PREVALENCE OF CANKER ON SEEDLINGS OF CITRUS (*CITRUS* SPP.) IN SELECTED AREAS OF BANGLADESH AND IT'S MANAGEMENT

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CERTIFICATE

This is to certify that the thesis entitled**,** ***"PREVALENCE OF CANKER ON SEEDLINGS OF CITRUS (CITRUS SPP.) IN SELECTED AREAS OF BANGLADESH AND IT'S MANAGEMENT"*** 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 **MAMUN-OR-RASHID** bearing Registration No**. 06-01961** 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: 5th May, 2013
Place: Dhaka, Bangladesh

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TO

MY BELOVED PARENTS

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ABSTRACT

Effect of different weather parameters on canker development on seedlings of citrus were studied during January, 2011 to December, 2012 in different citrus growing nurseries of Bangladesh with an effort to develop suitable management strategies for controlling citrus canker. Important bacterium *Xanthomonas axonopodis* pv. *citri* was isolated from the canker infected part of citrus seedlings 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. A positive correlation was observed between the incidence and severity of 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 and the lowest incidence and severity was recorded in the month of January at Dhaka. *In vitro* and field evaluation of antibacterial chemicals indicated that Mancozeb (Indofil M-45) and Copper compounds (Champion and Cupravit) were highly effective against *Xanthomonas axonopodis* pv. *citri*. *In vitro* evaluation of biocontrol agents revealed that *Bacillus subtilis* was highly effective against the bacterium followed by *Pseudomonas fluorescens.*

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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
WP	=	Wettable Powder
hr	=	Hour (s)
cv.	=	Cultivar (s)
i.e.	=	That is
T	=	Treatment
ft	=	Feet (s)
pv.	=	Pathovar
var.	=	Variety
mm	=	Milimiter
µl	=	Microliter
µm	=	Micrometer

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
BER	=	Bangladesh Economic Review
USA	=	United State of America
NA	=	Nutrient Agar (media)
YDCA	=	Yeast Dextrose Calcium carbonate Agar (media)
PDA	=	Potato Dextrose Agar (media)
KB	=	King's B
YMA	=	Yeast Malt Agar (media)
PSI	=	Per Square Inch
ANOVA	=	Analysis of variances

LSD	=	Least Significant Difference
CV%	=	Percentages of Co-efficient of Variance

CHAPTER I

INTRODUCTION

Citrus (*Citrus* spp.) is one of the most important, popular and nutritious fruit crop in the world as well as in Bangladesh, which belongs to the family Rutaceae. It has a great demand due to it's nutritive value, aroma and taste. Considering the multipurpose use, the demand of citrus is increasing day by day. Bangladesh is a developing country and has dense population of 151.6 million having 968 people/km^2 (BER, 2012). It produces less than 30% of the fruits needed to meet the minimum daily requirements for it's population. About 91% of it's 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). Annual citrus production in the world is 115 million tons (FAO, 2012). 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); Brurning 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 & 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 socioeconomic 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 (Kishun and Chand, 1991). It can be differentiated by growth and colony morphology on different media (Schaad, 1992). It produce bright yellow colony on both GYCA and YDC medium (Yenjerappa, 2009) and light yellow colony with clear zone surround them on SX medium (Vudhivanich, 2003).

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, rainfall and relative humidity on the incidence and severity of citrus canker has been focused by many researchers worldwide [Hossain (2011); Chowdhury (2009); 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)]. Temperatures 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).

Management of canker on seedlings of citrus has been investigated by many researchers (Singh *et al.*, 2005; Canteros, 2004; Graham and Leite, 2004; Das and Shyam, 2003; Dixon *et al.*, 2000; Gottwald and Timmer, 1995; Civerolo, 1981; Stall *et al.*, 1981 and Koizumi, 1977). Prophylactic sprays of copper oxychloride or other copper containing compounds provide protection against initial infection of citrus canker during growth flushes and fruit development (Das, 2003; Muraro *et al.*, 2001; Leite and Mohan, 1990 and Stall *et al.*, 1980). But the indiscriminate use of agrochemicals leads to degradation of the ecosystem, may induce pathogen resistance to the pesticide, and may cause human and animal health problems (Huang, 1997). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides (Sutton, 1996). *Bacillus subtilis* and *Pseudomonas fluorescens* are most effective antagonist against *Xanthomonas axonopodis* pv. *citri* (Kalita *et al.*, 1996). *Bacillus subtilis* shows biological activity against phytopatogenic bacteria by producing peptide antibiotics (Backman *et al.*, 1997 and Kloepper, 1997). Biochemical studies by Valasubramanian *et al.* (1994) showed efficient

strains of *Pseudomonas fluorescens* produce an antibiotic phenazine-1-carboxylic acid (PCA), which hinders the growth of plant pathogenic bacteria.

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 of management this disease in nursery. However, only a few reports are available in respect of prevalence, isolation, identification and management of this disease in the country. Therefore, attempt should be put forward to study the prevalence of this disease occuring on citrus seedlings and it's management in selected nurseries of Bangladesh.

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

1. Survey on the prevalence of canker on seedlings of citrus in selected nurseries of Bangladesh.
2. To identify the pathogen (s) associated with the disease.
3. To study the epidemiology in relation to the effect of temperature, relative humidity and rainfall on incidence and severity of citrus canker in nursery.
4. To find out a suitable management strategies for controlling citrus canker in nursery.

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

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.

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 (Chowdhury, 2009).

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.

2.2. Symptomology

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.

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 it's pathogenicity

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 XS 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.

Kishun and Chand (1991) reported that *Xanthomonas* was negative in nitrate reduction, urease oxidative, fermentative metabolism of glucose and acid from adonitol and sorbitol. The bacterium was positive in 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. Disease epidemiology

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.

Eshetu and Sijam (2007) observed that citrus canker severity was significantly correlated with temperature, 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 temperatures of 25-35^0C. The estimated minimum and maximum temperatures 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 temperatures, 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 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.

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, rain fall 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, rain fall and relative humidity were recorded in Asam.

In an experiment 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.

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.

2.5. Evaluation of disease management strategy

2.5.1. Chemical management

Behlau *et al.* (2010) studied the effects of copper sprays on annual and polyetic progress of citrus canker caused by *Xanthomonas citri* pv. *citri* in the presence of the Asian citrus leafminer (*Phyllocnistis citrella*) in a commercial orchard in northwest Parana state, Brazil. They observed although citrus canker incidence increased during each of the seasons studied, it decreased over the whole study period, more so in copper treated trees than in water sprayed controls. Copper treatment reduced disease incidence compared with controls in every year. Copper treatment also reduced estimated initial disease incidence and epidemic growth rates every year.

Behlau *et al.* (2008) investigated the benefit of wind breaks and copper sprays for control of citrus canker caused by *Xanthomonas axonopodis* pv. *citri* in southern Brazil. Control of canker was evaluated as incidence and severity of lesions on foliage and by the effect on premature leaf and fruit drop for three production seasons. Effect of the treatments on fruit production was evaluated as incidence of citrus canker on prematurely abscised fruits and harvested fruits. They found copper application significantly reduced damage to foliage and fruit, while windbreaks made little contribution to disease control. Copper sprays increased fruit yield for 3 years. This could be attributed to lower incidence of fruit with lesions and fewer fruits abscised due to canker infection. Incidence and severity on the leaves were inversely related to the number of fruits harvested per tree and directly related to the number of fruits abscised per tree.

Behlau *et al.* (2007) conducted an experiment to study citrus canker incidence and severity under natural conditions in an orchard of Pera Rio sweet orange (*Citrus sinensis*) planted in Brazil. Chemical control, by using copper oxychloride sprays and windbreak protection were evaluated to reduce citrus canker incidence and severity on leaves. Levels of disease incidence and severity were evaluated monthly using specific diagrammatic scales. The coefficient of determination (R^2) between incidence and severity levels was also determined. While copper sprays significantly reduced citrus canker on

leaves, windbreak did not contribute significantly to disease control. After 29 assessments, plants subjected to frequent copper sprays showed AUDPC values for citrus canker incidence which were 43.5% lower than those observed on plants not protected with chemical sprays. The same result was observed for citrus canker severity. After 18 assessments, plants sprayed with copper showed AUDPC values for severity which were 37.1% lower than those for the control plants. In both years, the values of R^2 between incidence and severity levels were higher than 0.80 ($p<0.01$).

Meneguim *et al.* (2007) conducted a study to determine the sensitivity of *Xac* strains to copper as well as to a mixture of copper with mancozeb. The highest copper concentration where *Xac* grew was 50 µg/ml. However, 45.5% of the bacterial strains from orchards with regular sprays of copper compounds grew in the presence of 50 µg copper/ml. In contrast, only 13.4% of the strains from citrus orchards that never received copper sprays grew in such a copper concentration. Mixing mancozeb with copper increased the tolerance of *Xac* to copper.

Koller *et al.* (2006) reported that copper treatments were not effective in controlling citrus canker at high inoculum concentration, but at low inoculum concentration, both Bordeaux mixture and copper oxychloride controlled the disease.

Maher *et al.* (2005) tested toxicants (streptomycin sulfate, Dithane M-45 [mancozeb], Agrimycin-100, Vitavax [carboxin], Benlate [benomyl] and Cobox [copper oxychloride]) at 1% concentration against multiplication of *Xanthomonas axonopodis* pv. *citri* (*in vitro*). They observed Agrimycin-100, Streptomycin sulfate, Vitavax and Dithane M-45 were more effective compared to other toxicants. Agrimycin-100, streptomycin sulfate, Dithane M-45 and Vitavax were further studied *in vitro* against the growth of X. *axonopodis* pv. *citri* at 0.01, 0.1 and 1% concentration. All the toxicants inhibited the multiplication of the bacterium at all concentrations, however

streptomycin sulfate was the most effective among the toxicants used while Agrimycin-100, Vitavax and Dithane M-45, in the order, were effective against the multiplication of bacterium at 0.01, 0.1 and 1% concentration. They also sprayed Streptomycin sulfate, Agrimycin-100, Vitavax, Dithane M-45 and Benlate at 0.2% concentration on the field grown citrus plants and then inoculated with *X. axonopodis* pv. *citri* for the control of citrus canker disease. Streptomycin sulfate, Vitavax, Dithane M-45 and Agrimycin-100 in the order proved effective also in reducing the disease intensity compared to the inoculated control.

Ren *et al.* (2005) conducted an experiment with 4 years old trees of Hongjianhcheng orange cultivar and 21 fungicides for control of the canker pathogen strain HCS-1 (*Xanthomonas axonopodis* pv. *citri*). They evaluated among the 21 fungicides for control of HCS-1 the best one was spraying 1000x diluted solution of 10% streptomycin and then the 1000x diluted solution of 53.8% Kocide [copper hydroxide] suspension. Both control effectiveness could reach 43.0 - 34.6% for the leaf canker.

Graham and Leite (2004) observed that prophylactic sprays of copper oxychloride (or other copper containing compounds) provide protection against initial infection in canker endemic areas during growth flushes and early fruit development (fruit approximately 2-6 cm diameter).

Pan (2004) tested the effect of different concentration of 77% Kocide [copper hydroxide] solutions on 11 to 13 years old trees of Sijiyou pomelo cultivar. The 500x dilution of 77% Kocide was the best one for control of pomelo canker (*Xanthomonas citri*). Spraying 500x dilution of solution of 77% Kocide wettable powder to the summer shoots could get 100% of the shoot leaves without infection. Spraying 500x dilution of 77% Kocide wettable powder removed infection. The efficacy could reach 98.2%.

Verona *et al.* (2004) studied the relationship between the frequency of copper oxychloride sprays and the incidence of citrus canker in sweet orange leaves

caused by *Xanthomonas axonopodis* pv. *citri*. They found control of citrus canker with 4 sprays of copper oxychloride at 30 day intervals during the growth season was satisfactory.

Das (2003) reported that timely application of protective copper containing and/or antibiotic sprays were generally the most effective means of disease management.

Zhong and Ling (2002) reported that citrus canker occurrence had a close relationship with the daily mean temperature. When a daily temperature of 12^0C occured for 10-15 days, the spring shoots and fruitlets were attacked. The experiments also showed that spraying spring 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) investigated that citrus canker (*Xanthomonas axonopodis* pv. *citri*) appeared in late April and early May, the rampant period being mid May to early June and found the occurrence was correlated with the temperature. They also observed spraying with 500 fold and 400 fold solution of 77% copper hydroxide and 60% chlorothalonil solution resulted the efficient control of the disease.

Ravikumar *et al.* (2001) reported that streptomycin sulfate (500 ppm) sprayed either alone or in combination with copper oxychloride (2000 ppm) was very effective in reducing the disease severity (2.23-2.51 number of lesions per fruit), followed by combination spray of streptocycline (500 ppm)+copper oxychloride (2000 ppm) (3.22) compared to the control (8.29).

Chen *et al.* (2000) evaluated some fungicide against citrus canker. They treated citrus canker affected plant with different concentrations of compounds including 56% cuprous oxide, Agro-streptomycine, 77% copper hydroxide (WP) and 50% shajunwang (WP). Eleven days after treatment the rate of

infection of citrus canker (*Xanthomonas axonopodis* pv. *citri*) on leaves and fruit were investigated. They showed that the best treatment was 50% shajunwang which achieved up to 94.5% control.

Jadeja *et al.* (2000) conducted field experiments to test the efficacy of various chemical treatments for the control of canker (*Xanthomonas axonopodis* pv. *citri*) of citrus at the Horticultural farm of Gujarat Agricultural University, Junagadh, Gujarat, India. Six years old acid lime (Kagzi lime) trees were treated with Bordeaux paste [Bordeaux mixture], Ridomil MZ 72 [metalaxyl + mancozeb], streptomycin sulphate + copper oxychloride, fosetyl Al [fosetyl], and aureofungin sol, alone and in various combinations. The results revealed that all the treatments with a foliar spray reduced canker incidence significantly except for aureofungin sol. Among these higher canker control was achieved with foliar application of streptomycin sulphate + copper oxychloride.

Lai (2000) studied the infection dynamics of *Xanthomonas axonopodis* pv. *citri* (citrus canker) and it's prevention and control by various pesticides (based on laboratory tests). The tests showed Kocide (copper hydroxide) and Baijuntong had good control effects.

Kale *et al.* (1994) suggested that for better control of canker, spraying of streptocycline + Copper oxychloride (0.1%) should preferably be done at 7 days or 15 days interval.

Leite and Mohan (1990) observed that sprays of copper oxychloride or other copper containing compounds reduce the prevalence of Xac infection in citrus field.

2.5.2. Management with bioagents

Nemeckova (2011) detected and isolated *Bacillus* sp. by methods based on the resistance of spores to heating or ethanol and after heating at 85^0C for 10 minutes they inoculated the sample on the reference medium which was shown antagonistic activity against some plant pathogen.

Yenjerappa (2009) conducted an experiment to evaluate the antagonistic effect of five biocontrol agents viz. *Trichoderma viride, Trichoderma harzianum, Pseudomonas fluorescens, Pseudomonas putida* and *Bacillus subtilis* against the growth of *Xanthomonas axonopodis* by inhibition zone assay method. Among the five biocontrol agents, *Bacillus subtilis* and *Pseudomonas fluorescens* were found significantly superior in inhibiting the growth of the pathogen with an inhibition zone of 16.6 and 16.1 mm diameter. Other biocontrol agents viz. *Trichoderma viride, Trichoderma harzianum* and *Pseudomonas putida* were ineffective as they failed to inhibit the growth of *X. axonopodis.*

Manonmani *et al*. (2007) conducted an experiment to maximize the biocontrol efficacy of antagonists by integrating diverse bioagents viz. *Pseudomonas fluorescens*, vermicompost extracts and plant extracts. The efficacy of combined application of bioagents in controlling the canker disease of citrus was observed under controlled environmental condition. Biocontrol as well as resistance induction efficacy of *P. fluorescens* were superior compared to other bioagents. Biocontrol efficacy of *P. fluorescens* was higher by recording percent disease index (PDI) of 18.3 and per cent reduction over control (PROC) was 57.7.

Monteiro *et al.* (2005) reported that *Bacillus subtilis* significantly inhibit the growth of *Xanthomonas in vitro*.

Three species of bacteria viz. *Bacillus subtilis, Bacillus polymixa, Pseudomonas fluorescens* and four species of fungi viz. *Aspergillus terreus, Trichoderma viridae, Trichoderma harzianum, Serratia marcescens* isolated from the phylloplane of citrus variety Assam lemon (citrus lemon) inhibited the growth of *Xanthomonas campestris* pv. *citri*, the incitant of citrus canker, when tested by agar plug method *in vitro* (Kalita *et al.*, 1996). Among these bioagents, *Bacillus subtilis* was found most effective antagonist producing

largest inhibition zone followed by *Pseudomonas fluorescens* and *Aspergillus terreus*.

Bacillus spp. involved in the control of plant diseases through a variety of mechanisms such as competition, systemic resistance induction and antibiotic production. The mechanism of antibiosis has been shown to be one of the most important to control plant diseases (Tomashow and Weller, 1996).

Chand *et al.* (1991a) reported the antagonistic activity of *Bacillus subtilis* against *Xanthomonas campestris in vitro*.

Unnamalai and Gnanamanickam (1984) reported the inhibiting effect of *Pseudomonas fluorescens* on the growth of *Xanthomonas citri*.

