STUDY ON THE EFFECT OF SALT STRESS ON CITRUS CANKER CAUSED BY *XANTHOMONAS AXONOPODIS* PV. *CITRI* AND EFFICACY OF SOME PHYTOHORMONES IN INDUCING RESISTANCE AGAINST THE DISEASE

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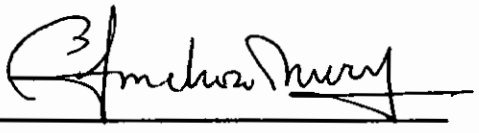

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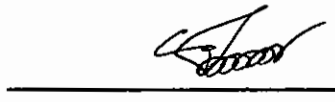

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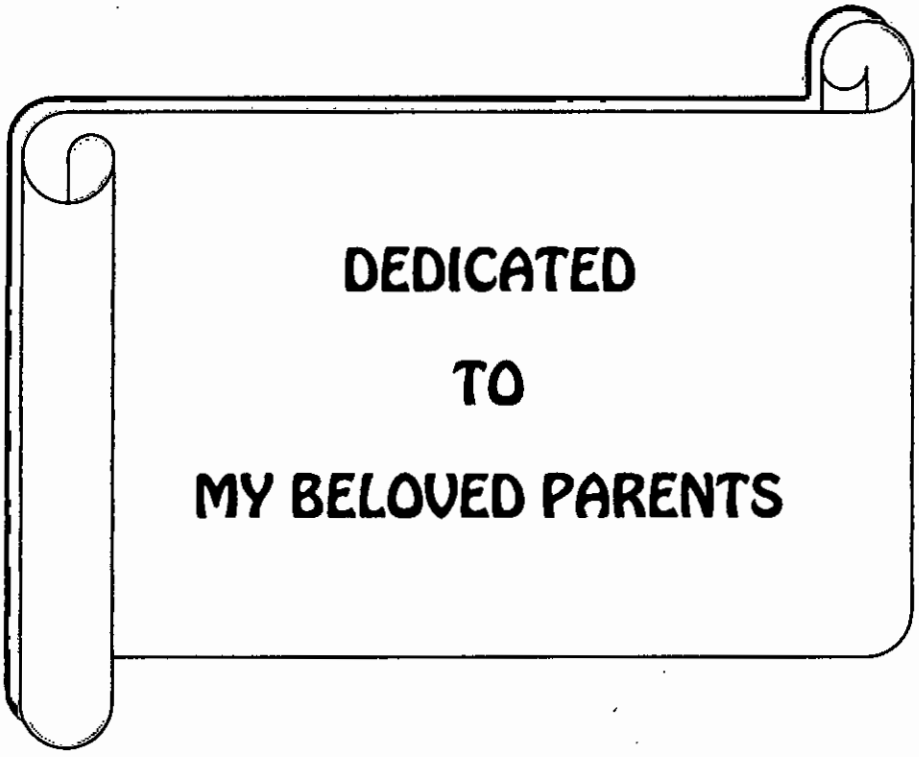

DEDICATED

TO

MY BELOVED PARENTS

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All praises are devoted to Almighty Allah, the most gracious, the most merciful and the supreme ruler of the universe, who enabled the author to complete the thesis successfully for the degree of Master of Science (MS) in Plant pathology.

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STUDY ON THE EFFECT OF SALT STRESS ON CITRUS CANKER CAUSED BY *XANTHOMONAS AXONOPODIS* PV. *CITRI* AND EFFICACY OF SOME PHYTOHORMONES IN INDUCING RESISTANCE AGAINST THE DISEASE

