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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *XANTHOMONAS CITRI* PV. *CITRI* CAUSING CITRUS CANKER, AND ITS *IN VITRO* CONTROL USING BOTANICALS

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ABSTRACT

Xanthomonas citri pv. *citri* (Xcc), which causes citrus canker—the most devastating disease of citrus—has been studied through biochemical analysis, morphological characterization, and in vitro management using selected botanicals. The laboratory research was conducted at the Central Laboratory of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. A total of 100 fruit and 300 leaf samples were collected from three markets and four nurseries in the Dhaka district, and an Xcc pathogenicity test was performed. Eighteen isolates from infected fruits and twenty-one isolates from infected leaves were characterized. The isolates showed varied reactions in the development of symptoms. In the biochemical test, the pathogen tested negative for Gram staining and Kovac's test but was positive for the KOH test. All isolates were rod-shaped, with colony colors ranging from yellow to pale yellow and a mucoid surface. The isolates Xcc-f-1, Xcc-f-2, Xcc-f-4, and Xcc-f-6 were found to be highly virulent, developing typical symptoms characterized by white crystalline callus formation at the point of inoculation within 7 to 10 days. Similarly, the isolates Xcc-l-2, Xcc-l-3, Xcc-l-4, Xcc-l-6, Xcc-l-7, Xcc-l-9, and Xcc-l-12 were also highly virulent, exhibiting typical symptoms with white crystalline callus formation at the point of inoculation within 7 to 10 days. Among the six botanical extracts evaluated for their efficacy against Xcc, *Zingiber officinale* (ginger) demonstrated the highest inhibition zone diameters of 15.87 mm and 9.40 mm at 1:1 (25g) and 1:2 (100g) concentrations, respectively, followed by *Azadirachta indica* at a 1:4 concentration.

Keywords: Citrus canker, *Xanthomonas citri*, eco-friendly management, Morphology, Biochemistry

INTRODUCTION

Citrus belonging to the family Rutaceae is the most important nutritious fruit crop in the world and in Bangladesh (Solaiman *et al*., 2015). Citrus includes five groups of cultivated citrus: sweet oranges, mandarins, grapefruits, pommel, and the oft-grouped lemons and limes (Donkersley *et al*., 2018). In Bangladesh, the totalacreage under citrus cultivation is about 5,995 ha while the total production is around 136,756 mt (BBS, 2012). Different species of citrus grown in theworld suffer from more than 100 diseases (Klotz, 1973). In Bangladesh, twelve diseases are known to occur in citrus. Characterized by their distinct aroma and delicious taste, citrus fruits have been recognized as an important food and integrated as part of our daily diet, playing key roles in supplying energy and nutrients and in health promotion (Liu *et al*., 2012). Green lemon needs a humid and warm climate. Egypt, Africa, Mexico, and the West of India are the main producers of the lemon. Mexico and India are two major producers of green lemons (Salahvarzi *et al*., 2016).

Citrus canker disease occurs in most citrus-growing countries around the world. The production of citrus fruits, however, is threatened by bacterial canker caused by *Xanthomonas citri* subsp. *citri* (Xcc) (Islam *et al*., 2019). Grapefruit, sweet oranges like pineapple, Hamlin, Mexican limes, lemons, trifoliate orange, and their hybrids are severely affected by *Xanthomonas citri* (Al-Dulaimi *et al*., 2018). Symptoms include leaf spotting, fruit rind blemishing, defoliation, shoot dieback, and fruit drop in favorable environmental conditions conducive to pathogen proliferation (Das, 2003). The primary symptoms of citrus canker are leaf and twig-spotting (Islam *et al*., 2014). This disease has a serious economic impact on citrus production

worldwide (Jalan *et al*., 2013). Still, now four types of citrus canker are found worlwide. Canker A (Asiatic canker) is found in Asia, South America, Oceania, and the USA; canker B (Cancrosis B) in South America (Carrera, 1933); canker C (Mexican lime cancrosis) in Brazil (Schaad *et al*., 2005); and canker D (citrus bacteriosis) in Mexico (Rodriguez *et al*., 1985). In biochemical analysis, Xcc gave a positive result in the KOH solubility test, starch hydrolysis test, catalase test, asculine hydrolysis, urease production, milk proteolysis, tween 80 lypolysis, gelatine liquefaction test, salt tolerant test, tobacco hypersensitivity reaction and gives a negative result in oxidase test (Kishun and Chand, 1991).