CHAPTER III
MATERIALS AND METHODS

Four experiments were carried out throughout the research period in order to study the disease of canker on seedlings of citrus. The experiments were as follows:

I. Survey on the disease of canker on seedlings of citrus in selected nurseries of Bangladesh.
II. Isolation and identification of causal organism(s) of canker of citrus.
III. Epidemiological survey on the prevalence of canker on seedlings of citrus.
IV. Evaluation of suitable management strategies for controlling citrus canker in nursery.

3.1. Experiment I. Survey on the disease of canker on seedlings of citrus in selected nurseries of Bangladesh

3.1.1. Location of survey area

Prevalence of canker on seedlings of citrus was surveyed in eight nurseries of Dhaka, Gazipur, Barisal and Khagrachari (Appendix-I).

3.1.2. Selection of Nursery

The eight nurseries of four districts were surveyed:

Name of district	Name of nursery
Dhaka	Green orchid nursery, Agargaon Barisal nursery, Savar
Gazipur	Gazipur nursery, Gazipur Laxmipur nursery, Gazipur
Barisal	Sarchina nursery, Barisal Riyad nursery, Barisal
Khagrachari	Hill research center, Khagrachari Ramghar nursery, Ramghar

3.1.3. Age and number of seedlings

30 seedlings of citrus of 1 year old were taken for each nursery of Bangladesh.

3.1.4. 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.

3.2. Experiment II: Isolation and identification of causal organism

3.2.1. Collection of diseased specimen

Diseased leaves were collected from the infected plants representing the different areas of survey. 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-II) was prepared according to the method followed by Schaad (1988). For the preparation of 1 liter NA medium at first 15 g bacto agar was taken in an Erlenmeyer flask containing 1000 ml distilled water. 5 g peptone and 3 g beef extract were then added to it. For mixing properly the nutrient agar was shaken thoroughly for few minutes. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.2.3. Preparation of Nutrient Broth (NB)

Nutrient broth (Appendix-II) was prepared according to the method followed 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-II) was prepared according to the method followed 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 containing 1000 ml distilled water. All the ingredients were mixed well and heated them. 20 g $CaCO_3$ then added to it and finally it was autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.2.5. Preparation of SX Agar

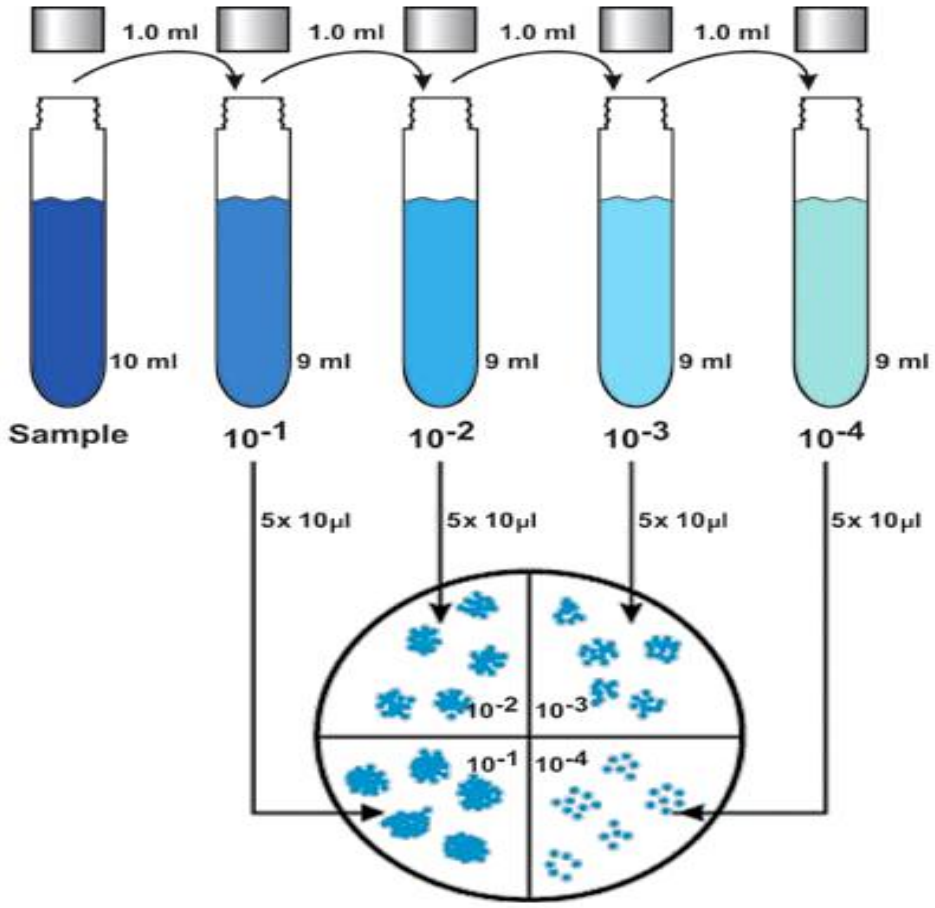
SX agar (Appendix-II) was prepared according to the method followed by Goszczynska *et al.* (2000). For the preparation of 1 liter SX agar medium 10 g soluble potato starch, 1 g beef extract, 5 g NH_4Cl, 2 g K_2HPO_4, 0.4 ml methyl

violet 2B (1% in 20% ethanol), 2 ml methyl green (1% in water) and 15 g agar were taken in an Erlenmeyer flask containing 1000 ml distilled water. Then it 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 to it. Finally it was 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 leaves were washed under running water. Then the young lesions with green healthy portion of diseased leaves were cut into small pieces. Surface sterilize were done by dipping them in 5% sodium hypochlorite solution for 2-3 minutes. Then washed them 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. One ml of this stock solution was transferred with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-1} dilution. Similarly, final dilution was made up to 10^{-4} (Flow chart 1).



Flow chart 1. Dilution plate technique for isolation of Bacteria

After preparing different dilution, 0.1 ml of each dilution was spreaded over NA plate previously dried (to remove excess surface moisture) at three replications as described by Goszczynska and Serfontein (1998). Spreading was done with the help of a glass-rod. 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 small 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).

3.2.6.3.1.a. Gram's staining

A small drop of sterile water was placed on a clean microscope slide. Part of a young colony (18-24 hrs old) was removed with a cold, sterile loop from the nutrient agar medium and the bacteria were smeared on to the slide that was very thin. The thinly spreaded bacterial film was air dried. Underside of the glass slide was heated by passing it four times through the flame of a sprit lamp for fixing the bacteria on it. Then the slide was flooded with crystal violet solution for 1 minute. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then it was flooded with lugol's iodine solution for 1 minute. After that it was decolorized with 95% ethanol for 30 seconds and again rinsed with running tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined at 40x and 100x magnification using oil immersion.

3.2.6.3.1.b. KOH solubility test

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

3.2.6.3.2. 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

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

3.2.6.3.2.b. Catalase test

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

3.2.6.3.2.c. Oxidase test

At first 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 placed on a petri dish. Then a part of a colony was removed with a sterile toothpick and smeared onto the moistened oxidase disk and observed up to 60 seconds whether it changed colour to dark purple or not.

3.2.6.3.2.d. Citrate utilization test

Pure colony of bacterium was streak inoculated on to the simmon's citrate agar slant with the help of a sterile transfer loop. Then it was incubated at 30^0C for 24 hours in incubation chamber. Aftetr incubation it was observed to determine colour changed from green to bright blue.

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

Pure colony of bacterium was stub inoculated into the tube containing motility indole urease agar (MIU) media with the help of a sterile transfer loop without touching the bottom of the tube. Then the tube was incubated at 30^0C for 48 hours in incubation chamber and observed the way of bacterial movement.

3.2.6.3.2.f. Gelatine liquefaction test

One loop-full bacterial culture was stub inoculated into the tube containing 12% (w/v) gelatine with the help of a sterile transfer loop. Then it was incubated at 30^0C for 24 hours. Gelatin liquefied microorganism was determined by the formation of liquid culture after keeping it at 5^0C in refrigerator for 15 minutes.

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 in each test tube. 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 and 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 pure culture of bacteria grown on NA plate. Then the test tubes were transferred in incubating shaker 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 after cooling, pure colony of bacterium was streak inoculated on the plate with the help of a sterile transfer loop. Then it was incubated at 30^0C for at least 24 hours in incubation chamber and observed the colony characters.

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 after cooling, pure colony of bacterium was streak inoculated on the plate with the help of a sterile transfer loop. Then it was incubated at 30^0C for at least 24 hours in incubation chamber and observed the colony characters.

3.2.6.3.3.c. Growth on selective SX agar media

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

3.2.6.3.4. Tobacco hypersensitivity reaction

To determine the pathogenic nature of the isolates, hypersensitivity reaction was studied on tobacco (*Nicotiana rustica)* plants by injection infiltration technique developed by Klement and Goodman (1967). An aliquot of the inoculums suspension of bacterium (approximately 10^8 cfu/ml) was prepared in sterile distilled water from a 24 hours culture plate. Then the lower surface of a mature tobacco leaf was infiltrated by pressing a syringe containing the

suspension against the leaf and distilled water was used as a negative control. Then it was observed for 72 hours.

3.2.6.4. Pathogenicity Test

Citrus plant (Kagozi lemon) grown on pot under greenhouse condition was used for examining the pathogenicity of *Xanthomonas axonopodis* pv. *citri* as per method described by Lin *et al.* (2008). To prepare inoculum, bacterial cells grown overnight in NA broth and resuspended in sterile distilled water to a concentration of approximately 10^8 cfu/ml. Then an aliquot of the inoculums suspension was injected forcedly into the lower surface of citrus leaf using a sterile syringe and distilled water was used as a negative control. Then it was observed for 15 days. Visual symptoms were recorded and examined. To confirm Koch's postulates, bacteria were then reisolated from the infected area.

3.3. Experiment III: Epidemiology of disease incidence and severity

3.3.1. Survey period

Altogether eight surveys were made during the period from January, 2011 to December, 2012; where first, second, third, fourth, fifth, sixth, seventh and eighth surveys were made in January, 2011; April, 2011; July, 2011; October, 2011; January, 2012; April, 2012; July, 2012 and October, 2012 respectively. The times of data colletion were determined on the basis of variations in temperature, relative humidity and rainfall during the growing seasons.

3.3.2. Data collection during survey

During the survey in the nurseries, total number of citrus seedlings as well as number of seedlings that infected with citrus canker was recorded. Then 30 seedlings were randomly selected for counting diseased leaves and disease free leaves. Moreover, five leaves per plant were randomly selected to determine the disease severity.

3.3.3. Determination of disease incidence and disease severity

Every seedling along with canker infected was counted in the nursery for calculating the incidence of the disease and then it was expressed in

percentage. Disease incidence of canker on seedlings of citrus was determined by the following formula:

$$\text{Percent Plant Infection} = \frac{\text{Number of diseased plants}}{\text{Number of total plants observed}} \times 100$$

Percent disease incidence (PDI) of foliar diseases was determined by the following formula (Rai and Mamatha, 2005):

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

The disease severity was recorded by using the following scale developed by Anonymous (2006).

Grade	Percent leaf infection
0	0.00
1	Up to 1
2	>1-10
3	>10-20
4	>20-40
5	>40-100

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of individual disease ratings}}{\text{Total number of leaves examined} \times \text{Maximum grade}} \times 100$$

3.4. Experiment IV: Evaluation of suitable management strategies for controlling citrus canker in nursery

3.4.1. Experimental site

The study relating to determine the effective management strategies for controlling citrus canker was carried out in Disease diagnostic laboratory and Nursery, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.4.2. Experimental period

The experiment was carried out from January, 2011 to December, 2012.

3.4.3. *In vitro* evaluation

3.4.3.1. Evaluation of antibacterial chemicals

3.4.3.1.1. Preparation of sterile swabs

A supply of cotton swabs on wooden applicator sticks was prepared. The swabs were held in a dry tube and were sterilized in the autoclave (Vandipitte *et al*., 1991).

3.4.3.1.2. Bioassay of antibacterial chemicals against the bacteria

Bioassay of antibacterial chemicals against the bacteria was done by well diffusion method measuring the inhibition zone (Anon, 1996). At first, two test tubes each containing 10 ml nutrient broth were taken which were inoculated with 48 hours old pure culture of bacteria grown on NA plate and another test tube containing only 10ml nutrient broth was taken as control. Then the test tubes were transferred in incubating shaker machine maintaining 30^0C temperature and 150 rpm for 24 hours. 4 holes of 4 mm in diameter were punched into the same NA plate maintaining equal distance. After shaking, the broth culture was spreaded uniformly on it with the help of sterile cotton swabs. Then different volumes of chemical suspension at different concentration (0.1%, 0.2%, 0.3% and 0.4%) were added into the hole each at three replications. In case of control only sterile water was used instead of chemical. The plates were then incubated at 30^0C in incubation chamber. Zone of inhibition around the holes were measured and recorded after every 24 hours for 5 days.

Table 1. Name of different antibacterial chemicals used in bioassay

Trade Name	Active ingredient	Chemical name	Concentration (%)			
Bavistin 50 WP	Carbendazim	Mythyl-2-benzimidazole carbamate	0.1	0.2	0.3	0.4
Cupravit 50 WP	Copper oxychloride	Copper chloride oxide hydrate	0.1	0.2	0.3	0.4

Sulcox 50 WP	Copper oxychloride	Copper chloride oxide hydrate	0.1	0.2	0.3	0.4
Champion 77 WP	Copper hydroxide	Copper hydroxide	0.1	0.2	0.3	0.4
Indofil M-45	Mancozeb	N-(2,6 dimethyl phenyl)-N (methoxyacetyl)-alanine methyl ester (C14H21NO4)	0.1	0.2	0.3	0.4
Dithane M-45	Mancozeb	N-(2,6 dimethyl phenyl)-N (methoxyacetyl)-alanine methyl ester (C14H21NO4)	0.1	0.2	0.3	0.4

3.4.3.2. Evaluation of bioagents

Five biocontrol agents viz. *Bacillus subtilis*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, *Trichoderma harzianum* and *Aspergillus flavus* were evaluated against *Xanthomonas axonopodis* pv. *citri. Bacillus subtilis, Pseudomonas fluorescens* and *Rhizobium leguminosarum* were obtained from rhizosphere soil samples collected from Horticultural Farm, Sher-e-Bangla Agricultural Unversity, Dhaka. *Trichoderma harzianum* was collected from Bangladesh Agricultural Research Institute (BARI), Gazipur and *Aspergillus flavus* was collected from Plant Pathology Laboratory, Sher-e-Bangla Agricultural Unversity (SAU), Dhaka.

3.4.3.2.1. Preparation of Potato Dextrose Agar (PDA)

Potato dextrose agar (PDA) medium (Appendix-II) was prepared as described by Islam (2009). For the preparation of 1 liter PDA medium 200 g peeled potato extract, 20 g dextrose and 17 g agar were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes. The media were acidified with 30 drops of 50% lactic acid per 250 ml medium to avoid the contamination of bacteria.

3.4.3.2.2. Preparation of Potato Dextrose Broth

Potato dextrose broth (Appendix-II) was prepared as described by Islam (2009). For the preparation of 1 liter potato dextrose broth, 200 g peeled potato extract and 20 g dextrose were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes. The media were acidified with 30 drops of 50% lactic acid per 250 ml medium to avoid the contamination of bacteria.

3.4.3.2.3. Purification of antagonistic fungi

A small colony from collected *Trichoderma harzianum* and *Aspergillus flavus* were transferred on PDA plate with the help of sterile needle. The inoculated PDA plates were incubated for 3-7 days at room temperature. Retransferring was done to get purified colony.

3.4.3.2.4. Preservation of antagonistic fungi

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

3.4.3.2.5. Preparation of King's B (KB) Media

King's B media (Appendix-II) was prepared according to the method followed by Goszczynska *et al.* (2000). For the preparation of 1 liter King's B medium 20 g proteose peptone No. 3 (Difco.), 15 ml glycerol, 1.5 g K_2HPO_4 (anhydrus), 1.5 g $MgSO_4.7H_2O$ and 15 g bacto agar were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.4.3.2.6. Preparation of Yeast Malt Agar (YMA) Media

Yeast malt agar medium (Appendix-II) was prepared according to the method followed by Goszczynska *et al.* (2000). For the preparation of 1 liter yeast malt agar media 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose and 20 g bacto agar were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.4.3.2.7. Isolation and purification of antagonistic bacteria from soil

Isolation of antagonistic bacteria from soil was done by dilution plate technique as described by Goszczynska and Serfontein (1998).

3.4.3.2.7.a. Preparation of working sample

For every dilution of soil samples, working sample was prepared from the composite sample that was made after the soil sample collection.

3.4.3.2.7.b. Preparation of soil suspension

One gram of soil was placed in the test tube containing 9 ml of sterile water and stirred thoroughly for few minutes in order to obtain a uniform dilute soil suspension. This was used as stock solution. One ml of this stock solution was transferred with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-1} dilution. Similarly, final dilution was made up to 10^{-4}. For isolation of *Bacillus subtilis,* stock solution was heated at 85^0C for 10 minutes (Nemeckova *et al*., 2011) as only spore forming bacteria are heat tolerant.

