MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *Xanthomonas citri* CAUSING CITRUS CANKER AND ITS *in-vitro* CONTROL WITH BOTANICALS

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CERTIFICATE

This is to certify that the thesis entitled **"MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *Xanthomonas citri* CAUSING CITRUS CANKER AND ITS *in-vitro* CONTROL WITH BOTANICALS"** submitted to the department of Plant Pathology, faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, in partial fulfillment of the requirements for the degree of **Master of Science (MS) in PLANT PATHOLOGY**, embodies the result of a piece of bona fide research work carried out by Registration No.: **19-10061,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dated:
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Dedicated To

My Beloved Family

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SAU, Dhaka **The Author**

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BY

FERDOUSI BEGUM

ABSTRACT

Xanthomonas citri pv. *ctitri* (Xcc), causing citrus canker - the most devastating disease of citrus, has been studied through biochemical analysis, morphological characterization and *in-vitro* management by selected botanicals. The laboratory works has been done at Central Laboratory of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. In biochemical test, the pathogen reacted on Gram Staining and Kovac's negative but the isolate was positive for KOH. All isolates were found rod shaped, with colony colour ranging from yellow to pale yellow with mucoid surface. Altogether 100 fruits and 300 leaves samples were collected from three markets and four nurseries of Dhaka districts, respectively and pathogenicity of *Xanthomonas citri* was tested. The isolates showed varied reactions in the symptoms development. In total, eighteen isolates from infected fruits and twenty one isolates from infected leaves were characterized. The isolates viz. Xcc-F-1, Xcc-F-2, Xcc-F-3, Xcc-F-4, Xcc-F-5 and Xcc-F-6 were found highly virulent and produced typical symptoms at the point of inoculation within 13 to 15 days while isolates Xcc-F-1 to Xcc-F-21 developed symptoms within 12 to 15 days in leaves. Six botanical extracts namely, Neem (T_1), Tulsi (T_2), Ginger (T_3), Turmeric (T_4), Garlic (T_5), and Onion (T_6) were evaluated for their efficacy against *Xanthomonas citri*. *Zingiber officinale* (Ginger) showed the highest 15.87 ± 0.0 mm and 9.40 ± 0.0 mm diameter inhibition zone against the isolated bacteria in 1:1 (25g) and 1:2 (100g) concentrations, respectively followed by 7.50 ± 0.0 mm diameter recorded in *Azadiricta indica* in 1:4 concentration. *Allium sativum* extract showed the lowest 8.0, 6.70 and 5.83 mm diameter of inhibition against the isolated bacteria in all concentrations. The present investigation concludes that there exist pathological and biochemical variations amongst the different isolates of *Xanthomonas citri* pv. *citri* and the tested botanicals showed promising performances in control the pathogen *in- vitro.*

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LIST OF ABBREVIATIONS

Full Name	Abbreviations
And	&
And Others	*et al*.
At the Rate of	@
Bangladesh Bureau of Statistics	BBS
Centimeter	Cm
Colony Forming Unit	cfu
Coefficient of Variance	CV
Degree Celsius	°C
Degrees of Freedom	df.
Etcetera	etc.
Gram	g
Hectare	ha
Hour (s)	hr
That is	i.e
Journal	J.
Kilogram	kg
Kilogram per hectare	Kg/ha
Least Significant Difference	LSD
Meter	m

Mililiter	ml
Millimeter	mm
Pathovar	pv.
Percentage	%
Parts per Million	ppm
Sher-e-Bangla Agricultural University	SAU
Species	spp.
Treatment	T
Videlicet (namely)	viz.
Xanthomonas citri	*Xcc*

CHAPTER 1

INTRODUCTION

Citrus is a genus of flowering plants belonging to the family Rutaceae (Islam *et al*., 2019). It is the most important nutritious fruit crops of the world as well as in Bangladesh (Solaiman *et al*., 2015). *Citrus*, also known as *agrumes* (sour fruits) by the Romance word, is one of the world's major fruit crops with global availability and popularity contributing to human diets (UNCTAD, 2004). Citrus is world's leading fruit crop with annual production of approximate 60 million megatons. These include five groups of cultivated citrus: sweet oranges, mandarins, grapefruits, pommel and the oft-grouped lemons and limes (Donkersley *et al*., 2018). In Bangladesh, the total acreage under citrus cultivation is about 5,995 ha while the total production is around 136,756 mt (BBS, 2012). Various factors are responsible for lower citrus production in Bangladesh. Among them, plant disease is one of the major influential factors. Different species of citrus grown in the world suffers from more than 100 diseases (Klotz, 1973). In Bangladesh, twelve diseases are known to occur in citrus. Characterized by the 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 humid and warm climate. Egypt, Africa, Mexico, and West of India are main producers of the lemon. Mexico and India are two major producers of green lemon. (Salahvarzi, 2016).

Citrus canker disease occurs in most citrus growing countries around the world. Although canker in citrus was recognized as a new disease in 1912 in Florida, USA, the disease may have been present in India in the 1800s (Sharma, 2009). The production of citrus fruits, however, is threatened by bacterial canker disease. The causal agent is *Xanthomonas citri* subsp. *citri* (Xcc) (Islam *et al*., 2019). The causal bacterium *Xanthomonas citri* has distinct forms (A, B and C) based on geographical distribution and host range. 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). Secondary rotting organisms invade lesions, causing fruit rot. 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).

The bacterium *Xanthomonas citri* pv. *citri* is a rod-shaped, gram-negative, and has a single polar flagellum. Colonies on laboratory media are usually yellow due to '*Xanthomonadin*' pigment production. Still now four types of citrus canker are found. Canker A (Asiatic canker) is found in Asia, South America, Oceania and the USA (Carrera, 1933); 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). It gave positive result in 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 negative result in oxidase test (Kishun and Chand, 1991).

Citrus canker is mainly controlled by the application of various chemicals such as fungicides and bactericides at different times of the growing season. Management of the disease will not only increase the production of citrus but also earns a fair amount of foreign exchange. It could be managed by variety of ways including chemical control, resistance breeding, integration of cultural practices, etc (Phule and Mahavidyalay, 2021). Management of ACC 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. In order to avoid the deleterious effect of synthetic pesticides, an alternative approach for the control of plant pathogenic organisms is important to tackle this problem (Khan *et al*., 2018). Mahajan and Das (2003) reported plants and botanical as a potential source to control citrus canker.

Appropriate management of citrus canker has been investigated by many researchers (Singh *et al.,* 2005; Canteros, 2004; Graham and Leite, 2004; Das and Shyam, 2003; Dixon *et al.,* 2000;

Gottwald and Timmer, 1989; Civerolo, 1981). Understanding the ecological conditions of citrus canker proliferation, are also very important aspect (Riasat *et al.,* 2020). Genome editing has

been started for those genes which are sensitive to citrus canker (Jia *et al*., 2017). Moreover, the use of several plant by-products, which poses antimicrobial properties, on several pathogenic bacteria and fungi has been reported by many Researchers (Dorman and Deans, 2000; Parameswari and Latha, 2001; Rath *et al*., 2001; Britto and Senthilkumar, 2001; Bylka *et al*., 2004; Shimpi and Bendre, 2005; Kilani, 2006). Thus, botanicals might be a good option against *Xanthomonas citri*.

Considering the above facts and points the present research work was designed to achieve the following objectives:

1. To isolate, identify and characterize *Xanthomonas citri* pv. *citri* causing citrus canker.

2. To determine the efficacy of some selected botanicals against *Xanthomonas citri* pv. *citri.*

CHAPTER 2

REVIEW OF LITERATURE

Canker of citrus caused by *Xanthomonas citri pv. citri*, once deemed as a disease of minor importance, become a serious threat for citrus production in recent years. The disease assumed it's severity in all the growing areas of the world resulting severe yield losses both in terms of quality and quantity. The information available on this disease, pathogen and management strategies are very meagre. Hence, the literature to the canker of citrus along with information on related crops disease and pathogen are reviewed here as under.

2.1. Citrus diseases and symptomology of citrus canker

Dewdney and Graham (2014) reported that citrus canker, caused by the bacterium *Xanthomonas citri* pv. c*itri* is a leaf, fruit, and stem blemishing disease that affects most citrus. Grapefruit, Mexican lime, and some early oranges are highly susceptible to canker; Navel, Pineapple, and Hamlin oranges, as well as lemons and limes are moderately susceptible; mid-season oranges, Valencias, tangors, tangelos, and other tangerine hybrids and less susceptible; and tangerines are tolerant. Young lesions are raised on both leaf surfaces, particularly on the lower leaf surface. The pustules later become corky and crater-like, with raised margins, sunken centers and surrounded by a yellow halo. Fruit lesions vary in size because the rind is susceptible for a longer period of time, and more than one infection cycle can occur on fruit. Twigs and stem infections resemble those on fruits. The lesions are raised with a corky appearance and can support long-term survival of the bacterium. Older lesions may darken when they become colonized by saprophytic fungi such as a *Colletotrichum spp*.

Duncan *et al.*, (2014) observed that citrus fruit suffer from different diseases that may considerably affect the fruit crop by destroying the tree and/ or the fruit. World-wide, citrus is known to be infected by many diseases including citrus canker caused by the bacterium *Xanthomonas citri* pv. *citri,* huanglungbing (HLB) caused by the bacterium Candidates liberibacter asiaticus, stubborn disease (*Spiroplasma citri*), citrus variegation chlorosis (CVC) caused by Xylella fastidiosa, citrus bacterium spot *Xanthomonas alfalfa* pv. citrumelonis (*Xanthomonas axonopodis* pv. *citrumelo*), citrus black spot (*Guignardia citricarpa*), anthracnose (*Colletotrichum gloeosporioides*), brown spot (*Alternaria alternata*), citrus scab caused by the

fungus *Elsinoe fawcettii* (*Sphaceloma citri*). Other species of limited economic importance because they are more localized, include the sting nematode (*Belonolaimus longicaudatus*) and two species of lesion Nematode (*Pratylenchus coffeae* and *P. brachyurus*).

Rashid *et al.,* (2014) reported that symptom expression of citrus canker varies depending on the age of the lesions, the plant part affected and the species of citrus infected. On leaves, first appearance was small, blister-like lesions, usually on the abaxial surface. As leaf lesions aged, they turned gray to tan brown with an oily margin, usually surrounded by a yellow halo. The center of the lesion became raised and corky and was visible on both sides of the leaf. Leaf tissues in old lesion had died and fall out. The lesions in young twigs and stems were superficially similar to those on leaves but they were generally irregularly shaped. Lesions were raised with a corky appearance but there was no yellow halo.

Gill (2013) reported citrus canker symptoms include brown spots on leaves, often with an oily or water-soaked appearance. The spots (technically called lesions) are usually surrounded by a yellow halo, and they can be seen on both the upper and lower sides of the leaf. Similar symptoms can appear on fruit. Even stems can have symptoms with brown bumps or lesions. As the leaf sports age, they become crushed and tan surrounded by a yellow ring. Eventually, the tan tissue can fall out creating a hole through the leaf. Leaf sports have been described as looking like a cigarette burn surrounded by a yellow circle. The disease cause citrus trees to prematurely drop leaves and fruits, and it can cause dieback of twigs and branches. With time, trees quit producing fruit and decline in health. (These symptoms can also be caused by *Phytophthora root* rot, but the leaves do not have sports when the problem in root rot). The bacterium responsible for the disease is spread from infected trees to healthy trees by wind-driven rain or on contaminated tools, clothing and equipment. It can move long distances on equipment, in large storms such as hurricanes and by the movement of infected citrus materials. It is not transmitted by insects.

Sharma and Sharma, (2009) stated that the symptoms of the disease are observed on all the aerial parts including leaves, twigs and fruit. Although phylogenetically different strains of *Xanthomonas* cause citrus canker, the symptoms and signs elicited on susceptible hosts are the same.