For controlling citrus canker disease a variety of ways applied including chemical control, resistance breeding, integration of cultural practices, etc (Phule and Mahavidyalay, 2021). Management of Xcc relies on an integrated approach which includes: (i) replacement of susceptible citrus species with resistant material; (ii) production of disease-free nursery stock; (iii) reduction of pathogen spread by establishing windbreaks and fences around groves; (iv) preventative copper sprays (Leite and Mohan, 1990). However, continuous use of copper compounds leads to soil contamination (Roller, 1998), as well as to the emergence of copper-tolerant phytobacterial strains (Marco and Stall,1983), which in turn results in reduced efficacy of copper bactericides. Mixing mancozeb fungicides with copper bactericides (copper-mancozeb) increases their bactericidal efficacy (Canteros, 2004) however, full control is unattainable if weather conditions favor disease development. To avoid the deleterious effect of synthetic pesticides, an alternativeapproach to control is important to tackle this problem (Khan *et al*., 2018). Mahajan and Das (2003) reported plants and botanicals as a potential source to control citrus canker.

Appropriate management of citrus canker has been investigated by many researchers (Singh *et al.,* 2005). Understanding the ecological conditions of citrus canker proliferation is also a very important aspect (Riasat *et al.,* 2020). Genome editing and the use of several plant by-products pose antimicrobial properties on several pathogenic bacteria and fungi have been previously reported (Kilani, 2006). The research work was therefore designed to isolate, identify, and characterize *Xanthomonas citri* pv. *citri* causing citrus canker and to determine the efficacy of some selected botanicals against *Xanthomonas citri* pv. *citri* under *in vitro* condition.

MATERIALS AND METHODS

Isolation, identification, and characterization of *Xanthomonas citri* pv. *citri*.

Location of the sampling area

The prevalence of canker on fruits and leaves of citrus was surveyed in three wholesale markets namely Karwan Bazar, Mohammadpur Town Hall Kancha Bazar, Mohakhali Kancha Bazar and four nurseries in the Dhaka district namely Horticulture Centre, Falabithi, Asadgate, Dhaka, Shobuj Bangla Nursery, Agargaon, Dhaka, Krishibid Upakaran Nursery, Begum Rokeya Ave, Dhaka and Shanti Garden, Sher-e-Bangla Nagar, Dhaka (Table 1 & Table 2). In total 100 fruits and 300 diseased leaves were collected and brought to the laboratory for isolation purposes and further study.

Survey, symptom observation, and data collection

Four surveys were conducted during the period from July 2021 to October 2021. During the survey in the markets and nurseries, samples composed of 100 random fruits and leaves with characteristic symptoms were considered. Three samples were made representing three markets and four nurseries of the District of Dhaka. The total number of citrus fruits and leaves as well as the number of fruits and leaves that were infected with citrus canker was recorded. The whole procedure was done three times to get three replications. Symptoms of the disease were studied by visual observation (Agrios, 2006). The specimens were kept in the refrigerator at 4ºC by following the standard procedure of preservation of disease specimens until isolation was made.

Isolation, purification, and preservation of *Xanthomonas citri* in the laboratory

Then the young lesions with green healthy portions of diseased fruits were washed under running tap water and cut into small pieces. Surface sterilization was done by dipping them in 75% ethanol solution for 2-3 minutes. Then they were washed three times with sterile water. The cut pieces were then kept in a test tube containing 3-4 ml of sterile water and kept for 30 minutes for bacterial streaming and getting a stock. After preparing different dilutions, 0.1 ml of each dilution was spread over a nutrient agar (NA) plate (15 g bacto agar, 5 g peptone, 3 g beef extract, and 1000 ml distilled water) incubated at 28-30ºC maintaining three replications. The plates were observed after 24-48 hr. Purified cultures were maintained on NA media and one single orange-yellow colony was picked by a wire loop and streaked on another media plate for pure culture. The purified plate was kept in a refrigerator at 4ºC in screw-cap test tubes on NA slant for future use.