3.4.3.2.7.c. Isolation and purification of bacteria

After preparing different dilution, 0.1 ml of each dilution was spreaded over NA plate, King's B (KB) plate and YMA plate previously dried (to remove excess surface moisture) at three replications. Spreading was done with the help of a glass-rod. The inoculated plates were kept in incubation chamber at 30ºC. The plates were observed after 24 hr and 48 hr. Then single colony grown over the plate was restreaked on NA plate with the help of a loop to get pure colony.

3.4.3.2.8. Preservation of antagonistic bacteria

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

3.4.3.2.9. Identification of antagonistic bacteria

Identification of antagonistic bacteria was done by conducting studies on their morphological and biochemical features as per standard microbiological procedures.

3.4.3.2.9.a. Morphological characters

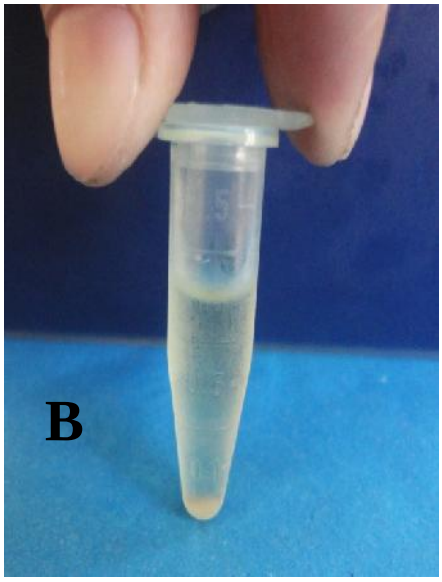
Morphological characteristics such as cell shape, gram’s reaction and pigmentation of antagonistic bacteria were studied by gram’s staining and KOH solubility test.

3.4.3.2.9.b. Biochemical characters

Different biochemical tests such as starch hydrolysis test, motility indole urease agar test, catalase test, oxidase test, **citrate utilization test** and gelatine liquefaction test were studied.

3.4.3.2.10. Screening of antagonistic organisms against *Xanthomonas axonopodis* pv. *citri*

Biocontrol agents were screened for their efficacy against the growth of *Xanthomonas axonopodis* pv. *citri* by well diffusion method measuring the inhibition zone (Yenjerappa, 2009). *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* were grown on nutrient broth and *Trichoderma harzianum* and *Aspergillus flavus* were grown on potato dextrose broth in incubating shaker machine maintaining 30ºC temperature and 150 rpm for 24 hours. Then the 1.5 ml of each culture were taken in eppendroff (EP) tubes which were then centrifuge in centrifuge machine at 1000 rpm for 15 minutes. Supernatant were found in upper part and pellet in lower part of EP tubes.



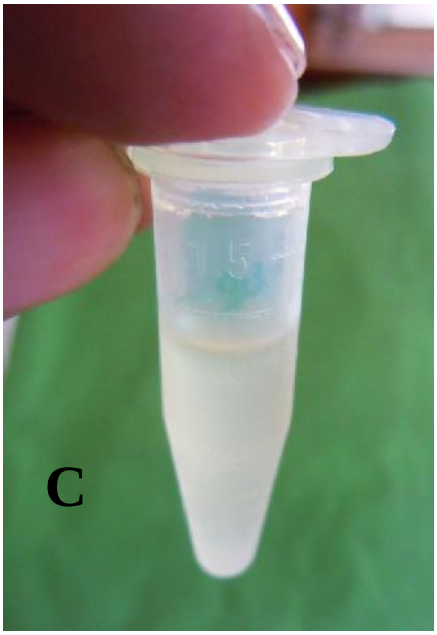


Plate 1. Procedure of collecting supernatant of different biocontrol agents

- **A.** Centrifuge machine
- **B.** Supernatant in upper part and pellet in lower part of an EP tube
- **C.** Only pure supernatant in EP tube

These supernatant were transferred to the fresh eppendroff tubes. Then the supernatant were dropped on the hole of previously swabbed plate with the pathogenic bacteria each at three replications. In case of control only sterile water was used instead of supernatant. The plates were then incubated at 30^0C in incubation chamber. Zone of inhibition around the holes were measured and recorded after every 24 hours for 5 days.

3.4.4. Field evaluation

3.4.4.1. Preparation of nursery soil

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 strelized earthen pots were filled up with the sterilized soil.

3.4.4.2. Raising of seedlings for management trial

Air layering of lemon (Kagozi lemon) seedling was done in the month of June, 2011. Shoots of 50-60 cm length from tip having 1.0 cm diameter were selected from healthy disease and insect free plants. The shoots were detached after 5 weeks and planted in the earthen pots.

3.4.4.3. Treatments

For the management of nursery diseases five different treatments were evaluated namely T_1 = Cupravit 50 WP spray as foliar application @ 0.3%, T_2 = Champion 77WP spray as foliar spray @ 0.2%, T_3 = Indofil M-45 spray as foliar application @ 0.3%, T_4 = Bavistin 50 WP spray as foliar application @ 0.3%, T_5 = Untreated control (Normal tap water was used). Fungicide solutions were prepared separately by taking requisite amount of fungicides for each dose. The fungicides were sprayed at 30 days interval by a hand sprayer. Precautions were taken with polythene barrier to avoid drifting of spray materials from one plant to neighboring plants. One seedling per pot and 5 seedlings per treatment were used. The data on incidence, severity and plant height were collected at one month interval before each spray schedule.

3.4.4.4. Experimental design and layout

The experiment was laid out in Randomized Complete Block Design (RCBD) with five replications.

3.4.4.5. Assessment of disease incidence, severity, % disease reduction over control and % increase of height over first count

Assessment of disease incidence and severity was calculated using the following formula described in experiment 3.3.3.

Perceent disease reduction (PDR) was calculated using the formula of Rai and Mamatha (2005) as:

$$\% \text{ Disease reduction (PDR)} = \frac{\text{PDI in control} - \text{PDI in treatment}}{\text{PDI in control}} \times 100$$

Percent height increase/decrease over first count was calculated using the formula:

$$\% \text{ Height increase/decrease over first count} = \frac{\text{Height at final count} - \text{Height at first count}}{\text{Height at final count}} \times 100$$

Percent height increase/decrease over control was calculated using the formula:

$$\% \text{ Height increase/decrease over control} = \frac{\text{Height increase in treatment} - \text{Height increase in control}}{\text{Height increase in control}} \times 100$$

3.4.4.6. Data collection

The data were recorded on the following parameters at 30 days interval as shown below:

a) Height of the seedlings (cm)

b) Total number of leaves/plant

c) Number of diseased leaves/plant

d) Percent diseased leaves/plant

e) Percent diseased leaf area/ plant

f) Number of branches/plant.

3.5. Meteorological data collection

Day to day meteorological data on temperature, relative humidity and rainfall were collected from Meterological Department, Agargaon, 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 2. Average temperature, relative humidity and rainfall of Dhaka, Gazipur, Barisal and Khagrachari from January, 2011 to December, 2012

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
Jan., 2011	16.88	17.89	73.80	72.90	0.52	0.49
Jan., 2012	18.90		72.00		0.46	
April, 2011	20.50	24.25	70.43	72.59	3.85	3.17
April, 2012	28.00		74.75		2.48	
July, 2011	30.50	29.57	83.50	83.50	5.50	4.49
July, 2012	28.63		83.50		3.47	

Oct., 2011	29.50	28.40	81.50	79.75	5.95	3.72
Oct., 2012	27.45		78.0		1.48	

3.6. Statistical analysis

Data on different parameters were analyzed in two factor randomized 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. To determine the effect of different climatic factors, viz. temperature, relative humidity and rainfall on the incidence and severity of canker on seedlings of citrus, correlation and regression analysis were performed.

CHAPTER IV

RESULTS

The results of the investigations undertaken on "prevalence of canker on seedlings of citrus in selected areas of Bangladesh and it's management" 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 2. A). 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 (Plate 2. B). 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 (Plate 2. C).

4.2. Isolation and purification of canker pathogen of citrus

The causal organism was isolated from the infected leafs showing typical symptoms of citrus canker. Isolation was done by employing the dilution plating 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. 1). Colonies were purified by restreaking the isolated colony on nutrient agar plate.

4.3. 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.

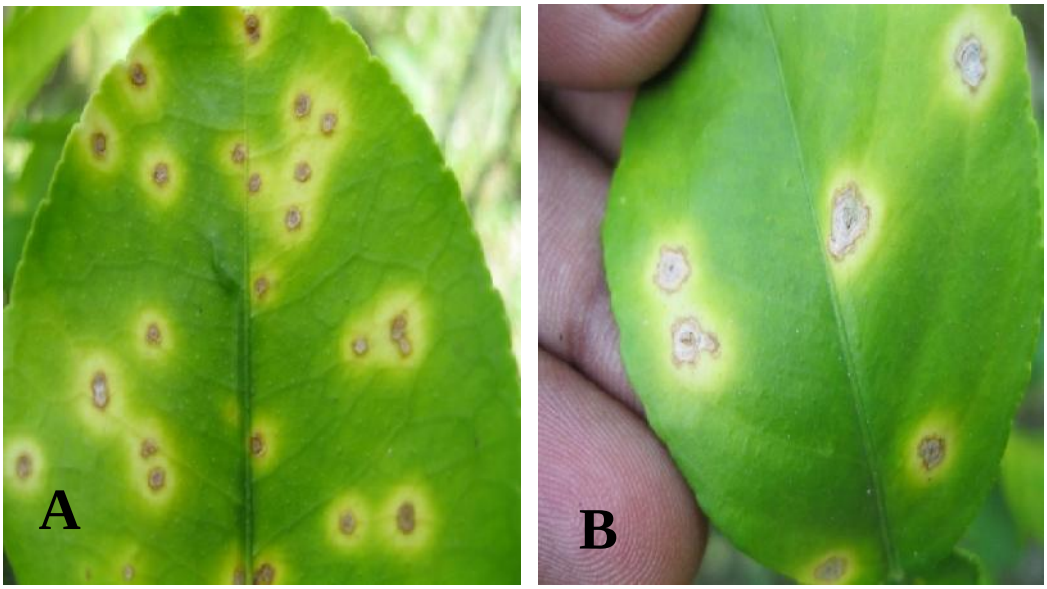


Plate 2. Symptoms of canker on seedlings of citrus

A. On leaf of citrus (early stage)

B. On leaf of citrus (old stage)

C. On twig of citrus

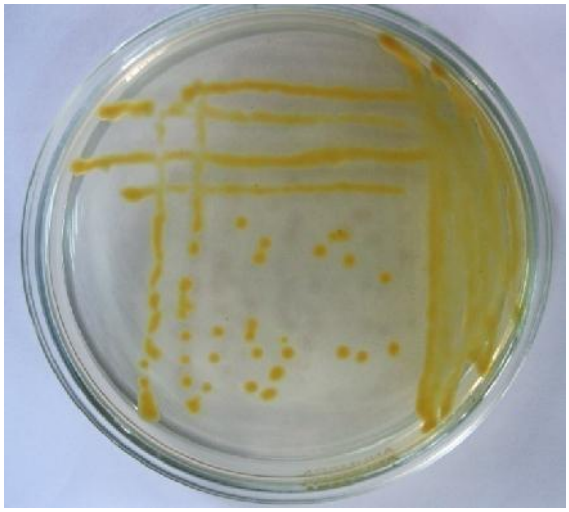


Fig. 1. Pure culture of *Xanthomonas axonopodis* pv. *citri* on NA plate

4.4. 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.

4.4.1. Morphological characters

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. The cells were readily stained with common stains such as crystal violet (Plate 3. A).

In KOH solubility test, a mucoid thread was lifted with the loop (Plate 3. B). Therefore the test was positive i.e., the bacterium was gram negative that supports the result of gram's staining test.

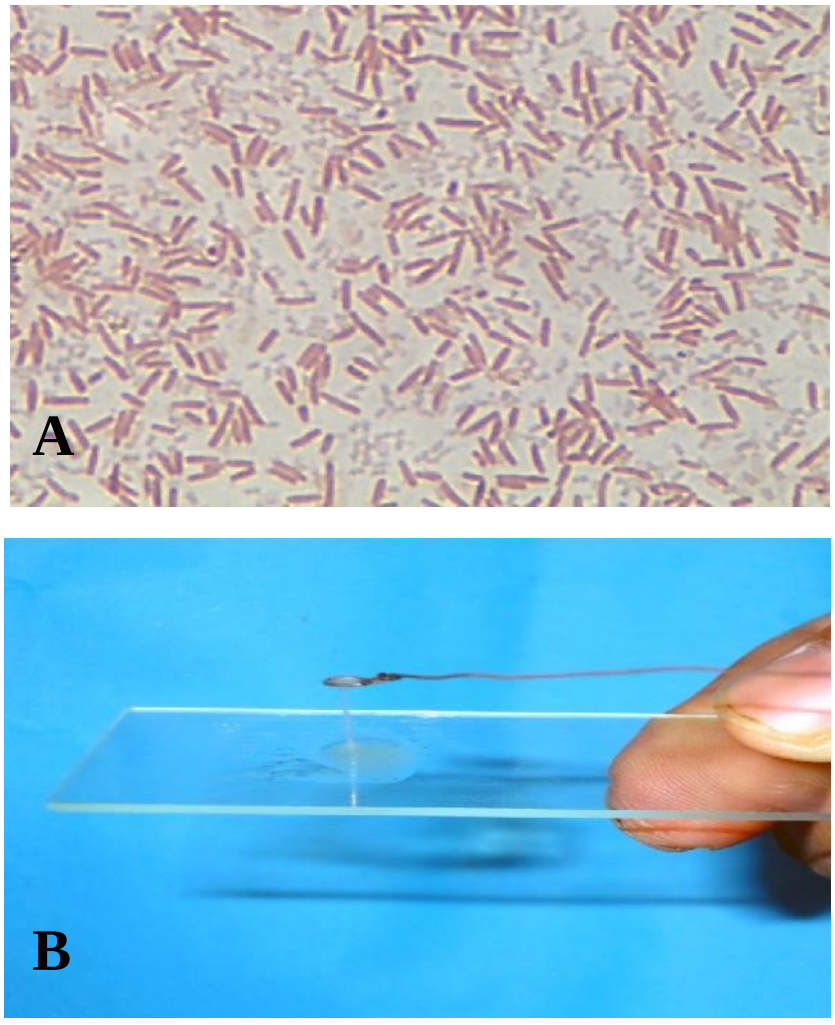


Plate 3. Morphological characters of *Xanthomonas axonopodis* pv. *citri*

A. Microscopic view of *Xanthomonas axonopodis* pv. *citri* after gram's staining at 125x magnification

B. KOH solubility test for *Xanthomonas axonopodis* pv. *citri*

4.4.2. Biochemical characters

Results obtained on various biochemical tests for the pathogen are presented in Table 3.

Table 3. 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 4. A).

In catalase test, after adding 3% H_2O_2 onto the colony of the bacterium bubbles were formed within a few seconds (Plate 4. 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 4. 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 4. 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 4. E). Thus the bacterium was motile (positive test).

In gelatine liquefaction test, gelatin was liquefied after 15 minutes of refrigeration at 5^{o}C (Plate 4. 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 4, Plate 4. G).

Table 4. Salt tolerance test for *Xanthomonas axonopodis* pv. *citri* in nutrient broth

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

4.4.3. Cultural characters

4.4.3.1. 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.

4.4.3.2. 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 5, Plate 5. A).

Circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies were found on YDCA medium (Table 5, Plate 5. B).

Bacterium exhibited very poor growth with light yellow to slightly blue, mostly circular, small, flattened, mucoid colonies on SX medium (Table 5, Plate 5. C).