Balestra *et al.,* (2008) proclaimed canker lesions as hyperplasia type, often surrounded by a water-soaked margin and yellow halo. Typical citrus canker lesions were found on 8 to 10 years old lime (*Citrus limetta*) trees in northern Somalia. Graham *et al,* (2004) found that the earliest symptoms on leaves appear as tiny, slightly raised blister-like lesions beginning around 9 days post-infection. As the lesions age, they first turn light tan, then tan to brown, and a water-soaked margin appears, often surrounded by a chlorotic halo. Defoliation becomes a problem as the disease intensifies. On twigs and fruit, citrus canker symptoms are similar: raised corky lesions surrounded by an oily or water-soaked margin.

Brunings and Gabriel (2003) observed on leaves that first appearance of *Xanthomonas citri* pv. *citri* was water soaked, 2-10 mm, similarly small sized, circular spots, usually on the abaxial surface. On leaves, stems, thorns and fruits, circular lesions became raised and blister-like, growing into white or yellow spongy pustules. These pustules then darkened and thickened into a light tan to brown corky canker, which was rough to the touch. On stems, pustules coalesced to split the epidermis along the stem length, and occasionally grindling of young stems may occur. Older lesions on leaves tend to have more elevated margins and were at times surrounded by a yellow chlorotic halo (that may disappear) and a sunken centre.

Verniere *et a*l. (2003) studied the Asiatic Citrus Canker (ACC) and found expression of symptom depend on temperature whereas relative humidity had no effect om disease suppression.

Vudhivanich (2003) observed that canker lesions at first were small, slightly raised, round, light green spots. Later, they became grayish white, rupture, and appear corky with brown, sunken centre.

Braithwaite *et al.,* (2002) observed that yellow/brown, raised and corky lesions were formed on leaves, twigs and fruits of cultivated citrus which darkened and developed central depressions with age. The edges of the lesions remained raised and were frequently surrounded by a chlorotic halo.

Zhong and Ling, (2002) observed that citrus canker lesions start appearing after 15-20 days after bud burst as pinpoint oily looking spots. Initially, the lesions are surrounded by a yellowish halo. A more reliable diagnostic symptom of citrus canker is the water-soaked margin that develops

around the necrotic tissue, which is easily detected with transmitted light. Signs of the pathogen are generally evident in older lesions as masses of rod shaped bacteria streaming from the edges of thinly cut lesion sections under the microscope.

Swarup *et al.,* (1991) noted that citrus canker lesions were first appeared as pin-point spots that became small, slightly raised pustules or blister-like eruptions. Initially, those appear on the lower leaf surface. Subsequently, the blisters became visible on the upper leaf surface. The young lesions were usually translucent due to water-soaking of the tissue. Lesions were initially circular or irregular, light colored at first and became tan or brown later.

Stall and Seymour (1983) reported that on the fruits, the lesions are almost similar to those on leaves and have a crater like depression in the center and extend to 1 mm in depth. The lesions can vary in size; with time such lesions become rough and raised and develop a brown to dark brown color. Further, the presence of a large number of lesions on the fruit surface may result in small and misshapen fruits especially when the infection is early.

2.2. Characteristics of *Xanthomonas citri*

Arshiya *et al.,* (2014) found that the different strains of *Xanthomonas citri* bacteria isolated from citrus canker were positive for starch hydrolysis, gelatin liquefaction, aesculin hydrolysis and tween 80 lipolysis, H_2S production, urease production, while all strains showed oxidase test negative.

Rashid *et al.,* (2014) isolated *Xanthomonas axonopodis pv. citri* from the canker infected part of citrus seedlings and identified by studies on morphological, biochemical and culture features of the bacteria. They observed that the bacterium was gram negative, rod shaped and showed positive results in KOX solubility test, starch hydrolysis test, catalase test, citrate utilization test, motility indole urease agar (MIU) test, gelatine liquefaction test and negative result in oxidase test. It produced circular, flattened or slightly raised, yellow to bright colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened colonies on SX medium.

Jabeen *et al.,* (2012) noticed 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 SX medium the bacteria gave light yellow, mucoid, round and

smooth colonies (1mm in diameter) while whitish, mucoid and smooth colonies were observed on Wakimoto medium.

Yenjerappa (2009) conducted an experiment to study the growth of *Xanthomonas citri* on different growth media and found that modified D-5 medium was significantly superior in promoting the luxurious growth of the pathogen followed by yeast extract nutrient agar medium. Colonies of the bacterium on MD-5 and YNA medium appeared as circular to irregular, flattened, color less to light yellow, occurred singly or rarely in aggregate. Colonies of similar morphology with glistening character and bright yellow colour were observed on both GYCA and YDC medium. Circular to irregular, slightly raised, mucoid colonies were recorded on nutrient agar and starch agar medium. XTS agar supported the moderated growth of the bacterium with minute, slightly raised, circular, creamy white coloured colonies. Bacterium exhibited very poor growth with dull white and slightly raised colonies character on BSCAA medium. He also revealed that *Xanthomonas axonopodis* liquefied the gelatine, hydrolysed the starch, positive for H_2S production, catalase and oxidase, utilized various carbon sources viz glucose, fructose, sucrose, dextrose and produced mild acid from these carbon sources but did not utilize lactose, maltose, mannose and mannitol.

Balestra *et al.,* (2008) isolated yellow, *Xanthomonas* like mucoid, convex colonies on YDC medium which were purified and stored on YDC slants. Upon conducting pathogenicity tests, they also observed symptoms typical of *Xanthomonas citri* on inoculated plants.

Vudhivanich (2003) isolated *Xanthomonas axonopodis pv. citri* from diseased citrus by tissue transplanting method on SX agar. After incubated for 48 hours at room temperature (30°C), the light yellow colony developed from plants tissue with clear zone surround them.

Braithwaite *et al.,* (2002) detected that gram negative *Xanthomonas citri pv citri* produced yellow pigmented, mucoid colonies on yeast dextrose agar, which were also isolated from the leaf lesions. They conducted pathogenicity test on potted citrus (*Citrofortunella mitis*) plants. Water-soaked lesions, 2-3 mm diameter, developed at the inoculation sites after 10 days and the bacteria were consistently re-isolated from the affected tissues.

Braithwaite *et al.,* (2002) observed that gram negative *Xanthomonas axonopodis pv. citri* produced yellow pigmented, mucoid colonies on agar plates with glucose or any other form of sugar. Presence of sugar makes the colonies very mucoid due to the production of extracellular polysaccharide slime. While the yellow colour of the colonies is the result of *Xanthomonadin* pigment production.

Gottwald and Graham (1992) observed that the concentrations less than 10^4 cfu/ml of *Xanthomonas* were insufficient to cause infection on unwounded citrus leaves under an impact pressure of 8.05 kPa, however 106cfu/ml gave consistent and successful infection.

Chand and Kishun (1991) reported that *Xanthomonas* produce mucoid, circular, convex, yellow, round, glistening and raised colonies on nutrient agar medium and on SX agar, pathogen produced a clear starch digestion zone.

Kishun and Chand (1991) reported that *Xanthomonas* was negative in nitrate reduction, urease oxidative, fermentative metabolism of glucose and acid from adonitol and sorbitol. The bacterium was positive in KOH solubility, gelatin liquefaction, hydrolysis of Tween 80, H_2S production, starch hydrolysis, indole production, growth at 3.5 percent NaCl, sucrose utilization, milk proteolysis and acid from most of the sugars.

Chand and Pal (1982) studied biochemical characteristics of *Xanthomonas* pv. *citri* and they found that bacterial cells were positive for hydrolysis of starch, aesculin, casein, liquefaction of gelatin and production of tyrosinase, catalase, reducing substance from sucrose, and hydrogen sulfide. The bacterium was negative for nitrate reduction, indole production and for methyl red test.

Starr and Stephens (1964) reported that genus *Xanthomonas* consisted of gram negative, rod shaped, polarly flagellated bacteria whose members were commonly occurred as serious plant pathogens. Colonies were typically yellow in color due to the presence of a particular carotenoid pigment identified through relatively simple screening procedures.

Goto (1992) found that the minimal dose of *Xcc* necessary for stomatal infection was 10^5 cfu/ml and that for wound infection, about 10^2 to 10^3 cells/ml were required.

Hingorani and Singh (1959) reported that nutrient agar, yeast glucose chalk agar and potato cylinders are the best media for the cultivation of *Xanthomonas axonopodis* because of luxuriant growth obtained on them. Colonies on nutrient agar were filiform, slightly, pale yellow and odoueless. Similar characters were also found on yeast glucose chalk agar with an exception that colour of the colony was bright yellow in the beginning and gradually changed to quite dark brown with age. They also described that the bacterium utilize xylose, glucose, mannose, galactose, sucrose, lactose and raffinose but not maltose, glycerine and salicin when grown in Durham's fermentation tubes containing one percent carboohydrates in a peptone free synthetic liquid medium. Ammonia was produced in peptone water after 15 days. Nitrites, hydrogen sulphide and indole were produced. Starch was hydrolysed, methyl red and voges proskauer tests gave negative results. Growth on gelatine slabs was good. Stratiform type of liquefaction commenced after 48 hours and completed within 21 days. The yellow colour of the growth on gelatine gradually changed from usual bright yellow to dark brown on yeast glucose chalk agar and cooked potato.

2.3. I*n- vitro* control of *Xanthomonas citri* and use of botanicals

Compendium (2021) reported that the interactions between *X. citri* and antagonistic bacteria including *Bacillus subtilis*, *Pantoea agglomerans*, *Pseudomonas syringae* and *P. fluorescens* have been reported *in vitro* and *in vivo*. However, the practical usefulness of these bacteria in controlling the pathogen has not been proved.

Hussain *et al.,* (2010) stated that diffusates, from various plants such as forest trees, shrubs, herbs, fruit seeds, etc. and from various parts of *Phyllanthus emblica, Accacia nilotica, Sapindus mukorossis* and *Terminakia chebula* which exhibited an inhibition zone of 4.83 to 6 mm at 50 g/liter, appeared to be the most effective diffusate against *Xanthomonas citri* pv. *citri*.

Khodakaramian *et al.,* (2008), observed that biological control of citrus bacterial canker, *Xanthomonas citri* pv. *citri,* was carried out by *pseudomonas* strains (Putida and fluorescent) in vitro and in green house. On in vitro based evaluations, strains having high potential for

inhibition along with antagonistic activities were selected against *X. citri* for green house evaluation as the disease was reduced by the selection between 23.8 and 64%.

Vudhivanich (2008) found that crude extract of *Chebulic myrobalan* fruit at 50, 000 ppm spraying before inoculation and after that 3 times every 7 days, decreased wound sizes. Average wound size at 15, 20 and 30 days were 0.62, 0.97 and 1.40 mm while in the control treatment was 0.97, 1.84 and 3.00 mm, respectively.

Sun *et al.,* (2004) were found that the presence of citrus canker on key/Mexican lime (*C. aurantiifolia*) and alemew (*Citrus macrophylla*) trees, the colonies, isolated from different infected portions, resembled the Asiatic group of *Xanthomonas citri* pv. *citri* (*Xac*-A) strains, in terms of growth characters on nutrient agar plates.

Das (2003) said in his work that a wide range of physiological, biochemical, serological, molecular and pathogenic variation was found among strains of bacteria associated with citrus canker. However, a better understanding of the pathogenic specialization and proper identification of *Xac* strains are needed.

Das (2003) said that in canker-free citrus producing areas, strict quarantine measures are practised aimed at excluding the pathogen. When the canker bacterium is introduced into such an areas (as it was in Florida, USA in 1910, 1984 and 1995) eradication campaign is conducted by uprooting and burning allsuspected and infected trees. Some strains of bacteria viz., *Pseudomonas syringae, Erwinia herbicola, Bacillus subtilis* and *Pseudomonas fluorescence* isolated from citrus phylloplane were reported to be antagonistic in vitro to the canker pathogen

Akhtar *et al.,* (1997), fount that diffusates, from various plants such as forest trees, shrubs, herbs, fruit seeds, etc. and from various parts of *Phyllanthus emblica*, *Accacia nilotica*, *Sapindus mukorossis* and *Terminakia chebula* which exhibited an inhibition zone of 4.83 to 6 mm at 50 g/liter, appeared to be the most effective diffusate against *Xanthomonas citri* pv. *citri*. These diffusates of higher plants having increased antimicrobial activity could be used for managing citrus canker disease as possessing both protective and curative actions.