ABSTRACT

Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Xac) is currently the most prominent disease impacting world citrus production. A study was conducted to investigate the effect of salt stress on bacterial canker pathogen and the efficacy of some selected phytohormones in inducing resistance against canker disease of citrus. The study was conducted during the period of January to December, 2015 in the experimental site of department of Plant Pathology at Sher-e-Bangla Agricultural University. The putative causal organism of citrus canker was identified as *Xanthomonas axonopodis* pv. *citri* on the basis of morphological, biochemical and cultural features. Citrus plants were exposed to salt stress by irrigating with 50 mM, 100 mM, 150 mM, and 200 mM salt concentration on weekly basis and inoculated with Xac. Abiotic stress influenced the defense response to pathogen and thus lesion number and size, disease incidence and severity were gradually increased with the increase of salt concentration. At the same time physiological performance of the plant such as net assimilation rate, chlorophyll content, stomatal conductance and transpiration rate were increased while intercellular CO_2 concentration was decreased up to 30 days after inoculation (DAI) at 100 mM salt concentration. Beyond this range bacterial performance to modulate plant homeostasis was reduced. Application of five selected phytohormones viz. salicylic acid (SA), jasmonic acid (JA), 3-indoleacetonitrile (IAN), folic acid (FA) and nicotinic acid (NA) curtailed disease development. Among the chemicals SA showed the highest performance in case of increasing latent period and decreasing lesion number and size, disease incidence and severity followed by JA, IAN, FA and NA compared to control. None of these phytohormones showed *in-vitro* antibacterial properties against Xac bacterium. SA treated plants showed the highest accumulation of endogenous total phenolic compound and potassium content in leaves cell followed by JA, INA, FA and NA compared to control. This provides evidence that the phytohormones had the ability to induce resistance against bacterial canker pathogen.

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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

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

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 States of America

NA = Nutrient Agar (media)

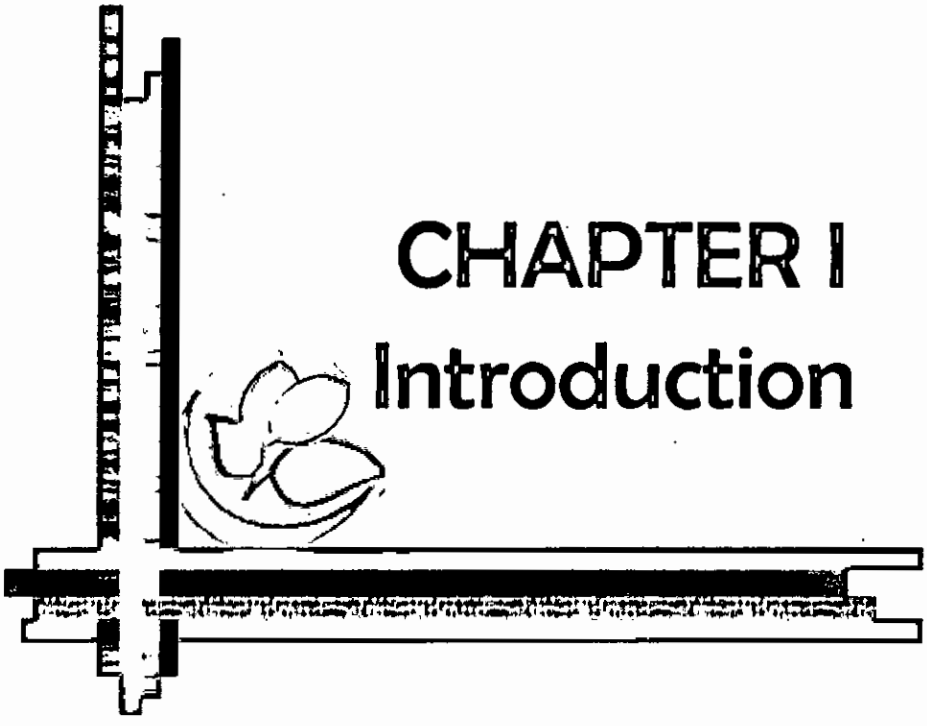
YDCA = Yeast Extract Dextrose Calcium carbonate Agar (media)

