PREVALENCE OF *Xanthomonas axonopodis* pv. *citri* ON CITRUS (*Citrus* spp.) AND EFFECT OF ENVIRONMENTAL FACTORS ON CITRUS CANKER DEVELOPMENT

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This is to certify that the thesis entitled, *"PREVALENCE OF Xanthomonas axonopodis pv. citri ON CITRUS (Citrus spp.) AND EFFECT OF ENVIRONMENTAL FACTORS ON CITRUS CANKER DEVELOPMENT"* submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of *MASTER OF SCIENCE IN PLANT PATHOLOGY* embodies the results of a piece of *bona fide* research work carried out by **ZURKANI-AL-REFAYEE** bearing Registration No**. 07- 02454** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

Dated:20 November, 2014
Place: Dhaka, Bangladesh

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TO

MY BELOVED PARENTS

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ABSTRACT

Prevalence of *Xanthomonas axonopodis* pv. *citri* on citrus causing canker on citrus fruits transported from different districts of Bangladesh to the wholesale markets of Dhaka was studied during September 2012 to May 2014. Survey revealed that the highest incidence and severity of canker of citrus were recorded on the fruits transported from Chittagong in the month of September and May, respectively and the lowest incidence and severity were recorded on the fruits transported from Gazipur and Munshiganj, respectively in the month of January. A positive correlation was observed between the incidence and severity of canker with temperature, relative humidity and rainfall. The bacterium *Xanthomonas axonopodis* pv. *citri* was isolated from the canker infected part of citrus fruits and it's morphological, biochemical and cultural features were studied. The bacterium was gram negative, rod shaped and showed positive results in KOH 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 yellow colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened colonies on SX medium. Study on the effect of environmental factors on canker affected citrus seedlings indicated that leaf infection (%) by canker pathogen was positively correlated with temperature, relative humidity and rainfall. It increased & decreased with the increasing & decreasing of temperature, relative humidity and rainfall. In case of the effect of light intensity highest incidence & severity of the disease were found when all the wavelengths of light less than 400 nm were blocked and lowest incidence & severity were found when all the wavelengths of light less than 340 nm were blocked.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	=	Percentage
et al.	=	And others
spp.	=	Species
J.	=	Journal
No.	=	Number
viz.	=	Namely
df.	=	Degrees of freedom
&	=	And
etc.	=	Etcetera
^{0}C	=	Degree Celsius
@	=	At the rate of
cm	=	Centimeter
cfu	=	Colony forming unit
ppm	=	Parts per million
NaCl	=	Sodium chloride
Kg	=	Kilogram
g	=	Gram

ml	=	Milliliter
hr	=	Hour (s)
cv.	=	Cultivar (s)
i.e.	=	That is
T	=	Treatment
ft	=	Feet (s)
pv.	=	Pathovar
var.	=	Variety
mm	=	Milimiter

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

SAU	=	Sher-e-Bangla Agricultural University
BAU	=	Bangladesh Agricultural University
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
UNCTAD	=	United Nations Conference of Trade and Development
USA	=	United State of America
NA	=	Nutrient Agar (media)
YDCA	=	Yeast Dextrose Calcium Carbonate Agar (media)
WPR	=	World Population Review
ANOVA	=	Analysis of variances
LSD	=	Least Significant Difference
CV%	=	Percentages of Co-efficient of Variance
SX Agar	=	Semi-selective *Xanthomonas* spp. Agar

CHAPTER I

INTRODUCTION

Citrus (*Citrus* spp. L.) is one of the most important, popular and nutritious fruit crops in the world as well as in Bangladesh. It belongs to the family Rutaceae and has a great demand due to its nutritive value, aroma and taste. It is thought to be originated in Indian sub continent because of maximum genetic diversity are grown in this region (Sohi and Kapoor, 1990). Considering the multipurpose use, the demand of citrus is increasing day by day. Bangladesh is a developing country and has dense population of 158.5 million having 964.42 people/km^2 (WPR, 2014). It produces less than 30% of the fruits needed to meet the minimum daily requirements for it's population. About 91% of its people are suffering from the deficiency of vitamin-C (Haque, 2005). Citrus serves as a potential source of vitamins and minerals (Alam *et al*., 2003) especially vitamin-C. It also has some medicinal and digestive value too (Reuther *et al*., 1967). **Total annual citrus production in the world was estimated at over 123 million tons in the period 2009-2010** (UNCTAD, 2013). Bangladesh produces only 21 thousand metric tons citrus fruits every year according to a study of 2009-2010 (BBS, 2010). Eight species of citrus are grown in Bangladesh. Among them, lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and pummelo (*Citrus grandis*) are commonly cultivated in our country. As the hilly and high land remains fallow round the year, there is a great opportunity to extend citrus cultivating area in the country.

Among the various factors, plant diseases play an important role in lowering the yield. It has been estimated that the production could be increased at least by 28% if the crop could be protected against various seedling diseases (Chowdhury, 2009). Now-a-days, the demands of seedlings are very high in nurseries because healthy seedlings are prime need and basic raw material for establishment of an orchard. Citrus plants are very much prone to the attack of numerous diseases. 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 different species of citrus seedlings. Most of the commercial citrus species grown in nursery around the world including Bangladesh are suffering from a bacterial disease, citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Graham *et al.,* 2004; Gottwald *et al.,* 2002 and Koizumi, 1985). The disease is endemic in many tropical and subtropical citrus growing areas (Goto, 1992) and has been spread to most citrus producing areas of the world. The disease is believed to have originated in South East Asia which was first found around 1912, spread throughout the southeastern U.S. on imported seedlings from Japan (Schoulties *et al*., 1987). Intensive research on citrus canker is being carried out throughout the world which has been reviewed by Sahi *et al.* (2007); Zekri *et al.* (2005); Brunings and Gabriel (2003); Schubert and Sun (2003); Gottwald *et al.* (2001); Bergamin-Filho *et al.* (2000); Gabriel *et al.* (2000); Stall and Civerolo (1991); Sohi and Kapoor (1990); Civerolo (1984); Stall and Seymour (1983); Chand and Pal (1982) and Rossetti (1977). In addition to tree debilitation and losses in quality and quantity of fruit, citrus canker results in devastating socio-economic and political impacts because of the market standards for fresh fruit and perceptions of possible inoculums transmission on the fresh fruit product.

The bacterium (*Xac*) is rod shaped measuring 1.5-2.0 x 0.5-0.75 µm, gram negative and has a single polar flagellum (Vudhivanich, 2003). Colonies on culture media are usually yellow as a result of xanthomonadin pigment production. It gives positive result in KOH solubility test, starch hydrolysis test, catalase test, **citrate utilization test,** motility indole urease agar (MIU) test, gelatine liquefaction test, salt tolerant test, tobacco hypersensitivity reaction and gives negative result in oxidase test (Chand and Kishun, 1991). It can be differentiated by growth and colony morphology on different media (Schaad, 1992).

Young fruits and leaf tissues are more susceptible than mature tissues. Additionally, wounding induced by the larvae of the Asian citrus leaf miner

(*Phyllocnistis citrella stainton*) increases infection by *X. axonopodis* pv. *citri* during the flush periods (Schubert *et al.*, 2001; Gottwald, 1997; Cook, 1988; Sinha *et al.*, 1972; Sohi and Sandhu, 1968 and Nirvan, 1961).

Environmental factors play an important role in the susceptibility of citrus plants to canker. The effects of temperature, light intensity, rainfall and relative humidity on the incidence and severity of citrus canker has been focused by many researchers worldwide [Gill (2013); Hossain (2011); Bock *et al.* (2010); Serrano *et al.* (2010); Chowdhury (2009); Chung *et al.* (2009); Eshetu and Sijam (2007); Pruvost *et al.* (2002); Timmer *et al.* (2000); Smith *et al.* (1997); Gottwald *et al.* (1988); Whiteside (1988); Civerolo (1985); Danós *et al*. (1984); Reddy (1984); Ramakrishnan (1954) and Peltier and Frederich (1926)]. Temperature between 15 to 20°C and 35 to 40°C are conducive for infection and development of citrus canker disease (Pria *et al.*, 2006). The disease is mostly prevalent in area with more than 1000 mm rainfall per year (Verniere *et al.*, 2003). The pathogen is dispersed by splashing rain and winds in excess of 8.0 ms^{-1} (Gottwald *et al.*, 1989; Serizawa and Inoue, 1976 and Serizawa *et al.*, 1969). The disease is severe in high light intensity (Gill, 2013; Serrano *et al.*, 2010; Chung *et al.*, 2009).

Although a huge number of nurseries are engaged in producing citrus seedlings, they fail to produce quality seedlings due to lack of their knowledge about this disease. Moreover, the nursery people do not have adequate knowledge about the disease. However, only a few reports are available in respect of prevalence, isolation, identification and effect of physical factors on this disease in the country. Therefore, attempt should be put forward to study the prevalence of this disease occurring on citrus crops in different regions of Bangladesh.

Considering the above facts, the present research program has been designed with the following objectives:

1. To survey on the prevalence of *Xanthomonas axonopodis* pv. *citri* on citrus in some selected wholesale markets of Dhaka, Bangladesh.
2. To identify the pathogen (s) associated with the disease.
3. To study the effect of different environmental factors (temperature, relative humidity, rainfall and light intensity) on citrus canker disease development.

CHAPTER II
REVIEW OF LITERATURE

Canker of citrus caused by *Xanthomonas axonopodis* 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 pertaining to the canker of citrus along with information on related crops disease and pathogen are reviewed here as under.

2.1. Survey and report on occurrence of canker of citrus

Rashid *et al.* (2014) conducted a survey in eight nurseries of Bangladesh during January 2012 to December 2013. The survey revealed that the highest

incidence (72.22%) and severity (27.55%) of canker of citrus was recorded in the month of July at Khagrachari and the lowest incidence (28.33%) and severity (5.55%) was recorded in the month of January at Dhaka. A positive correlation was observed between the incidence and severity of canker with temperature, relative humidity and rainfall.

Hossain (2011) studied the nursery diseases of citrus in Bangladesh during the period of 2010-2011. He recorded canker, scab and die-back diseases in different citrus growing areas of Bangladesh.

Chowdhury (2009) observed that citrus suffers from many diseases at all stages of growth of which scab, canker, die-back, citrus greening are considered as important diseases of seedlings. These are widely occurring throughout the citrus growing countries in the world. It has been estimated that the production could be increased at least by 28% if the crop could be protected against various seedling diseases.

Burhan *et al.* (2007) observed the incidence of citrus canker (*Xanthomonas axonopodis* pv. *citri*) on 15 cultivars of sweet orange (*Citrus sinensis*) in Pakistan and found all cultivars were more or less infected with citrus canker. The trend in intensity of diseased leaves and lesions per leaf was partially similar in cultivars.

Derso *et al.* (2007) conducted a survey in eight states of Malaysia and found incidence of 36.5% and severity of 15.2% on leaves, while incidence of 18.7% and severity of 7.5% on fruits. Field host range included Mexican lime (*Citrus aurantiifolia*), pomelo (*C. grandis*) and kaffier lime (*C. hysterix*). Canker severity was significantly correlated ($r = 0.7041$, $p = 0.011$) with temperature but not with rainfall, altitude and tree age.

Eshetu and Sijam (2007) conducted surveys in small-scale farms, commercial plantations, backyard orchards and nurseries in Ethiopia between August and November, 2004 to quantitatively determine the occurrence, distribution,

intensity and host range of citrus canker caused by *Xanthomonas axonopodis* pv. *citri*. Incidence on leaves was determined by counting the total number of leaves and expressed as a proportion of leaves with at least one lesion. Incidence on fruits was determined on attached fruits, recorded as the presence or absence of symptoms. Severity was also measured on leaves and fruits. They observed that 71.4% of the leaves had at least one lesion and 26.8% of the leaf area was infected.

Bal and Dhiman (2005) observed citrus canker (*Xanthomonas axonopodis* pv. *citri*) as serious disease in Kinnow mandarin nursery plants. The relationship between the disease development and environmental factors was studied and canker was found to build up during the first week of June the onset of rains. The highest incidence of citrus canker (73.3%) was recorded during the second week of September. Canker showed a positive correlation with temperature, relative humidity and rain. The period from July to September was identified as the most conducive for the development of citrus canker.

Citrus canker likely native to Southeast Asia and India has now spread worldwide into to warm, moist, citrus-growing coastal regions. The pathogen is now also established in Australia, Japan, the Middle East, Africa, Papua New Guinea and elsewhere in the Pacific and the Americas (Kumar *et al*. 2004).

Barbosa *et al.* (2001) conducted surveys in commercial groves in Sao Paulo and Minas Gerais, Brazil, during 1999 to evaluate the incidence and distribution of citrus canker (caused by *Xanthomonas axonopodis* pv. *citri*). Citrus canker presented a highly aggregated distribution with highest incidence in the northwest zone (4.32%) followed by the centre (0.51%) and north zone (0.18%) while no incidence was observed in the south zone.

Singh *et al*. (1997) conducted a survey at Panjab in India and disease incidence recorded on 3 citrus species viz. rough lemon (*Citrus jambhiri*) 76.50-80.10%, sweet orange (*C. sinensis*) 10.8-20.30% and Kinnow (*C. nobiilus* × *C. deiicisa*) 46.25-81.07%. The highest disease incidence was recorded in the sitb-

mounlarnous zone on rough lemon (81.10%) and Kinnow (80.07%) and the lowest incidence was recorded on sweet orange 10.80% in the arid irrigated zone.