Akhtar *et al.,* (1997) observed that diffusates (by using agar diffusion assay) of *Phyllanthus emblica*, *Acacia nilotica*, *Sapindus mukorossi* and *Terminalia chebula* inhibit the bacterium and

exhibited an inhibition zone measuring 4.83-6.00 mm at 50 g/l. These diffusates (50, 20, 10 g/l) also reduce the number of lesions on detached leaves and fruits of grapefruit, thus exhibiting protective as well as curative actions.

Pabitra *et al.,* (1996) have observed *in vitro* inhibition of *X. campestris* pv. *citri* by *Bacillus subtilis*, *B. polymyxa*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Aspergillus terreus*, *Trichoderma viride* and *Trichoderma harzianum* isolated from phylloplane of lemon.

Ota (1983) found a strain of *Pseudomonas syringae* antagonistic to *Xanthomonas citri* pv. *citri* which also prevented enlargement of lesions on infected leaves in citrus plants.

CHAPTER 3

MATERIALS AND METHODS

Two experiments were conducted throughout the research period in order to study the canker on citrus are as follows:

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

ii. Determination of efficacy of some selected botanical extracts against *Xanthomonas citri* pv. *citri.*

3.1. Experiment 1. Isolation, identification and characterization of *Xanthomonas citri* pv. *citri*.

3.1.1. Location of the area

Prevalence of canker on fruits and leaves of citrus was surveyed in three wholesale markets and four nurseries in Dhaka district. Three wholesale market and nurseries are as follows:

Whole sale markets	Nurseries
1. Karwan Bazar	1. Horticulture Centre, Falabithi, Asadgate, Dhaka
2. Mohammadpur Town Hall Kancha Bazar	2. Shobuj Bangla Nursery, Agargaon, Dhaka
3. Mohakhali Kancha Bazar	3. Krishibid Upakaran Nursery, Begum Rokeya Ave, Dhaka
	4. Shanti Garden, Sher-e-Bangla Nagar, Dhaka

In total 100 fruits and 300 diseased leaves were collected and brought to the laboratory for isolation purpose and further study.

3.1.2. Observation of the symptoms

Symptoms of the disease were studied by visual observation as described by Agrios (2006), Brunings and Gabriel (2003) and Civerolo (1981). Sometimes hand lens was used for critical observation of the disease. Identification of the disease was finally confirmed through isolation and different biochemical test.

3.1.3. Experimental period

Experiment was made during the period from July, 2021 to October, 2021. First survey was made in July, 2021 second survey was made in August, 2021 third survey was made September, 2021 and forth survey was made in October, 2021.

3.1.4. Data collection during experiment

During the survey in the markets and nurseries, samples composed of 100 random fruits and leaves were considered. Three samples were made representing three markets and four nurseries of the District of Dhaka. Total number of citrus fruits and leaves as well as number of fruits and leaves that were infected with citrus canker was recorded. The whole procedure was done three times to gets three replications.

3.1.5. Experimental site

Laboratory works were conducted in the laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.1.6. Collection and preparation of disease specimen

Fruit and leave samples water (Figure 1 and Figure 2) with characteristic symptoms were collected from markets and nurseries. The specimens were kept in the refrigerator at 4ºC by following standard procedure of preservation of disease specimens until isolation was made.

3.1.7. Isolation and purification of *Xanthomonas citri*

The diseased samples were washed under running tap water. Then the young lesions with green healthy portion of diseased fruits were cut into small pieces. Surface sterilization were done by dipping them in 75% ethanol solution for 2-3 minutes. Then they were washed three times with

sterile water. After surface sterilization the cut pieces were kept in a test tube containing 3-4 ml of sterile water and kept for 30 minutes for bacterial streaming and getting stock. After prepared different dilution, 0.1 ml of each dilution was spread over nutrient agar plate previously dried (to remove excess surface moisture) with micropipette at three replications. A bacterial suspension of each specimen was grown on nutrient agar plates and incubated at 28-30ºC. The plates were observed after 24-48 hr. Purified cultures were maintained on NA media one of single orange-yellow colony was picked by wire loop and streaked on another media plate for pure culture.

3.1.8. Preservation of canker pathogen of citrus

After purification of bacteria on nutrient agar plates, it was kept in refrigerator at 4ºC in screw-cap test tubes on NA slant for future use.

3.1.9. Nutrient Agar (NA) and Broth (NB) preparation

Nutrient agar media and nutrient broth were prepared according to the method followed by Schaad (1988). To prepare the nutrient agar at first 15 g bacto agar was taken in an Erlenmeyer flask containing 1000 ml distilled water. Then 5 g peptone and 3 g beef extract were added to it. The nutrient agar was shaken thoroughly for few minutes for mixing properly. In case of nutrient broh preparation, 5g peptone and 3 g beef extract were taken in the flask containing 1000ml distilled water. The mixture was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.1.10. Identification of the pathogen

Identification of the pathogen causing citrus canker was determined by conducting studies on morphological, biochemical and cultural features of the pathogen as per standard microbiological procedures.

3.1.11. Pathogenicity test (Inoculation and symptom development)

Collected citrus fruits and leaves were used for studying the pathogenicity of *Xanthomonas citri* pv. *citri*. The test was conducted by following the method described by (Lin *et al*., 2008). For the preparation of sample, it kept at 28°C for 24 hr and it was re-suspended in sterile ± inoculum, bacterial cells were grown overnight in NA broth at distilled water. Then an aliquot of the inoculams suspension was injected into the lower surface of citrus leaf with the help of a sterile

syringe. Distilled water was used as a negative control. 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.

Pure isolates of the bacterium were grown on nutrient agar plates and incubated at 28 ℃. Bacterial cells were then harvested in sterile distilled water by using sterile glass rod and the bacterial suspension was adjusted described by (Hamida *et al*., 2020). Morphological characteristics of the pathogen such as cell shape, gram's reaction and pigmentation were investigated as per the standard procedures described by (Islam *et al*., 2014).

3.1.12. Preparation of inoculums

Xanthomonas citri pv. citri bacteria were collected from pure culture that was preserved in small screw-cap test tubes on NA slant at 4ºC temperature in the refrigerator. Then bacteria suspension was prepared with sterile distilled water. The suspension was sieved through a double layer of cheese cloth to remove any kind of dirt. One drop of tween-20 (polyoxyethylene 20 sorbitan monolaurate) was added to the suspension to maintain uniform dispersion of bacterial cell in suspension.

3.1.13. Inoculation

Inoculation of bacteria was done by syringe inoculation method (insulin syringe). 10^6 cfu/ml unit of bacterial suspension was inoculated in each of the fruits and leaves. Six healthy fruits and twelve healthy leaves were inoculated. Every fruits and leaves were marked by permanent marker pen. The inoculated fruits and leaves were covered with cotton to maintain moisture content and prevent natural contamination with other Microorganisms. Humid condition was maintained gently spraying sterilized distilled water on fruits and leaves surface. Inoculation was done in 13 September.

3.1.14. Data collection

The first data was taken 10 days after inoculation and that was 23 September, 2021. Data was taken as the percent disease infection of the previously inoculated and marked fruits and leaves. Data was taken after every five days and it was taken at five consecutive periods.

3.1.15. Morphological characterization

Morphological characteristics of the pathogen such as cell shape, gram's reaction and pigmentation were studied as per the standard procedures described by Schaad, (1992); Gerhardt, (1981) and Bradbury, (1970).

3.1.16. Accession of collected test materials

Leaves and fruits of green lemon collected from various locations of Dhaka district were collected, placed in plastic bags, and brought to the laboratory for bacterial isolation (Table 1 and Table 2).

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

Isolates	**Locations**
Xcc-f-1	Karwan Bazar
Xcc-f-2	Karwan Bazar
Xcc-f-3	Karwan Bazar
Xcc-f-4	Karwan Bazar
Xcc-f-5	Karwan Bazar
Xcc-f-6	Karwan Bazar
Xcc-f-7	Mohammadpur Town Hall Kancha Bazar
Xcc-f-8	Mohammadpur Town Hall Kancha Bazar
Xcc-f-9	Mohammadpur Town Hall Kancha Bazar
Xcc-f-10	Mohammadpur Town Hall Kancha Bazar
Xcc-f-11	Mohammadpur Town Hall Kancha Bazar
Xcc-f-12	Mohammadpur Town Hall Kancha Bazar
Xcc-f-13	Mohakhali Kancha Bazar
Xcc-f-14	Mohakhali Kancha Bazar
Xcc-f-15	Mohakhali Kancha Bazar
Xcc-f-16	Mohakhali Kancha Bazar
Xcc-f-17	Mohakhali Kancha Bazar
Xcc-f-18	Mohakhali Kancha Bazar

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

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

3.1.16.1. 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.

3.1.16.2. 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).

3.1.17. Biochemical characterization

Different tests such as Gram Staining, KOH test, Pathogenicity test, KOVACS' Oxidase tests, etc. were performed with the fresh growth of isolates (Ali *et al.*, 2017; Chaity *et al*., 2019; Mubeen *et al*., 2015).

3.1.17.1. 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; Bradbury, 1970).

3.2. Experiments II. *In -vitro* Control of *Xanthomonas citri* pv. *citri* by Botanical

3.2.1. Experimental site

Laboratory works were conducted in the laboratory of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.2.2. Experimental period

The *in vitro* investigation was carried out in the month of July to December, 2021.

3.2.3. Botanical treatments used in the experiment

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

Table 3. Efficacy of botanicals in controlling of *Xanthomonas citri in-vitro*

Treatments (Botanical)	**Common Name**	**Scientific Name**	**Parts of Plant 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

3.2.4. Preparation of botanical extracts

Fresh plant parts namely *Azadirachta indica (*Neem leaf), *Ocimum indica (*Tulsi leaf), *Zingiber officinale* (Ginger rhizome*) Curcuma longa (*Turmeric rhizome*), Allium sativum (*Garlic clove*), Allium cepa (*Onion bulb) were used as treatments (Table 3). Visibly injured organs were discarded. Desired concentration of botanicals 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, preparation extract, each botanical was dipped in one per cent 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), mixture-cum grinder. The mixture was kept undisturbed at room temperature (28°C) for 18 hrs. in 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 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. Sensitivity of the different isolates was tested by modified paper disc diffusion technique (Negi and Kumar, 2015).

3.2.5. *In-vitro* experiment

The effectiveness of these plant extracts was tested by disc diffusion technique (Negi and Kumar, 2015). 100 µl of bacterial suspension (1×10^8 cfu/ml) were spread onto the surface of 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 on to 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 extracts against the bacterium.

3.2.6. Statistical analysis

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

CHAPTER 4

RESULTS

4.1. 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 (Figure 1A). The center of the lesion was raised and corky. In some leaves tissues in old lesion were died and fall out. The lesions in fruits were superficially similar to those on leaves but they were found irregularly shaped. Lesions in fruits were raised with corky appearance but there was no yellow halo at mature stage (Figure 1 B)

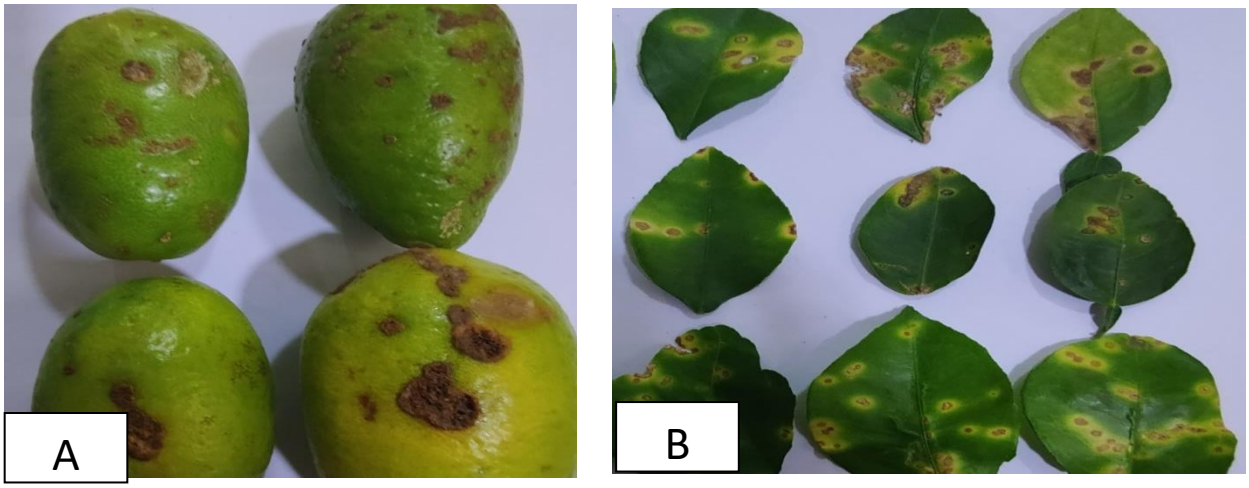

Figure 1. Infected citrus showing canker symptoms (A) Fruits (B) Leaves

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

Total twenty one isolates of *Xanthomonas citri* pv. *citri* were isolated from infected leaf of citrus and eighteen isolates were observed from infected fruit of citrus those were collected from different locations in Dhaka district. The isolates were purified by 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 streaking method, single colonies were found and partially identified based on colony morphology. The colonies were creamy white in color.