PSI = Per Square Inch

ANOVA = Analysis of variances

LSD = Least Significant Difference

CV% = Percentages of Co-efficient of Variance



CHAPTER I
Introduction

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INTRODUCTION

Citrus belonging to the family Rutaceae, is one of the most paramount nutritious fruit crops of the world as well as in Bangladesh. It has gained popularity because of its nutritive value, taste, aroma and economic importance among the people and is still booming (Whitney and Rolfes, 1999). There is considerable evidence that citrus foods may help reduce the risk, or retard the progression, of several serious diseases and disorders such as cardiovascular disease, cancer, anemia etc (Harats *et al.*, 1998 and Reuther *et al.*, 1967). Annual total citrus production in the world is 117 million tons among which lime and lemon production is 6.8 million tons (FAO, 2014). Bangladesh is ranked 52 in the world and has a 0.1% share in citrus production worldwide (FAOSTAT, 2012). In Bangladesh, the total acreage under citrus cultivation is about 5,995 ha while the total production is around 144,000 mt (BBS, 2015). In 2014-2015 fiscal year Bangladesh has exported 60 thousand 180 kg citrus in Europe alone and the amount is USD 2,06,111 (EPB, 2015). 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. There is a large scope of expanding citrus production in our country. Various factors are responsible for lower citrus production in Bangladesh. Among them, plant disease is one of the major influential factors. Different species of citrus grown in the world suffers from more than 100 diseases (Klotz, 1973). Among them twelve diseases are known to occur in different species of citrus in Bangladesh (FAO, 2014). Citrus canker is the most widespread and prevalent disease betwixt these diseases (Graham *et al.*, 2004; Gottwald *et al.*, 2002 and Koizumi, 1985). The disease caused by the bacterium *Xanthomonas axonopodis.* It is originated in Southeast Asia, is extremely persistent when it becomes established in an area (Wolf, 2016). 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. Citrus canker is distributed over thirty countries of the world (Das, 2003). This disease hampers citrus production not only by impeding quality of produces but also reducing economic value of the fruits. According to USDA approximately 50 million dollars per year are spending for management of this disease (USDA, 2014). The bacterium *Xanthomonas*

axonopodis pv. *Citri* is a rod-shaped, gram-negative, and has a single polar flagellum. Colonies on laboratory media are usually yellow due to 'xanthomonadin' pigment production. Still now four types of citrus canker are found. Canker A (Asiatic canker) is found in Asia, South America, Oceania and the USA (Carrera, 1933); canker B (Cancrosis B) in South America (Carrera, 1933); canker C (Mexican lime cancrosis) in Brazil (Schaad *et al.*, 2005); and canker D (citrus bacteriosis) in Mexico (Rodriguez *et al.*, 1985). It gives positive result in KOH solubility test, starch hydrolysis test, catalase test, asculine hydrolysis, urease production, milk proteolysis, tween 80 lypolysis, gelatine liquefaction test, salt tolerant test, tobacco hypersensitivity reaction and gives negative result in oxidase test (Kishun and Chand, 1991). The bacterium produces 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). Asian citrus leaf miner is the vector of this pathogen wounding induced by the larvae increases infection by *X. axonopodis* pv. *citri* during the flush periods (Schubert *et al.*, 2001; Gottwald *et al*, 1997; Cook, 1988; Sinha *et al.*, 1972; Sohi and Sandhu, 1968 and Nirvan, 1961). Environmental factors play foremost role in the susceptibility of citrus plants to canker. 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). *Xanthomonas axonopodis* pv. *citri* contains a gene encoding a PNP-like protein, XacPNP, that shares significant sequence similarity and identical domain organization with plant PNPs but has no homologues in other bacteria (Gottig *et al.*, 2008). Still now Xac is the only bacteria that can produce XacPNP gene and these PNPs (Plant Natriuretic Peptides) are mobile protein signaling molecules those are secreted into the apoplast particularly under conditions of biotic and abiotic stress to regulate plant homeostasis (Wang *et al.*, 2011). During salt stress condition plants up regulate PNPs which helps to maintain morphological and physiological growth in plants (Rafudeen *et al.*, 2003).

In nature, plants are often exposed to a combination of different stress factors. Among those wide variety of abiotic and biotic stressors, salinity and pathogens are important factors affecting plant health and productivity. Salt stress presents an increasing threat to worldwide agriculture. Salinity is a build-up of soluble salts (Levy and Syvertsen, 2004) which causes adverse morphological, physiological, and biochemical effects in

different organs of citrus plants through an increased concentration of sodium and chloride (Raveh and Levy, 2005; Camara-Zapata *et al.*, 2004; Rachmilevitch *et al.*, 2004 and Zekri, 2004; Boman, 1993; Maas, 1993 and Banuls and Millo, 1992). Prevalence of citrus diseases is increased under salt stress condition. It has been suggested by Afek, (1993); Blaker, (1986); Dann *et al.*, (1998); and Willers and Holmden, (1980). NaCl has severe and various effect on citrus amid many other salts because it liberates 60% more ions (Aranda *et al.*, 1998). It is known that exposure to one stressor can alter plant response to the subsequent stress and both positive and negative interactions between abiotic and biotic stresses have been reported (Desprez-Loustau *et al.*, 2006 and Knight *et al.*, 1998). Although the impact of individual stressors e.g. drought, salinity, chilling, pathogen infection have been extensively studied, little is known about how a combination of different stresses, applied simultaneously or sequentially, affects plants. Saline stress can reduce or enhance disease severity. Enhanced resistance of barley (*Hordeum vulgare*) against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) was induced by salt stress (Wiese *et al.* 2004). However, the resistance of tomato to *Pseudomonas syringae* pv. *tomato* was not affected by salinity (Thaler and Bostock, 2004) and in Arabidopsis stress-triggered increased concentration of abscisic acid induced susceptibility to *P. syringae* pv. *tomato* (Mohr and Cahill, 2007). In cucumber salt stress increase disease severity and induce susceptibility to *Pseudomonas syringae* pv. *lachrymans* (Chojak *et al.*, 2012). Besides salinity stress inhibit defense mechanism of citrus plant against *Phytophthora* pathogen (Blaker and Mcdonald, 1986).