2.2. Symptomology

Dewdney and Graham (2014) reported that citrus canker, caused by the bacterium *Xanthomonas axonopodis* pv. *citri*, 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 are 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 fruit. 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 *colletotrichum* spp.

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 spots age, they become crusted and tan surrounded by a yellow ring. Eventually, the tan tissue can fall out creating a hole through the leaf. Leaf spots have been described as looking like a cigarette burn surrounded by a yellow circle. The disease causes citrus trees to prematurely drop leaves and fruit, 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 spots when the problem is 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.

Balestra *et al.* (2008) reported hyperplasia type lesion, often surrounded by a water-soaked margin and yellow halo, typical of citrus canker were found on 8 to 10 years old lime (*Citrus limetta*) and grapefruit (*Citrus paradisi*) trees in northern and southern Somalia, respectively.

Brunings and Gabriel (2003) observed on leaves that first appearance of *Xanthomonas axonopodis* 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 girdling 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 al.* (2003) studied the Asiatic Citrus Canker (ACC) and found expression of symptom depend on temperature where as relative humidity had no effect on disease suppression.

Lesions of citrus canker at first were small, slightly raised, round, light green spots. Later, they became grayish white, rupture, and appear corky with brown, sunken centre. The margins of the lesions were often surrounded by a yellowish halo (Vudhivanich, 2003).

Braithwaite *et al.* (2002) observed citrus canker as yellow/brown, raised and corky lesions 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.

Lesions of citrus canker 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 about 7 days after infection. 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. Lesions were light coloured at first and became tan or brown. As lesions developed, the epidermis ruptured and the lesions became spongy or corky. The lesions finally became crater-like with a raised margin and sunken centre. The centre of large, old lesions cracked and/or dropped out (Swarup *et al.*, 1991).

2.3. Isolation and identification of the pathogen and its pathogenicity

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 cultural features of the bacteria. They observed that the bacterium was gram negative, rod shaped and showed positive results in KOH 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 yellow colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened colonies on SX medium.

Jabeen *et al.* (2012) observed that after 48-72 h of incubation at 28^{o}C, *Xanthomonas* gave yellow, circular, smooth, convex and viscous bacterial colonies on yeast dextrose calcium carbonate agar medium (YDCA). On SX medium the bacteria gave light yellow, mucoid, round and smooth colonies (1 mm in diameter) while whitish, mucoid and smooth colonies were observed on Wakimoto medium.

Yenjerappa (2009) conducted an experiment to study the growth of *Xanthomonas axonopodis* 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, colourless 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 moderate 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 gelatin, 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, xanthomonad 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 *X. 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 (30^0C), the light yellow colony developed from plant tissue with clear zone surround them.

Braithwaite *et al.* (2002) observed that gram negative *Xanthomonas axonopodis* 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.

In an experiment Gottwald and Graham (1992) found 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 10^6 cfu/ml gave consistent and successful infection.

In 1991, Chand and Kishun 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.

Chand and Kishun (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 H_2S production, starch hydrolysis, KOH solubility, gelatin liquefaction, hydrolysis of Tween 80, sucrose utilization, indole production, growth at 3.5 percent NaCl, milk proteolysis and acid from most of the sugars.

Chand and Pal (1982) studied on biochemical characteristics of *Xanthomonas axonopodis* 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.

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 (Starr and Stephens, 1964).

Goto (1962) found that the minimal dose of *Xac* 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 raised, glistening, pale yellow and odourless. 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 utilizes xylose, glucose, mannose, galactose, sucrose, lactose and raffinose but not maltose, glycerine and salicin when grown in Durham’s

fermentation tubes containing one percent carbohydrates in a peptone free synthetic liquid medium. Ammonia was produced in peptone water after 15 days. Nitrites, hydrogen sulphide and indole were not produced. Starch was hydrolysed, methyl red and voges proskauer tests gave negative results. Growth on gelatin slabs was good. Stratiform type of liquefaction commenced after 48 hours and completed within 21 days. The yellow colour of the growth on gelatin gradually changed from usual bright yellow to dark brown on yeast glucose chalk agar and cooked potato.

2.4. Effect of environmental factors on disease development

Dewdney and Graham (2014) reported that major citrus canker outbreaks generally occur when new shoots are emerging or when fruit are in the early stages of development, especially if a major rainfall event occurs during this critical time. Frequent rainfall in warm weather, especially storms, contributes to disease development. Citrus canker is mostly a cosmetic disease, but when conditions are highly favorable for infection, it causes defoliation, shoot die-back, and fruit drop. Leaf susceptibility is complicated by the Asian leaf miner. The galleries caused by leaf miner larvae do not heal quickly and increase leaf susceptibility. This results in leaves with highly susceptible wounds for long periods of time, through which the bacterium can infect the leaf. The number and size of lesions are greatly increased and result in many times the inoculum pressure in a grove compared to citrus canker in the absence of leaf miner.

Rashid *et al.* (2014) observed that there was a positive correlation between the incidence and severity of citrus canker with temperature, relative humidity and rainfall. Survey revealed that the highest incidence and severity of canker of citrus was recorded in the month of July at Khagrachari, where temperature, relative humidity and rainfall was highest and the lowest incidence and severity was recorded in the month of January at Dhaka, where temperature, relative humidity and rainfall was lowest.

Gill (2013) reported 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. Disease increases with high temperature, high light intensity and high relative humidity.

Bock *et al.* (2010) reported that citrus canker (caused by the bacterial pathogen *Xanthomonas citri* pv. *citri*) dispersed predominantly in rain splash. To simulate dispersal in splash and to investigate the effect of wind speed on infection, young plants of *Swingle citrumelo* were exposed to sprayed inoculum at different wind speeds. Wind was generated using an axial fan and a pressurized sprayer delivered the inoculum spray. In the five experiments, higher wind speeds (>10 ms^{-1}) consistently resulted in higher incidence and severity of citrus canker developing. More disease was associated with visible injury as the wind speed increased. They also found the concentration of the Xcc inoculum increased the incidence and severity of citrus canker. Reducing wind speed in citrus groves with the aid of wind breaks may contribute to a reduction in the severity of an epidemic by reducing dispersal and infection events.

Serrano *et al.* (2010) reported that citrus canker becomes severe in high light intensity, high temperature, high rainfall and low tree vigor.

Christiano *et al.* (2009) conducted an experiment under controlled condition to assess the influence of temperature and leaf wetness duration on infection and subsequent symptom development of citrus canker. The combined effect of temperature (15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 42°C) and leaf wetness duration (0, 4, 8 12, 16, 20 and 24 h) on infection and development of Asiatic citrus canker (*Xanthomonas axonopodis* pv. *citri*) on Tahiti lime plant was examined in growth chambers. No disease developed at 42°C and zero hours of leaf wetness. Periods of leaf wetness as short as 4 h were sufficient for citrus canker infection. However, longer leaf duration wetness (24 h) did not result in much increase in the incidence of citrus canker, but led to twice the number of

lesions and four times the disease severity. Temperature was the greatest factor influencing disease development. At optimum temperatures (25 to 35°C), there was 100% disease incidence. Maximum disease development was observed at 30 to 35°C, with up to a 12-fold increase in lesion density, a 10-fold increase in lesion size and a 60-fold increase in disease severity

Chung *et al.* (2009) reported that citrus is susceptible to a large number of diseases caused by plant pathogens. Economic losses due to plant diseases can be severe in Florida. Among other citrus diseases citrus canker is common in subtropical regions with summer rains and has been found in numerous countries in South America, Asia, and Africa and in Australia. High temperature, high light intensity and stress all favor symptom expression of canker on citrus. All citrus cultivars are susceptible to canker at least some degree. Lemons, grapefruits, limes, and mandarins are especially susceptible and late maturing varieties, such as Valencia.

Eshetu and Sijam (2007) observed that citrus canker severity was significantly correlated with temperature and high light intensity but not with rainfall, elevation or tree age.

Khan and Abid (2007) observed that environmental conditions i.e., relative humidity, rainfall, maximum temperature, minimum temperature and wind speed were correlated with citrus canker disease severity. The disease development had a significant correlation with relative humidity and rain fall and negative correlation with maximum temperature. The disease development increased with the increase in rain fall and relative humidity and decreased with the increase in maximum temperature. But wind speed and minimum temperature did not affect the disease development significantly. The environmental data were split into two parts keeping in view the intensity of disease i.e., April-May-June and July-August-September. Comparison showed that in the first three months, there was no significant effect of observed environment variables on the disease development. In data of July-August-

September, it was found that there was a significant correlation of maximum temperature and relative humidity with disease development.

Pria *et al.* (2006) conducted an experiment under controlled condition to assess the influence of temperature and leaf wetness duration on infection and subsequent symptom development of citrus canker in sweet orange. The quantified variables were incubation period, disease incidence, disease severity, mean lesion density and mean lesion size at temperatures of 12, 15, 20, 25, 30, 35, 40 and 42^0C and leaf wetness durations of 0, 4, 8, 12, 16, 20 and 24 hrs. Symptoms did not develop at 42^0C. The relationship between citrus canker severity and leaf wetness duration was explained by a monomolecular model, with the greatest severity occurring at 24 hrs of leaf wetness, with 4 hrs of wetness being the minimum duration sufficient to cause 100% incidence at optimal temperature of 25-35^0C. The estimated minimum and maximum temperature for the occurrence of disease were 12^0C and 40^0C, respectively.

Bal and Dhiman (2005) observed that citrus canker was found to build up during the first week of June with the onset of rains. They also observed that highest incidence of citrus canker was during the second week of September. The disease showed a positive correlation with temperature, relative humidity and rainfall.

Das (2003) reported that warm, humid, cloudy climate, along with heavy rainfall and strong wind promotes citrus canker.

Verniere *et al.* (2003) studied the Asiatic citrus canker (ACC) and found expression of symptom depend on temperature and light intensity where as relative humidity had no effect on disease expression.

Khan *et al.* (2002) studied to evaluate the influence of maximum and minimum air temperature, rainfall, relative humidity, wind speed and clouds on citrus canker disease (*Xanthomonas campestris* pv. *citri* [*X. axonopodis* pv. *citri*]) development on three citrus cultivars (Kinnow, Lime and Feutrell's early)

grown at two sites, i.e., University of Agriculture, Faisalabad and Post-graduate Agricultural Research Station, Faisalabad, Pakistan during July-September, 2001. Out of the six environmental variables, they found minimum temperature and wind speed significantly influenced the citrus canker disease development at both sites.

Zhong and Ling (2002) studied the occurrence of citrus canker disease *Xanthomonas axonopodis* pv. *citri* during 1995-1998. Results indicated that 15-20 days after bud burst, disease symptoms started to appear. Citrus canker occurrence had a close relationship with the daily mean temperature. When a daily temperature of 12°C occurred for 10-15 days, the spring shoots and fruit lets were found to be attacked. Experiments showed that spraying shoots with 77% kocide (Copper hydroxide) wettable powder at 20-30 days after bud burst and spraying summer-autumn shoots at 10-15 days after bud burst provided good citrus canker control.

FU and XU (2001) showed that citrus canker (*Xanthomonas axonopodis* pv. *citri*) appeared in late April and early May; the rampart period being mid May to early June. Because higher the temperature, the earlier the occurrence. Spraying with different fungicides showed that a 500 fold solution of 77% copper hydroxide and 60% chlorothanil solution respectively, resulted in the efficient control of the disease.

Srivastava *et al*. (1997) studied on the epidemiology of *X. citri* and found that disease incidence was severe at 29-29.4^{0}C temperature, 80-90.5% relative humidity and 8.9-9.97 mm rainfall. With decrease in temperature, rainfall and relative humidity, there was considerable reduction of disease intensity by the end of September.

Kalita *et al*. (1995) reported that highest incidence of citrus canker was in August, although the incidence was also in June and September because in these months the highest temperature, rainfall and relative humidity were recorded in Asam.

Timmer *et al.* (1991) observed that between 10^4 and 10^6 cells of *Xanthomonas* were exuded from lesions when exposed to a period (less than 1 hour) of wetting or rainfall that can causes infection on citrus plant.

Reddy (1990) reported that canker infected leaves, twigs and branches constitute the source of inoculam to spread the disease from season to season. Since the infected leaves drop off early and the bacteria perish rapidly in the soil.

Sothiosorubini *et al.* (1986) observed that ideal condition for infection of citrus plants by *Xanthomonas campestris* pv. *citri* were 30˚C temperature and 100 percent relative humidity. The pathogen gained entry through wounds and occasionally through natural openings. Infection took 7 days to develop from time of inoculation under laboratory condition and 18 days in field.

Aiyappa (1958) reported that all cultivated varieties of citrus and some wild species in Karnataka were highly susceptible to canker possibly due to heavy rainfall, high humidity and low temperature.

Ramakrishnan (1954) reported that young tissues of the plant were readily affected. In the nursery stages sweet oranges and other varieties were also infected by canker. High humidity, temperature between 20-35^{0}C and the presence of moisture on the host surface for 20 minutes or more favored the incidence of the disease.