4.3. Pathogenicity test

The pathogenic variability amongst the six isolates of fruits and twelve isolates of leaves of *Xanthomonas citri* pv. *citri*, were studing (Table 4 and Table 5). All were found susceptible to all the isolates *Xanthomonas citri* pv. *citri*,. The isolates *Xanthomonas citri* pv. *citri* showed varied reaction in the symptoms development. The isolates viz. *Xcc-f-1, Xcc-f-2, Xcc-f-4, xcc-f-6* were found highly virulent in 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 case of leaves, pathogenic ability of all different isolates of *Xanthomonas citri* pv. *citri* 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, 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 *Xanthomonas citri* pv. *citri* was done on the basis of the symptoms development on fruits and leaves and days taken for appearance of the symptoms as No canker (-), Weak canker (+), Moderate canker (++), Strong canker (+++) as presented in Table 4 and Table 5. The Atiq *et.al.,* (2007), Katkar *et al*., (2016) and Jabeen *et al*., (2011) also confirmed the bacterium in similar manner as performed in this study.

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	Mohammadpur Town Hall Kancha Bazar	9	+++	Strong Canker
Xcc-f -5	Mohakhali Kancha Bazar	14	+	Weak Canker
Xcc-f -6	Mohakhali Kancha Bazar	7	+++	Strong Canker

No canker (-), Weak canker (+), Moderate canker (++), Strong canker (+++).

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 Bangla Nursery	10	+++	Strong Canker
Xcc-l-5	Shobuj Bangla Nursery	15	+	Weak Canker
Xcc-l-6	Shobuj Bangla Nursery	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

No canker (-), Weak canker (+), Moderate canker (++), Strong canker (+++).

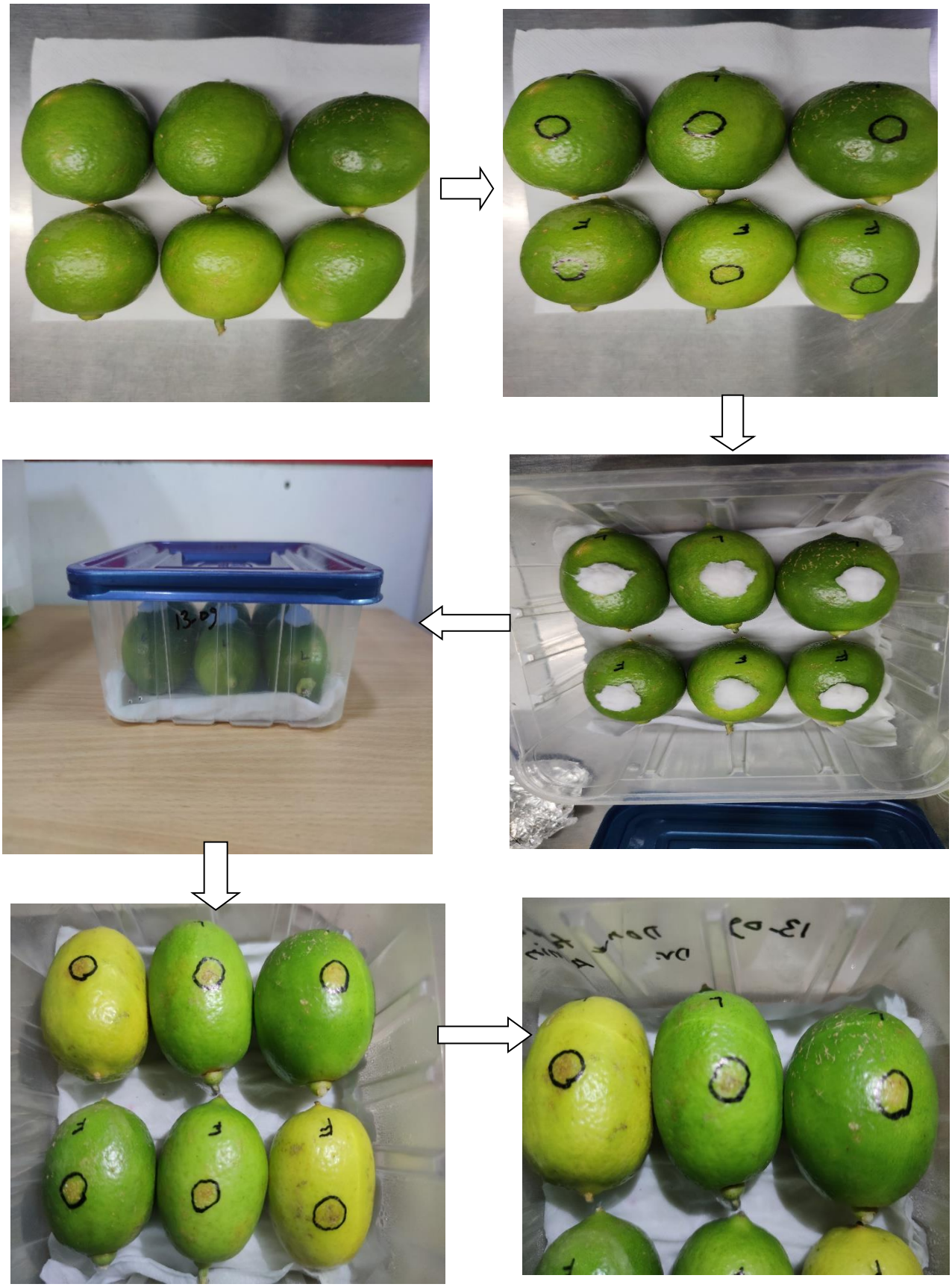

Figure. 2. Inoculation of *Xanthomonas citri* pv. *citri* and symptoms developed on fruits.

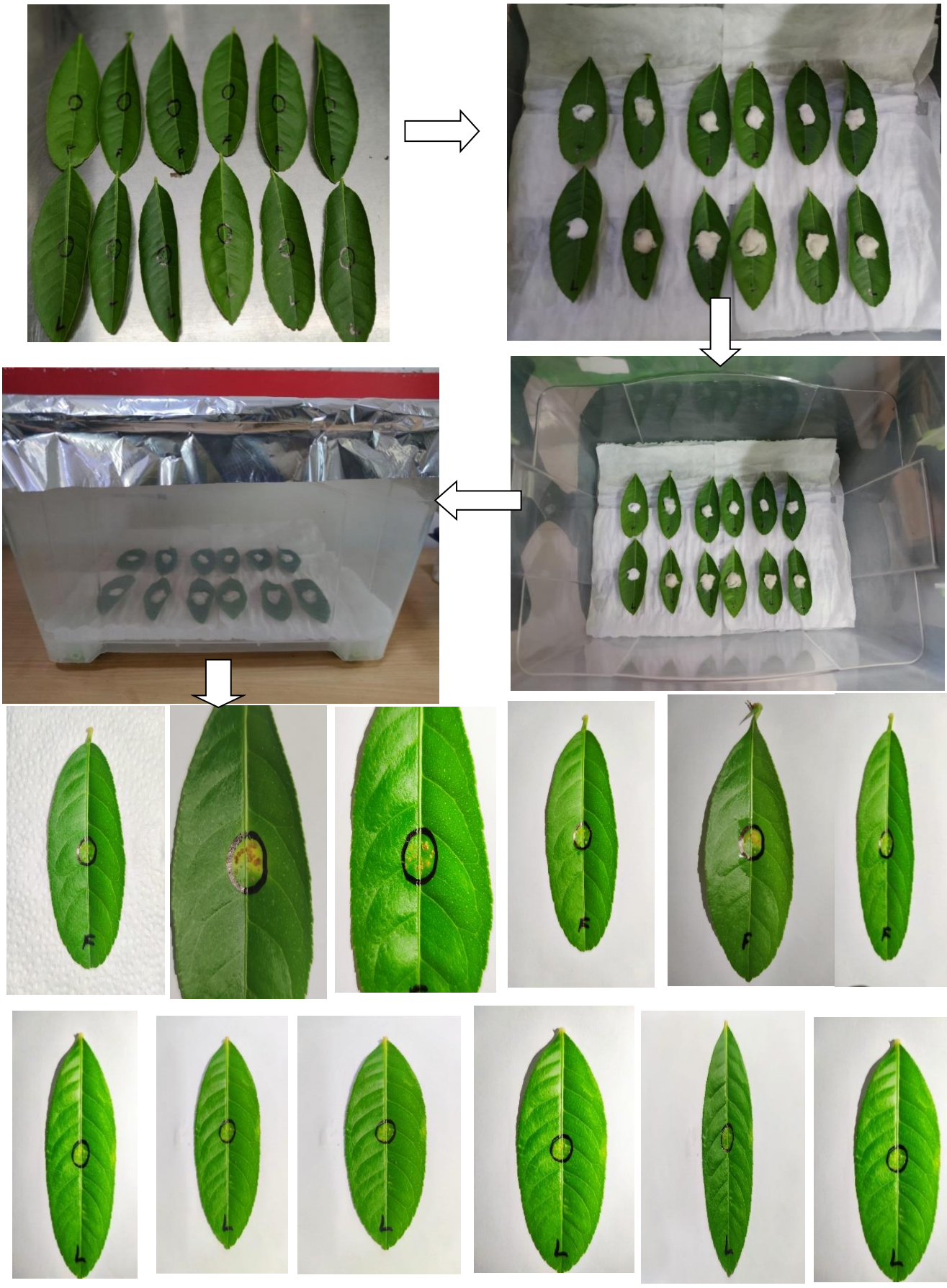

Figure 3. Inoculation of Xanthomonas citri pv. citri and symptoms developed on leaves

4.4. Biochemical Characteristics of *Xanthomonas citri pv. citri*

Biochemical characteristics of isolates were studied in order to check similarity of biochemical features with genus *Xanthomonas* by subjecting to various biochemical tests as shown in (Table 6).

In gram staining results indicated, Gram-negative bacteria have a thinner layer (10% of cell envelope), and are stained pink with safranin. Here, the isolated bacteria found to gram negative, motile and rod shaped under light microscope at 100 X magnification. After adding the Kovac's reagent, bacteria did not produce red/pink color band on the top of the tube and H_2S was not produced as no black precipitation was formed. In Kovac's test, medium containing filter paper and oxidizing agent reagent did not produce any color (Table 6).

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 in color	-ve	Gram staining showed gram negative bacteria
Kovac's	No color formation	-ve	Bacteria did not produce any characteristic color showed gram negative bacteria
KOH	Thread like	+ve	Thread like slime when picked up with a inoculam loop showed gram negative bacteria

4.5. 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 (Figure. 5) 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 leaves isolates. The unique opaque yellow colour colonies were obtained in the medium. The yellow colour was due to the production of *Xanthin* produced by the genus *Xanthomonas*. Colony colour of the bacterium was pale yellow to yellow while the shape and size of the colony were medium, convex and mucoid. (Figure. 6). Table. 7 and Table. 8 are showed the morphological characteristics of the citrus canker pathogens found on fruits and leaves, respectively.

