetitive PCR based detection of Genetic Diversity in *Xanthomonas axonopodis* pv. *citri* Strains. J. Appl. Biol. Biotech. 2(01):017-022.

BBS. 2010.Yearbook of Agricultural Statistics. Bangladesh Bureau of Statistics, Ministry of Planning, Govt. of the Peoples Republic of Bangladesh.

CABI. 2006. *Crop protection compendium.* Wallingford, UK.

Coletta, F. H.D., Takita, M.A, Souza, A.A., Neto J.R., Destefano,. S.A.L., Hartung, J.S. and Machado, M.A. 2006. Primers based on the rpf gene region provide improved detection of *Xanthomonas axonopodis* pv. *citri* in naturally and artificially infected citrus plants. J. Appl. Microbio. 100 (2): 279–285.

Cubero, J., Graham, J.H. 2002.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. 68:1257-1264.

DAE. 2014. Final report on Pest Risk Analysis (PRA) on citrus under Strengthening Phytosanitary Capacity in Bangladesh Project (SPCB), DAE, Khamarbari, Farmgate, Dhaka.

Das, A.K. and Singh, S. 2000. 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, 1054-1056.

Golmohammadi, M., Cubero, J., Peñalver, J.M., Quesada, J.M., López, M.M. and Llop, P. 2007. Diagnosis of *Xanthomonas axonopodis* pv. *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods. J. Appl. Microbiol. 103 (6): 2309-2315.

Haider, M., Ahmad, Z., Shakeel, Q., Shafiq, S., Chand, S. A., Modassar, W., Ahsan, M., Abdul-Qadir, M. and Kharal, H. (2020). Biochemical characterization of Xanthomonas axonopodis pv. citri; A major impediment to Citrus orchards. International Journal of Biosciences.16.3. 512-517

Hartung, J., Daniel, J.F. and Pruvost, O. 1993. Detection of *Xanthomonas campestris* pv. *citri* by the Polymerase Chain Reaction Method. Applied and Environmental Microbiology 59(4):1143-8.

Hartung, J.S., Pruvost, O.P., Villemot, I. and Alvarez, A.1996. Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-polymerase chain reaction assay. Phytopathology 86 (1): 95-101.

Hussain, K., Nawaz, K., Majeed, A., Haq, I.U. and Lin, F. 2010. Molecular and biochemical characterization of *Xanthomonas axonopodis* pv. *citri* pathotypes. African J. Microbiol. 9:9092-9095.

Hussain, K., Nawaz, K., Majeed, A., Ikram-ul-Haq, F. Lin. (2011). Molecular and biochemical characterization of Xanthomonas axonopodis pv. citri pathotypes. African J. Biotech. 9(54), 9092.

Irobi, O.N. and Banso, A.1994 Effects of crude leaf extracts of *Acalyphatorta* against some anaerobic bacteria. Journal of Ethnopharmacology. 43: 63-65.

Islam, M.A., Mazumdar, R.M., Islam, S., Alam, M.J. and Urmee, S.A. 2014 Isolation, identification and *in-vitro* antibiotic sensitivity pattern of citrus canker causing organism *Xanthomonas axonopodis.* J. Advanced Life Sci. 1(4):215-222.

Mavrodieva, V., Levy, L. and Gabriel, D.W. 2004. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology. 94 (1): 61-68.

Mubeen, M., Arshad, H.M.I., Iftikhar, Y., Ullah, I and Bilqees, I. 2015. Bio-chemical characterization of *Xanthomonas axonopodis* pv. *citri:* a gram negative bacterium causing citrus canker. International Journal of Science and Nature. 6(2):151-154.

Ordax, M., Biosca, E., Wimalajeewa, S,López M., and Marco-Noales, E. 2009. Survival of *Erwinia amylovora* in mature apple fruit calyces through the viable but non-culturable (VBNC) state. J. appl. microbio. 107(1): 106-116.

Praba, S.K. and Kumaresan, R. (2014). Efficacy of antimicrobial activity of aqueous garlic (Allium sativum) extract against different bacterial species. Journal of Chemical and Pharmaceutical Research. 6(10), 677.