CHAPTER III

MATERIALS AND METHODS

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

1. Survey on the prevalence of *Xanthomonas axonopodis* pv. *cirti* on citrus in some selected wholesale markets of Dhaka, Bangladesh.

2. Isolation, purification and identification of causal organism(s) of canker of citrus**.**
3. Study on the effect of environmental factors on citrus canker disease development.

3.1. Experiment I. Survey on the prevalence of *Xanthomonas axonopodis* pv. *citri* on citrus in some selected wholesale markets of Dhaka, Bangladesh

3.1.1. Location of survey area

Prevalence of canker on fruits of citrus was surveyed in three wholesale markets of Dhaka. Fruits from three districts were surveyed at each of the markets.

3.1.2. Selection of markets and districts

Three wholesale markets of Dhaka were selected according to their size and popularity. They were:

1. Karwanbazar wholesale market
2. Savar wholesale market
3. Shyambazar wholesale market

Three districts were surveyed from where the fruits were brought to the markets. The districts were selected based on the availability of regional fruits. They were:

1. Munshiganj
2. Gazipur
3. Chittagong

3.1.3. 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 were used for critical observation of the disease. Identification of the disease was finally confirmed through isolation and different biochemical test

3.1.4. Survey period

Altogether twelve surveys were made during the period from September 2012 to May 2014. Where first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh and twelfth surveys were made in 15th September 2012; 30th September 2012; 15th January 2013; 30th January 2013; 15th May 2013; 30th May 2013; 15th September 2013; 30th September 2013; 15th January 2014; 30th January 2014 and 15th May 2014; 30th May 2014 respectively. The times of data collection were determined on the basis of variations in temperature relative humidity and rainfall during the growing seasons.

3.1.5. Data collection during survey

During the survey in the markets, samples composed of 50 random fruits were considered. Three samples were made representing three of the selected districts. Total number of citrus fruits as well as number of fruits that are infected with citrus canker was recorded to determine disease incidence. The whole procedure was done three times to get three replications. Then again 20 fruits were randomly selected to determine the disease severity.

3.1.6. Determination of disease incidence and disease severity

Every fruit along with canker infected was counted in the market for calculating the incidence of the disease and then it was expressed in percentage.

Disease incidence of canker on fruits of citrus was determined by the following formula (Rai and Mamatha, 2005):

$$\text{Percent Disease Incidence (Fruits)} = \frac{\text{Number of diseased fruits}}{\text{Number of total fruits observed}} \times 100$$

Percent Disease Severity (PDI) was determined by the following formula (Rai and Mamatha, 2005):

$$\text{Percent Disease Severity (Fruits)} = \frac{\text{Area of fruit tissue infected by disease}}{\text{Total area of inspected fruit}} \times 100$$

3.1.7. Meteorological data collection

Day to day meteorological data of the selected three districts on temperature, relative humidity and rainfall were collected from Meteorological Department, Sher-e-Bangla Nagar, Dhaka-1207. The data taken were analyzed for calculating monthly mean of minimum and maximum temperature, relative humidity and rainfall throughout the study period of the respective locations.

Table 1. Average temperature, relative humidity and rainfall of Munshiganj, Gazipur and Chittagong from September, 2012 to May, 2014

Month	Average Temp. (°C)	Mean Average Temp. (°C) of 2 years	Average relative humidity (%)	Mean average relative humidity (%) of 2 years	Average Rainfall (cm)	Mean average rainfall (cm) of 2 years
2012 ,Sep	28.23	28.68	83.40	83.86	31.42	31.83
2013 ,Sep	29.14		84.33		32.23	

2013 ,.Jan	17.51	17.97	68.43	69.34	0.92	3.89
2014 ,.Jan	18.43		70.25		0.86	
2013 ,May	29.12	28.73	77.50	77.87	32.44	32.15
2014 ,May	28.34		78.25		31.87	

3.1.8. Statistical analysis

Data on different parameters were analyzed in three factor randomized complete block design (RCBD) through computer software MSTAT-C (Anonymous, 1989). To determine the level of significant differences and to separate the means within the parameters, Duncan's Multiple Range Test (DMRT) and Least Significant Difference (LSD) test were performed.

3.2. Experiment II. Isolation and identification of causal organism

3.2.1. Collection and preparation of diseased specimen

Fruit samples with characteristic symptoms were collected from the markets representing the selected districts. The specimens were kept in the refrigerator at 4^0C by following standard procedure of preservation of disease specimens until isolation was made.

3.2.2. Preparation of Nutrient Agar (NA)

Nutrient agar media (Appendix-I) was prepared by following the method used by Schaad (1988). For 1 liter NA medium preparation firstly 15 g bacto agar was taken in an Erlenmeyer flask, which contains 1000 ml distilled water. After that 5 g peptone and 3 g beef extract were added to it. The nutrient agar was shaken thoroughly for few minutes to mix the materials properly. All the glassware like petridish, conical flask, test tubes etc. and other materials were wrapped with brown paper. Then it was autoclaved at 121^0C under 15 PSI pressure for 15 minutes (Plate 1-B).

3.2.3. Preparation of Nutrient Broth (NB)

Nutrient broth (Appendix-I) was prepared by following the method used by Schaad (1988). For the preparation of 1 liter nutrient broth 5 g peptone and 3 g beef extract were taken in the Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.2.4. Preparation of Yeast Extract Dextrose Calcium Carbonate Agar (YDCA)

Yeast extract dextrose calcium carbonate agar (Appendix-I) was prepared by following the method used by Goszczynska *et al.* (2000). For the preparation of 1 liter yeast extract dextrose calcium carbonate agar medium 10 g yeast extract, 20 g D-glucose and 15 g bacto agar were taken in an Erlenmeyer flask, that contains 1000 ml of distilled water. All of the ingredients were mixed and heated well. Then 20 g $CaCO_3$ was added to it. After that it was autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.2.5. Preparation of SX Agar

SX agar (Appendix-I) was prepared by following the method used by Goszczynska *et al.* (2000). For the preparation of 1 liter SX agar medium 10 g soluble potato starch, 1 g beef extract, 5 g NH_4Cl, 2 g K_2HPO_4, 0.4 ml methyl violet 2B (1% in 20% ethanol), 2 ml methyl green (1% in water) and 15 g agar were taken in an Erlenmeyer flask, that contains 1000 ml of distilled water. Then the mixture was autoclaved at 121^0C under 15 PSI pressure for 15 minutes. After that, it was cooled to 50^0C and 2ml cycloheximide (100 mg/ml in ethanol) was added. Finally they were mixed well.

3.2.6. Method of isolation and identification of causal organism

3.2.6.1. Isolation and purification of canker pathogen of citrus

The diseased samples were washed under running water. Then the young lesions with green healthy portion of diseased fruits were cut into small pieces. Surface sterilize were done by dipping them in 5% sodium hypochlorite 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.

Bench of the laminar airflow (Plate 1-C) was cleaned with Hexisol. Then it was provided with UV for 20 minutes. After completion of autoclaving the materials were transferred in the laminar airflow chamber. 9ml sterile water was taken in 5 test tubes to make dilution of 1:10, 1:100, 1:1000, 1:10000, 1:100000. One ml of the stock solution was transferred with the help of sterile pipette into each of the test tubes and shaken thoroughly to make the dilution properly (Plate 1-A). The NA media was poured in Petri dish.

After preparing different dilution, 0.1 ml of each dilution was spread over NA plate previously dried (to remove excess surface moisture) with micropipette at three replications as described by Goszczynska and Serfontein (1998). Spreading was done with the help of a glass-rod. Then the plate was covered with the lid and sealed with paper tape. By this way 15 petri dishes were prepared with all of the dilutions. Then the petri dishes were wrapped with paper tape and all the petridishes were marked with dilution number and date (Plate 1-D)

The inoculated NA plates were kept in incubation chamber at 30^0C. The plates were observed after 24 hrs and 48 hrs. Then single colony grown over NA plate was restreaked on another plate with the help of a loop to get pure colony.

3.2.6.2. Preservation of canker pathogen of citrus

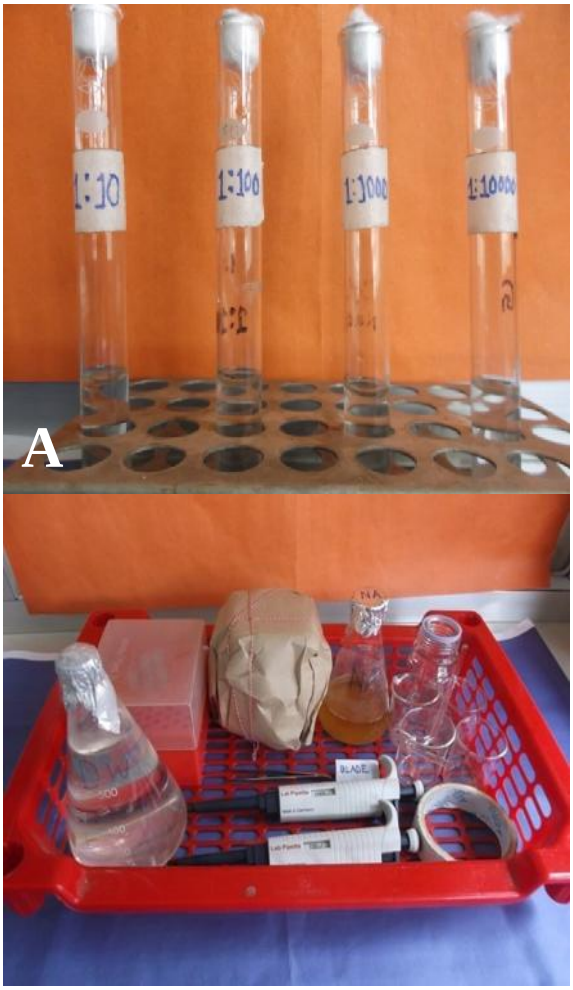
After purification of bacteria on NA plate, it was kept in refrigerator at 4^0C in screw-cap test tubes on NA slant for future use.

3.2.6.3. 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.

3.2.6.3.1. Morphological characters

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).



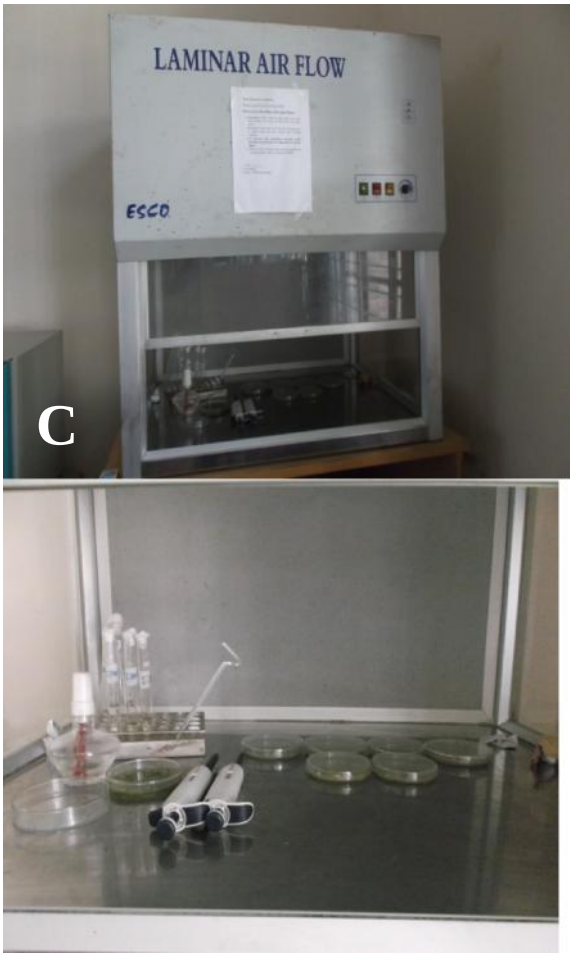


Plate 1: Isolation of the pathogen

A. Dilution plate procedure
B. Glassware and other equipments before autoclaving
C. Laminar Air Flow chamber
D. Isolation procedure

3.2.6.3.1.a. Gram's staining

A small drop of sterile water was put on a clean microscope slide. A young colony part (18-24 hrs old) was removed by a cold, sterile loop from the nutrient agar medium and the bacteria were smeared on to the slide. Then the

spreaded bacterial film was air dried. After that the underside of the glass slide was heated by passing it four times through the flame of a sprit lamp to fix the bacteria on it. Then the slide was flooded by crystal violet solution for 1 minute. Then it was rinsed under running tap water for a few seconds and the remaining excess water was removed by air. Then it was flooded by lugol's iodine solution for 1 minute. After that it was decolorized by 95% ethanol for 30 seconds and then again rinsed with running tap water and air dried. Then it was counterstained by 0.5% safranine for 10 seconds. Then it was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined under 40x and 100x magnification using oil immersion.

3.2.6.3.1.b. KOH solubility test

A small drop of 3% KOH (aqueous) was placed on a microscope glass slide. A young colony part (18-24 hrs old) was removed from the NA plate with the help of a cool, sterile loop. Then 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).

3.2.6.3.2. Biochemical characters

Different biochemical tests such as starch hydrolysis test, catalase test, oxidase test, **citrate utilization test,** motility indole urease agar (MIU) test, gelatine liquefaction test and salt tolerant test were studied as per the methods described by Schaad (1992) and Salle (1961).