Identification and pathogenicity test (Inoculation and symptom development)

Xcc was identified based on morphological, biochemical, and cultural features of the pathogen as per standard microbiological procedures. Healthy citrus fruits and leaves were used for studying the pathogenicity of Xcc. The test was conducted by following the method described by (Lin *et al*., 2008). Bacterial cells were grown overnight in NA broth in the distilled water at 28°C for 24 hr. The bacterial suspension was sieved through a double layer of cheesecloth to remove any kind of dirt and the

concentration was adjusted. One drop of tween-20 (polyoxyethylene 20 sorbitan monolaurate) was added to the suspension to maintain uniform dispersion of bacterial cells in suspension. Then an aliquot of the inocula suspension was injected (10^6 cfu/ml) into the lower surface of the citrus leaf and fruit with the help of a sterile syringe (Fig. 1 & Fig. 2). Distilled water was used as a negative control. Six healthy fruits and twelve healthy leaves were inoculated. All fruits and leaves were marked with a permanent marker pen. The inoculated fruits and leaves were covered with cotton to maintain moisture content and prevent natural contamination with other Microorganisms. The humid condition was maintained by gently spraying sterilized distilled water on the fruits and leaves' surface. After that, it was observed for 15 days. Visual symptoms were recorded and examined. To confirm Koch's postulates, bacteria were re-isolated from the infected area.

Morphological characterization

Morphological characteristics of the pathogen such as cell shape, gram's reaction, and pigmentation were studied as per the standard procedures.

Gram staining

Gram staining test was used to differentiate bacterial species into gram-positive and gram-negative, based on the physical properties of their cell walls. Gram staining was carried out according to Chaudhry and Rashid (2011) method. Crystal violet, ethanol, iodine, and safranin were used. At first, the isolated bacterial culture was heat-fixed onto a glass slide. Then crystal violet was added to the bacterial sample and incubated for 1 min. After washing the slide, iodine was added in the medium. Then safranin was used to counterstaining. After all these steps the slide was used to observe under the light microscope at 100 X using oil immersion (Fig. 3).

KOH test

A single drop of 3% KOH (aqueous) was placed on a glass slide. One loop full of a single colony (18-24 hrs old) was taken from the NA plate using a cooled, sterile loop and it was mixed with KOH solution until an even suspension was obtained. The loop was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials as described by Suslow *et al.* (1982).

Biochemical characterization

Different tests such as Gram Staining, KOH test, Pathogenicity test, KOVACS' Oxidase tests, etc. were performed with the fresh growth of isolates (Chaity *et al*., 2019).

Kovacs' oxidase test

A drop of 1% Kovacs' reagent (1g Tetramethyl-p-phenylenediamine Dihydrochloride in100 ml distilled water) was placed on the center of Whitman filter paper no.1 and loop full of Xcc inoculum was gently rubbed on the filter paper. Positive control was also maintained (Kovacs, 1956).

In-vitro control of *Xanthomonas citri* pv. *citri* by botanicals

Botanical treatments used in the experiment

Botanical plant-based extracts were used as treatments to evaluate their efficacy against *Xanthomonas citri*. Six botanicals were used in the present study and the information regarding the botanicals and specific parts/parts used for evaluation against *Xanthomonas citri* pv.*citri* is presented in Table 3.

Preparation of botanical extracts

The desired concentration of botanical extracts was freshly prepared in sterile distilled water. 100 grams of dried material of each plant parts were thoroughly washed under running tap water and shade dried. Before, the preparation extract, each botanical was dipped in one percent ethanol for one minute. The extracts were prepared by grinding 100 g of washed bulb/rhizome/ fruit of different species in 100 ml distilled water (for aqueous extract) in, mixture-cum grinder. The mixture was kept undisturbed at room temperature (28°C) for 18 hrs. in a sterile flash. Extracts were passed through two layers of cheesecloth and the filtrates were then collected in 50 ml round bottom flasks and their bacterial activity against the citrus canker bacterium. These were then filtered through Whatman No.1 filter paper using volumetric flasks (100 ml capacity). After filtration, the extract was evaporated in the water bath until 100 ml extract was left in the container. For 1:0.50 (w/v) and 1:0.25 (w/v), 100 gm plant materials were dissolved in 50 ml and 25 ml sterile distilled water, respectively. The sensitivity of the different isolates was tested by a modified paper disc diffusion technique (Negi and Kumar, 2015).