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

Isolates	**Shapes**	**Size**	**Colour**	**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 infectedleaves

Isolates	**Shapes**	**Size**	**Colour**	**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
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

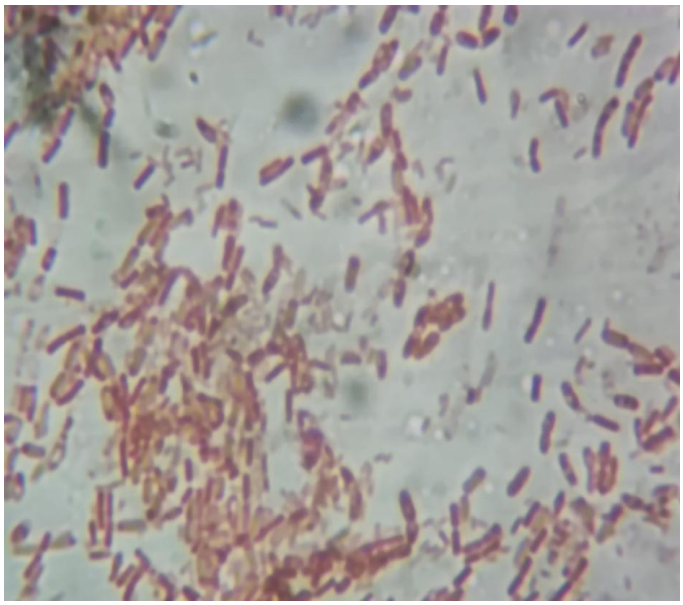

Figure 4. Microscopic view of *Xanthomonas citri* pv. *citri* after gram's staining at (X 100).

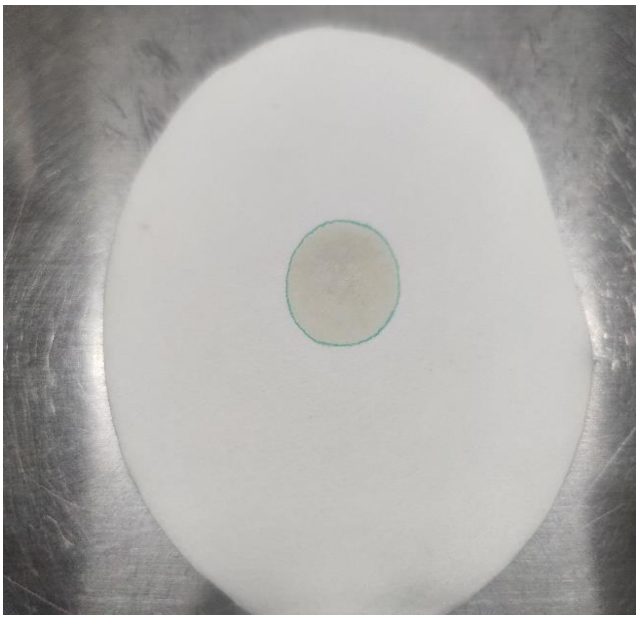

Figure 5. *Xanthomonas citri* gram negative bacterium that give no colour formation after 60 s in Kovac's test.

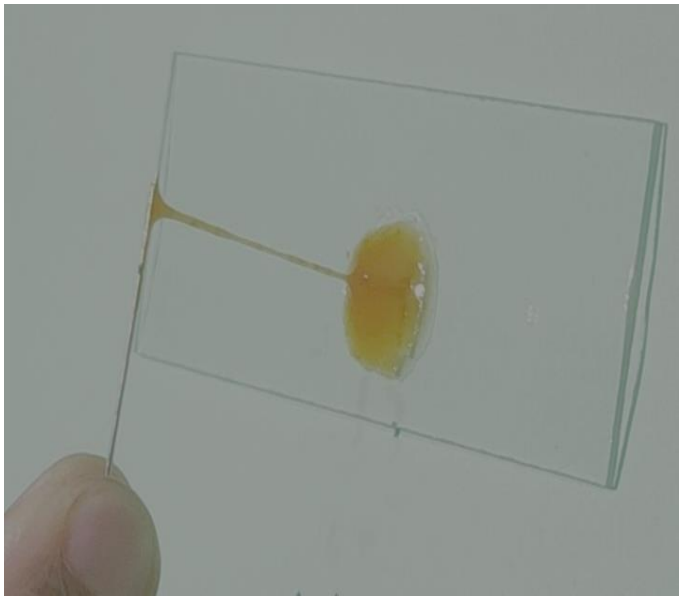

Figure 6. Gram negative bacteria form thread like slime when picked up with a inoculum loop in KOH test.

Figure 7. Cultural characteristics of *Xanthomonas citri* on Nutrient agar media.

4.6. Efficacy of Botanicals

Antibacterial activities of six different plant extracts were determined against the isolated bacteria. *Zingiber officinale* (Ginger) showed 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 *Azadiricta indica* in 1:4 (25g) concentration. *Allium sativum* extract showed lowest 8.0, 6.70 and 5.83 mm diameter of zone of inhibition against the isolated bacteria in all concentration levels. On the other hand, the extract of *Curcuma longa, Ocimum indica and Allium cepa* were showed comparatively similar result against pathogen isolates. The result was given in Table 9.

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
S.E. (±)		0.609	0.80	2.03
LSD (0.05%)		6.22	2.44	1.32

In column means value having similar letter (s) are statistically similar and those having dissimilar letter(s) differ significantly at .05 level of significance.

CHAPTER 5
DISCUSSION

In the present study, 100 fruit and 300 leave samples were collected from three markets and four nurseries of Dhaka districts for detection and further study of canker disease. The disease recorded in the present study based on the visual symptoms following the description of Bruning and Gabriel (2003), Agrios (2006) and Civerolo (1981). The disease also reported by Hossain (2011) in the citrus growing areas of Bangladesh. The disease recorded in the present study had also been reported on citrus fruits from several other countries (Burhun *et al*., 2007; Eshetu and Sijam, 2007; Bal and Dhiman, 2005; Graham *et a*l., 2004. Awasthi *et al*., (2005) reported citrus canker was the major problem in the nursery. The causal organism of canker of citrus was isolated from infected fruits and leaves following standard dilution plate technique using nutrient agar medium. Repeated isolation from the infected samples well separated, typical, yellow, convex, mucoid, colonies of bacterium on nutrient agar medium after 48 hours of incubation at 28°C. The pathogen also been reported by researchers throughout the world (Vudhivanich, 2003, Kale *et al.,*1994 and Qui and Ni, 1988). 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 canker of citrus (*Xanthomonas citri pv. citri)* was identified by conducting studies on its morphological, biochemical and cultural features as per standard microbiological procedures. The isolated bacteria were considered as gram negative enteric bacteria. Similar outcome were obtained by Kajal *et al.,* (2021), Ali *et al.,* (2017) and Soares *et al.,* (2010), with *Xanthomonas citri* pv. *citri* in medium. Total twenty one isolates of *Xanthomonas citri* pv. *citri* were isolated from infected leaf of citrus and eighteen isolates were observed from infected fruit of citrus. By streaking method, single colonies were found and partially identified based on colony morphology. The colonies were creamy white in color. The procedure was followed by Isokar *et al*., (2020), Prakash and Karmegam (2012) and Schaad (1988). Bacteria isolated from *Citrus aurantifolia* by Abubaker *et al.,* (2016) also showed similar results by different biochemical test. After gram staining under compound microscope at 100 X 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's staining test i.e., the bacteria was gram negative. Similar result in KOH solubility test was found by Kishun and Chand (1991). Braithwaite *et al* (2002), Schaad (1988), Gerhardt (1981), Bradbury (1970) and Starr and Stephens (1964) also reported that *Xanthomonas axonopodis pv.citri* as gram negative, rod shaped bacterium. In the present study the bacterium (*Xanthomonas axonopodis pv. citri)* showed positive results in KOH solubility test and negative result in oxidase test. Similar result has also been reported by Yenjerappa (2009), Kishun and Chand (1991) and Chand and Pal (1982). 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 bacteria gave light yellow, mucoid, round and smooth colonies. A similar result has also been reported by many researchers (Balestra *et al*., 2008; Vudhivanich, 2003 and Braithwaite *et al*., 2002). Our results confirmed the work of Mubeen *et al.,* (2015), Islam *et al.,* (2014) and Hussain *et al.,* (2010) 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. The Katkar *et al.,* (2016) and Jabeen *et al.,* (2011) also confirmed the bacterium in similar manner as performed in this study. Katkar *et al.,* (2016) observed that the fifteen isolates of *Xcc* on the basis of symptoms development on leaves and day taken for 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, pin prick 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 pin prick method was found to be more efficient in detached leaf assay produced large size lesion.

The efficacy of botanical extracts were varied significantly in the present study. The highest zone of inhibition was found in Ginger against isolated bacteria followed by Neem extracts in 1:4 concentration. These results are in consonance with the findings of several previous workers. Yenjerappa (2009) reported that, *Allium sativum* extract at 10 per cent 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 were tested against *Xanthomonas citri* pv. *citri* by Ramesh (2015) and 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 *Xanthomonas. citri* pv. *citri* earlier by several workers (Leksomboon *et al.,* (2001); Prakash and Karmegam (2012); Giri *et al.,* (2008); Vudhivanich, (2003); Raju *et al.,* (2013).

CHAPTER 6

SUMMARY AND CONCLUSION

Citrus (*Citrus spp.*) is an important fruit crop grown all over the world. It belongs to the family Rutaceae. Though the demand of citrus is increasing day by day it's production in terms of area and yield is not satisfactory due to lack of knowledge about the diseases of citrus and the pathogens that are responsible for those diseases. Fruits of citrus are vulnerable attack by various diseases in Bangladesh especially citrus canker, but least concrete information regarding their distribution, incidence, severity, epidemiology and management is available.

Citrus canker manifested itself in the form of small, blister-like lesions on the abaxial surface of infected leaves. As the disease progress, they turned gray to tan brown with an oily margin surrounded by a yellow halo. The center of the lesion became raised and corky and was visible on both sides of the leaf. Leaf tissues in old lesion had died and fall out. The lesions in fruits were superficially similar to those on leaves but they were generally irregularly shaped. Infected leaves and fruits having typical symptoms were collected from naturally infected plant and isolated the pathogen in the laboratory by following dilution plating method using nutrient agar medium. The causal organism was purified by re-streaking on nutrient agar medium with single colony and confirmation was done by pathogenicity test. The bacterium was gram negative, rod shaped with rounded ends. It showed negative result in oxidase test and positive result in KOH solubility test. On NA medium the bacterium appeared as circular, mucoid, convex, yellow to pale yellow colour. On the basis of morphological, biological and cultural characteristics it can be terminated that the pathogen was *Xanthomonas citri* pv. *citri.*

In the pathogenicity test, it was 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. The result of the present study concludes that there is variability present among the different isolates of *Xanthomonas citri* pv. *citri*.

A laboratory experiment was carried out to find the efficacy of some botanicals against citrus canker pathogen *Xanthomonas citri* pv. *citri.* Management of plant diseases through plant extracts are a remarkable and economical method. Antibacterial activities of six different plant extracts namely *Azadirachta indica (*Neem leaf)*, Ocimum indica (*Tulsi leaf)*, Zingiber officinale* (Ginger rhizome*), Curcuma longa (*Turmeric rhizome*), Allium sativum (*Garlic clove*), Allium cepa (*Onion bulb) were determined against the isolated bacteria. *Zingiber officinale* (Ginger) showed 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 *Azadiricta indica* in 1:4 (25g) concentration. *Allium sativum* extract showed lowest 8.0, 6.70 and 5.83 mm diameter of zone of inhibition against the isolated bacteria in all concentration levels. On the other hand, the extract of *Curcuma longa*, *Ocimum indica and Allium cepa* were showed comparatively similar result against pathogen isolates. The presented work provides the information on potentiality of biocontrol agents to reduce the canker disease of citrus plants with comparatively safe and an easy-to-use tool. After the inoculation of pathogen *in-vitro* conditions, the fruits and leaves developed symptoms within 12 to 16 days and the reaction varies significantly i.e., weak and strong. In case of botanicals treatments, highest zone of inhibition was observed in *Zingiber officinale* whereas *Allium sativm* showed lowest inhibition zone. In different biochemical media test, bacterial isolates of *Xanthomonas citri* showed no reaction in both gram staining and Kovac's test and positive in KOH test respectively. Isolates were response differently in fruits and leaves in regards of morphological and cultural characteristics i.e., yellow to pale yellow in color and convex and mucoid shape. Literature survey indicated that no much work is done about the use of bio-control agent against bacteria causing citrus canker in our country; so the climate, atmosphere, environmental factors are different from different geographical locations. It was concluded that biochemical and molecular characterization of *Xanthomonas citri* pv. *citri* is necessary for the identification and control measures of citrus canker disease in Bangladesh.