3.2.6.3.2.a. Starch hydrolysis test

Pure colony of bacterium was spot inoculated on nutrient agar plate containing 0.2% soluble starch for starch hydrolysis test. Then it was incubated at 30^0C for 48 hours in an incubation chamber. After incubation the plate was flooded with lugol's iodine solution. Then it was observed whether a clear zone appeared around the colony or not.

3.2.6.3.2.b. Catalase test

A few drops of 3% H_2O_2 (Hydrogen peroxide) was added with 48 hours old pure culture of bacterial colony grown on NA plate. Then it was observed whether bubbles were produced within a few seconds or not.

3.2.6.3.2.c. Oxidase test

For oxidase test oxidase disk containing 1 ml 1% aqueous w/v solution of NNN'N-tetramethyl-p-phenylene-diamine-dihydrochloride solution was soaked in sterile water and then it was placed on a petri dish. Then a young colony part was removed with a sterile toothpick and smeared onto the moistened oxidase disk and observed up to 60 seconds whether the colour is changed to dark purple or not.

3.2.6.3.2.d. Citrate utilization test

At first pure colony part of bacterium was streak inoculated on the simmon's citrate agar slant with the help of a sterile loop. Then it was incubated for 24 hours at 30^0C in an incubation chamber. After incubation it was observed whether the colour is changed from green to bright blue.

3.2.6.3.2.e. Motility indole urease agar (MIU) test

At first pure colony part of bacterium was stub inoculated into a test tube that contains motility indole urease agar (MIU) media with the help of a sterile loop without touching the bottom of the tube. After that the tube was incubated for 48 hours at 30^0C in an incubation chamber. Then the way of the bacterial movement was observed.

3.2.6.3.2.f. Gelatine liquefaction

One loop-full pure culture of bacterium was stub inoculated into a test tube that contains 12% (w/v) gelatine with the help of a sterile loop. Then it was incubated for 24 hours at 30^0C in an incubation chamber. Then it was kept at 5^0C in refrigerator for 15 minutes. Gelatin liquefied microorganism was determined by the formation of liquid culture.

3.2.6.3.2.g. Salt tolerant test

At first 7 test tubes were taken for 1%, 2%, 3%, 4%, 5%, 6% and 7% NaCl. Then 10 ml nutrient broth was poured into each of the test tubes. For preparing 1% NaCl solution, 0.1 g NaCl was mixed in 10 ml NA broth. Similarly 2%, 3%, 4%, 5%, 6% and 7% NaCl was prepared by adding 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 g NaCl in each 10 ml NA broth respectively. The pH was adjusted at 7.0. Another test tube containing only 10 ml NA broth was taken as control. Finally all the test tubes were autoclaved. After that, the 1%, 2%, 3%, 4%, 5%, 6% and 7% salt broths were inoculated with 48 hours old colony part of bacteria grown on NA plate. Then the test tubes were transferred to shaker incubator machine maintaining 30^0C temperature and 150 rpm. Data were recorded after every 24 hours for 7 days.

3.2.6.3.3. Cultural characters

Schaad (1992) reported that most of pathovars of *Xanthomonas* can be differentiated by growth and colony morphology on different media. Growth characteristics of the pathogen were studied by using various differential, selective and semi-selective media.

3.2.6.3.3.a. Growth on nutrient agar (NA) media

Nutrient agar (NA) medium was poured into a sterile Petri dish and cooled. Then pure culture of bacterium was streak inoculated on the plate with the help of a sterile loop. Then it was incubated for at least 24 hours at 30^0C in incubation chamber and the colony characters were observed.

3.2.6.3.3.b. Growth on semi-selective yeast extract dextrose calcium carbonate agar (YDCA) media

Yeast extract dextrose calcium carbonate agar (YDCA) medium was poured into a sterile Petri dish and cooled. Then pure culture of bacterium was streak inoculated on the plate with the help of a sterile loop. Then it was incubated for

at least 24 hours at 30^0C in incubation chamber and the colony characters were observed.

3.2.6.3.3.c. Growth on selective SX agar media

SX agar medium was poured into a sterile Petri dish and cooled. Then pure culture of bacterium was streak inoculated on the plate with the help of a sterile loop. Then it was incubated for at least 24 hours at 30^0C in incubation chamber and the colony characters were observed.

3.2.6.3.4. Tobacco hypersensitivity reaction

For the determination of the pathogenic nature of the isolates, hypersensitivity reaction was studied on tobacco (*Nicotiana rustica)* plants by injection infiltration technique, that was developed by Klement and Goodman (1967). An aliquot of the inoculums suspension of bacterium (approximately 10^8 cfu/ml) was prepared from a 24 hours culture plate in sterile distilled water. Then the lower surface of a mature tobacco leaf was infiltrated with the help of a syringe containing the suspension. Distilled water was used as a negative control. After that it was observed for 72 hours.

3.2.6.4. Measurement of optical density (OD)

3.2.6.4.1. Broth culture preparation

Broth culture of *Xanthomonas axonopodis* pv *citri* was made on 100ml conical flask. At first 0.05g peptone and 3.3 g beef extract were diluted in 100ml water. Then one loopfull of *Xanthomonas axonopodis* pv *citri* bacteria from pure culture was inoculated in the suspension of conical flask. 4 conical flasks were inoculated. Then it was kept in shaker incubator at 30^0c for 72 hours.

3.2.6.4.2. Optical density measurement

After every 12 hours sample was taken from conical flask and optical density was measured by Spectrophotometer at 600nm wavelength. Data was taken at

every 12 hours and it was taken at 6 consecutive periods. That means the first data was taken at the first 12 hours, the second data was taken at the second 12 hours that means at 24 hours. Like this data was taken consecutively at 36, 48, 60 and 72 hours. After 12 hours sample was taken from conical flask and optical density was measured by Spectrophotometer at 600nm wavelength.

3.2.6.5. Pathogenicity Test

Citrus plant grown on pot under greenhouse condition was used for studying the pathogenicity of *Xanthomonas axonopodis* pv. *citri.* The test was conducted by following the method described by Lin *et al.* (2008). For the preparation of inoculum, bacterial cells were grown overnight in NA broth and it was resuspended in sterile distilled water to a concentration of approximately 10^8 cfu/ml. Then an aliquot of the inoculums 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 reisolated from the infected area.

3.3. Experiment III. Study on the effect of environmental factors on canker disease development

3.3.1. Effect of temperature, relative humidity and rainfall

3.3.1.1. Plant sample collection

Three healthy, vigor and disease-free plant samples were collected from a nursery named Green orchid nursery which is situated on Sher-e-Bangla Nagar, Dhaka.

3.3.1.2. Experimental Site

The experiment was conducted in the Disease diagnostic laboratory and Nursery, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.3.1.3. Experimental Period

The experiment was conducted during the period of July 2014 to August 2014.

3.3.1.4. Preparation of pot

The substratum was prepared by mixing soil, sand and well decomposed cow dung in the proportion of 2:1:1 and sterilized with 5 ml formalin (40%) diluted with 200 ml water for 4 kg soil (Dashgupta, 1988). The prepared soil was heaped in square block. Soil heap was then covered by a polythene sheet for 48 hr to make the soil free from soil borne inocula. After 4 days of treatment, surface sterilized earthen pots were filled up with the sterilized soil. No chemical fertilizers were used in the pot soil.

3.3.1.5. Planting of seedlings

One seedling per pot was planted and they were properly covered with soil. Plants were watered whenever necessary.

3.3.1.6. Preparation of Inoculums

Xanthomonas axonopodis pv. *citri* bacteria were collected from pure culture that was preserved in small screw-cap test tubes on NA slant at 4^0C temperature in the refrigerator. Then bacterial suspension were 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. Optical density (OD) value was fixed at 0.5. Then serial dilution 10^2 was prepared. The concentration of bacterial suspension was adjusted to 10^7 cfu/ml.

3.3.1.7. Inoculation

Inoculation of bacteria was done by syringe inoculation method (insulin syringe). One unit of bacterial suspension was inoculated in each of the leaves. 5 healthy leaves were inoculated from each of the plants. Every leaf was marked by threads. The inoculated plants were covered with polyethylene sheet to maintain relative humidity (% RH) and also to prevent natural contamination with other Microorganisms. Humid condition was maintained gently spraying

sterilized distilled water on leaf surface. Inoculation was done in 15th July 2014.

3.3.1.8. Data collection

The first data was taken 10 days after inoculation and that was 25th July 2014. Data was taken as the percent disease infection of the previously inoculated and marked leaves. Temperature, relative humidity, and rainfall were recorded every day. Data was taken after every five days and it was taken at 5 consecutive periods. The mean temperature, relative humidity and rainfall of every last five days were calculated and compiled with data sheet.

3.3.1.9. Determination of Leaf infection (%)

Leaf infection (%) was determined by the following formula (Rai and Mamatha, 2005):

$$\text{Percent leaf infection} = \frac{\text{Number of diseased leaves}}{\text{Number of total leaves observed}} \times 100$$

3.3.2. Study on the effect of light intensity (different wavelengths of light) on disease incidence and disease severity

3.3.2.1. Plant sample collection

Eighteen healthy, vigor and disease-free plant samples were collected from different nurseries of Dhaka city.

3.3.2.2. Collection of films that can block different wavelengths of light

Five films were collected from Yamagata University, Japan. One of the films cannot block any wavelengths of light. It was implemented in control environment. The other four films can block light with the wavelengths of 340nm, 350nm, 360nm and 400nm.

3.3.2.3. Experimental Site

The experiment was conducted in the Central laboratory, Department of Plant Pathology and Horticulture field, Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.3.2.4. Experimental Period

The experiment was conducted during the period of June 2014 to August 2014.

3.3.2.5. Different experimental steps

The following steps were conducted by following the procedures of Experiment III

1. Preparation of pot
2. Planting of seedlings
3. Preparation of inoculums
4. Inoculation

Inoculation was done in 9th June, 2014.

3.3.2.6. Procedure of the experiment

After inoculation plants were transferred randomly into different blocks located in the Horticulture field, Department of Horticulture, Sher-e-Bangla Agricultural University. Six blocks were used to conduct the experiment. Three plants were set with uniform spacing into each of the blocks. Blocks were marked as open, control, A, B, C and D (Plate 2). Each of the blocks (without Open block) were covered by different films. Each individual film can block a selected range of wavelength of light (Table 2). Water was sprayed on the plants under the films regularly to keep the optimum moisture content.

Table 2. Efficiency of different films implemented into different blocks on blocking different wavelengths of light

Name of blocks	Range of wavelengths of light that could be blocked by the films implemented into different blocks
Open	No film was used

Control	Film was used but it was not able to block any wavelength of light
A	Able to block any wavelength less than 340nm
B	Able to block any wavelength less than 350nm
C	Able to block any wavelength less than 360nm
D	Able to block any wavelength less than 400nm

Plate 2: Experimental plot (Effect of light intensity on incidence and severity)

I. Total experimental plot with different blocks (Open, Control, A, B, C and D)

II. A single block covered by film

3.3.2.7. Data collection

Data were taken at three consecutive periods. The first, second and third data were taken at 20 days after inoculation, at 40 days after inoculation and at 60 days after inoculation and they were taken at the date of 29th June 2014; 19th July 2014 and 8th August 2014. Data were taken as the disease incidence (%) and disease severity (%) of the previously inoculated and marked leaves (Five leaves per plant).

3.3.2.8. Determination of disease incidence and disease severity

During the experiment, total number of leaves that are inoculated in a plant as well as number of leaves that are infected with citrus canker in that plant was recorded to determine the Disease Incidence. Total area of the leaf and area of leaf tissue infection was calculated to determine Disease Severity. Disease incidence and disease severity were recorded 10 days after inoculation.

Percent Disease Incidence (PDI) of foliar diseases was determined by the formula (Rai and Mamatha, 2005):

$$\text{Percent Disease Incidence (Leaves)} = \frac{\text{Number of diseased leaves on each plant}}{\text{Number of total leaves on each plant}} \times 100$$

Percent Disease Severity (PDS) was determined by the following formula (Rai and Mamatha, 2005):

$$\text{Percent Disease Severity (Leaves)} = \frac{\text{Area of leaf tissue infected by disease}}{} \times 100$$

Total area of inspected leaf

CHAPTER IV

RESULTS

The results of the investigations undertaken on "Prevalence of *Xanthnonas axonopodis* pv. *citri* on citrus and the effect of environmental factors on citrus canker development" during the study period are presented as below.

4.1. Symptomatology

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 (Plate 3 A). The center of the lesion became raised and corky. Leaf tissues in old lesion had died and fall out (Plate 3 B). The lesions in fruits and were superficially similar to those on leaves but they were generally irregularly shaped. Lesions in fruits were raised with a corky appearance but there was no yellow halo at mature stage (Plate 3 C).

4.2. Survey on determining the disease incidence and disease severity of canker of citrus

4.2.1. Incidence and severity of citrus canker at some selected wholesale markets of Dhaka, Bangladesh from September 2012 to May 2014

Incidence of citrus canker varied from markets to markets and that ranged from 17.37% to 17.63% (Table 3). Highest incidence was recorded at Shyambazar wholesale market and Savar wholesale market (17.63%) and lowest was recorded at Karwanbazar wholesale market (17.37%). Severity of citrus canker

also varied markets to markets and that ranged from 9.037% to 9.778% (Table 3).