In-vitro experiment

The effectiveness of these plant extracts was tested by the disc diffusion technique (Negi and Kumar, 2015). 100 µl of bacterial suspension (1×10^8 cfu/ml) were spread onto the surface of the nutrient agar plate using sterile cotton swabs. Sterile filter paper discs (5 mm) were dipped briefly in the respective botanical extracts and were then applied onto the surface of the inoculated nutrient agar plates. Discs impregnated in botanical extracts were used as positive controls, while sterile distilled water-treated discs were used as a negative control. The treated plates were incubated at 28° C for 48 h and the developing inhibition zones were observed and measured to determine the relative efficacy of each botanical extract against the bacterium.

Table 1. Sources of *Xanthomonas citri* pv. *citri* isolates isolated from citrus fruits

Isolates	Locations
Xcc-f-1, *Xcc*-f-2, *Xcc*-f-3, *Xcc*-f-4, *Xcc*-f-5, *Xcc*-f-6 (Sub-total = 6)	Karwan Bazar
Xcc-f-7, *Xcc*-f-8, *Xcc*-f-9, *Xcc*-f-10, *Xcc*-f-11, *Xcc*-f-12 (Sub-total = 6)	Mohammadpur Town Hall Kancha Bazar
Xcc-f-13, *Xcc*-f-14, *Xcc*-f-15, *Xcc*-f-16, *Xcc*-f-17, *Xcc*-f-18 (Sub-total = 6)	Mohakhali Kancha Bazar
Total number of isolates = 18	Location = 3

Table 2. Sources of *Xanthomonas citri* pv. *citri* isolates isolated from citrus leaves

Isolates	Locations
Xcc -l-1, *Xcc* -l -2, *Xcc* -l -3, *Xcc* -l -4, *Xcc* –l-5, *Xcc* -l -6, *Xcc* -l -7 (Sub-total=7)	Horticulture Center, Falabithi, Asadgate, Dhaka
Xcc -l – 8, *Xcc* -l -9, *Xcc* -l -10, *Xcc* -l -11 (Sub-total=4)	Shobuj Bangla Nursery, Agargaon, Dhaka
Xcc -l -12, *Xcc* -l -13, *Xcc* -l 14, *Xcc* -l -15 (Sub-total=4)	Krishibid Upakaran Nursery, Begum Rokeya Ave, Dhaka 1207
Xcc -l -16, *Xcc* -l -17, *Xcc*-l -18, *Xcc*-l -19, *Xcc*-l -20, *Xcc*- l -21 (Sub-total=6)	Shanti Garden, Sher-E-Bangla Nagar, Dhaka 1207
Total isolates = 21	Total location = 4

Table 3. Botanical extract used in controlling of *Xanthomonas citri in-vitro*

Treatments (Botanical)	Common Name	Scientific Name	Plant parts used
T_1	Neem	*Azadirachta indica*	Leaf
T_2	Tulsi	*Ocimum indica*	Leaf
T_3	Ginger	*Zingiber officinale*	Rhizome
T_4	Turmeric	*Curcuma longa*	Rhizome
T_5	Garlic	*Allium sativum*	Clove
T_6	Onion	*Allium cepa*	Bulb

Table 4. Pathogenicity Test of *Xanthomonas citri* pv. *citri* on fruits

Isolates	Locations	Days to initiation of symptoms	Symptoms	Reactions
Xcc-f-1	Karwan Bazar	8	+++	Strong Canker
Xcc-f -2	Karwan Bazar	10	+++	Strong Canker
Xcc-f -3	Mohammadpur Town Hall Kancha Bazar	13	+	Weak Canker
Xcc-f-4	MohammadpurTown Hall Kancha Bazar	9	+++	Strong Canker
Xcc-f -5	Mohakhali Kancha Bazar	14	+	Weak Canker
Xcc-f -6	Mohakhali Kancha Bazar	7	+++	Strong Canker

Statistical analysis

All the above experiments of the present study were conducted in triplicate for consistency of results and statistical purposes. The data were expressed as mean and standard error (Mean ± SE) and analyzed by analysis of variance (ANOVA) through Statistix.10 software. The data were calculated using Microsoft Excel 2010 software.

RESULTS AND DISCUSSION

Symptomology

On leaves small, blister-like lesions were observed. In aged lesions gray to tan brown with an oily margin surrounded by a yellow halo were also found. The center of the lesion was raised and corky. In some leaves tissues in old lesions died and fell out. The lesions in fruits were superficially similar to those on leaves but they were found irregularly shaped. Lesions in fruits were raised with a corky appearance but there was no yellow halo at the mature stage.