Appropriate management of citrus canker has been investigated by many researchers (Singh *et al.*, 2005; Canteros, 2004; Graham and Leite, 2004; Das and Shyam, 2003; Dixon *et al.*, 2000; Gottwald and Timmer, 1989; Civerolo, 1981; Stall *et al.*, 1981 and Koizumi, 1977). Still now no pesticide has been found effective against this disease. Besides this agro pesticides are noxious for environment (Huang, 1997). Therefore concentration is focused on propitious method of disease management that will be friendly for environment as well as for mankind (Sutton, 1996). Induced resistance (IR) in plant by spraying various phytohormones is one of the best alternative methods of disease management. Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges (VanLoon

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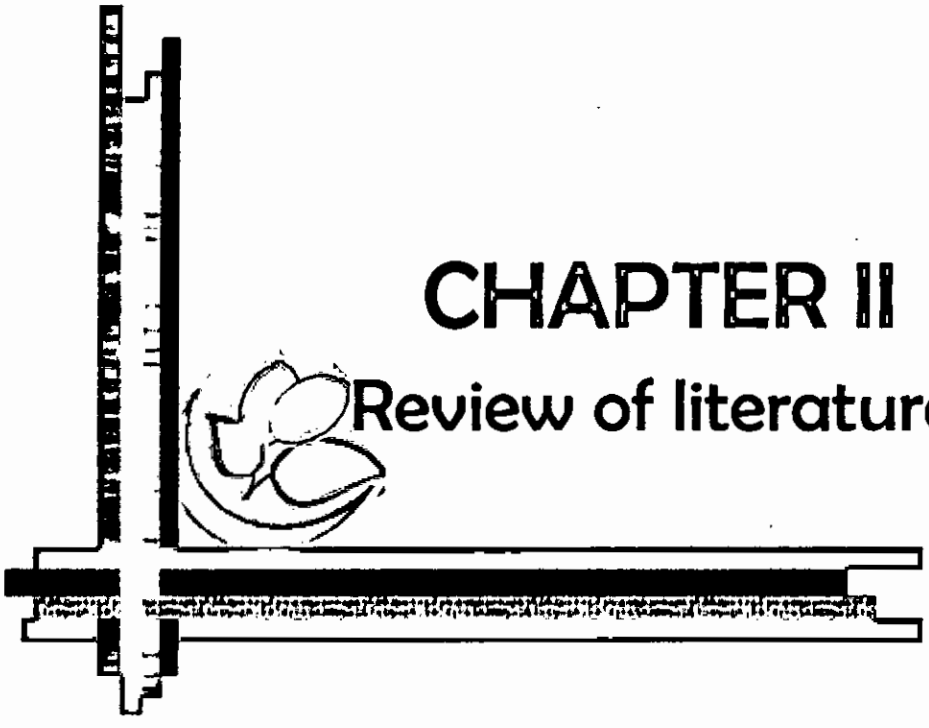
et al., 1998). The two most clearly defined forms of induced resistance are Systemic acquired resistance (SAR) and Induced systemic resistance (ISR) which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved, as demonstrated in model plant systems (Yan *et al.*, 2002; Maleck *et al.*, 2000; Schenk *et al.*, 2000; Knoester *et al.*, 1999; Pieters *et al.*, 1996, Uknes *et al.*, 1992; Ward *et al.*, 1991. ISR is induced by jasmonic acid and ethylene molecules signaling pathway and it is salicylic acid independent, hence no PR protein are synthesized (Choudhary *et al.*, 2007; Yan *et al.*, 2002 and Knoester *et al.*, 1999). On the other hand SAR is induced by salicylic acid signaling pathway and also synthesized PR protein. This protein functions as antimicrobial cell wall degradation of pathogen. They also possess lytic chitinase and peroxidase enzymes which are functional against pathogen. SAR once induced remains active for long time (Vallad and Goodman, 2004). Phytohormones such as salicylic acid, jasmonic acid, indoles, nicotinic acid, folic acid play critical role in induced resistance against plant diseases (Konan *et al.*, 2014; Li and Wang, 2007; Wang and Liu, 2012; Esmailzadeh *et al.*, 2008; Meir *et al.* 1998; Creelman, *et al.*, 1995; Conrath *et al.*, 1995; Hoffland *et al.*, 1995; Cohen *et al.*, 1993; and Chaudhry *et al.*, 1994). Salicylic acid (SA), a plant hormone plays an important role in induction of plant defense against a variety of biotic and abiotic stresses through morphological, physiological and biochemical mechanisms. It has important roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also induces specific changes in leaf anatomy and chloroplast structure. SA is involved in endogenous signaling, mediating in plant defense against pathogens (Hayat and Ahmad, 2007). Exogenous application of SA in plant reduces disease severity and incidence (Hadi and Balali, 2010; Yao and Tian, 2005 and Saikia *et al.*, 2003). Jasmonic acids are a class of lipidic plant hormones, synthesized from linolenic acid present in the chloroplast membrane. They are involved in development of abiotic stress responses and plant-microbes interactions in defense and symbiosis (Carlos, 2011; Galis *et al.*, 2009 and Delker *et al.*, 2006). Foliar application of JA induced the generation of a wide range of secondary plant metabolites, like alkaloids, terpenoids, flavonoids, coumarins, stilbenoids, hydroxycinnamic acids, and so forth. (Faurie *et al.*, 2009 and Belhadj *et al.*, 2006). 3-indolylacetonitrile (IAN) is a naturally occurring plant growth hormone (auxin) (Jones *et al.*, 1952). This anti-virulence compounds have been suggested as alternative ways to fight infectious diseases because unlike antimicrobials, anti-