Plate 3. Symptoms of citrus canker

A. Leaf (early stage)

B. Leaf (old stage)

C. On fruit

Highest severity was recorded at Savar wholesale market (9.778%) and lowest was recorded at Karwanbazar wholesale market (9.037%). Incidence and severity of citrus canker recorded in all the selected markets were statistically similar.

Table 3. Incidence and severity of citrus canker at some selected wholesale markets of Dhaka, Bangladesh from September 2012 to May 2014

Name of markets	Citrus canker	
	Disease Incidence (%) (Sept ,2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Karwanbazar wholesale market	17.37 a	9.037 a
Savar wholesale market	17.63 a	9.778 a
Shyambazar wholesale market	17.63 a	9.222 a
LSD (0.05)	6.712	3.077
CV (%)	23.35	20.10

4.2.2. Incidence and severity of citrus canker of the fruits of different districts of Bangladesh from January 2013 to September 2014

Incidence of citrus canker varied from districts to districts and that ranged from 17.26% to 17.70% (Table 4). Highest incidence (17.70%) was recorded at Chittagong and lowest (17.26%) was recorded at Gazipur. Severity of citrus canker also varied from districts to districts and that ranged from 9.037% to 9.630% (Table 4). Highest severity (9.630%) was recorded on the fruits of Chittagong and lowest (9.037%) was recorded on the fruits of Gazipur. Incidence and severity of citrus canker recorded in all the districts were statistically similar.

Table 4. Incidence and severity of citrus canker of the fruits of different districts of Bangladesh from January 2013 to September 2014

Name of districts	Citrus canker	
	Disease Incidence (%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Munshiganj	17.67 a	9.370 a
Gazipur	17.26 a	9.037 a
Chittagong	17.70 a	9.630 a
LSD (0.05)	6.712	3.077
CV (%)	23.35	20.10

4.2.3. Incidence and severity of canker in different growing seasons citrus of Bangladesh during September 2012 to May 2014

Incidence of citrus canker varied significantly from September 2012 to May 2014 and that ranged from 4.59% to 25.48% (Table 5). Highest (25.48%) incidence was recorded in the month of September (2012 & 2013) and lowest incidence (4.593%) was recorded in the month of January (2013 & 2014). Severity of canker of citrus also varied significantly from September 2012 to May 2014 and that ranged from 2.18% to 13.63% (Table 5). Highest (13.63%) severity was recorded in the month of May (2013 & 2014) and lowest (2.18%) severity was recorded in the month of January (2013 & 2014).

Table 5. Incidence and severity of citrus canker during September 2012 to May 2014 of Bangladesh

Name of months	Citrus canker	
	Disease Incidence (%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
September	25.48 a	12.22 a
January	4.59 b	2.18 b
May	22.56 a	13.63 a
LSD (0.05)	6.712	3.077
CV (%)	23.35	20.10

4.2.4. Incidence and severity of citrus canker of the fruits of different districts of Bangladesh at some selected wholesale markets of Dhaka from September 2012 to May 2014

Incidence of citrus canker varied from districts to districts as well as markets to markets and that ranged from 15.67% to 19.00% (Table 6). Highest (19.00%) incidence was recorded on the fruits of Munshiganj at Karwanbazar wholesale market. Lowest (15.67%) incidence was recorded on the fruits of Gazipur at Karwanbazar wholesale market. Severity of citrus canker also varied significantly from districts to districts as well as markets to markets and that ranged from 8.44% to 10.44% (Table 6). Highest (10.44%) severity was recorded on the fruits of Chittagong at Shyambazar wholesale market and lowest severity (8.44%) was recorded on the fruits of Gazipur at Shyambazar wholesale market. Incidence and severity recorded on the fruits of all the districts of all the markets were statistically similar.

Table 6. Incidence and severity of citrus canker of the fruits of different districts of Bangladesh at some selected wholesale markets of Dhaka from September 2012 to May 2014

Name of markets	Name of districts	Citrus canker	
		Disease Incidence (%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Karwanbazar wholesale market	Munshiganj	19.00 a	9.33 a
	Gazipur	15.67 a	8.78 a
	Chittagong	17.44 a	9.00 a
Savar wholesale market	Munshiganj	17.89 a	10.00 a
	Gazipur	18.22 a	9.89 a
	Chittagong	16.78 a	9.44 a
Shyambazar wholesale market	Munshiganj	16.11 a	8.78 a
	Gazipur	17.89 a	8.44 a
	Chittagong	18.89 a	10.44 a
LSD (0.05)		6.712	3.077
CV (%)		23.35	20.10

4.2.5. Incidence and severity of citrus canker during different growing seasons at some selected wholesale markets of Dhaka, Bangladesh

Incidence of citrus canker varied significantly from season to season as well as market to market and that ranged from 4.44% to 25.89% (Table 7). Highest (25.89%) incidence was recorded in the month of September (2012 & 2013) at Shyambazar wholesale market. Lowest (4.44%) incidence was recorded in the month of January (2013 & 2014) at Karwanbazar wholesale market. Severity of

citrus canker also varied significantly from season to season as well as market to market and that ranged from 2.11% to 13.89% (Table 7). Highest (13.89%) severity was recorded in the month of May (2013 & 2014) at Shyambazar wholesale market and lowest severity (2.11%) was recorded in the month of January (2013 & 2014) at Karwanbazar wholesale market.

Table 7. Incidence and severity of citrus canker during September 2012 to May 2014 at some selected wholesale markets of Dhaka, Bangladesh

Name of markets	Name of months	Citrus canker	
		Disease Incidence (%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Karwanbazar wholesale market	September	25.11 a	11.78 a
	January	4.44 b	2.11 b
	May	22.56 a	13.22 a
Savar wholesale market	September	25.44 a	13.22 a
	January	4.56 b	2.33 b
	May	22.89 a	13.78 a

Shyambazar wholesale market	September	25.89 a	11.67 a
	January	4.78 b	2.23 b
	May	22.22 a	13.89 a
LSD (0.05)		6.712	3.077
CV (%)		23.35	20.10

4.2.6. Incidence and severity of citrus canker during different growing seasons of the fruits of different districts of Bangladesh

Incidence of citrus canker varied significantly from season to season as well as district to district and that ranged from 4.11% to 26.00% (Table 8). Highest (26.00%) incidence was recorded in the month of September (2012 & 2013) on the fruits of Chittagong and lowest (4.11%) incidence was recorded in the month of January (2013 & 2014) on the fruits of Gazipur. Severity of citrus canker also varied significantly from season to season as well as district to district and that ranged from 2.00% to 13.78% (Table 8). Highest (13.78%) severity was recorded in the month of May (2013 & 2014) on the fruits of Chittagong and lowest severity (2.00%) was recorded in the month of January (2013 & 2014) on the fruits of Gazipur.

Table 8. Incidence and severity of citrus canker during different growing seasons of the fruits of different districts of Bangladesh

Name of districts	Name of months	Citrus canker	
		Disease Incidence (%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Munshiganj	September	25.56 a	12.33 a
	January	4.89 b	2.11 b
	May	22.56 a	13.67 a
Gazipur	September	24.89 a	11.67 a
	January	4.11 b	2.00 b

	May	22.78 a	13.44 a
Chittagong	September	26.00 a	12.67 a
	January	4.78 b	2.44 b
	May	22.33 a	13.78 a
LSD (0.05)		6.712	3.077
CV (%)		23.35	20.10

4.2.7. Incidence and severity of citrus canker during different growing seasons of the fruits of different districts of Bangladesh at some selected wholesale markets of Dhaka

Incidence of citrus canker varied significantly from season to season and district to district as well as market to market and that ranged from 3.67% to 28.67% (Table 9). Highest (28.67%) incidence of citrus canker was recorded in the month of September (2012 & 2013) on the fruits of Chittagong at Shyambazar wholesale market. Lowest (3.67%) incidence was recorded in the month of January (2013 & 2014) on the fruits of Gazipur at Savar wholesale market. Severity of citrus canker also varied significantly from season to season and district to district as well as market to market and that ranged from 1.67 % to 15.67% (Table 9). Highest (15.67%) severity of citrus canker was recorded in the month of May (2013 & 2014) on the fruits of Chittagong at Shyambazar wholesale market and lowest severity (1.67%) was recorded in the month of January (2013 & 2014) on the fruits of Munshiganj at Shyambazar wholesale market.

Table 9. Incidence and severity of citrus canker during different growing seasons of the fruits of different districts of Bangladesh at some selected wholesale markets of Dhaka

Name of markets	Name of districts	Name of months	Citrus canker	
			Disease Incidence(%) (Sept 2012 – May 2014)	Disease Severity (%) (Sept 2012 – May 2014)
Karwanbazar wholesale market	Munshiganj	September	27.67 ab	12.33 bcd
		January	5.33 c	2.00 e
		May	24.00 ab	13.67 abcd
	Gazipur	September	22.00 ab	11.00 cd
		January	4.00 c	2.00 e
		May	21.00 b	13.33 abcd
	Chittagong	September	25.67 ab	12.00 bcd
		January	4.00 c	2.33 e
		May	22.67 ab	12.67 abcd
Savar wholesale market	Munshiganj	September	25.67 ab	13.33 abcd
		January	5.33 c	2.67 e
		May	22.67 ab	14.00 abc
	Gazipur	September	27.00 ab	13.33 abcd
		January	3.67 c	2.00 e
		May	24.00 ab	14.33 ab
	Chittagong	September	23.67 ab	13.00 abcd
		January	4.67 c	2.33 e
		May	22.00 ab	13.00 abcd
Shyambazar wholesale market	Munshiganj	September	23.33 ab	11.33 bcd
		January	4.00 c	1.67 e
		May	21.00 b	13.33 abcd
	Gazipur	September	25.67 ab	10.67d
		January	4.67 c	2.00 e
		May	23.33 ab	12.67 abcd
	Chittagong	September	28.67 a	13.00 abcd
		January	5.67 c	2.67 e
		May	22.33 ab	15.67 a
LSD (0.05)			6.712	3.077
CV (%)			23.35	20.10

4.2.8. Effect of weather components on the incidence and severity of canker on the fruits of citrus during September 2012 to May 2014

Incidence of citrus canker was influenced by temperature, relative humidity and rainfall. Highest incidence (25.48%) was recorded in the month of September (2012 & 2013) when the average temperature, relative humidity and rainfall were 28.68°C, 83.86% and 31.83mm, respectively (Fig. 1). On the other hand, lowest incidence (4.59%) was recorded in the month of January (2013 & 2014) having average temperature, relative humidity and rainfall 17.97°C, 69.34% and 3.89mm, respectively. In the month of May (2013 & 2014) incidence was 23.35% when the temperature, relative humidity and rainfall were 28.73°C, 77.87% and 32.15mm, respectively.

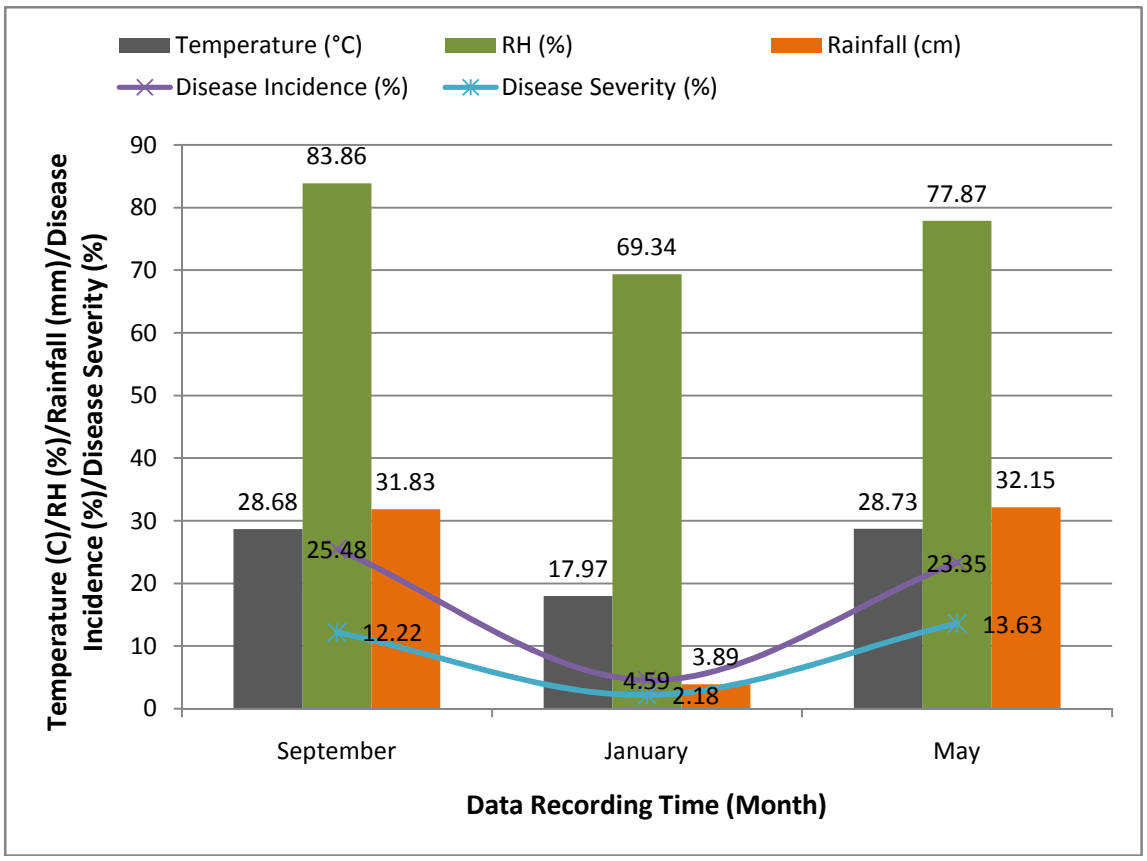


Figure 1. Effect of weather components on the incidence and severity of canker on fruits of citrus during September 2012 to May 2014

Severity of citrus canker was also influenced by temperature, relative humidity and rainfall. Highest severity (13.63%) was recorded in the month of May (2013 & 2014) when the average temperature, relative humidity and rainfall

were 28.73°C, 77.87% and 32.15 mm, respectively. Lowest severity (2.18%) was recorded in the month of January (2013 & 2014) having average temperature, relative humidity and rainfall 17.97°C, 69.34% and 3.89 mm, respectively. In the month of September (2013 & 2014) severity was 12.22% when the temperature, relative humidity and rainfall were 28.68 °C, 83.86 % and 31.83 mm, respectively.