Isolation and purification of *Xanthomonas citri* pv *citri in vitro* from diseased specimen

Total twenty-one isolates of *Xanthomonas citri* pv. *citri* was isolated from the infected leaf of citrus and eighteen isolates were observed from infected fruit of citrus that were collected from different locations in Dhaka district. The isolates were purified by the streak plate method. Pale yellow to yellow-pigmented bacterial colonies were formed on nutrient agar medium after 72 hours of incubation at 28 ± 2^0C which were identical to *Xanthomonas citri* pv. *citri*. These isolates were maintained on NA slants and used for further study. By the streaking method, single colonies were found and partially identified based on colony morphology. The colonies were creamy white.

Pathogenicity test

The pathogenic variability amongst the six isolates of fruits and twelve isolates of leaves of Xcc were studied (Table 4 and Table 5). All were found susceptible to all the isolates Xcc. The isolates Xcc showed varied reactions in the development of the symptoms. The isolates viz. *Xcc-f-1, Xcc-f-2, Xcc-f-4, xcc-f-6* were found highly virulent in the development of typical symptoms i.e. white crystalline callus formation at the point of inoculation within 7 to 10 days. The isolates *Xcc-f-3, Xcc-f-5* were found less virulent as they developed symptoms after 13 to 16 days of inoculation.

However, in the case of leaves, the pathogenic ability of all different isolates of Xcc were conformed and found that isolates *Xcc-l*-1, *Xcc-l*-5, *Xcc-l*-8, and *Xcc-l*-10 were found less virulent as they developed symptoms after 13 to 16 days of inoculation on the other hand, the remaining isolates *Xcc-l-2, Xcc-l-3, Xcc-l-4, Xcc-l-6, Xcc-l-7, Xcc-l-9, Xcc-l-12* were showed highly virulent in development of typical symptoms i.e. while crystalline callus formation at the point of inoculation within 7 to 10 days. The categorization of isolates of Xcc was done based on the development of the symptoms on fruits and leaves and days taken for the appearance of the symptoms as No canker (-), Weak canker (+), Moderate canker (++), Strong canker (+++) as presented in Table 4 and Table 5. Katkar *et al*., (2016) also confirmed the bacterium similarly as performed in this study.

Biochemical Characteristics of *Xanthomonas citri pv. citri*

The biochemical characteristics of isolates were studied to check the similarity of biochemical features with genus *Xanthomonas* by subjecting them to various biochemical tests as shown in (Table 6). In gram staining results indicated, that Gram-negative bacteria have a thinner layer (10% of cell envelope), and are stained pink with safranin. Here, the isolated bacteria were found to be gram-negative, motile, and rod-shaped under a light microscope at 100 X magnification. After adding the Kovac's reagent, bacteria did not produce a red/pink color band on the top of the tube and H2S was not produced as no black precipitation was formed. In Kovac's test, the medium containing filter paper and oxidizing agent reagent did not produce any color (Table 6).

Morphological Characteristics of *Xanthomonas citri* pv. *citri*

Eighteen isolates were collected from infected fruits (Table 7). All the isolates were rod shaped and medium-sized. Eleven isolates were yellow i.e.; isolates number *Xcc-f*-1, *Xcc-f*-2, *Xcc-f*-4, *Xcc-f*-5, *Xcc-f*-8, *Xcc-f-9*, *Xcc-f*-10, *Xcc-f*-11, *Xcc-f*-12, *Xcc-f*-14 and *Xcc-f*-17 in color at the test and other i.e., *Xcc-f*-3, *Xcc-f*-6, *Xcc-f*-7, *Xcc-f*-13, *Xcc-f*-15, *Xcc-f*-16 and *Xcc-f*-18 were found pale yellow in color. Elevation, margin and surface of all isolates were convex even and mucoid respectively. Twenty one isolates were collected from infected leaves in which fourteen were recorded yellow in color (*Xcc-l*-1*, Xcc-l*-2*, Xcc-l-3, Xcc-l-5, Xcc-l*-6*, Xcc-l*-8*, Xcc- l*-11*, Xcc-l*-12*, Xcc-l*-13*, Xcc-l*-15*, Xcc-l*-16*, Xcc-l*-17*, Xcc-l*-18 *and Xcc-l*-19*)* and *Xcc-l*-4*, Xcc-l-7, Xcc-l*-9*, Xcc-l*-10*, Xcc-l*-14*, Xcc-l*-20 *and Xcc-l*-21 were in pale yellow. Cultural characteristic includes colony, shape, margin, size, elevation and pigmentation of isolates were studied by using Nutrient Agar as a basel cultural medium in both fruits and leaf isolates. The unique opaque yellow color colonies were obtained in the medium. The yellow color was due to the production of *Xanthin* produced by the genus *Xanthomonas*. The colony color of the bacterium was pale yellow to yellow while the shape and size of the colony were medium, convex, and mucoid. Table. 7 and Table. 8 showed the morphological characteristics of the citrus canker pathogens found on fruits and leaves, respectively.