CHAPTER 7

REFERENCES

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 Journal of North America*, **7**(5): 254-265.

Agrios, G. N. (2006). *Xanthomonas*. In: Plant Pathology. 5th ed. Academic Press. San Diego, CA: 671-672.

Akhtar. MA., Rahber, Bhatti, MH. and Aslam, M. (1997). Antibacterial activity of plant diffusates against *Xanthomonas campestris* pv. *citri. International Journal of Pest Management,* **43**: 149-153.

Al-Dulaimi, F., AL-Kaisse, A. A., Al-rubaye, L. and Abdulwadood, M. (2018). First report of citrus bacterial canker caused by *Xanthomonas axonopodis* pv. *citri* in iraq. *Journal of Biotechnology Research Center*, **12**(2): 24-31.

Ali, M. R., Hasan, M. F., Lia, R. S., Akter, A., Sumi, M. S. E., Hossain, M. F. and Sikdar, B. (2017). Isolation and characterization of a canker disease causing pathogen from *Citrus aurantifolia* and evaluation of its biological control measure. *Journal of Entomology 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. *Journal of Applied Biology and Biotechnology*, **2**(01): 017-022.

Awasthi, D. P., Sarkar, S., Mishra, N. K. and Kaisar, S. A. K. M. (2005). Disease situation of some major fruit crops in new alluvial plains of west Bengal. Environ. Ecol. **235**: 497-499.

Bal, R. S. and Dhiman, J. S. (2005). An analysis of synchronous progress of canker. Scab complex in kinnow nursery. Plant Dis. Res. **20** (2): 147-150.

Balestra, G. M., Sechler, A., Schuenzel, E. and Schaad, N. W. (2008). First report of citrus canker caused by *Xanthomonas citri* in Somalia. Plant Disease, **92**(6): 981.

BBS (2012). Bangladesh Bureau of Statistics. Statistical Year Book of Bangladesh. Statistics Division, Ministry of Planning, Government of the Peoples Republic of Bangladesh: 375.

Bradbury, J. F. (1970). Isolation and preliminary study of bacteria from plants. *PANS Pest Articles & News Summaries*, **16(**4): 632-637.

Braithwaite, M., Leite, R. P. J., Smith, J. J., Boa, E. and Saddler, G. S. (2002). First report of citrus canker caused by *Xanthomonas campestris pv. citri* on *Citrus sinensis* in Bolivia. Plant Pathology, **51**(3): 383-385.

Britto, S. J. and Senthilkumar, S. (2001). Antibacterial activity of *Solanum incanum* L. leaf extracts. *Asian Journal of Microbiology, Biotechnology & Environmental Sciences*, **3**(1): 65-66.

Brunings, A. M. and Gabriel, D. W. (2003). *Xanthomonas citri*: breaking the surface. *Molecular Plant Pathology*, **4**(3): 141-157.

Burhan, M., Choudhary, N. A., Ishfaq, M. and Sarwar, M. (2007). Incidence of citrus canker (*Xanthomonas citri pv. citri)* on orange cultivars in nursery. Int. J. Agr. Biol. **9** (3): 533-534.

Bylka, W., Szaufer-Hajdrych, M., Matławska, I. and Goślińska, O. (2004). Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris* L. *Letters in applied microbiology*, **39**(1), 93-97.

Canteros, B. I. (2004). Management of citrus canker in Argentina. A review. In *Proceedings of the International Society for Citriculture*.

Carrera, C., (1933). Informe preliminar sobre una enfermedad nueva comprobada en los citrus de Bella Vista (Corrientes). Bol. Mens. Min. Agric. Nac. Buenos. Aires.**34**:275-280.

Chaity, A. S., Chowdhury, M. E. K., Hasan, M. F., Islam, M. A., Haque, M. F. and Sikdar, B. (2019). Isolation, characterization and control of canker disease causing bacteria of selected citrus plants in Rajshahi, Bangladesh. *European Journal Biotechnology and Bioscience*, **7**: 9-17.

Chand, R., Kishun, R. and Singh, S. J. (1991). Characterization of bacteriophage parasitizing on *Xanthomonas campestris pv. viticola*. *Indian J. Exptl. Biol.* **29:** 180-181.

Chand, R. and Kishun, R. (1991). Studies on bacterial blight (*Xanthomonas campestris pv. punicae)* of pomegranate. *Indian Phytopath*. **44:** 370-371.

Chand, J. N. and Pal, V. (1982). Citrus canker in India and its management. In: Problems of citrus diseases in India (S.P. Raychaudhuri and Y.S. Ahlawat, Eds.). Surabhi Printers and Publishers, New Delhi, 21-26.

Chaudhry, Z. and Rashid, H. (2011). Isolation and characterization of Ralstonia solanacearum from infected tomato plants of Soan Skesar valley of Punjab. *Pakistan Journal of Botany*, **43(**6): 2979-2985.

Civerolo, E. L. (1981). Citrus bacterial canker disease: An overview. Proc. Intn. Soc.Citric. 1: 390-394.

Compendium, I. S. (2021). *Xanthomonas citri* (citrus canker).

Das, A. K. (2003). Citrus canker- A review. *Journal of Applied Horticulture*, **5**(1): 52-60.

Das, A. K. and Shyam, S. (2003). Integration of chemicals and cultural practices for management of bacterial canker (*Xanthomonas axonopodis pv. citri*) in acid lime (*Citrus aurantifolia*). Indian J. Agric. Sci. **73**(10): 570-571.

Dewdney, M. M. and Graham, J. H. (2014). Florida Citrus Pest Management Guide: Citrus Canker (EDIS). *The Institute of Food and Agricultural Sciences (IFAS). University of Florida*, 182.

Dixon, W. N., Levy, L. and Gottwald, T. R. (2000). Proceedings of the International Citrus Canker Research Workshop, Ft. Pierce, FL, USDAARS.

Donkersley P, Silva F, Carvalho C, Al-Sadi AM. and Elliot S (2018). Biological, environmental and socio-economic threats to citrus limeproduction. *Journal of Plant Diseases and Protection*, **125**:339–356

Dorman, H. D. and Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of applied microbiology*, **88**(2), 308-316.

Duncan, L.W., Noling J.W. and Inserra, R.N. (2014). Florida citrus pest management Guide: Nematodes. University of Florida cooperative Extension service: 59- 61.

Eshetu, D. and Sijam, K. (2007). Citrus canker: a new disease of Mexican lime (*Citrus aurantiifolia*) and sour orange (*C. aurantium*) in Ethiopia. *Fruits Paris*. **62** (2): 89-98

Gerhardt, P. (1981). Manual of methods of General Bacteriology. American Society of Microbiology Washington, D.C:127.

Gill, D. (2013). Citrus canker: A serious bacterial disease. *Louisiana Department of Agriculture and Forestry. State of Louisiana. USA.*

Giri, G. K., Gade, R. M., Gulhane, A. R. and Das, S. (2008). Efficacy of bioagents, botanicals and chemicals against citrus canker (*Xanthomonas axonopodis* pv. *citri.*). *Journal of Plant Disease Sciences*, **3**(2): 249-250.Graham, J. H. and Leite, R. P. J. (2004). Lack of control of citrus canker by induced systemic resistance compounds. *Plant Disease*, **88**(7): 745-750.

Graham, J. H., Gottwald, T. R., Cubero, J. and Achor, D. S. (2004). *Xanthomonas axonopodis pv. citri.* factors affecting successful eradication of citrus canker. Molecular Plant Pathology. **5**(1): 1-15.

Goto, M. (1962). Studies on Citrus Canker, I. Bull. Fac. Agric. Shizuoka University, **12**: 3-72.

Gottwald, T. R. and Graham, J. H. (1992). A device for precise and nondisruptive stomatal inoculation of leaf tissues with bacterial pathogens. Phytopathology. **82**: 930-935.

Gottwald, T. R., Timmer, L. W. and Mcguire, R. G. (1989). Analysis of disease progress of citrus canker in nurseries in Argentina. *Phytopathology*, **79**: 1276- 1283

Hamida, E. A., Elhassanb, S. M. and Abubakerc, M. Y. A. (2020). Use of Botanicals Against Citrus Canker (*Xanthomonas axonopodis* pv. *citri*) Affecting Lime (*Citrus aurantifolia* Swingle) in Sudan. *Journal of Horticultural Science and Technology*, **3**(4): 93-97.

Haider, M.F., Ahmad, Z., Shakeel, Q.,Shafiq, S., Chand, S.A., Saddiq, W.M., Ahsan, M.A. and Kharal, M.(2020). Biochemical characterization of *Xanthomonas axonopodis* pv. *citri;* A major impediment to Citrus orchards. International Journal of Biosciences, **16** (3): 512-517.

Hingorani, M. K. and Singh, N. J. (1959). *Xanthomonas sp.* Nov. *On Punica granatum L. Indian J. Agric. Sci.* **29:** 45-48

Hossain, I. (2011). Nursery diseases of some selected fruit species in Bangladesh. *Eco-friendly Plant Disease Management Laboratory, Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh*, *24*.

Hussain, K., Nawaz, K., Majeed, A., Haq, I. U., Lin, F., Ali, K. and Raza, G. (2010). Molecular and biochemical characterization of *Xanthomonas axonopodis* pv. *citri* pathotypes. *African Journal of Biotechnology*, **9**(54): 9092-9095.

Islam, M. N., Ali, M. S., Choi, S. J., Hyun, J. W. and Baek, K. H. (2019). Biocontrol of citrus canker disease caused by *Xanthomonas citri* subsp. *citri* using an endophytic Bacillus thuringiensis. *The Plant Pathology Journal*, **35**(5): 486.

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*. *Advancements in Life Sciences*, **1**(4): 215-222.

Isokar, S., Bramhankar, S.B., Das, A., Thakur, K.D., Pillai, T., Dinkwar and Kharat, V. (2020). Morphological, biochemical and molecular characterization of *Xanthomonas citri* subsp. *citri* isolates from different agroclimatic zones of Maharashtra. *International Journal of Chemical Studies, 8*: 62-70.

Jalan, N., Kumar, D., Yu, F., Jones, J. B., Graham, J. H. and Wang, N. (2013). Complete genome sequence of *Xanthomonas citri* subsp. *citri* strain Aw12879, a restricted-host-range citrus canker-causing bacterium. *Genome Announcements*, **1**(3): e00235-13.

Jabeen, R., Iftikhar, T. and Batool, H. (2012). Isolation, characterization, preservation and pathogenicity test of *Xanthomonas oryzaepv. oryzae* causing BLB disease in rice. Pakistan Journal of Botany, 44(1): 261-265.

Jabeen R, Saeed UR, Rahman, Rais A. Evaluating BLB resistance/aggressiveness in rice through best inoculum concentration, inoculation and application methods. Pak. J Bot. (2011); **43**(5): 2635-2638.

Jadhav, R. R., More, A. S., Apet, K. T.and Dandnaik, B. P. (2018). In vitro Bioefficacy of Different Antibiotics, Fungicides and Botanicals against (*Xanthomonas axonopodis* pv. *citri*.) causing

Bacterial canker of Kagzi Lime. *International Journal of Current Microbiology and Applied Sciences*, **6**: 1693-1699.

Jia, H., Zhang, Y., Orbović, V., Xu, J., White, F. F., Jones, J. B. and Wang, N. (2017). Genome editing of the disease susceptibility gene Cs LOB 1 in citrus confers resistance to citrus canker. *Plant Biotechnology Journal*, **15**(7): 817-823.