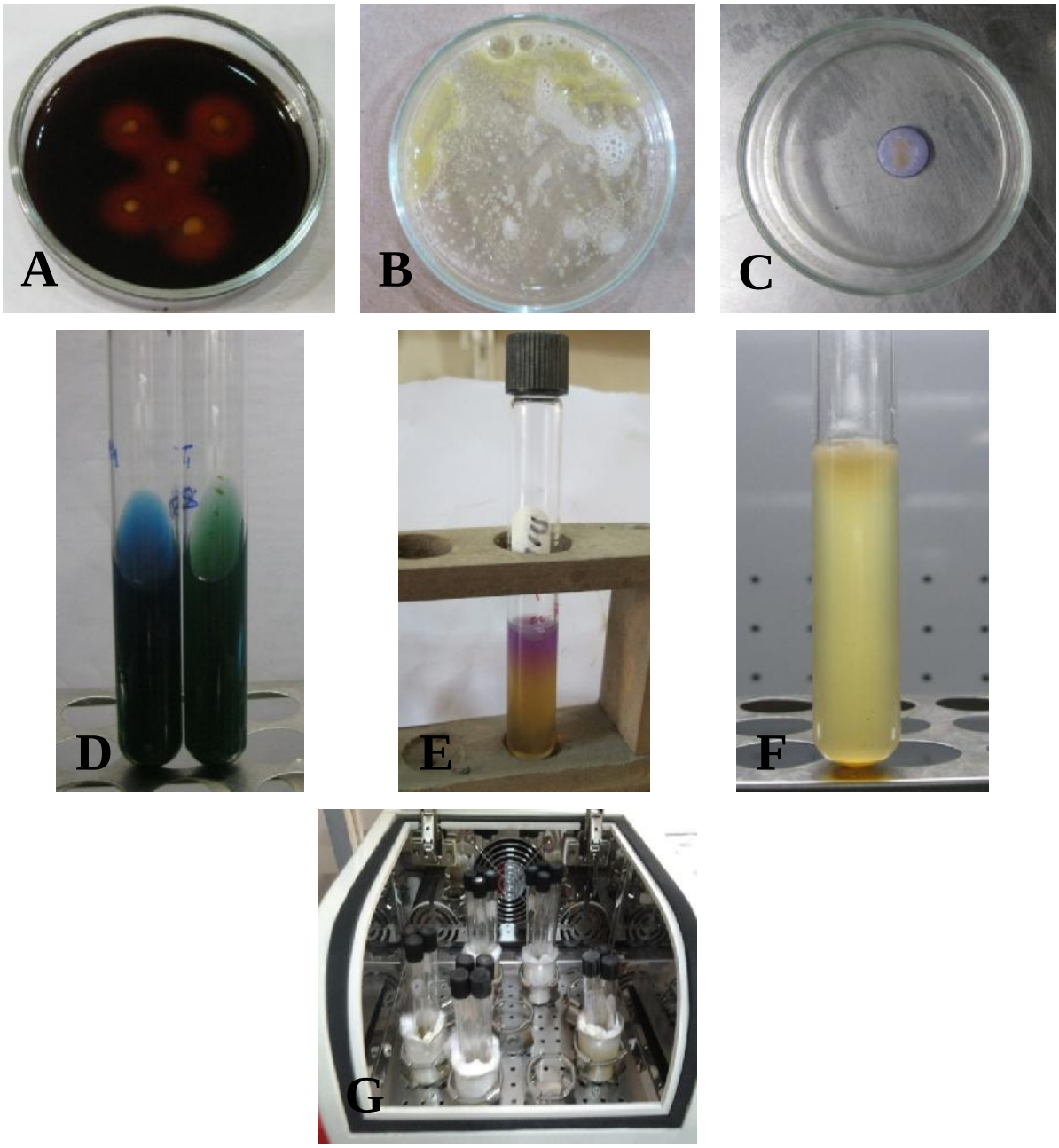


Plate 4. Biochemical characters of *Xanthomonas axonopodis* pv. *citri*

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 in shaker incubator

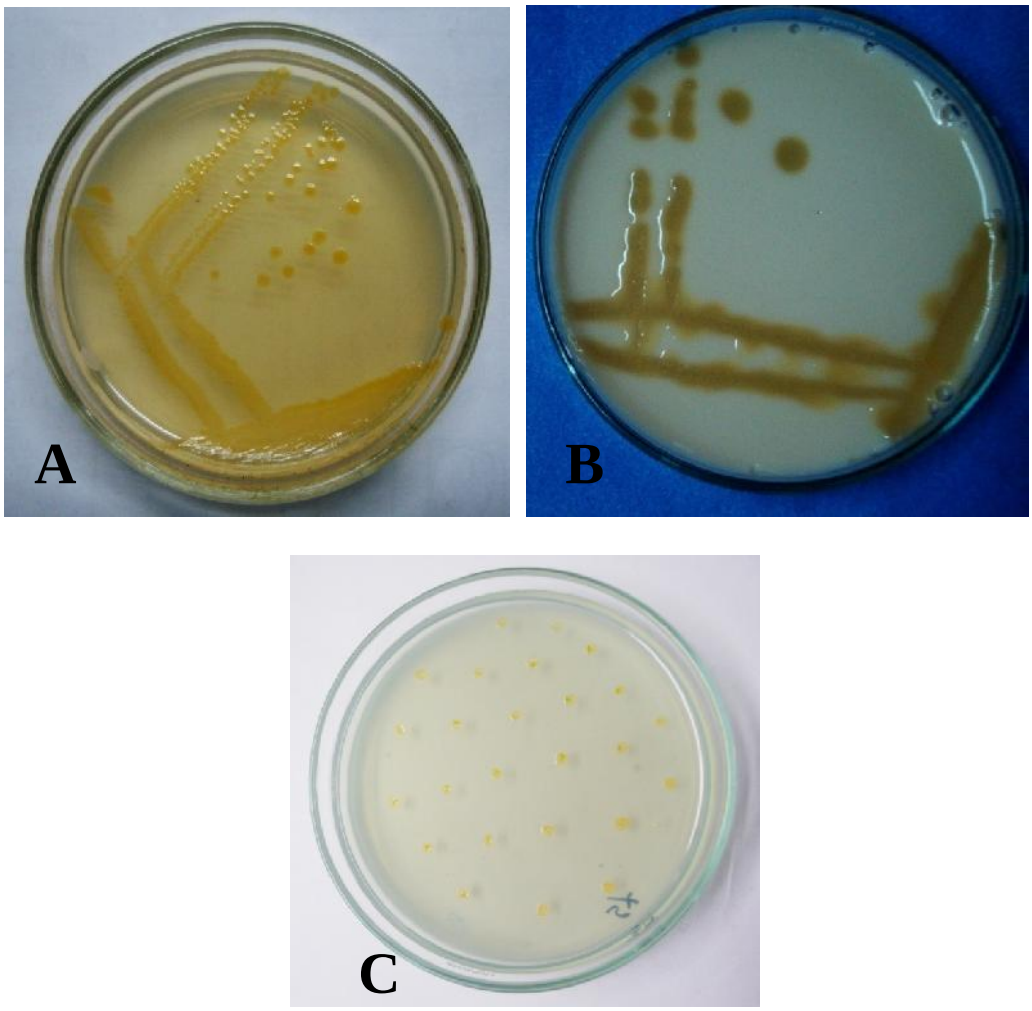


Plate 5. Cultural characteristics of *Xanthomonas axonopodis* pv. *citri* on different growth media

A. On Nutrient agar (NA) medium

B. On Yeast extract nutrient agar (YDCA) medium

C. On SX agar medium

Table 5. 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

4.5. 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 6. A), the infiltrated area became dry and necrotized within 48 hours. Thus bacteria showed positive reaction (Plate 6. B). There was no change in case of control (Plate 6. C).

4.6. 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 7). 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 6. 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)
- **C.** Tobacco leaf (control)

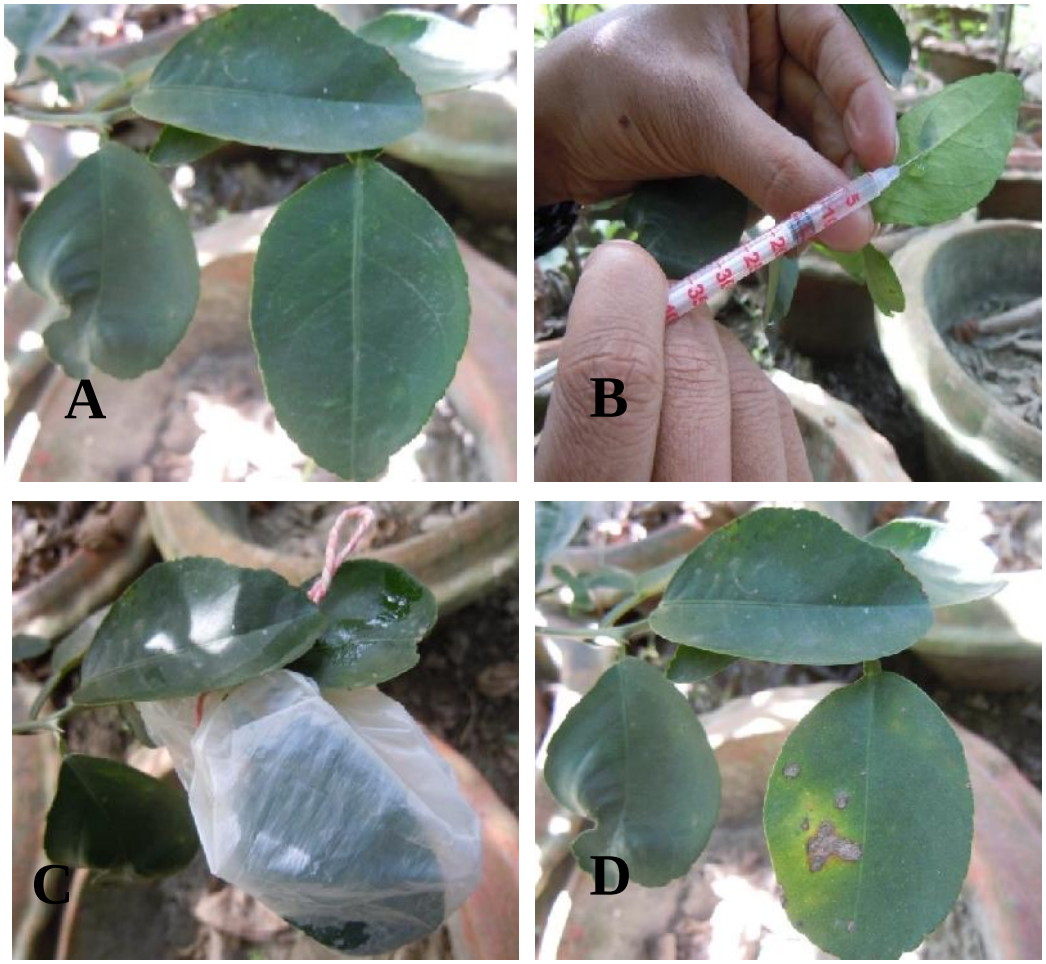


Plate 7. Pathogenicity test

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

4.7. Epidemiology of disease incidence and severity

4.7.1. Incidence and severity of citrus canker at different experimental locations of Bangladesh during January, 2011 to October, 2012

Incidence of canker of citrus varied from location to location and that ranged from 40.97 to 49.30% (Table 6). Highest incidence was recorded at Khagrachari (49.30%) and lowest was recorded at Dhaka (40.97%). Severity of canker of citrus also varied from location to location and that ranged from 13.79 to 17.52% (Table 6). Highest severity was recorded at Khagrachari (17.52%) and lowest was recorded at Dhaka (13.79%).

Table 6. Incidence and severity of citrus canker at different locations of Bangladesh from January, 2011 to October, 2012

Location	Canker of citrus	
	% Disease Incidence (Jan., 2011-Oct., 2012)	% Disease Severity (Jan., 2011-Oct., 2012)
Dhaka	40.97 b	13.79 c
Gazipur	46.94 a	15.77 b
Barisal	48.75 a	14.69 bc
Khagrachari	49.30 a	17.52 a
$LSD_{(p\leq0.05)}$	4.80	1.70
CV%	6.25	6.25

Each data represents the mean value of two nurseries at each location of two years.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

4.7.2. Incidence and severity of citrus canker in different growing seasons of Bangladesh during January, 2011 to October, 2012

Incidence of canker of citrus varied significantly from January, 2011 to October, 2012 and that ranged from 30.97 to 63.89% (Table 7). Highest (63.89%) incidence was recorded in the month of July (2011 & 2012) and lowest (30.97%) was recorded in the month of January (2011 & 2012). Severity of canker of citrus also varied significantly from January, 2011 to October, 2012 and that ranged from 6.86 to 24.26% (Table 7). Highest (24.26%) severity was recorded in the month of July (2011 & 2012) and lowest (6.86%) was recorded in the month of January (2011 & 2012).

Table 7. Incidence and severity of citrus canker during January, 2011 to October, 2012 of Bangladesh

Time of data collection	Canker of citrus	
	% Disease Incidence (Jan., 2011-Oct., 2012)	% Disease Severity (Jan., 2011-Oct., 2012)
January	30.97 d	6.86 d
April	48.89 b	19.33 b
July	63.89 a	24.26 a
October	42.22 c	11.33 c
$LSD_{(p\leq 0.05)}$	4.90	1.63
CV%	6.25	6.25

Each data represents the mean value of two nurseries at each location of two years.
Values followed by the same letter within a column are not significantly different ($p\leq 0.05$) according to Duncan's multiple range test.

4.7.3. Incidence and severity of citrus canker during different growing seasons at different experimental location

Incidence of canker of citrus varied significantly from season to season as well as location to location and that ranged from 28.33 to 72.22% (Table 8). Highest (72.22%) incidence of canker of citrus was recorded in the month of July (2011 & 2012) at Khagrachari. Lowest (28.33%) incidence was recorded in the month of January (2011 & 2012) at Dhaka. Statistically similar incidence (28.89%) was recorded in the month of January (2011 & 2012) at Gazipur. Severity of canker of citrus also varied significantly from season to season as well as location to location and that ranged from 5.55 to 27.55% (Table 8). Highest (27.55%) severity of canker of citrus was recorded in the month of July (2011 & 2012) at Khagrachari and lowest severity (5.55%) was recorded in the month of January (2011 & 2012) at Dhaka.

Table 8. Incidence and severity of citrus canker during January, 2011 to October, 2012 at different experimental locations of Bangladesh

Location	Time of data collection	Canker of citrus	
		% Disease Incidence (Jan., 2011-Oct., 2012)	% Disease Severity (Jan., 2011-Oct., 2012)
	January	28.33 h	5.55 j

Dhaka	April	47.22 de	17.33 f
	July	55.00 c	21.50 de
	October	33.33 gh	10.77 h
Gazipur	January	33.89 g	7.33 i
	April	49.44 de	20.00 e
	July	64.44 b	24.66 b
	October	40.00 f	11.11 h
Barisal	January	28.89 gh	6.55 ij
	April	51.11 cd	18.22 f
	July	63.89 b	23.33 bc
	October	51.11 cd	10.66 h
Khagrachari	January	32.78 gh	8.00 i
	April	47.77 de	21.77 cd
	July	72.22 a	27.55 a
	October	44.44 ef	12.77 g
$LSD_{(p\leq 0.05)}$		4.90	1.63
CV(%)		6.25	6.25

Each data represents the mean value of two nurseries at each location of two years.
Values followed by the same letter within a column are not significantly different ($p\leq 0.05$) according to Duncan's multiple range test.

4.7.4. Effect of weather components on the incidence and severity of canker on seedlings of citrus during January, 2011 to October, 2012

Incidence of canker of citrus was influenced by average temperature, relative humidity and rainfall. Highest incidence (63.89%) was recorded in the month of July (2011 & 2012) when the average temperature, relative humidity and rainfall were 29.57°C, 83.50% and 4.49 cm, respectively (Fig. 2). On the other hand, lowest incidence (30.97%) was recorded in the month of January (2011 & 2012) having average temperature, relative humidity and rainfall 17.89°C, 72.90% and 0.49 cm, respectively. In the month of April (2011 & 2012) incidence was 48.89% when the temperature, relative humidity and rainfall were 24.25°C, 72.59% and 3.17cm, respectively and in the month of October

(2011 & 2012) incidence was 42.22% when the temperature, relative humidity and rainfall were 28.40°C, 79.75% and 3.72 cm, respectively.

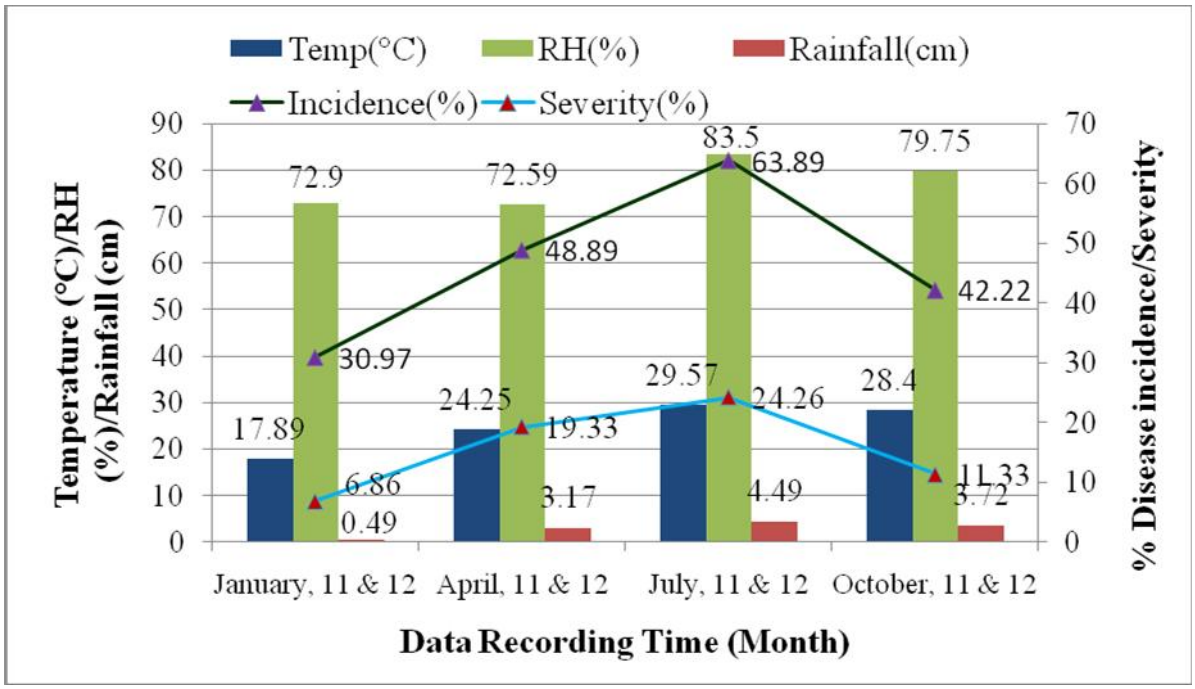


Fig. 2. Effect of weather components on the incidence and severity of canker on seedlings of citrus during January, 2011 to October, 2012

Severity of canker of citrus was also influenced by average temperature, relative humidity and rainfall. Highest severity (24.26%) was recorded in the month of July (2011 & 2012) when the average temperature, relative humidity and rainfall were 29.57°C, 83.50% and 4.49 cm, respectively. On the other hand, lowest severity (6.86%) was recorded in the month of January (2011 & 2012) having average temperature, relative humidity and rainfall 17.89°C, 72.90% and 0.49 cm, respectively. In the month of April (2011 & 2012) severity was 19.33% when the temperature, relative humidity and rainfall were 24.25°C, 72.59% and 3.17cm, respectively and in the month of October (2011 & 2012) severity was 11.33% when the temperature, relative humidity and rainfall were 28.40°C, 79.75% and 3.72 cm, respectively.