Table 5. Pathogenicity Test of *Xanthomonas citri* pv. *citri* on leaves

Isolates	Locations	Days to initiation of symptoms	Symptoms	Reactions
Xcc-l-1	Horticulture center	13	+	Weak Canker
Xcc-l-2	Horticulture center	9	+++	Strong Canker
Xcc-l -3	Horticulture center	7	+++	Strong Canker
Xcc-l-4	Shobuj BanglaNursery	10	+++	Strong Canker
Xcc-l-5	Shobuj BanglaNursery	15	+	Weak Canker
Xcc-l-6	Shobuj BanglaNursery	10	+++	Strong Canker
Xcc-l-7	Krishibid Upakaran Nursery	8	+++	Strong Canker
Xcc-l-8	Krishibid Upakaran Nursery	15	+	Weak Canker
Xcc-l-9	Krishibid Upakaran Nursery	7	+++	Strong Canker
Xcc-l-10	Shanti Garden	14	+	Weak Canker
Xcc-l-11	Shanti Garden	15	+	Weak Canker
Xcc-l-12	Shanti Garden	9	+++	Strong Canker

Figure 1. Inoculation of *Xanthomonas citri* pv. *citri* and symptoms developed on fruits.

Figure 2. Inoculation of *Xanthomonas citri* pv. *citri* and symptoms developed on leaves.

Table 6. Responses of the Isolated Bacteria in Different Biochemical Test Media

Name of the Test	Appearance	Reactions	Remarks
Gram Staining	Small, Rod-shaped, pink color	-ve	Gram staining showed gram-negative bacteria
Kovac's	No color formation	-ve	Bacteria did not produceany characteristic color showed gram-negative bacteria
KOH	Thread-like	+ve	Thread-like slime when picked up with an inoculum loop showed gram-negative bacteria

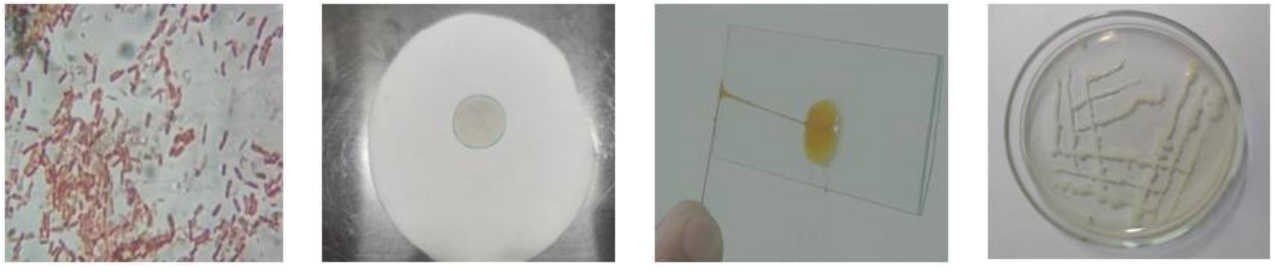
Table 7. Morphological Characteristics of *Xanthomonas citri* pv. *citri* isolated from infected fruits

Isolates	Shapes	Size	Color	Elevation	Margin	Surface	Gram Staining	Kovac's Test	KOH Test
Xcc-f-*1*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*2*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*3*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*4*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*5*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*6*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*7*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*8*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*9*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*10*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*11*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*12*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*13*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*14*	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*15*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*16*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*17*	Rod	Medium	yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-f -*18*	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve

Table 8. Morphological Characteristics of *Xanthomonas citri* pv. *citri* isolated from infected leaves