Kajal, G.,Sangita, A. and Rajan, W. (2021). Isolation and study of bacteriophage of citrus canker causing bacterial pathogen. International Journal of Life Sciences, **9** (2):175-180.

Katkar, M., Raghuwanshi, K. S., Chimote, V. P. and Borkar, S. G. (2016). Pathological, bio-chemical and molecular diversity amongst the isolates of *Xanthomonas axonopodis* pv. *citri* causing citrus canker in acid lime from different agro-climatic region of India. *International Journal of Environment, Agriculture and Biotechnology*, **1**(2): 2456-2478.

Kale, K. B., Kolte, S. O. and Peshney, N. L. (1994). Economics of chemical control of citrus canker caused by *Xanthomonas campestris* pv. *citri* under field conditions. *Indian Phytopathology*, **47**(3): 253-255.

Khan, W.A., Atiq, M., Sahi, S.T., Khan, A.A., Ali, S., Younas, M., Ali, Y., Bashir M.R. and Sajid, M. (2018). Induction of resistance against citrus canker through chemicals and plant activators. Advances in Zoology and Botany, **6**(1): 26-30.

Khodakaramian, A., Heydari, A. and Balestra, G. M. (2008). Evaluation of *Pseudomonads* bacterial isolates in biological control of citrus bacterial canker disease. *International Journal of Agricultural Research*, **3**(4): 268-272.

Kilani, A. M. (2006). Antibacterial assessment of whole stem bark of Vitex doniana against some enterobactriaceae. *African Journal of Biotechnology*, **5**(10).

Kishun, R. and Chand, R. (1991). Outbreak of black rot of radish in India. Indian Journal of Plant Pathology, **9**(1and 2): 7-13.

Klotz, L.J. (1973). Color Hand Book of Citrus Disease. University of California, Division of Agricultural Science, 122.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature*, **178** (4535): 703-703.

Leite Jr, R. P. and Mohan, S. K. (1990). Integrated management of the citrus bacterial canker disease caused by *Xanthomonas campestris* pv. *citri* in the State of Paraná, Brazil. *Crop Protection*, **9**(1): 3-7.

Leksomboon, C., Thaveechai, N. and Kositratana, W. (2001). Potential of plant extracts for controlling citrus canker of lime. *Agriculture and Natural Resources*, **35**(4): 392-396.

Lin, H. C., Chang, H. and Tzeng, K. C. (2008). Characterization of novel strains of citrus canker Bacterium from citrus in Taiwan. *台灣農業研究*, **57**(4), 265-278.

Liu, Y., Heying, E. and Tanumihardjo, S. A. (2012). History, global distribution, and nutritional importance of citrus fruits. *Comprehensive reviews in Food Science and Food Safety*, **11**(6):530-545.

Mahajan, A. and Das, S. (2003). Plants and microbes- Potential source of pesticide for future. Pesticide Information, **28**(4): 33-38

Marco, G. M. and Stall, R. E. (1983). Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris pv. vesicatoria* that differ in sensitivity to copper (No. RESEARCH).

Mubeen, M., Arshad, H. M., Iftikhar, Y., Irfan Ullah, M. 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.

Negi, A. and Kumar, P. 2015. Antibacterial effect of plants extracts and antibiotics on *Xanthomonas axonopodis pv. citri in-vitro*. Trends in Biosciences, 8(9): 2374-2376.

Ota T (1983) Interaction in vitro and in vivo between *Xanthomonas campestris* pv. *citri* and antagonistic *Pseudomonas sp.* Annals of the Phytopathological Society of Japan, **49**: 308

Pabitra K, Bora LC, Bhagabati KN. and Kalita P (1996) Phylloplane microflora of citrus and their role in management of citrus canker. *Indian Phytopathology*, **49**: 234-237.

Parameswari, C. S. and TULASI LATHA, A. (2001). Antibacterial activity of *Ricinus communis* leaf extract. *Indian drugs*, **38**(11): 587-588.

Pathak, V. N. (1980). Diseases of fruit crops. *Diseases of fruit crops*:12-22.

Peterson, R. A., Johnson, G. I., Schipke, L. G. and Cooke, A. W. (1986) Chemical control of stem end rot in mango. In *III International Mango Symposium*, **29:** 304-311.

Phule, R. S. S. S. M. and Mahavidyalay, P. (2021). Isolation and study of bacteriophage of citrus canker causing bacterial pathogen. *International Journal of Life Sciences*, **9**(2): 175-180.

Ploet, R. C., Zentmyer, G. A., Nishijima, W. T., Rohrbach, K. G. and Ohr, H. D. (1994). *Compendium of tropical fruit diseases*. The American Phytopathological Society: 34-44.

Polek, M. (2007). *Citrus bacterial canker disease and Huanglongbing (citrus greening)*. UCANR Publications.

Prakash, M. and Karmegam, N. (2012). In-vitro antibacterial activity of certain plant extracts against plant disease causing bacteria isolated from citrus plant. *International Journal of Current Microbiology and Applied Sciences*, **1**(1): 1-11.

Qui, C. H., & Ni, B. Q. (1988). A preliminary test on the control of citrus canker with monomolecular forming substance (MMFS) and other chemicals. *Fujian Agril. Sci. Technol*, *1*, 19.

Raju, J., Benagi, V. I., Jayalakshmi, K., Angadi, S. G., Basha, H. and Giri, M. S. (2013). In-vitro evaluation of bioprospectus for bacterial blight of pomegranate. *BIOINFOLET-A Quarterly Journal of Life Sciences*, **10(**2): 710-713.

Ramesh, I. (2015). Biological management of *Xanthomonas axonopodis* pv. *Punicae* (Hingorani and Singh) Vauterin *et al.*, causing bacterial blight of *punica granatum* L. M.Sc thesis, Univ. of Hortic. Sci., Bagalkot. Karnataka.

Rashid, M., Chowdhury, M. S. M., & Sultana, N. (2014). Prevalence of canker on seedlings of citrus (*Citrus spp*.) in selected areas of Bangladesh and its management. *The Journal of Plant Pathology*, **114:** 177-187.

Rath, C. C., Dash, S. K. and Mishra, R. K. (2001). In vitro susceptibility of Japanese mint (*Mentha arvensis* L.) essential oil against five human pathogens. *Indian Perfumer*, **45**(1): 57-62.

Riasat, A., Ghazanfar, M. U. and Raza, W. (2020). Interaction of citrus canker with Phyllocnistis citrella stainton. *Pakistan Journal of Phytopathology*, **32**(1): 01-07.

Rodriguez, G. S., Garza, L. J. G., Stapleton, J. J. and Civerolo, E. L., (1985). Citrus bacteriosis in Mexico. *Plant Disease*, **69** (9): 808-810.

Roller, W. (1998). Chemical Approaches to Managing Plant Pathogens. In Handbook of Pest Management : 359-398. CRC Press.

Sahi, S. T., Ghazanfar, M. U., Afzal, M., Rashed, A. and Habib, A. (2007). Incidence of citrus canker disease caused by *Xanthomonas campestris* pv. *citri* (Hasse) dows on Kinnow (*Citrus reticulata*) and its chemotherapy. *Pakistan Journal of Botany*, **39(**4): 1319.

Salahvarzi, A., Mostaghim, T. and Reza Khani, M. (2016). Production of Noncarbonated Lemon Orange Juice. *European Online Journal of Natural and Social Sciences*, **5**(2): 504.

Schaad, N. C., Postnikova, E., Lacy, G. H., Sechler, A., Agarkova, I., Stromberg, P. E., Stromberg, V. K. and Vidavar, A. K., (2005). Emended classification of Xanthomonad pathogens on citrus. Systematic Appllied Microbiology, **29:** 690-695.

Schaad, N. W. (1988). Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. *American Phytopathological Society*, St. Paul, Minnesota : 85-197.

Shahid, M. M., Sahi, S. T., GHAZANFAR, M. U., Inam, M., HAQ, I., HAQ, Y. I. and AHMAD, T. (2005). Evaluation of different toxicants against *Xanthomonas campestris* pv. *citri* (Hasse) Dows. *Int. J. Agric. Biol*, **7:** 121-124.

Sharma, S. K. and Sharma, R. R. (2009). Citrus canker approaching century: A review. *Tree and Forestry Science and Biotechnology*, **2**: 54-56.

Shimpi, S. R. and Bendre, R. S. (2005). Stability and antibacterial activity of aqueous extracts of *Ocimum canum* leaves. *Indian perfumer*, **49**(2): 225.

Singh,S.J. (1998). Advances in Diseases of fruit crops in India.Kaltani Publisher: 197-199.

Singh, D., Singh, R. S. and Kaur, K. (2005). Biological management of citrus scab in Panjab. Plant Dis. Res. **20**(1): 76-82.

Solaiman, A. M., Nishizawa, T., Nayem, S. A., Mohsin, S. M. and Chowdhury, M. S. M. (2015). Effect of Partially UV-Blocking Films on *Xanthomonas axonopoides* Pv. *Citri* Causing Citrus (*Citrus aurantifolia*) Canker. *World Journal of Agricultural Science*, **11**: 202-209.

Soares, M. R., Facincani, A. P., Ferreira, R. M., Moreira, L. M., de Oliveira, J. C., Ferro, J. A. and Gozzo, F. C. (2010). Proteome of the phytopathogen *Xanthomonas citri* subsp. *citri*: a global expression profile. *Proteome Science*, **8**(1): 1-11.

Starr M. P. and Stephens. W. L. (1964). Pigmentation and taxonomy of the genus *Xanthomonas.* Journal of Bacteriology, **87**: 293-302.

Stall, R. E. and Seymour, C. P. (1983). Canker, a threat to citrus in the Gulf-Coast states. *Plant Disease*, **67**(5), 581-585.

Sun, X., Stall, R. E., Jones, J. B., Cubero, J., Gottwald, T. R., Graham, J. H. and Sutton, B. (2004). Detection and characterization of a new strain of citrus canker bacteria from Key/Mexican lime and alemow in South Florida. *Plant disease*, **88**(11): 1179-1188.

Suslow, T. V., Schroth, M. N. and Isaka, M. (1982). Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathology (USA)*.

Swarup, S., Feyter, R., Brlansky, R. H. and Gabriel, D. W. (1991). A pathogenicity locus from Xanthomonas citri enables strains from several pathovars of *X. campestris* to elicit canker like lesions on citrus. *Phytopathology*. **81**: 802-809.

[UNCTAD] United Nations Conference on Trade and Development (2004). Market Information in the Commodities Area: Information on citrus fruit. Available from: http://r0.unctad.org/infocomm/anglais/orange/ sitemap.htm. Accessed 2011 June.

Verniere, C. J., Gottwald, T. R. and Pruvost, O. (2003). Disease development and symptom expression of *Xanthomonas axonopodis pv. citri* in various citrus plant tissues. *Phytopathol.* **93** (7): 832-843.

VM Kharat, SB Bramhankar, KD Thakur, ss Isokar, Tini Pillai and GT Dinkwar (2020). Management of citrus canker bacterium by using botanicals and chemicals. *International Journal of Chemical Studies*; **8** (2): 1878-1882

Vudhivanich S (2008) Decreasing symptom development of citrus canker by Thai herbal crude extract. Proceedings of the 46th Kasetsart University Annual Conference, 29 January – 1 February 2008, Kasetsart, 489-496.

Vudhivanich, J. (2003). Efficacy of Thai herbal extract for growth inhibition of *Xanthomonas axonopodispv. citri*, the bacterial canker of citrus. Kasetsart Journal of Natural Science, **37**: 445 – 452.

Yenjerappa, S. T. (2009). Epidemiology and management of bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*. Ph.D. Thesis. Department of Plant Pathology, University of Agricultural Sciences, Dharwad.

Zhong, D. Z. and Ling, N. C. (2002). Observation on the occurrence regulation of citrus canker disease and its control. *South China Fruits*, **31**(2), 20-21.

APPENDICES

Appendix I. Preparation of culture media and reagents

The compositions of the media used in this thesis work are given below. Unless otherwise mentioned all media were autoclaved at 121°C for 15 minutes at 15 Ib pressure.