4.7.5. Relationship between weather factors and incidence as well as severity of canker on seedlings of citrus

Correlation and linear regression analysis were performed to determine the relationship between different components of climatic factor (temperature,

relative humidity and rainfall) and the incidence as well as severity of canker on seedlings of citrus (Table 9). From the correlation studies it was revealed that the temperature was positively correlated to both incidence (r = 0.635) and severity (r = 0.465) of canker on seedlings of citrus. Similarly, the incidence (r = 0.488, r = 0.752) and severity (r = 0.26, r = 0.63) of canker on seedlings of citrus were also positively correlated with both relative humidity and rainfall, respectively.

Table 9. Linear regression analysis of the effect of seasonal average temperature, relative humidity and rainfall on the incidence and severity of canker on seedlings of citrus

Climatic factors	Slope (b)		Coefficient of determination (R^2)		Probability (P)	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
Temperature	2.077	1.010	0.635	0.465	0.159	0.279
Relative humidity	1.797	0.745	0.488	0.260	0.261	0.463
Rainfall	6.865	3.573	0.752	0.630	0.090	0.162

4.8. Evaluation of management strategies for controlling citrus canker

4.8.1. *In vitro* evaluation

4.8.1.1. *In vitro* evaluation of antibacterial chemicals

4.8.1.1.1 Efficacy of antibacterial chemicals against *Xanthomonas axonopodis* pv. *citri* at different days after incubation

In case of Cupravit 50WP, highest inhibition zone (19.67 mm) was observed after 48 hours, 72 hours and 96 hours of incubation when concentration was 0.3% and 80 µl/well was used. Statistically similar result was observed after 24 hours of incubation when concentration was 0.2% and 100 µl/well was used with inhibition zone (19.00 mm). On the other hand, no inhibition zone was observed in control condition (Table 10).

In case of Sulcox 50 WP, highest inhibition zone (20.33 mm) was observed after 48 hours of incubation when concentration was 0.3% and 100 µl/well was used followed by after 24 hours and 72 hours of incubation at same

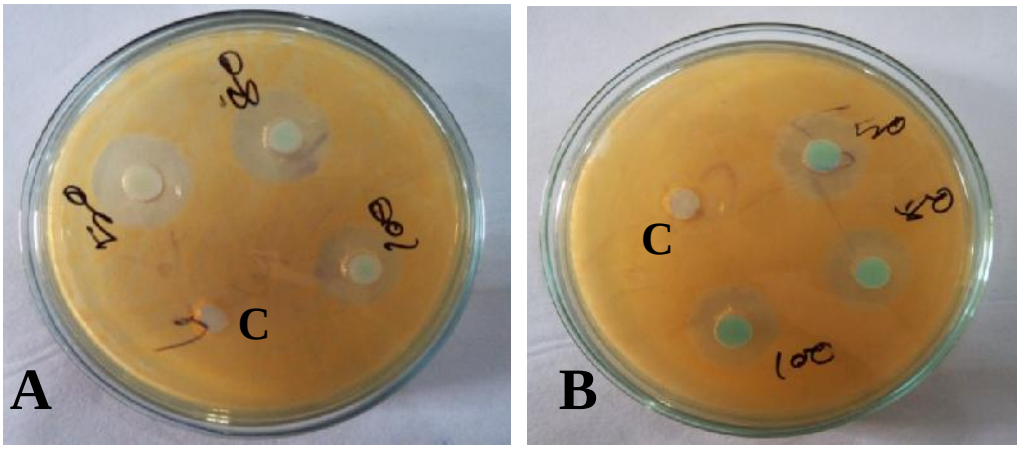
concentration and same volume with inhibition zone (19.33 mm). On the other hand, no inhibition zone was observed in control condition (Table 11).

In case of Champion 77 WP, highest inhibition zone (20.67 mm) was observed after 48 hours of incubation when concentration was 0.2% and 100 µl/well was used followed by after 24 hours of incubation at same concentration and same volume with inhibition zone (20.33 mm). On the other hand, no inhibition zone was observed in control condition (Table 12).

In case of Indofil M-45, highest inhibition zone (37.67 mm) was observed after 48 hours of incubation when concentration was 0.3% and 80 µl/well was used followed by after 72 hours of incubation at same concentration and same volume with inhibition zone (35.00 mm). On the other hand no inhibition zone was observed in control condition (Table 13).

In case of Dithane M-45, highest inhibition zone (29.00 mm) was observed after 48 hours of incubation when concentration was 0.3% and 80 µl/well was used followed by after 24 hours of incubation at same concentration and same volume with inhibition zone (26.00 mm). On the other hand, no inhibition zone was observed in control condition (Table 14).

In case of Bavistin 50 WP, highest inhibition zone (9.67 mm) was observed after 24 hours of incubation when 100 µl/well was used at both 0.3% and 0.4% concentration and when 80 µl/well was used at 0.4% concentration. On the other hand, no inhibition zone was observed after 48 hours, 72 hours, 96 hours and 120 hours of incubation in all the treatments along with control condition (Table 15).



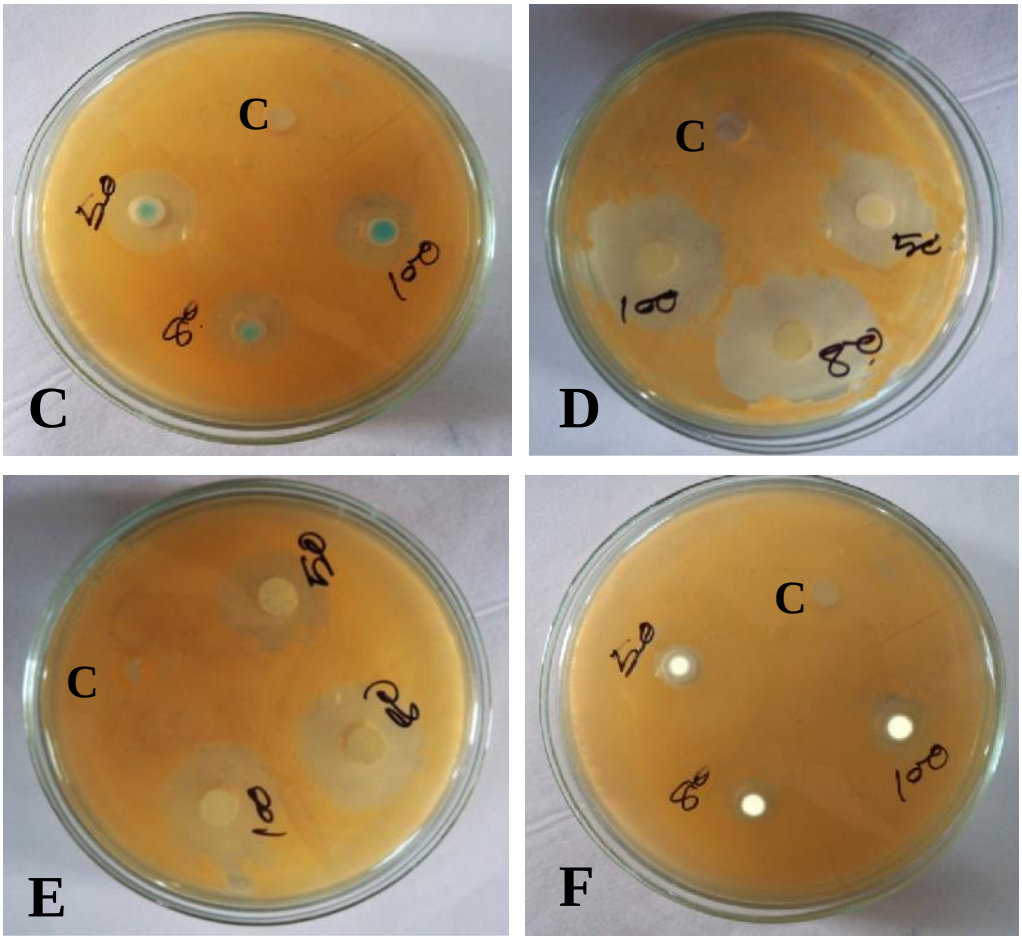


Plate 8. Bioassay of antibacterial chemicals against the bacteria

A. Cupravit 50 WP
B. Sulcox 50 WP
C. Champion 77WP
D. Indofil M-45
E. Dithane M-45
F.Bavistin 50 WP

Table 10. Efficacy of Cupravit 50 WP against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	8.33 g	9.33 g	9.00 g	8.33 f	7.67 g
0.2		14.67 de	15.33 de	15.67 d	13.33 e	12.33 e
0.3		15.33 cde	16.00 cd	16.00 cd	15.00 d	13.67 cd
0.4		16.00 bcd	16.33 cd	16.67 bcd	16.33 bcd	13.67 cd
0.1		10.33 f	11.67 f	10.67 f	9.67 f	8.33 g
0.2		16.00 bcd	16.33 cd	15.67 d	15.33 cd	13.00 de

0.3	80	18.33 a	19.67 a	19.67 a	19.67 a	17.00 a
0.4		17.00 abc	17.00 bcd	17.00 bcd	17.00 b	15.67 b
0.1	100	13.67 e	14.33 e	13.00 e	13.00 e	11.00 f
0.2		17.67 ab	19.00 a	18.33 ab	16.67 bc	14.67 bc
0.3		16.33 bcd	18.33 ab	17.67 bc	16.33 bcd	15.67 b
0.4		17.00 abc	17.33 bc	17.33 bcd	15.00 d	14.00 cd
0.1	Control	0.00 h	0.00 h	0.00 h	0.00 g	0.00 h
0.2		0.00 h	0.00 h	0.00 h	0.00 g	0.00 h
0.3		0.00 h	0.00 h	0.00 h	0.00 g	0.00 h
0.4		0.00 h	0.00 h	0.00 h	0.00 g	0.00 h
$LSD_{(p\leq 0.05)}$		1.69	1.52	1.63	1.46	1.00
CV %		8.86	7.56	8.27	7.89	6.08

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq 0.05$) according to Duncan's multiple range test.

Table 11. Efficacy of Sulcox 50 WP against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	11.00 g	12.83 g	11.83 e	11.83 e	11.17 d
0.2		14.00 f	16.00 ef	16.00 c	14.00 c	13.33 bc
0.3		16.67 cde	18.00 bcd	18.33 ab	18.00 a	16.33 a
0.4		16.00 de	18.00 bcd	18.33 ab	16.67 ab	15.67 a
0.1	80	14.00 f	14.00 g	13.67 d	12.00 de	11.00 d
0.2		15.33 ef	17.00 de	17.33 bc	16.33 b	15.33 a
0.3		17.67 bc	18.00 bcd	18.00 ab	17.00 ab	16.67 a
0.4		17.33 bcd	17.67 cd	17.67 abc	17.67 ab	15.00 ab
0.1	100	16.00 de	15.33 f	13.33 de	13.33 cd	13.00 c
0.2		17.33 bcd	18.33 bc	17.67 abc	17.00 ab	15.33 a
0.3		19.33 a	20.33 a	19.33 a	18.00 a	15.67 a
0.4		18.67 ab	19.00 b	18.33 ab	17.00 ab	15.00 ab
0.1	Control	0.00 h	0.00 h	0.00 f	0.00 f	0.00 e
0.2		0.00 h	0.00 h	0.00 f	0.00 f	0.00 e
0.3		0.00 h	0.00 h	0.00 f	0.00 f	0.00 e
0.4		0.00 h	0.00 h	0.00 f	0.00 f	0.00 e
$LSD_{(p\leq 0.05)}$		1.39	1.18	1.54	1.42	1.65
CV %		6.80	5.48	7.32	7.12	9.02

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq 0.05$) according to Duncan's multiple range test.

Table 12. Efficacy of Champion 77 WP against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	17.67 d	18.00 cd	17.67 cd	16.67 bcd	15.67 bcd
0.2		19.67 abc	20.00 ab	19.67 ab	17.67 abc	17.00 ab
0.3		17.67 d	18.00 cd	17.33 d	16.33 cd	16.33 abc
0.4		18.33 cd	19.00 bcd	18.00 bcd	15.33 d	14.67 cd
0.1	80	19.33 abc	19.33 abc	19.33 abc	17.67 abc	16.67 ab
0.2		20.00 ab	20.00 ab	20.00 a	18.00 abc	17.33 ab
0.3		19.00 abcd	19.33 abc	19.67 ab	18.33 abc	17.33 ab
0.4		18.33 cd	17.67 d	17.67 cd	15.33 d	14.33 d
0.1	100	20.00 ab	20.00 ab	19.67 ab	18.33 abc	16.67 ab
0.2		20.33 a	20.67 a	20.00 a	19.67 a	18.00 a
0.3		19.33 abc	18.67 bcd	18.67 abcd	17.00 bcd	17.00 ab
0.4		18.67 bcd	19.33 abc	19.33 abc	18.67 ab	16.33 abc
0.1	Control	0.00 e	0.00 e	0.00 e	0.00 e	0.00 e
0.2		0.00 e	0.00 e	0.00 e	0.00 e	0.00 e
0.3		0.00 e	0.00 e	0.00 e	0.00 e	0.00 e
0.4		0.00 e	0.00 e	0.00 e	0.00 e	0.00 e
$LSD_{(p\leq0.05)}$		1.41	1.38	1.69	1.85	1.60
CV %		5.87	5.71	7.05	8.46	7.67

Each data represents the mean value of three replications.

Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

Table 13. Efficacy of Indofil M-45 against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	23.00 f	25.33 e	21.33 h	21.33 g	21.33 d
0.2		24.00 f	26.00 e	25.33 ef	23.67 ef	22.33 d
0.3		31.00 bc	33.00 b	32.67 b	30.33 ab	30.33 a
0.4		29.33 c	30.67 bc	29.33 cd	29.33 abc	26.67 b
0.1	80	25.00 ef	25.33 e	22.00 gh	21.67 fg	21.67 d
0.2		27.00 d	29.00 cd	29.00 cd	27.67 c	24.67 c
0.3		34.33 a	37.67 a	35.00 a	31.00 a	29.33 a
0.4		29.33 c	30.00 c	29.00 cd	28.67 bc	26.00 bc
0.1		24.67 f	25.33 e	23.67 fg	22.00 fg	21.67 d

0.2	100	26.67 de	26.67 de	26.67 e	25.33 de	23.00 d
0.3		32.67 ab	33.33 b	30.33 c	30.33 ab	29.67 a
0.4		29.33 c	31.33 bc	27.33 de	27.33 cd	27.33 b
0.1	Control	0.00 g	0.00 f	0.00 i	0.00 h	0.00 e
0.2		0.00 g	0.00 f	0.00 i	0.00 h	0.00 e
0.3		0.00 g	0.00 f	0.00 i	0.00 h	0.00 e
0.4		0.00 g	0.00 f	0.00 i	0.00 h	0.00 e
$LSD_{(p\leq0.05)}$		1.88	2.54	1.92	2.05	1.63
CV %		5.30	6.83	5.50	6.12	5.08

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

Table 14. Efficacy of Dithane M-45 against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	14.67 d	17.33 e	15.67 e	13.33 d	0.00 h
0.2		19.00 c	21.67 cd	19.67 cd	17.67 c	15.33 ef
0.3		25.00 ab	28.33 a	25.00 a	23.00 a	19.00 bc
0.4		23.67 b	26.00 b	22.33 b	22.00 a	17.00 cde
0.1	80	15.33 d	17.00 e	15.33 e	13.67 d	12.33 g
0.2		19.33 c	21.67 d	19.33 cd	18.67 bc	16.67 de
0.3		26.00 a	29.00 a	25.33 a	23.67 a	21.33 a
0.4		24.00 ab	26.00 b	22.33 b	20.00 b	17.00 cde
0.1	100	15.67 d	20.33 d	17.67 de	15.00 d	13.67 fg
0.2		23.67 b	24.00 bc	21.00 bc	19.33 bc	18.33 bcd
0.3		25.67 ab	25.67 b	25.33 a	22.33 a	19.67 ab
0.4		24.00 ab	24.00 bc	21.67 bc	19.33 bc	17.33 cde
0.1	Control	0.00 e	0.00 f	0.00 f	0.00 e	0.00 h
0.2		0.00 e	0.00 f	0.00 f	0.00 e	0.00 h
0.3		0.00 e	0.00 f	0.00 f	0.00 e	0.00 h
0.4		0.00 e	0.00 f	0.00 f	0.00 e	0.00 h
$LSD_{(p\leq0.05)}$		1.97	2.17	2.34	1.95	1.95
CV %		7.29	7.32	8.85	8.10	9.84

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

Table 15. Efficacy of Bavistin 50 WP against *Xanthomonas axonopodis* pv. *citri*

Conc. %	Volume (µl)	Inhibition zone (mm)				
		24hr	48hr	72hr	96hr	120hr
0.1	50	0.00 e	0	0	0	0
0.2		0.00 e	0	0	0	0
0.3		7.67 d	0	0	0	0
0.4		8.67 bc	0	0	0	0
0.1	80	0.00 e	0	0	0	0
0.2		7.67 d	0	0	0	0
0.3		9.33 ab	0	0	0	0
0.4		9.67 a	0	0	0	0
0.1	100	0.00 e	0	0	0	0
0.2		8.33 cd	0	0	0	0
0.3		9.67 a	0	0	0	0
0.4		9.67 a	0	0	0	0
0.1	Control	0.00 e	0	0	0	0
0.2		0.00 e	0	0	0	0
0.3		0.00 e	0	0	0	0
0.4		0.00 e	0	0	0	0
$LSD_{(p\leq0.05)}$		0.74	0	0	0	0
CV %		9.98	0	0	0	0

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

4.8.1.1.2. Comparative efficacy of antibacterial chemicals against *Xanthomonas axonopodis* pv. *citri* at different days after incubation

After 24 hours of incubation, among six antibacterial chemicals Indofil M-45 showed the highest inhibition zone (34.33 mm) at 0.3% concentration when 80 µl/well was used where Bavistin 50 WP showed the inhibition zone (9.67 mm) at 0.3% concentration when 100 µl/well was used (Table 16).