Isolates	Shapes	Size	Color	Elevation	Margin	Surface	Gram Staining	Kovac's Test	KOH Test
Xcc-l-1	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-2	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-3	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-4	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-5	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-6	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-7	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-8	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-9	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-10	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-11	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-12	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-13	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve

Isolates	Shapes	Size	Color	Elevation	Margin	Surface	Gram Staining	Kovac's Test	KOH Test
Xcc-l-14	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-15	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-16	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-17	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-18	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-19	Rod	Medium	Yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-20	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve
Xcc-l-21	Rod	Medium	Pale yellow	Convex	Even	Mucoid	-ve	-ve	+ve



A **B** **C** **D**

Fig. 3. A. Microscopic view of *Xanthomonas citri* pv. *citri* (Xcc) after gram's staining (x100), B. No color formation after 60 s in Kovac's test of gram-negative bacterium Xcc, C. Gram-negative bacterium Xcc forms thread-like slime when picked up with inoculum loop in KOH test, and D. Cultural characteristics of Xcc on nutrient agar media.

Table 9. Inhibitory effects of different botanical extracts on *Xanthomonas citri* pv. *citri*

Botanicals		Inhibitory zone (mm)		
Scientific name	**Common name**	**1:1 (w/v)**	**1:2 (w/v)**	**1:4 (w/v)**
Azardiricta indica	Neem	11.03 b	9.33 a	7.50 a
Ocimum indica	Tulsi	9.50 b	8.90 ab	6.40 ab
Zingiber officinale	Ginger	15.87 a	9.40 a	6.63 ab
Curcuma longa	Turmeric	11.75 ab	8.43 abc	7.03 ab
Allium sativum	Garlic	8.00 b	6.70 c	5.8 3b
Allium cepa	Onion	11.37 b	7.20 bc	6.67 ab
Control	Water	0.00	0.00	0.00
CV (%)		11.17	11.77	22.14
LSD (0.05%)		6.22	2.44	1.32

In column means values having a similar letter (s) are statistically similar and those having a dissimilar letter(s) differ significantly at a 0.05 level of probability.

Efficacy of Botanicals against Xcc in vitro

Antibacterial activities of six different plant extracts were determined against the isolated bacteria (Table 9). *Zingiber officinale* (Ginger) showed the highest 15.87 ±0.0mm and 9.40 ±0.0mm diameter of zone of inhibition against the isolated bacteria in 1:1 (100g) and 1:2 (50g) concentrations respectively followed by 7.50±0.0mm diameter recorded in *Azadirachta indica* in 1:4 (25g) concentration. *Allium sativum* extract showed the lowest 8.0, 6.70, and 5.83 mm diameter zone of inhibition against the isolated bacteria in all concentration levels. On the other hand, the extractof *Curcuma longa, Ocimum indica,* and *Allium cepa* showed comparatively similar results against pathogen isolates.

The disease recorded in the present study is based on the visual symptoms following the description by Agrios (2006). Hossain (2011) also reported the disease in the citrus-growing areas of Bangladesh. The disease recorded in the present study has also been reported on citrus fruits from several other countries (Burhun *et al*., 2007). Researchers throughout the world have also reported the pathogen (Vudhivanich, 2003). Chand and Kishun (1991) reported that

Xanthomonas produce mucoid, circular, convex, yellow, round, glistening, and raised colonies on nutrient agar medium. Lin *et al*. (2008) isolated the bacterial pathogen from the canker-infected fruits and leaves and proved pathogenicity.

The causal agent of the canker of citrus (Xcc*)* was identified by conducting studies on its morphological, biochemical, and cultural features as per standard microbiological procedures. The isolated bacteria were considered gram-negative enteric bacteria. Similar outcomes were obtained by Kajal *et al.,* (2021) with Xcc in the medium. A total of twenty-one isolates of Xcc were isolated from the infected leaf of citrus and eighteen isolates were observed from the infected fruit of citrus. By the streaking method, single colonies were found and partially identified based on colony morphology. The colonies were creamy white. The procedure was followed by Isokar *et al*., (2020). Bacteria isolated from *Citrus aurantifolia* by Abubaker *et al.,* (2016) also showed similar results by different biochemical tests. After gram staining under the compound microscope at 100X magnification with oil immersion, the bacterium was rod-shaped, cells appeared singly, pink in color, and capsulated.