4.3. Isolation and purification of canker pathogen of citrus

The causal organism was isolated from the infected fruits showing typical symptoms of citrus canker. Isolation was done by employing the dilution plate technique using nutrient agar medium. Repeated isolation from the infected plant parts yielded well separated, typical, yellow, convex, mucoid, colonies of bacterium on nutrient agar medium after 48 hours of incubation at 30^0C (Fig. 2). Colonies were purified by restreaking the isolated colony on nutrient agar plate.

4.4. Preservation of canker pathogen of citrus

Purified bacterium was kept in refrigerator at 4^0c in small screw-cap test tubes on NA slant, which served as a stock culture for further studies.

4.5. Identification of the pathogen

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

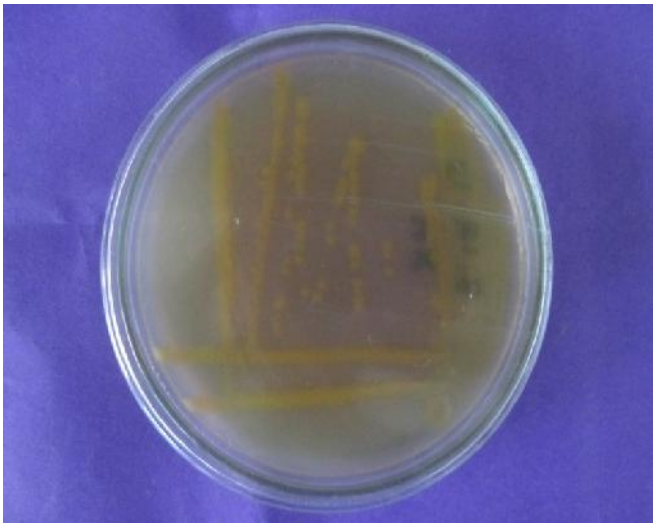


Fig. 2. Pure culture of *Xanthomonas axonopodis* pv. *citri* on NA plate

4.5.1. Morphological characters

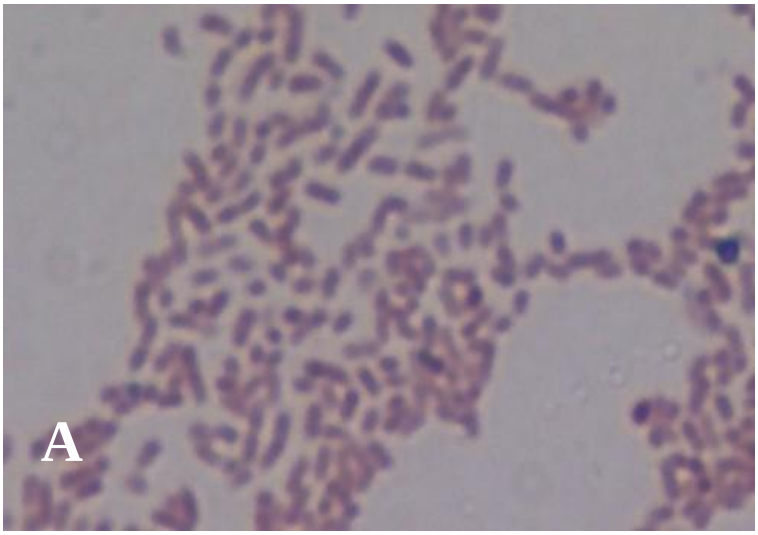
4.5.1.1. Morphological study of colony

The colony developed on NA media was circular, entire, yellow in colour, convex, filiform on NA slant, uniform fine turbidity on broth culture.

4.5.1.2. Morphological characters of the organism

Under the compound microscope at 100x magnification with oil immersion, the bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative and capsulated. The cells were readily stained with common stains such as crystal violet (Plate 4 A).

In KOH solubility test, a mucoid thread was lifted with the loop (Plate 4. B). Therefore the test was positive i.e., the bacterium was gram negative that supports the result of gram's staining test.



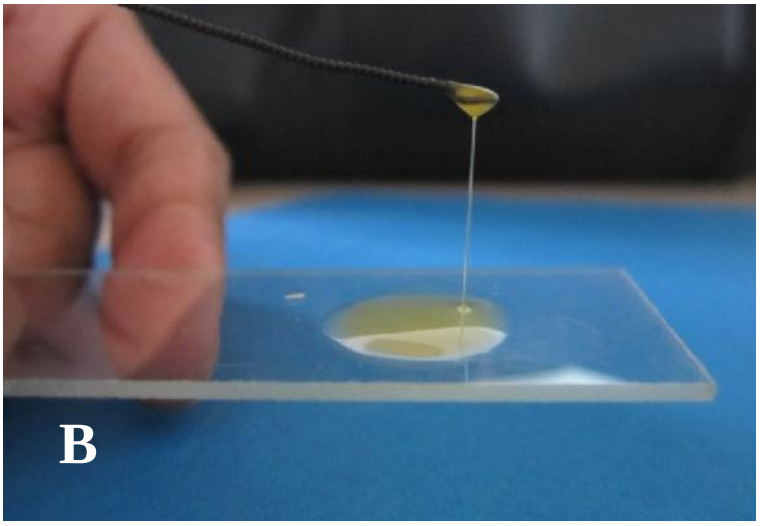


Plate 4. Morphological characters of *Xanthomonas axonopodis* pv. *citri*

A. Microscopic view of *Xanthomonas axonopodis* pv. *citri* after gram's staining at 100x magnification
B. KOH solubility test for *Xanthomonas axonopodis* pv. *citri*

4.5.2. Biochemical characters

Results obtained on various biochemical tests for the pathogen are presented in Table 10.

Table 10. Biochemical characteristics of *Xanthomonas axonopodis* pv. *citri*

Biochemical tests	Results
Starch hydrolysis test	+
Catalase test	+
Oxidase test	-
Citrate utilization test	+
Motility indole urease agar (MIU) test	+
Gelatine liquefaction test	+
Salt tolerant test	+

In starch hydrolysis test, after adding lugol's iodine a clear zone was formed around the bacterial colony indicated starch hydrolysis (amylase activity) i.e., the test was positive (Plate 5 A).

In catalase test, after adding 3% H_2O_2 onto the colony of the bacterium bubbles were formed within a few seconds (Plate 5 B), which revealed that the test was positive.

In oxidase test, after rubbing the bacterium onto the moistened oxidase disk, it did not form any color in oxidase disk (Plate 5 C), which revealed that the test was negative.

In **citrate utilization test,** after 24 hours of incubation green colour of simmon's citrate agar slant changed into a bright blue colour indicated the test was positive i.e., the bacterium used citrate as a carbon source for their energy (Plate 5 D).

In motility indole urease agar (MIU) test, after 48 hours of incubation it was found the bacterium migrated away from the original line of inoculation (Plate 5 E). Thus the bacterium was motile (positive test).

In gelatine liquefaction test, gelatin was liquefied after 15 minutes of refrigeration at 5^oC (Plate 5 F). Thus the bacterium showed the positive result.

In case of salt tolerance test, turbidity was formed after 24 hr, 48 hr and 72 hr up to 3% salt concentration in shaker incubator (Table 10, Plate 5 G).

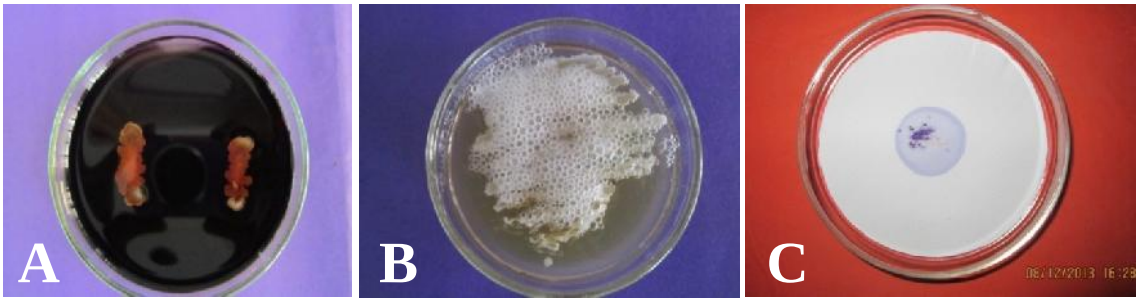
Table 11. Salt tolerance test for *Xanthomonas axonopodis* pv. *citri* in nutrient broth

Time	Salt tolerance					
	1%	2%	3%	4%	6%	7%
24hr	+	+	-	-	-	-
48hr	+	+	+	-	-	-
72hr	+	+	+	-	-	-

4.5.3. Cultural characters

4.5.3.1. Efficacy of different media in supporting the growth of the pathogen

Of the various media tested for the efficacy to support the growth of *X. axonopodis* pv. *citri*, nutrient agar (NA) medium was found significantly superior in promoting the luxurious growth of the pathogen as evidenced by the maximum recovery of bacterial colonies followed by yeast extract nutrient agar (YDCA) medium. Lowest amount of bacterial colonies were found on SX agar medium.



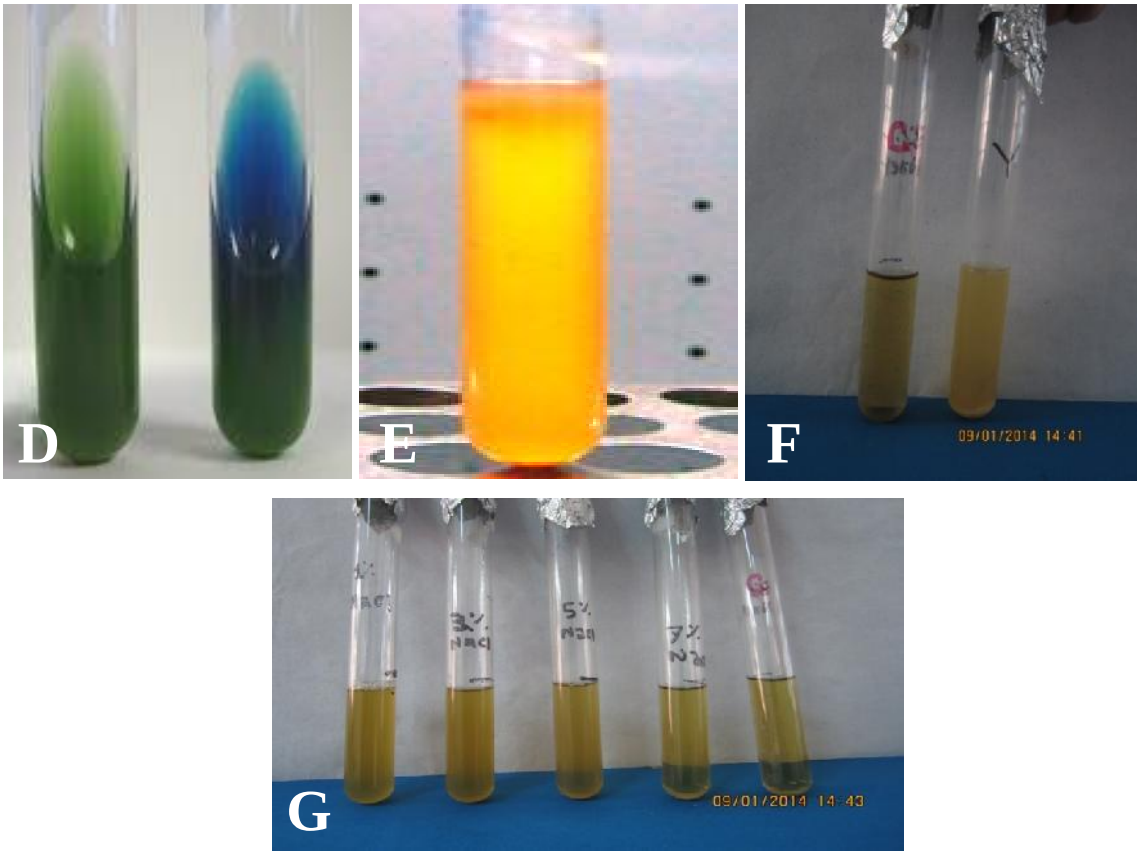


Plate 5. Biochemical characters of *Xanthomonas axonopodis* pv. *citri*

A. Starch hydrolysis test
B. Catalase test
C. Oxidase test
D. Citrate utilization test
E. Motility indole urease agar (MIU) test
F. Gelatine liquefaction test G. Salt tolerance test

4.5.3.2. Colony morphology on different growth media

Colonies of *X. axonopodis* pv. *citri* on NA medium appeared as circular, mucoid, convex, yellow to orange colour (Table 12).

Circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies were found on YDCA medium (Table 12).

Bacterium exhibited very poor growth with light yellow to slightly blue, mostly circular, small, flattened, mucoid colonies on SX medium (Table 12).

Table 12. Cultural characteristics of *Xanthomonas axonopodis* pv. *citri* on different solid media

Media	Colony characters		
	Colour	Shape	Appearance
NA	yellow to orange	Circular	mucoid, convex
YDCA	yellow to bright yellow	Circular	mucoid, flattened or slightly raised
SX	light yellow to slightly blue	mostly circular	mucoid, flattened

4.6. Tobacco hypersensitivity reaction

In case of tobacco hypersensitivity reaction, after infiltration the lower surface of a mature tobacco leaf by pressing a syringe containing the bacterial suspension against the leaf (Plate 7 A), the infiltrated area became dry and necrotized within 48 hours. Thus bacteria showed positive reaction (Plate 7 B). There was no change in case of control.

4.7. Pathogenicity test

For proving pathogenicity, the bacterial cell suspension (10^8 cfu/ml) was injected forcedly into the lower surface of citrus leaf (Kagozi lemon) as described in "Materials and Methods" chapter.

The characteristic symptoms were observed on citrus leaf after ten days of inoculation as small, blister-like lesions, which later on turned gray to tan brown surrounded by a yellow halo (Plate 8). Reisolations were carried out from these lesions and comparisons were made with the original culture to confirm the identity of the pathogen. Artificially inoculated plants yielded the bacterial colonies similar to the original ones.

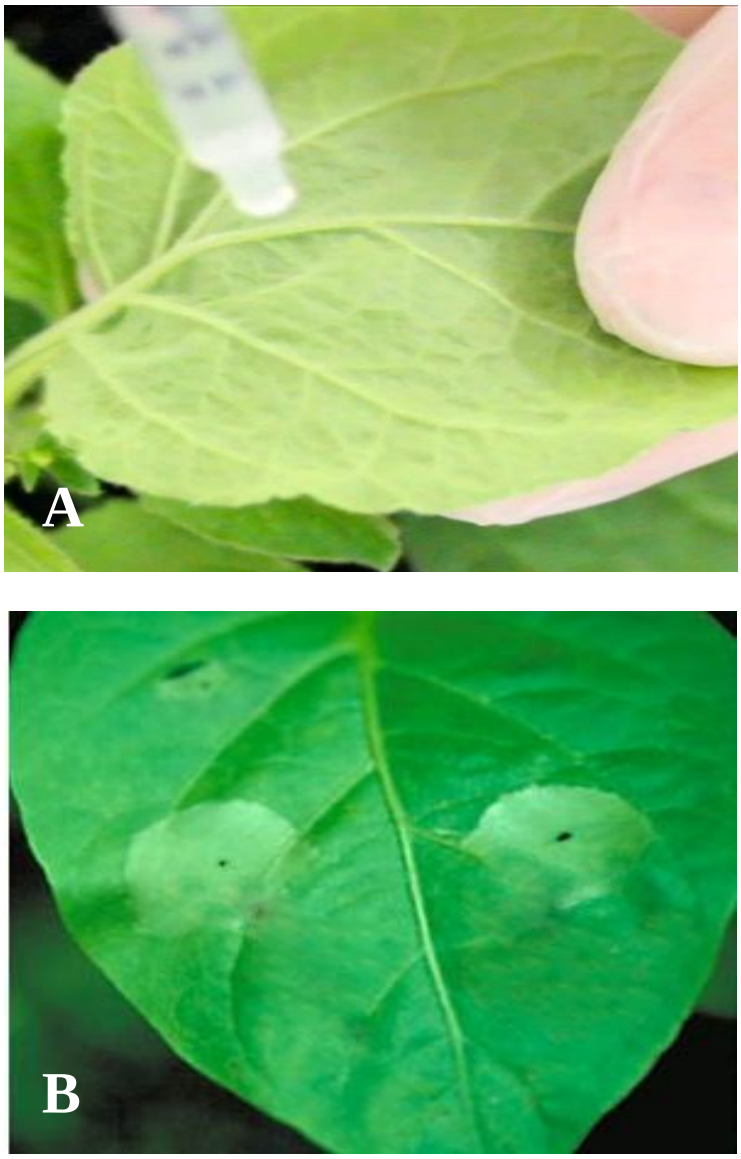


Plate 7. Tobacco hypersensitivity test

A. Inoculation of bacterial suspention into the lower surface of tobacco leaf with a sterile syringe
B. Tobacco hypersensitivity positive (*Xanthomonas axonopodis* pv. *citri* inoculated)

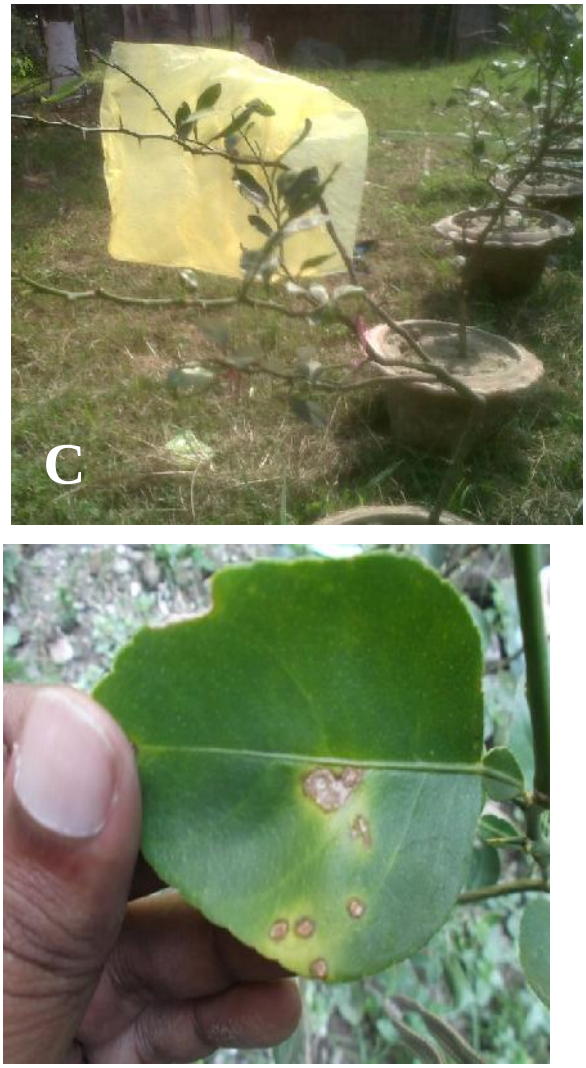


Plate 8. Pathogenicity test

A. Leaf before inoculation with bacteria
B. Inoculation of bacterial suspension into the lower surface of citrus leaf with a sterile syringe
C. Tagging and covering of inoculated leaf with polythene sheet
D. Symptom of canker observed on inoculated leaf

4.8. Study on the effect of environmental factors on citrus canker disease development

4.8.1. Effect of temperature, relative humidity and rainfall on leaf infection (%)

Leaf infection (%) increases with the increase of temperature and relative humidity and decreases with the decrease of temperature and relative humidity.

Table 13. Temperature (°C), relative humidity (%), rainfall (mm) and leaf infection (%) of canker on citrus seedlings during July 2014 to August 2014

Parameters		Leaf Infection (%)	Temperature (°C)	RH (%)	Rainfall (mm)
10 days after inoculation	Plant 1	4.5	30.5	72	31.2
	Plant 2	5	30.5	72	31.2
	Plant 3	5.5	30.5	72	31.2
15 days after inoculation	Plant 1	8	30.2	71	31.1
	Plant 2	9	30.2	71	31.1
	Plant 3	10	30.2	71	31.1
20 days after inoculation	Plant 1	12.25	28.5	69	30.3
	Plant 2	12	28.5	69	30.3
	Plant 3	11.75	28.5	69	30.3
25 days after inoculation	Plant 1	20.5	31.3	73	33.4
	Plant 2	21	31.3	73	33.4
	Plant 3	21.5	31.3	73	33.4
30 days after inoculation	Plant 1	21.75	26.5	68	28.2
	Plant 2	22	26.5	68	28.2
	Plant 3	22.25	26.5	68	28.2

Mean Leaf infection was 5% at 10 days after inoculation, when the last 5 days mean temperature was 30.5°C, relative humidity was 72% and rainfall was 31.2 mm (Fig. 3). Leaf infection was 9% at 15 days after inoculation, when temperature was 30.2°C, relative humidity was 71% and rainfall was 31.1 mm. Leaf infection was 12% at 20 days after inoculation, when temperature was 28.5°C, relative humidity was 69% and rainfall was 30.3 mm. Temperature, relative humidity and rainfall decreases till 20 days after inoculation. At 25 days after inoculation the mean temperature, relative humidity and rainfall

increases to 31.3°C, 73% and 33.4 mm, respectively and the leaf infection (%) suddenly increases to 21% with the increase of temperature, relative humidity and rainfall. At 30 days after inoculation the last 5 days mean temperature, relative humidity and rainfall again decreases to 26.5°C, 68%, 28.2 mm. Leaf infection was 22% at 30 days after inoculation. Development of leaf infection (%) was reduced with reduced temperature, relative humidity and rainfall.

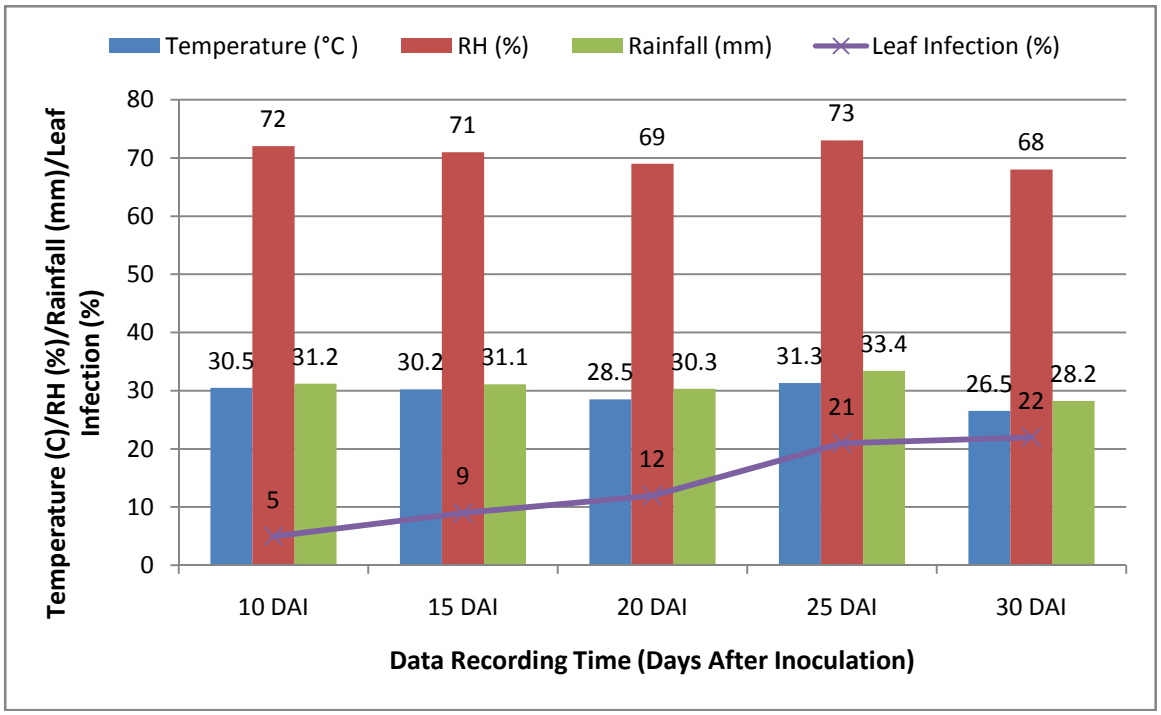


Fig. 3: Effect of Temperature, Relative Humidity and Rainfall on Leaf Infection (%) of citrus canker

4.8.2. Effect of light intensity on disease incidence and disease severity

4.8.2.1. Incidence of canker of citrus at different wavelengths of light from June 2014 to August 2014

Incidence of canker of citrus varied from blocks to blocks. At 20 days after inoculation it ranged from 10 % to 27% (Table 14), at 40 days after inoculation it ranged from 20 % to 47% and at 60 days after inoculation it ranged from 45 % to 77%. Highest incidence was recorded at block D in case of all the selected different periods after inoculation (27% at 20 days after inoculation, 47% at 40 days after inoculation and 77% at 60 days after inoculation) and lowest incidence was recorded at block A in case of all the selected different periods

after inoculation (10% at 20 days after inoculation, 20% at 40 days after inoculation and 45% at 60 days after inoculation).