After 48 hours of incubation, among six antibacterial chemicals Indofil M-45 showed the highest inhibition zone (37.67 mm) at 0.3% concentration when 80 µl/well was used where no inhibition zone was observed in case of Bavistin 50 WP at 0.3% concentration when 100 µl/well was used (Table 16).

After 72 hours of incubation, among six antibacterial chemicals Indofil M-45 showed the highest inhibition zone (35.00 mm) at 0.3% concentration when 80 µl/well was used where no inhibition zone was observed in case of Bavistin 50 WP at 0.3% concentration when 100 µl/well was used (Table 16).

After 96 hours of incubation, among six antibacterial chemicals Indofil M-45 showed the highest inhibition zone (31.00 mm) at 0.3% concentration when 80 µl/well was used where no inhibition zone was observed in case of Bavistin 50 WP at 0.3% concentration when 100 µl/well was used (Table 16).

After 120 hours of incubation, among six antibacterial chemicals Indofil M-45 showed the highest inhibition zone (29.33 mm) at 0.3% concentration when 80 µl/well was used where no inhibition zone was observed in case of Bavistin 50 WP at 0.3% concentration when 100 µl/well was used (Table 16).

In vitro bioassay of antibacterial chemicals revealed that highest inhibition zone showed by Indofil M-45 at 0.3% concentration with 80 µl/well than other chemicals used in the experiment (Table 16).

Table 16. Comparative efficacy of antibacterial chemicals against *Xanthomonas axonopodis* pv. *citri*

Antibacterial chemicals	Conc. (%)	Volume (µl)	Inhibition zone (mm)				
			24hr	48hr	72hr	96hr	120hr
Cupravit 50 WP	0.3	80	18.33 c	19.67 c	19.67 c	19.67 c	17.00 cd
Sulcox 50 WP	0.3	100	19.33 c	20.33 c	19.33 c	18.00 c	15.67 d
Champion 77 WP	0.2	100	20.33 c	20.67 c	20.00 c	19.67 c	18.00 c
Indofil M-45	0.3	80	34.33 a	37.67 a	35.00 a	31.00 a	29.33 a
Dithane M-45	0.3	80	26.00 b	29.00 b	25.33 b	23.67 b	21.33 b
Bavistin 50 WP	0.3	100	9.67 d	0.00 d	0.00 d	0.00 d	0.00 e
$LSD_{(p\leq0.05)}$			2.51	2.77	1.44	1.91	1.36
CV %			6.46	7.18	3.97	5.62	4.41

Each data represents the mean value of three replications.

Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

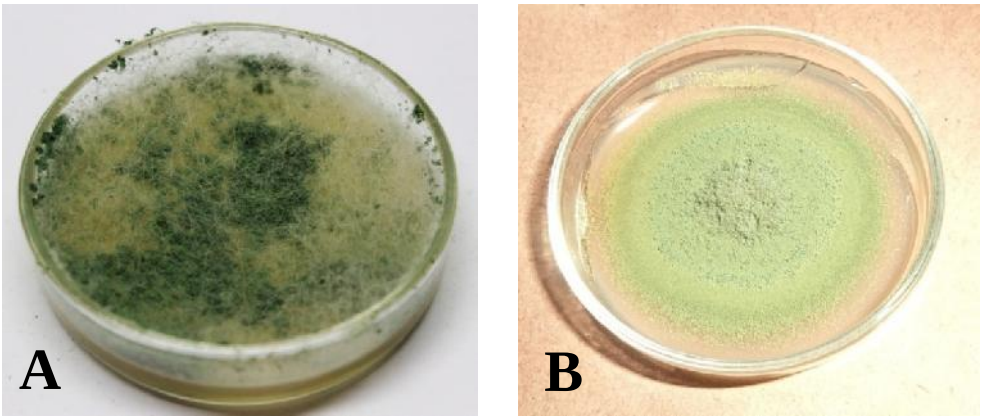
4.8.1.2. *In vitro* evaluation of bioagents

4.8.1.2.1. Purification of antagonistic fungi

Purification of *Trichoderma harzianum* and *Aspergillus flavus* were done on PDA plate by retransferring the colony from collected sample. *Trichoderma harzianum* formed concentric ring on PDA plate and colony appeared as white to light green at 2-4 days and dark green at 5 days (Plate 9. A). Colony of *Aspergillus flavus* grew rapidly on PDA plate that appeared as light sparse grey green to parrot green (Plate 9. B).

4.8.1.2.2. Isolation and purification of antagonistic bacteria

Antagonistic bacteria wsere isolated from soil by employing dilution plating technique. *Pseudomonas fluorescens* produces pink pigmentation on King's B (KB) plate after 48 hours of incubation at 30^0C (Plate 9. C). *Rhizobium leguminosarum* produced gummy white appearance on YMA plate. Repeated isolation yielded creamy white, round colonies of *Pseudomonas fluorescens* whose margins were uneven (Plate 9. D) on nutrient agar plate. *Bacillus subtilis* produced cream colour, flat and circular colonies with undulated margins (Plate 9. E), where *Rhizobium leguminosarum* produced white, fluidal and circular, convex colonies on nutrient agar plate (Plate 9. F).



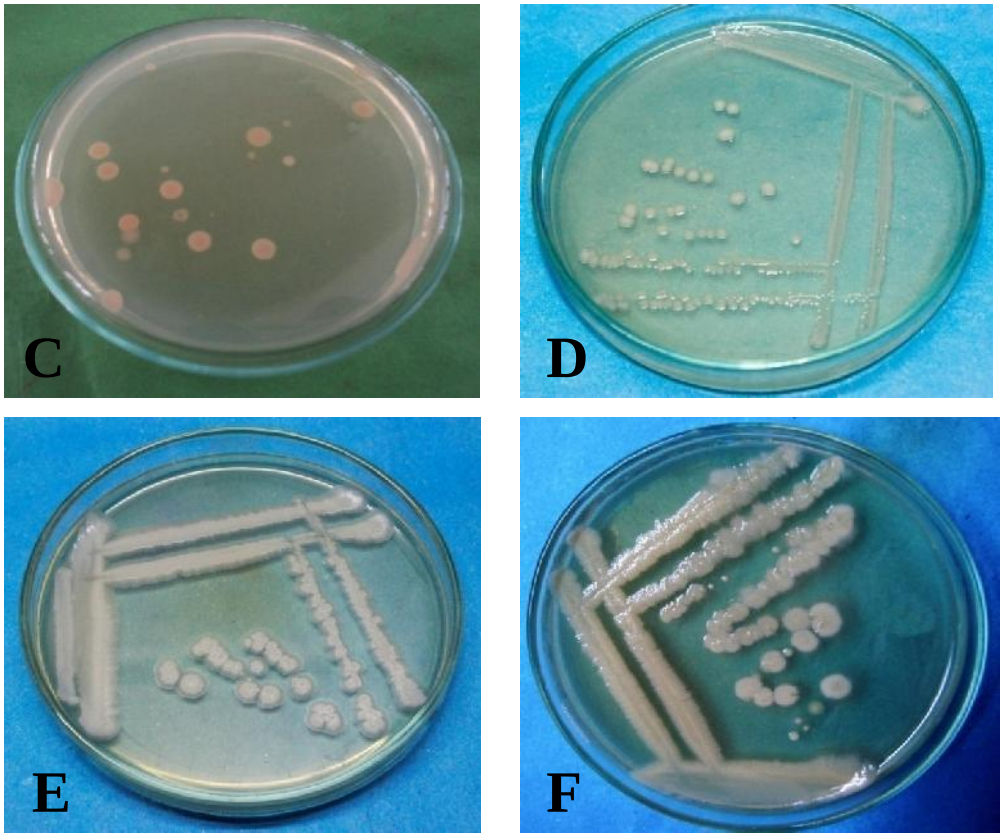


Plate 9. Pure culture of biocontrol agents

A. *T richoderma harzianum* on PDA plate

B. *A spergillus flavus* on PDA plate

C. *P seudomonas fluorescens* on King's B plate

D. *P seudomonas fluorescens* on NA plate

E. *B acillus subtilis* on NA plate

F. *R hizobium leguminosarum* on NA plate

4.8.1.2.3. Identification of antagonistic bacteria

Identification of antagonistic bacteria was done by conducting studies on their morphological and biochemical features as per standard microbiological procedures.

4.8.1.2.3.a. Morphological characters

Under the compound microscope at 125x magnification with oil immersion, *Bacillus subtilis* was gram positive, straight to slightly bent rod that appeared singly or in chains (Plate 10. A); *Pseudomonas fluorescens* was gram negative, straight to curved rod that appeared singly or in short chains (Plate 10. B) and *Rhizobium leguminosarum* was gram negative, rod shaped, pleomorphic that appeared singly or in cluster (Plate 10. C).

4.8.1.2.3.b. Biochemical characters

Results obtained on various biochemical tests for antagonistic bacteria are presented in Table 17.

Table 17. Biochemical characteristics of antagonistic bacteria

Biochemical tests	*Bacillus subtilis*	*Pseudomonas fluorescens*	*Rhizobium leguminosarum*
Starch hydrolysis test	+	+	-
Catalase test	+	+	+
Oxidase test	+	+	+
Citrate utilization test	+	+	+
Motility indole urease agar (MIU) test	+	+	+
Gelatine liquefaction test	+	+	+

+ = positive reaction

- = negative reaction

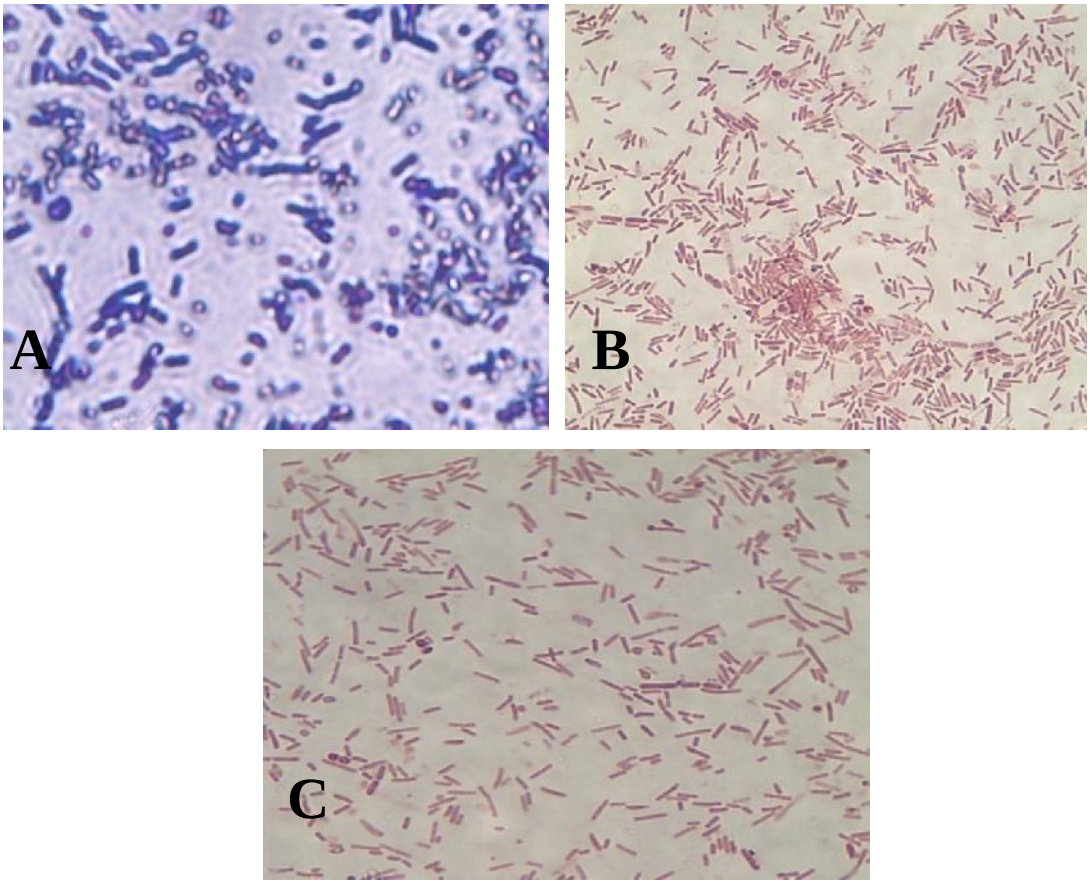


Plate 10. Microscopic view of antagonistic bacteria after gram staining at 125x magnification

A. *Bacillus subtilis*

B. *Pseudomonas fluorescens*

C. *Rhizobium leguminosarum*

In starch hydrolysis test, *Bacillus subtilis* and *Pseudomonas fluorescens* formed clear zone around the colony after adding Lugol's Iodine where *Rhizobium leguminosarum* do not produce any clear zone around the colony, which

revealed that the test was positive for *Bacillus subtilis* (Plate 11. A) and *Pseudomonas fluorescens* (Plate 11. B) and negative for *Rhizobium leguminosarum*.

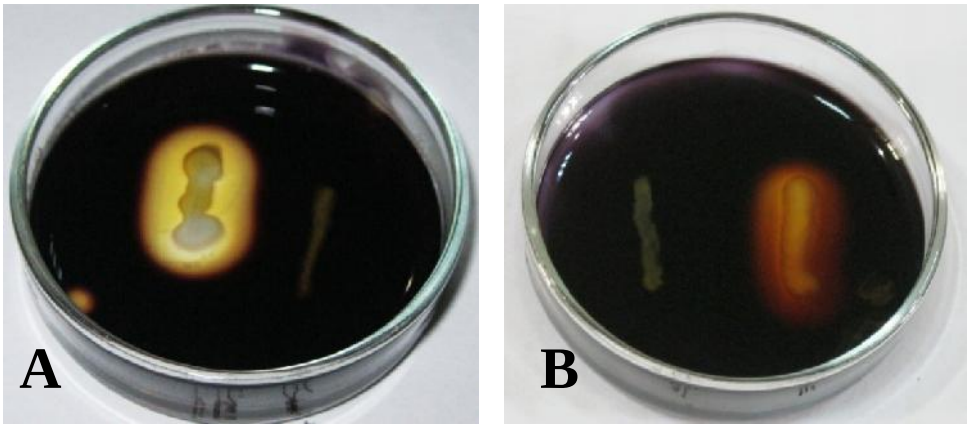
In catalase test, bubbles were formed by *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* after adding 3% H_2O_2 onto the colony within a few seconds (Plate 11. C-E), which revealed that the test was positive for all the three bacteria.

In oxidase test, after rubbing *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* onto the moistened oxidase disk, they formed dark purple color in oxidase disk (Plate 12. A-C), which revealed that the test was positive for all the three bacteria.

In **Citrate utilization test,** *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* changed green colour of simmon's citrate agar slant into a bright blue colour after 24 hours of incubation which indicated the test was positive for all the three bacteria i.e., bacteria used citrate as a carbon source for their (Plate 13. A-C).

In motility indole urease agar (MIU) test, after 48 hours of incubation *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* were migrated away from the original line of inoculation (Plate 13. D-F). Thus the bacteria were motile (positive test).

In gelatine liquefaction test, gelatin was liquefied after 15 minutes of refrigeration at 5^oC in all the three bacteria (Plate 14. A-C). Thus they show positive result.