Nutrient Agar (NA)

Beef extract (Difco): 3.0 g

Peptone (Difco): 5.0 g

Bacto agar: 15.0 g

Distilled water: 1000 ml

Nutrient Broth (NB)

Beef extract (Difco): 3.0 g

Peptone (Difco): 5.0 g

Distilled water: 1000 ml

KOH solubility reagent

3% aqueous solution of KOH was prepared from the KOH granules.

Gram's staining reagents

Gram's Crystal violet (Hucker's modification)

Solution A: Crystal violet (90% dye content): 2.0 g

Ethyl alcohol: 20.0 ml

Solution B: Ammonium oxalate: 0.8 g

Distilled water: 80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

Gram's Iodine (Gram's modification of Lugol's solution)

Iodine: 1.0 g

Potassium iodine (KI): 2.0 g

Distilled water: 300.0 ml

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%): 98 ml

Acetone: 2 ml

Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol): 10 ml

Distilled water: 100 ml

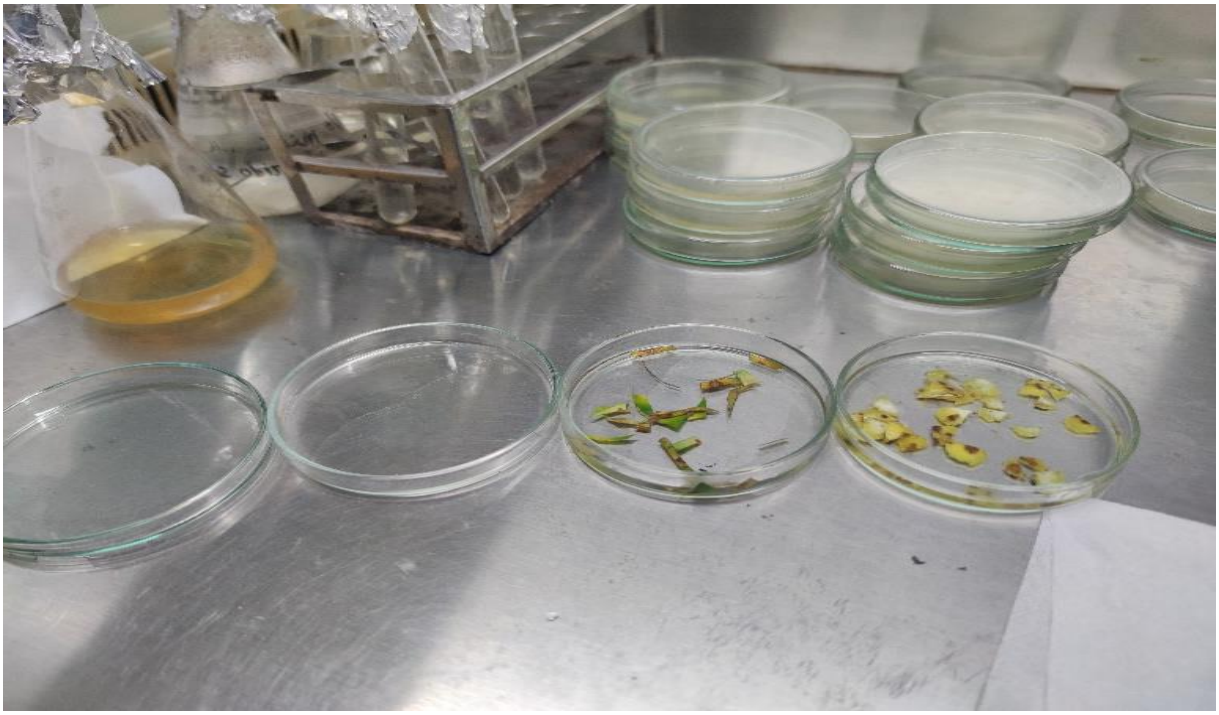

Figure 1. Isolates of plant parts from infected fruits and leaves

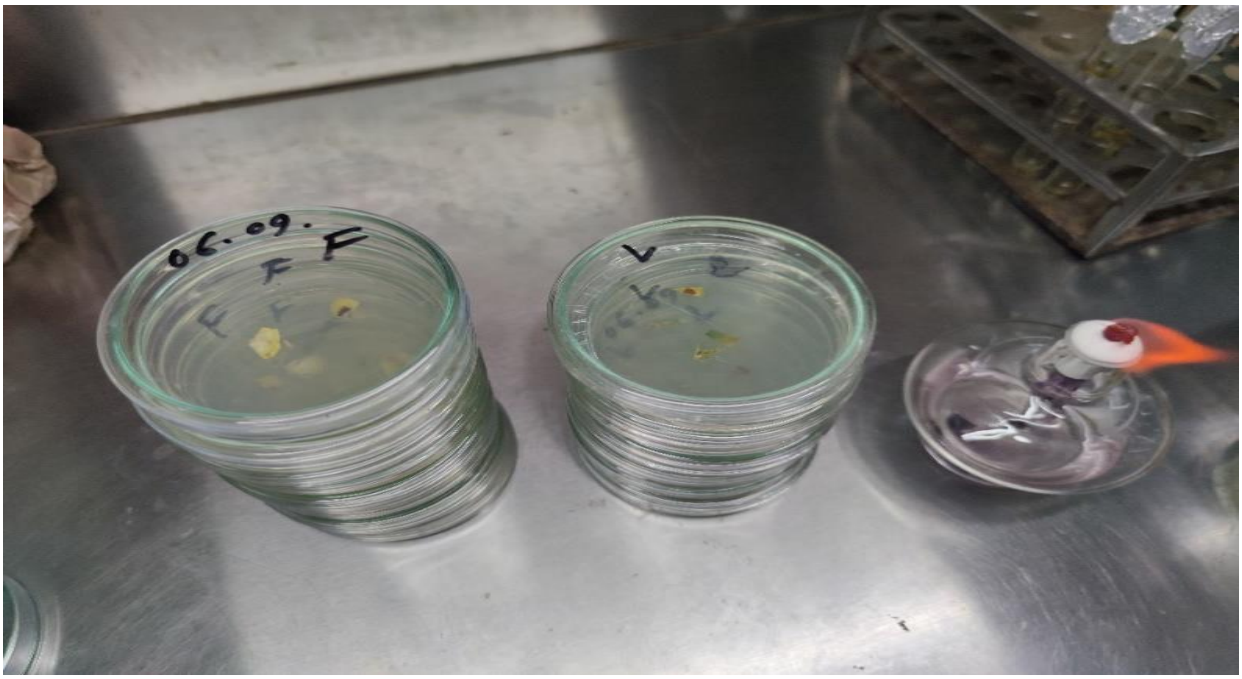

Figure 2. Pure isolates of the bacterium were grown on nutrient agar plates

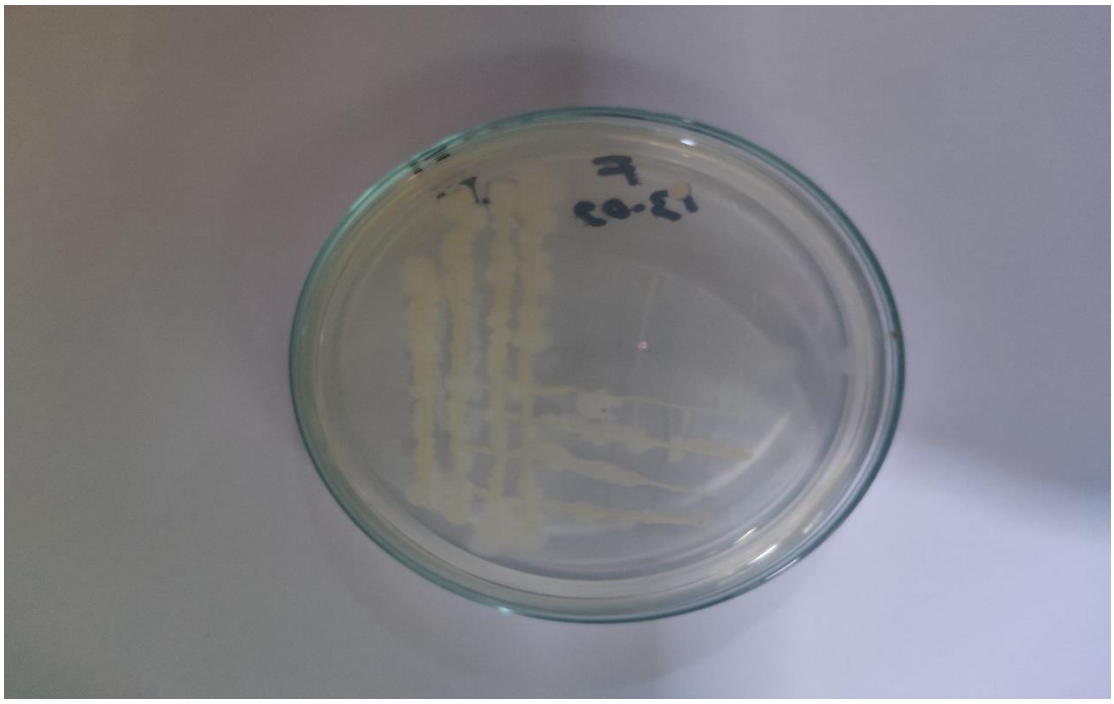

Figure 3. Pure culture of *Xanthomonas citri* on Nutrient Agar media

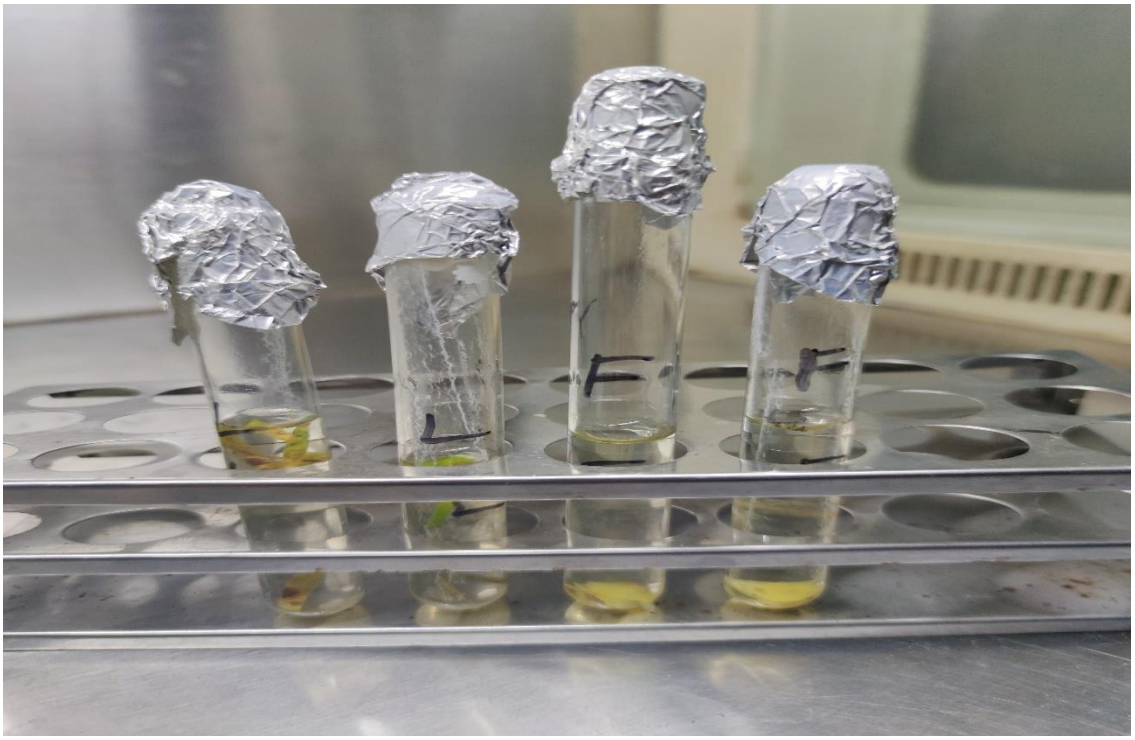

Figure 4. Sample collection into sterile distilled water for future use