Table 14. Incidence of canker of citrus at different wavelengths of light from June 2014 to August 2014

Name of blocks	Efficiency of films implemented into different blocks	Disease Incidence (%)		
		Days After Inoculation		
		20	40	60
Open	No film was used	21.00b	38.00b	65.00b
Control	Film cannot block any wavelength	17.00d	30.67d	55.00d
A	Can block wavelength <340nm	10.00f	20.00f	45.00f
B	Can block wavelength <350nm	13.00e	27.00e	49.00e
C	Can block wavelength <360nm	19.00c	33.00c	57.00c
D	Can block wavelength <400nm	27.00a	47.00a	77.00a
LSD (0.05)		1.779	1.677	1.779
CV (%)		5.61	2.89	1.72

4.8.2.1. Severity of canker of citrus at different wavelengths of light from June 2014 to August 2014

Severity of canker of citrus varied from blocks to blocks. At 20 days after inoculation it ranged from 5% to 14% (Table 15), at 40 days after inoculation it ranged from 13 % to 33% and at 60 days after inoculation it ranged from 21% to 38%. Highest severity was recorded at block D in case of all the selected different periods after inoculation (14% at 20 days after inoculation, 33% at 40 days after inoculation and 38% at 60 days after inoculation) and lowest severity was recorded at block A in case of all the selected different periods after inoculation (5% at 20 days after inoculation, 13% at 40 days after inoculation and 21% at 60 days after inoculation).

Table 15. Severity of canker of citrus at different wavelengths of light from June 2014 to August 2014

Name of blocks	Efficiency of films implemented into different blocks	Disease Severity (%)		
		Days After Inoculation		
		20	40	60
Open	No film was used	12.00b	27.00b	35.00b
Control	Film cannot block any wavelength	9.00c	21.00d	29.00d
A	Can block wavelength <340nm	5.00e	13.00f	21.00f
B	Can block wavelength <350nm	7.00d	19.00e	27.00e
C	Can block wavelength <360nm	10.00c	25.00c	31.00c
D	Can block wavelength <400nm	14.00a	33.00a	38.00a
LSD (0.05)		1.779	1.779	1.779
CV (%)		10.53	4.35	3.31

CHAPTER V
DISCUSSION

Three wholesale markets of Dhaka viz. Karwanbazar, Savar and Shyambazar was surveyed during the period of September 2012 to May 2014 for prevalence of canker on the fruits of citrus transported from three districts of Bangladesh viz. Munshiganj, Gazipur and Chittagong. Citrus canker was recorded as a common disease in all the surveyed markets. The disease recorded in the present study based on visual symptoms following the description of Brunings and Gabriel (2003), Agrios (2006) and Civerolo (1981). The disease had been reported by Hossain (2011) and Chowdhury (2009) in the citrus growing areas of Bangladesh. The disease recorded in the present study had also been reported on citrus fruits from different countries of the world (Burhan *et al.,* 2007; Eshetu and Sijam, 2007; Bal and Dhiman, 2005; Graham *et al.,* 2004; Gottwald *et al.,* 2002; Schubert *et al.,* 2001 and Koizumi, 1985). Awasthi *et al.* (2005) reported citrus canker was the major problem in the nursery.

In the present study, prevalence of canker on fruits of citrus varied in respect of markets and locations. Similar variation in prevalence of canker on seedlings of citrus in respect of nursery and location was recorded by Chowdhury (2009), Khan and Abid (2007), Das (2003) and Khan *et al.* (2002). In the present study, it was also observed that the incidence and severity of canker of citrus varied from location to location. These variations may be due to effect of environment of different agro-ecological zone. Highest incidence and severity of canker of citrus was recorded at Chittagong. Chittagong is a south eastern hilly district of Bangladesh. This high prevalence may be due to environmental effect of that particular agro-ecological zone.

Incidence and severity of canker of citrus was significantly influenced by average temperature, relative humidity, rainfall and light intensity. The climate of Bangladesh is characterized by high temperature, heavy rainfall, and often excessive humidity with fairly marked seasonal variations (Anonymous, 1995). Determining the effects of temperature, rainfall and relative humidity report on the incidence and severity of canker of citrus has been focused by many researchers worldwide (Hossain, 2011; Bock *et al.*, 2010; Chowdhury, 2009; Eshetu and Sijam, 2007; Khan and Abid, 2007; Pria *et al.*, 2006; Das, 2003; Khan *et al.*, 2002; Sothiosorubini *et al.*, 1986; Aiyappa, 1958 and Ramakrishnan, 1954).

During the survey, prevalence (incidence and severity) of canker of citrus was found to be increased in the month of September and May while decreased in the month of January. A positive correlation was observed between prevalence of canker with temperature, relative humidity and rainfall. With the increase of temperature, relative humidity and rainfall both the incidence and severity increased significantly. The result of the present study was in agreement with the report of Bal and Dhiman (2005). They found that citrus canker was build up during the first week of June with the onset of rains. They also observed that highest incidence of citrus canker was during the second week of September and the disease showed a positive correlation with temperature, relative

humidity and rainfall and hence the period from July to September was identified as the most conducive for the development of citrus canker. Khan and Abid (2007) reported that canker development increased with the increase in rainfall and relative humidity and decreased with the increase in maximum temperature.

The causal organism of canker of citrus (*Xanthomonas axonopodis* pv. *citri*) was isolated from infected fruits following standard dilution plate technique using nutrient agar medium. Repeated isolation from the infected plant parts yielded well separated, typical, yellow, convex, mucoid, colonies of bacterium on nutrient agar medium after 48 hours of incubation at 30^0C. The pathogen has also been reported by many 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 leaves and proved pathogenicity.

The causal agent of canker of citrus (*Xanthomonas axonopodis* pv. *citri*) was identified by conducting studies on its morphological, biochemical and cultural features as per standard microbiological procedures. After gram's staining under the compound microscope at 125x magnification with oil immersion, the bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) 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 (1992), Gerhardt (1981), Bradbury (1970) and Starr and Stephens (1964) also reported *Xanthomonas axonopodis* pv. *citri* as gram negative, rod shaped bacterium. In the present study the bacterium (*Xanthomonas axonopodis* pv. *citri*) showed positive results in starch hydrolysis test, catalase test, **citrate utilization test,** motility indole urease agar (MIU) test and gelatine liquefaction

test and negative result in oxidase test. Similar results has also been reported by Yenjerappa (2009), Kishun and Chand (1991) and Chand and Pal (1982). In the present study, it was observed that *Xanthomonas axonopodis* pv. *citri* produce circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened, mucoid colonies on SX medium. 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 48-72 h of incubation at 28^0C. In XS medium the bacterium 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). With the results of present study, it was observed that *Xanthomonas axonopodis* pv. *citri* can tolerate up to 3% salt concentration after 72 hours of incubation which is supported by Verniere *et al.* (1998). The present study revealed that after infiltration the lower surface of a mature tobacco leaf with *Xanthomonas axonopodis* pv. *citri*, the infiltrated area became dry and necrotized within 48 hours which conformed the pathogenic nature of the bacteria. A same result has also been reported by Swarup *et al.* (1992) and Klement *et al.* (1964).

Leaf infection (%) of canker on the seedlings of citrus was significantly influenced by average temperature, relative humidity and rainfall. After inoculation of the causal organism in the leaves of seedlings the leaf infection (%) development was very slow for the first 20 days. A day to day decreasing amount of temperature, relative humidity and rainfall was recorded during this period. At 25 days after inoculation a rapid increase of the leaf infection development was recorded. During this period increasing of temperature, relative humidity and rainfall was also recorded. At 30 days after inoculation leaf infection development was again reduced. A reduced amount of temperature, relative humidity and rainfall was recorded during this period. From this study it was found that leaf infection (%) development is positively

correlated with temperature, relative humidity and rainfall; it increases and decreases with the increasing and decreasing of temperature, relative humidity and rainfall, respectively. The result of the present study was in agreement with the report of Bal and Dhiman (2005). Khan and Abid (2007) reported that canker development increased with the increase in rainfall and relative humidity and decreased with the increase in maximum temperature. Determining the effects of temperature, rainfall and relative humidity report on the incidence and severity of canker of citrus has been focused by many researchers worldwide (Hossain, 2011; Bock *et al.*, 2010; Chowdhury, 2009; Eshetu and Sijam, 2007; Khan and Abid, 2007; Pria *et al.*, 2006; Das, 2003; Khan *et al.*, 2002; Sothiosorubini *et al.*, 1986; Aiyappa, 1958 and Ramakrishnan, 1954).

Incidence and severity of canker on the seedlings of citrus was significantly influenced by light intensity (Different wavelengths of light). Different amount of incidence (%) and Severity (%) on the seedlings was recorded under the films that can block different wavelengths of light. Highest incidence and highest severity both were recorded at block D. The film that was implemented into block D can block all the wavelengths of light that are less than 400nm. Lowest incidence and severity both were recorded at block A. The film that was implemented into block A can block all the wavelengths of light that are less than 340nm. The effect of light intensity on canker of citrus has been focused by many researchers worldwide (Gill, 2013; Serrano *et al.*, 2010; Chung *et al.*, 2009; Eshetu and Sijam, 2007; Verniere *et al.*, 2003).

CHAPTERVI

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 to attack by various diseases in Bangladesh especially citrus canker, but least concrete information regarding their distribution, incidence, severity, epidemiology and management is available. Therefore, the present study was designed to study the occurrence and prevalence of canker on seedlings of citrus and to study the correlation of canker development with environmental factors on the fruits of three districts that are transported to three wholesale markets of Dhaka.

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.

Survey conducted for two years revealed that incidence and severity of canker of citrus varied from location to location, season to season as well as market to market. Significant variations were observed in the incidence and severity under the variation of weather parameters. Highest (26.67%) incidence of canker of citrus was recorded in the month of September on the fruits

transported from Chittagong to Shyambazar wholesale market. Lowest (3.67%) incidence was recorded in the month of January on the fruits transported from Gazipur to Savar wholesale market. Highest (15.67%) severity was recorded in the month of May on the fruits transported from Chittagong to Shyambazar wholesale market and lowest severity (1.67%) was recorded in the month of January on the fruits transported from Munshiganj to Shyambazar wholesale market. A positive correlation was observed between the incidence and severity of canker of citrus with temperature, relative humidity and rainfall.

Causal organism of canker of citrus was isolated from the infected leaf by following dilution plating technique using nutrient agar medium. Causal organism of citrus canker was purified by restreaking on nutrient agar medium with single colony and confirmation was done by pathogenicity test. The pathogen was identified by it's morphological, biochemical and cultural features as per standard microbiological procedures. The bacterium was gram negative, rod shaped with rounded ends. It showed positive result in KOH solubility test, starch hydrolysis test, catalase test, **citrate utilization test,** motility indole urease agar (MIU) test, gelatine liquefaction test, salt tolerant test, tobacco hypersensitivity reaction and negative result in oxidase test. The bacterium appeared as circular, mucoid, convex, yellow to orange colour on NA medium; circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened, mucoid colonies on SX medium.

Present study revealed that the occurrence of canker of citrus is positively correlated with the environmental factors viz. temperature, relative humidity, rainfall and light intensity. An experiment to study the effect of light intensity on canker disease during this study period revealed that highest incidence & severity of the disease were found when all the wavelengths of light less than 400nm were blocked and lowest incidence & severity were found when all the wavelengths of light less than 340nm were blocked. Other parameters of epidemiology viz. leaf wetness period, vapor pressure deficit, sunshine hour,

microclimatic parameters including canopy temperature, relative humidity etc, should be critically evaluated to have profound effects on over wintering formation, germination and development of inoculums in different pathosystem and these should be critically studied for each host-pathogen system to find out the most appropriate time to combat the disease at minimum effort.

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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 121^0C for 15 minutes at 15 lb 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

SX Agar

Potato starch (Soluble)	10.0 g
Beef extract (Dico)	1.0 g
NH_4Cl	5.0 g
K_2HPO_4	2.0 g
Methyl violet 2B (1% in 20% ethanol)	0.4 ml
Methyl green (1% in water)	2.0 ml
Bacto agar	15.0 g
Cycloheximide	2.0 g
Distilled water	1000 ml

Yeast Extract Dextrose Calcium Carbonate Agar (YDCA)

Yeast extract	10.0 g
D-glucose	20.0 g
Ca CO_3	20.0 g
Bacto agar	15.0 g
Distilled water	1000 ml

Gelatine Liquefaction Media

Beef extract	3.0 g
Peptone	5.0 g
Gelatine	120 g
Distilled water	1000 ml

Simmon’s Citrate Agar

Magnesium sulphate	0.2 g
Sodium citrate	2.0 g
NaCl	5.0 g
Dipotassium Phosphate	1.0 g
Monopotassium Phosphate	1.0 g
Bromothymol blue	0.08 g
Bacto agar	20.0 g
Distilled water	1000 ml

Motility Indole Urease (MIU) Agar

Peptone	30.0 g
Urea	20.0 g
Monopotassium phosphate	2.0 g
NaCl	5.0 g
Phenol red	0.05 g
Bacto agar	4.0 g
Distilled water	1000 ml
pH	7.0

Starch hydrolysis media and reagent

Culture medium	
Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 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 iodide (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

Catalase reagent

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase reagent

1% aqueous solution of NNN’N-tetramethyl-p-phenylene-diamine dihydrochloride was prepared from the absolute solution.