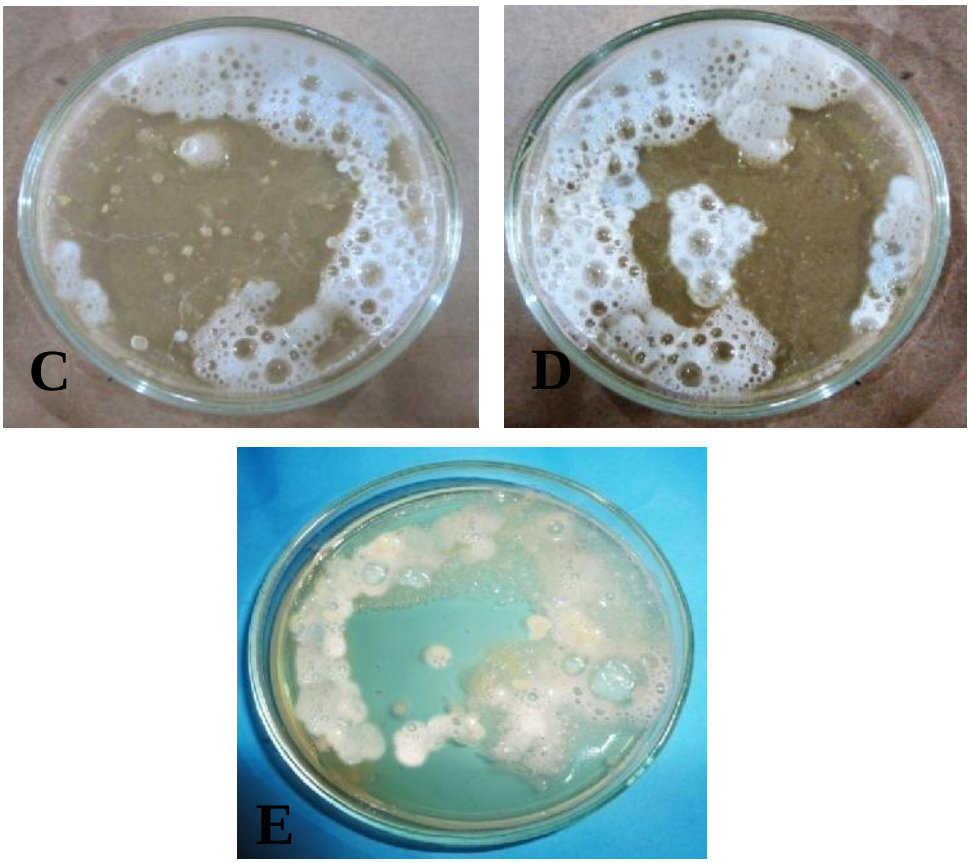


Plate 11. Biochemical tests for antagonistic bacteria (Starch hydrolysis test and Catalase test)

A. *Bacillus subtilis* in left side and *Rhizobium leguminosarum* in right side (Starch hydrolysis test)

B. *Rhizobium leguminosarum* in left side and *Pseudomonas fluorescens* in right side (Starch hydrolysis test)

C. *Bacillus subtilis* (Catalase test)

D. *Pseudomonas fluorescens* (Catalase test)

E. *Rhizobium leguminosarum* (Catalase test)

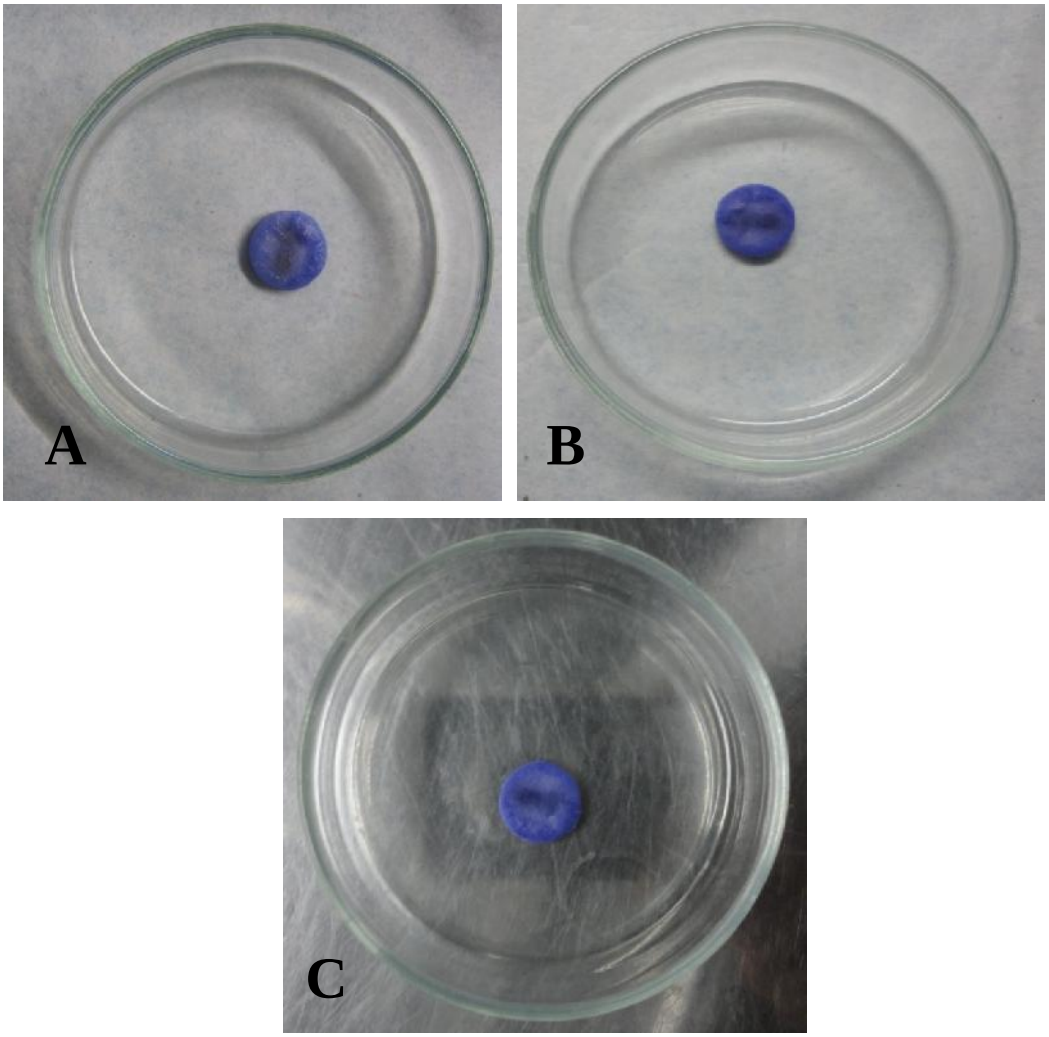


Plate 12. Biochemical tests for antagonistic bacteria (Oxidase test)

A. *Bacillus subtilis*

B. *Pseudomonas fluorescens*

C. *Rhizobium leguminosarum*

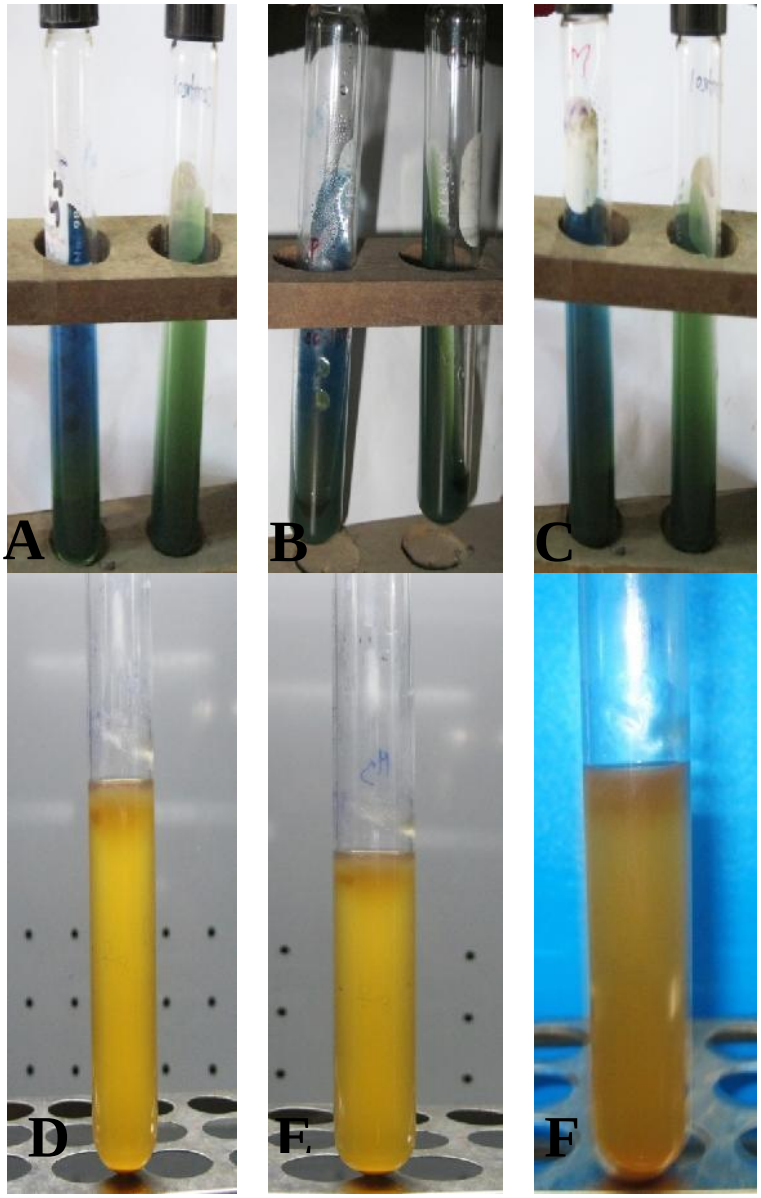


Plate 13. Biochemical tests for antagonistic bacteria (Citrate utilization test and MIU test)

A. *Bacillus subtilis* **(Citrate utilization test)**

B. *Pseudomonas fluorescens* **(Citrate utilization test)**

C. *Rhizobium leguminosarum* **(Citrate utilization test)**

D. *Bacillus subtilis* (MIU test)

E. *Pseudomonas fluorescens* (MIU test)

F. *Rhizobium leguminosarum* (MIU test)

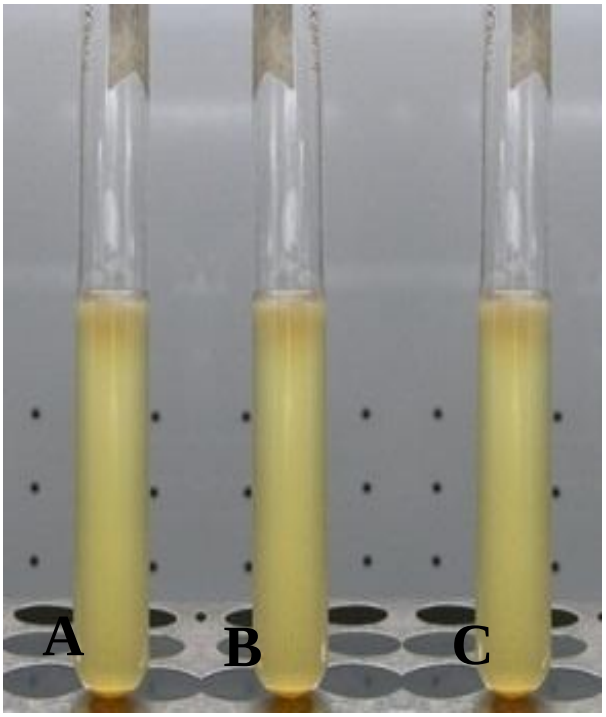


Plate 14. Biochemical tests for antagonistic bacteria (Gelatine liquefaction test)

A. *Bacillus subtilis*

B. *Pseudomonas fluorescens*

C. *Rhizobium leguminosarum*

4.8.1.2.4. Screening of antagonistic organisms against *Xanthomonas axonopodis* pv. *citri*

The study conducted revealed that among the five biocontrol agents, *Bacillus subtilis* and *Pseudomonas fluorescens* were found significantly superior in inhibiting the growth of *Xanthomonas axonopodis* pv. *citri*. *Bacillus subtilis* showed highest amount of inhibition zone (19.00 mm) after 72 hours of incubation followed by *Pseudomonas fluorescens* with inhibition zone (14.33 mm) after 48 hours of incubation. Other biocontrol agents viz. *Rhizobium leguminosarum*, *Trichoderma harzianum* and *Aspergillus flavus* were ineffective as they failed to inhibit the growth of *X. axonopodis* pv. *citri* (Table 18, Plate 15).

Table 18. Efficacy of bioagents against the growth of *Xanthomonas axonopodis* pv. *citri*

Bioagents	Inhibition zone (mm)				
	24hr	48hr	72hr	96hr	120hr
Bacillus subtilis	13.67 a	16.33 a	19.00 a	16.67 a	13.33 a
Pseudomonas fluorescens	11.33 b	14.33 b	12.67 b	10.67 b	8.667 b
Rhizobium leguminosarum	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c
Trichoderma harzianum	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c
Aspergillus flavus	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c
Control	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c
$LSD_{(p\leq0.05)}$	0.66	0.64	0.79	0.64	0.58
CV %	8.76	6.84	8.23	7.67	8.62

Each data represents the mean value of three replications.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

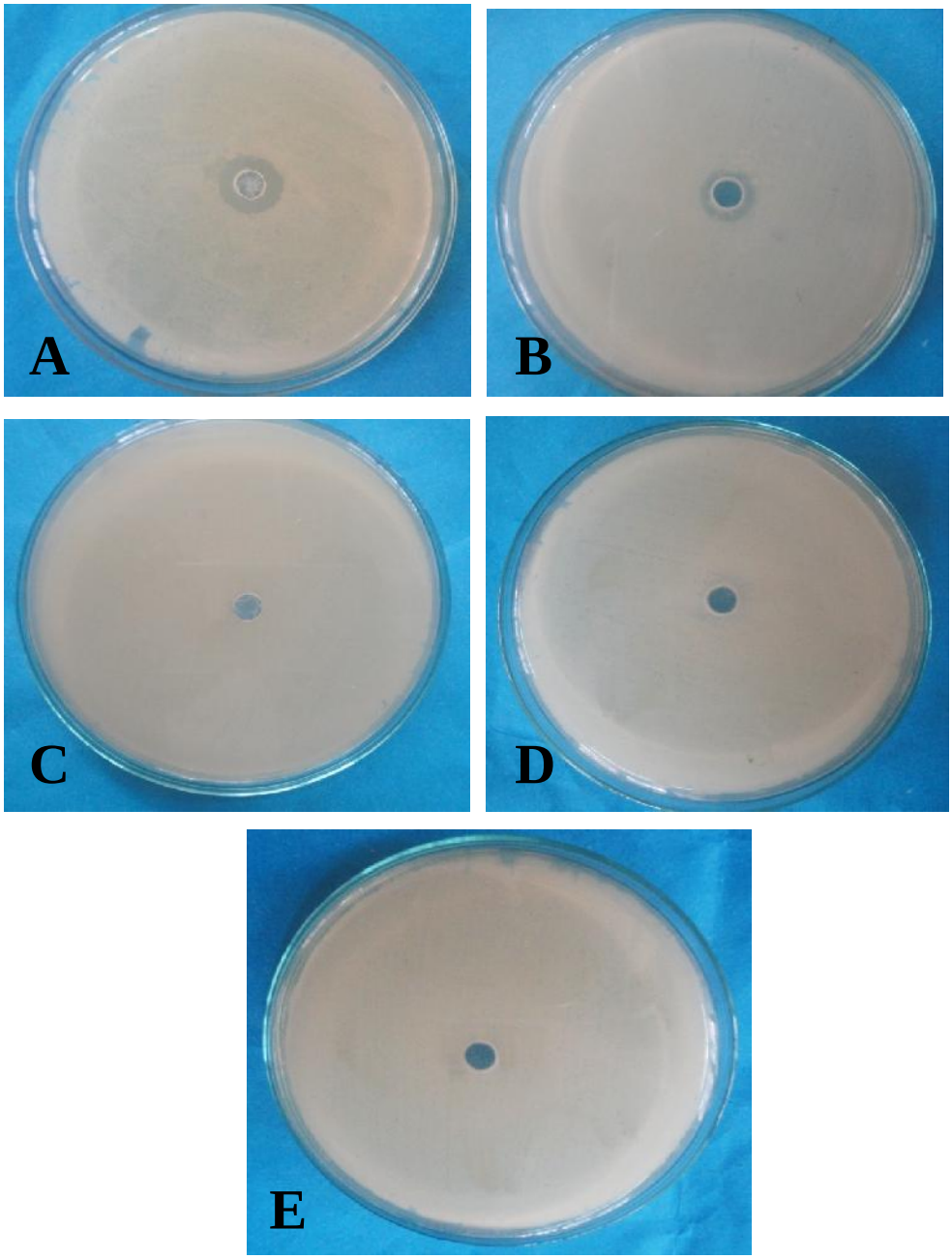


Plate 15. Screening of bioagents against *Xanthomonas axonopodis* pv. *citri*

- **A.** *Bacillus subtilis*
- **B.** *Pseudomonas fluorescens*
- **C.** *Rhizobium leguminosarum*
- **D.** *Trichoderma harzianum*
- **E.** *Aspergillus flavus*

4.8.2. Field evaluation

4.8.2.1. Effect of different antibacterial chemicals on the incidence of canker on seedlings of citrus

All the treatments significantly reduced the incidence of canker on seedlings of citrus over control during the period of October, 2011 to September, 2012 (Table 19). Out of all the treatments applied, except T_5 (untreated control) highest incidence (100%) was observed in the month of June, 2012; July, 2012; August, 2012 and September, 2012 in T_4 (Bavistin was applied as foliar spray @ 0.3%) and lowest incidence (0.00%) was observed in the month of January, 2012; February, 2012; March, 2012 and April, 2012 in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%), January, 2012, February, 2012 and March, 2012 in T_2 (Champion was applied as foliar spray @ 0.2%) and January, 2012 and February, 2012 in T_1 (Cupravit was applied as foliar spray @ 0.3%). Considering the mean incidence, highest incidence (74.83%) was recorded in T_5 (untreated control), which was statistically different from all other treatments and lowest incidence (41.22%) was observed in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%). In case of percent reduction of disease incidence over control, highest reduction (44.92%) was observed in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%) and lowest reduction (6.98%) was observed in T_4 (Bavistin was applied as foliar spray @ 0.3%). The order of efficiency of different management practices regarding percent reduction of canker incidence on seedlings of citrus were $T_3 > T_2 > T_1 > T_4$.