A mucoid thread was lifted with the loop in KOH solubility test that supports the result of gram'sstaining test i.e., the bacteria was gram negative. A similar result in KOH solubility test was found by Kishun and Chand (1991). Braithwaite *et al* (2002) also reported that *Xanthomonas axonopodis* pv. *citri* as a gram-negative, rod-shaped bacterium. In the present study, the bacterium (*Xanthomonas axonopodis* pv. *citri)* showed positive results in the KOH solubility test and negative results in the oxidase test. Similar results have also been reported by Yenjerappa (2009). Jabeen *et al.* (2012) observed that *Xanthomonas* gave yellow, circular, smooth, convex, and viscous bacterial colonies on yeast dextrose calcium carbonate agar medium (YDCA) after 48-72h of incubation at 28^{o}C. On NA medium the bacteriagave light yellow, mucoid, round, and smooth colonies. A similar result has also been reported previously (Balestra *et al*., 2008). Our results confirmed the work of Mubeen *et al.,* (2015) who used several biochemical tests to identify and characterize different strains of citrus canker causing bacteria. Arshiya *et al.,* (2014) also found that the different strains of *Xanthomonas axonopodis* pv. *citri* bacteria isolated from citrus canker were gram-negative, obligate aerobes and non-spore forming rod yellow giving convex round and mucoid colonies on YDC (Yeast, Dextrose, Calcium carbonate) agar medium. Our results confirmed the work of Mubeen *et al.,* (2015) who used gram reaction tests to identify and differentiate different pathotypes of citrus canker causing bacteria on biochemical test.

After the inoculation of bacteria, the symptoms of the disease were observed about 7 to 16 days depending upon the isolate. Initially the weak symptoms were observed like slightly raised small blister- like lesions. The symptoms started turning tan to brown and a water-soaked margin appeared around the leaves and fruits surrounded by yellow halo forming the visible lesions resembling canker symptoms later. Pathogenic ability of all different isolates *Xcc* were confirmed and found that isolates *Xcc-f*-1, *Xcc-f*-2, *Xcc-f*-4, and *Xcc-f*-6 were showed highly pathogenic to initiate minute canker lesion and fully developed symptoms (strong) after 7 to 10 days of inoculation. While *Xcc-f-3, Xcc-f-5* were found to produce very poor (weak) in producing (less virulent) virulent as they developed symptoms after 13 to 16 days of inoculation. However, in case of leaves, isolates *Xcc-l*-1, *Xcc-l*-5, *Xcc-l*-8 and *Xcc-l*-10 were found less virulent symptoms (weak) developed symptoms after 13 to 16 days of inoculation on the other hand, remaining

Isolates *Xcc-l-2, Xcc-l-3, Xcc-l-4, Xcc-l-6, Xcc-l-7, Xcc-l-9, Xcc-l-12* were showed highly virulent in development of typical symptoms (strong) i.e. while crystalline callus formation at the point of inoculation within 7 to 10 days. Katkar *et al.,* (2016) also confirmed the bacterium in a similar manner as performed in this study. Katkar *et al.,* (2016) observed that the fifteen isolates of *Xcc* based on symptoms development on leaves and day taken for the appearance of the symptoms as no canker (－), weak canker (＋), moderate canker (＋＋) and strong canker (＋＋＋) as presented. Jabeen *et al.* (2011) reported that three methods of inoculation, clipping, pinprick, and brushing were tested both on detached leaves and on attached leaves in vitro and in vivo experiments. All these methods were effective for artificial inoculation, but the pinprick method was found to be more efficient in detached leaf assay produced large-size lesions.

Like our study, Yenjerappa (2009) reported that *Allium sativum* extract at 10 percent concentration was significantly greater in efficacy than all other treatments followed by parthenium and lantana leaf extract and *Allium cepa* bulb. The *in-vitro* efficacy of 15 different botanicals was tested against Xcc and the results revealed that *Zingiber officinale* had recorded maximum average inhibition. *Zingiber officinale*, tulsi leaves extract *(Ocimum indica*.), neem seed oil, garlic extract, *Allium sativum* were reported antibacterial against Xcc earlier by Raju *et al.,* 2013.

CONCLUSION

Among the six botanical extracts evaluated for their efficacy against Xcc, *Zingiber officinale* (ginger) demonstrated the highest inhibition zone diameters of 15.87 mm and 9.40 mm at 1:1 (25g) and 1:2 (100g) concentrations, respectively, followed by *Azadirachta indica* at a 1:4 concentration.

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