4.8.2.2. Effect of different antibacterial chemicals on the severity of canker on seedlings of citrus

For the application of different antibacterial chemicals, significant variations in the severity of canker on seedlings of citrus were observed during the period of October, 2011 to September, 2012 (Table 20). Out of all the treatments applied, lowest severity (0.00-0.21%) was observed in the month of January, 2012 which was remain almost static in the month of February, 2012 and it was gradually increased in the following months up to July, 2012 and then gradually decrease up to November, 2012. Considering the mean severity, highest severity (3.89%) was recorded in T_5 (untreated control), which was

statistically different from all other treatments and lowest severity (1.08%) was recorded in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%). In case of percent reduction of disease severity over control, highest reduction (72.24%) was observed in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%) and lowest reduction (18.51%) was observed in T_4 (Bavistin was applied as foliar spray @ 0.3%). The order of efficiency of different management practices regarding percent reduction of canker severity on seedlings of citrus were $T_3 > T_2 > T_1 > T_4$.

4.8.2.3. Effect of different antibacterial chemicals on the height of citrus seedlings

Significant variations in the height increase over first count were found under different treatments (Table 21). Maximum height increase over first count (46.11%) was recorded in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%) and minimum height increase over first count (36.14%) was recorded in T_5 (untreated control). Maximum height increase over control (27.59%) was recorded in T_3 (Indofil M-45 was applied as foliar spray @ 0.3%) and minimum height increase over control (9.99%) was recorded in T_4 (Bavistin was applied as foliar spray @ 0.3%).

CHAPTER V

DISCUSSION

Citrus is now an emerging fruit crop of Bangladesh was surveyed in eight nurseries under four districts of Bangladesh viz. Green orchid nursery, Barisal nursery, Gazipur nursery, Laxmipur nursery, Sarchina nursery, Riyad nursery, Hill Research Center and Ramghar nursery during the period of January, 2011 to December, 2012. Citrus canker was recorded as a common disease in all the surveyed nurseries. 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 seedlings 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 as the major problem in the nursery.

In the present study the causal organism of canker of citrus (*Xanthomonas axonopodis* pv. *citri*) was isolated from infected leaves following standard dilution plating 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 it's 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).

Prevalence of canker on seedlings of citrus varied in respect of nursery and location. 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 Khagraachari. Khagraachari 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 and rainfall. The climate of Bangladesh is characterized by high temperature, heavy rainfall, and often excessive humidity with fairly marked seasonal variations (Anonymous, 1995). ANOVAs, correlation and linear regression analysis were performed to determine the relationship between different components of climatic factor (temperature, relative humidity and rainfall) and the incidence as well as severity of canker on seedlings of citrus. Gilling (1986) reported that ANOVAs has been the fundamental method used by plant pathologist to determine the correlation between the prevalence and environmental parameters. 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 April and July while decreased in the month of January and October. Correlation regression analysis of prevalence of canker of citrus along with environmental parameters revealed that this increase and decrease were due the effect of temperature, relative humidity and rainfall. 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 temperatures, 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.

Among the different antibacterial chemicals evaluated in the present *in vitro* investigation, Indofil M-45 [Mancozeb] (37.67 mm) and Dithane M-45 [Mancozeb] (26.00 mm) exhibited significantly superior efficacy in inhibiting the growth of *Xanthomonas axonopodis* pv. *citri* while Champion [Copper hydroxide] (20.33mm), Cupravit [Copper oxychloride] (19.67 mm) and Sulcox [Copper oxychloride] (20.33 mm) were found moderately effective and Bavistin [Carbendazim] (9.67 mm) was less effective. In field experiment, significant effect of different management practices on the incidence and severity of canker of citrus and plant height of citrus were observed. Application of control measures resulted in gradual decrease of the incidence and severity of diseases over untreated control. The highest reduction of incidence and severity of canker of citrus was observed in applying Indofil M-45 [Mancozeb] as foliar spray. Champion [Copper hydroxide] and Cupravit [Copper oxychloride] were found moderately effective in reduction of incidence and severity of canker of citrus and Bavistin [Carbendazim] was less effective. Maher *et al.* (2005) reported that Dithane M-45 [Mancozeb] was effective against the multiplication of bacterium as well as effective in reducing the disease intensity of citrus canker. Meneguim *et al.* (2007) reported that copper as well as mixture of copper with mancozeb were effective in controlling citrus canker. Behlau *et al.* (2010), Graham and Leite (2004), Pan (2004), Verona *et al.* (2004), Das (2003), Zhong and Ling (2002), Chen *et al.* (2000) and Leite and Mohan (1990) reported that copper containing chemicals were effective in controlling citrus canker.

Comparative efficacy of five different biocontrol agents viz. *Bacillus subtilis*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, *Trichoderma harzianum* and *Aspergillus flavus* were evaluated *in vitro* against the causal agent of canker of citrus. Among the different biocontrol agents evaluated in the present investigation, *Bacillus subtilis* (19.00 mm) and *Pseudomonas fluorescens* (14.33 mm) exhibited significantly superior efficacy in inhibiting the growth of *Xanthomonas axonopodis* pv. *citri* while other biocontrol agents viz. *Rhizobium leguminosarum*, *Trichoderma harzianum* and *Aspergillus flavus* were ineffective against *Xanthomonas axonopodis* pv. *citri.* The results obtained in the present study were in agreement with the reports of earlier workers (Yenjerappa, 2009; Manonmani *et al*., 2007; Monteiro *et al.*, 2005; Kalita *et al.*, 1996; Chand *et al.*, 1991a and Unnamalai and Gnanamanickam, 1984). Kalita *et al.* (1996) reported that among the bioagents, *Bacillus subtilis* was most effective antagonist producing largest inhibition zone followed by *Pseudomonas fluorescens* against *Xanthomonas axonopodis*.

CHAPTER VI

SUMMARY AND CONCLUSION

Citrus (*Citrus* spp.) belongs to the family Rutaceae is an important fruit crop grown all over the world. 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. Seedlings 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 parameters in eight nurseries in four selected districts namely, Dhaka, Gazipur, Barisal and Khagrachari and to study the effective management strategies of the disease.

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 young twigs and stems were superficially similar to those on leaves but they were generally irregularly shaped.

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.

Survey conducted for two years revealed that incidence and severity of canker of citrus varied from location to location as well as season to season. Significant variations were observed in the incidence and severity under the variation of weather parameters. Highest incidence (72.22%) and severity (27.55%) of canker of citrus was recorded in the month of July at Khagrachari and 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 of citrus with temperature, relative humidity and rainfall.

Significance of different management practices *in vitro* was evaluated by measuring the inhibition zone and field evaluation was done by observing the incidence and severity of the disease at one month interval before each spray schedule. *In vitro* as well as field evaluation of antibacterial chemicals indicated that Mancozeb (Indofil M-45) and Copper compounds (Champion and Cupravit) were highly effective against *Xanthomonas axonopodis* pv. *citri*. Among five different biocontrol agents, *in vitro* evaluation revealed that *Bacillus subtilis* was highly effective against *Xanthomonas axonopodis* pv. *citri* followed by *Pseudomonas fluorescens* where other biocontrol agents were ineffective.

Present study revealed that the occurrence of canker on seedlings of citrus is related to the temperature, relative humidity and rainfall. 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 inoculam 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. *Bacillus subtilis* and *Pseudomonas fluorescens* should be incorporated in citrus canker

management system as eco-friendly alternatives to the hazardous chemical compounds.

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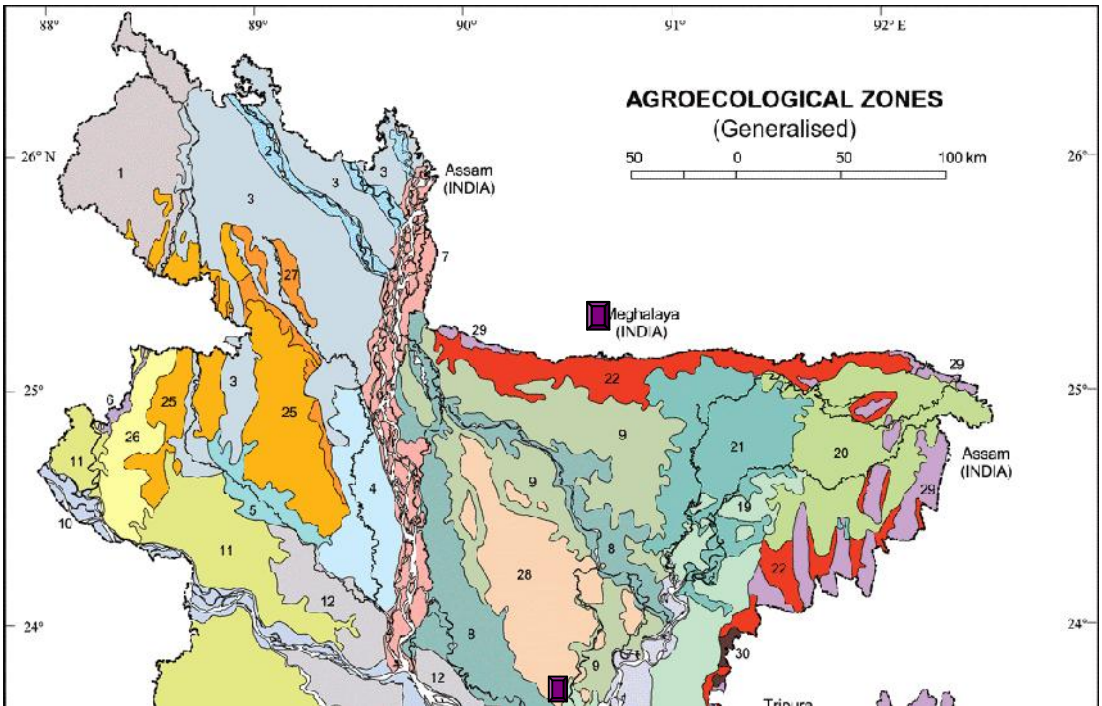
APPENDICES

Appendix I. Map showing the experimental site under study



- Position of experimental site

Appendix II. 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

Potato Dextrose Agar (PDA)

Peeled potato	200 g
Dextrose	20 g
Agar	17 g
Distilled water	1000 ml

Potato Dextrose Broth

Peeled potato	200 g
Dextrose	20 g
Distilled water	1000 ml

King's B (KB)

Proteose peptone No. 3 (Dico)	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (Anhydrous)	1.5 g
$MgSO_4.7H_2O$	1.5 g
Bacto agar	15.0 g
Distilled water	1000 ml

Yeast Malt Agar (YMA)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Bacto agar	20.0 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

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

KOH solubility reagent

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

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.

Table 19. Effect of different antibacterial chemicals on the incidence of canker of citrus during the growing period of October, 2011 to September, 2012

Treatments	% Disease Incidence									
	Oct., 2011	Nov., 2011	Dec., 2011	Jan., 2012	Feb., 2012	Mar., 2012	Apr., 2012	May 2012	Jun., 2012	Jul., 2012
T_1	66.00 c	52.67 c	36.00 b	0.00 c	0.00 c	11.33 c	34.00 c	39.33 c	96.00 b	97.33 a
T_2	66.00 c	50.67 c	35.33 b	0.00 c	0.00 c	0.00 d	33.33 c	38.00 c	94.00 b	94.00 b
T_3	60.67 d	36.67 d	22.67 c	0.00 c	0.00 c	0.00 d	0.00 d	34.00 d	89.33 c	88.67 d
T_4	90.67 b	69.33 b	60.67 a	39.33 b	38.67 b	41.33 b	47.33 b	48.00 b	100.00 a	100.00 a
T_5	100.00 a	100.00 a	63.33 a	42.67 a	43.33 a	44.00 a	50.67 a	53.98 a	100.00 a	100.00 a
$LSD_{(p\leq0.05)}$	2.74	3.21	3.03	2.17	2.17	2.00	2.95	3.38	2.97	2.45
CV (%)	2.66	3.87	5.19	9.85	9.85	7.71	6.65	5.91	2.31	1.90

Data represent the mean value of 5 (five) replications.
Values followed by the same letter within a column are not significantly different ($p\leq0.05$) according to Duncan's multiple range test.

T_1= Cupravit was applied as foliar spray @ 0.3%

T_2= Champion was applied as foliar spray @ 0.2%

T_3= Indofil M-45 was applied as foliar spray @ 0.3%

T_4= Bavistin was applied as foliar spray @ 0.3%

T_5= Untreated control

Table 20. Effect of different antibacterial chemicals on the severity of canker of citrus during the growing period of October, 2011 to September, 2012

Treatments	% Disease severity									
	Oct., 2011	Nov., 2011	Dec., 2011	Jan., 2012	Feb., 2012	Mar., 2012	Apr., 2012	May 2012	Jun., 2012	Jul., 2012
T_1	1.01 d	0.11 c	0.24 c	0.00 b	0.00 c	0.06 c	0.43 c	0.87 b	3.88 c	4.98 c
T_2	1.42 c	0.17 c	0.22 cd	0.00 b	0.00 c	0.00 d	0.41 c	0.83 b	3.74 c	4.76 c
T_3	0.73 e	0.08 c	0.20 d	0.00 b	0.00 c	0.00 d	0.00 d	0.06 c	3.28 d	3.91 d
T_4	2.69 b	1.84 b	0.39 b	0.19 a	0.54 b	0.73 b	0.81 b	0.87 b	4.96 b	9.56 b
T_5	3.57 a	3.30 a	0.44 a	0.21 a	0.80 a	0.81 a	1.02 a	1.02 a	7.09 a	10.32 a
$LSD_{(p\leq0.05)}$	0.25	0.14	0.04	0.04	0.04	0.04	0.07	0.09	0.15	0.23

CV (%)	9.79	9.52	9.44	9.14	5.00	6.30	9.89	9.36	2.34	2.53

Data represent the mean value of 5 (five) replications.
Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

T_1= Cupravit was applied as foliar spray @ 0.3%

T_2= Champion was applied as foliar spray @ 0.2%

T_3= Indofil M-45 was applied as foliar spray @ 0.3%

T_4= Bavistin was applied as foliar spray @ 0.3%

T_5= Untreated control

Table 21. Effect of different antibacterial chemicals on the height of citrus seedlings during the growing period of October, 2011 to September, 2012

Treatments	% Height (cm) of citrus seedling													
	Oct., 2011	Nov., 2011	Dec., 2011	Jan., 2012	Feb., 2012	Mar., 2012	Apr., 2012	May 2012	Jun., 2012	Jul., 2012	Aug., 2012	Sep., 2012	% Height (cm) increase from initial count	% Height (cm) increase or decrease over control
T_1	57.80 c	62.60 bc	66.20 b	70.60 b	74.40 b	77.40 b	82.00 b	85.80 b	90.20 c	94.60 c	98.80 c	104.4 c	44.64 b	23.52
T_2	60.40 b	64.60 ab	69.60 a	74.80 a	79.20 a	83.60 a	88.40 a	93.80 a	98.40 a	103.2 a	107.2 a	110.4 a	45.29 ab	25.32
T_3	58.20 c	63.00 bc	66.80 b	72.20 ab	78.20 a	83.40 a	88.20 a	91.80 a	96.20 b	100.2 b	104.4 b	108.0 b	46.11 a	27.59
T_4	58.80 bc	62.40 c	66.00 b	70.40 b	73.60 b	76.60 b	80.00 b	83.40 b	87.40 d	91.20 d	94.60 e	97.60 d	39.75 c	9.99
T_5	63.60 a	65.80 a	68.20 ab	71.20 b	74.60 b	79.20 b	82.60 b	85.80 b	89.60 c	93.60 c	97.00 d	99.60 d	36.14 d	
$LSD_{(p \leq 0.05)}$	1.64	1.91	2.06	2.64	2.32	3.09	2.57	2.44	2.19	2.24	1.70	2.08	0.95	
CV (%)	2.04	2.24	2.28	2.74	2.2	2.8	2.2	2.0	1.7	1.7	1.2	1.4	1.67	

					8	8	7	6	7	3	6	9		

Data represent the mean value of 5 (five) replications.
Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

T_1= Cupravit was applied as foliar spray @ 0.3%

T_2= Champion was applied as foliar spray @ 0.2%

T_3= Indofil M-45 was applied as foliar spray @ 0.3%

T_4= Bavistin was applied as foliar spray @ 0.3%

T_5= Untreated control