virulence compounds do not affect growth and so there is less chance of developing resistance (Lesic *et al.*, 2007 and Hentzer *et al.*, 2003). Foliar application of IAN reduced canker severity by inhibiting biofilm formation in citrus plants (Li and Wang, 2013). Folic acid was first isolated in and extracted from spinach leaves by Mitchell and others in 1941 (Mitchell *et al.*, 1941). As major carriers and donors of one-carbon units, folic acids are involved in a wide range of key metabolic functions including the biosynthesis of nucleic acids, amino acids and pantothenate, lignin formation and photorespiration (Hanson and Roje, 2001). These compounds are involved in phtotosynthesis, biochemical conversion of nitrogen and carbon, synthesis and catabolism of amino acids (Basset *et al.*, 2004; Cossins, 2000 and Brown *et al.*, 1985). Folic acid is active in plant in reduced form as tetrahydrofolic acid and tetrahydrofolic coenzyme (Stakhova *et al.*, 2000) and induces SAR in arabidiopsis against *Alternaria brassicicola* (Wittek *et al.*, 2015). Nicotinic acid and its derivatives are potent inducers of plant defense responses including the synthesis of pathogenesis-related (PR) proteins and the development of enhanced disease resistance. NA was found to improve the resistance of rice against *Magnaporthe grisea* and *Xanthomonas oryzae* (Smith and Metraux, 1991). It regulates oxidation and reduction process within the cell and produce secondary metabolites such as alkaloids (Behrman, 1976 and Mothes and Schutte, 1969). NA showed moderate defense against diseases in sugarcane, radish, cucurbits crops (Rabab *et al.*, 2013; Sundar *et al.*, 2012 and Hoffland *et al.*, 1995).

Climatic condition favors citrus production in Bangladesh. But quality production of citrus is hampered due to canker disease. Moreover less information are available in respect of epidemiology and management of this disease. Considering the above facts this research program has been designed with the following objectives:

1. To isolate and identify the pathogen (s) associated with the disease
2. To observe the influence of salt stress condition on the disease severity
3. To induce resistance in citrus against *Xathomonas axonopodis* pv. *citri* by using some phytohormones



CHAPTER II

Review of literature

CHAPTER II

REVIEW OF LITERATURE

Asiatic form of citrus canker (Canker A), caused by the bacterium *Xanthomonas axonopodis* pv. *citri,* is a leaf, fruit and stem blemishing disease that affects most citris. In recent years, this disease has become a major threat for citrus production worldwide. Researches have been done for many years on this disease and still it is ongoing. Wherefore, the literature related to the canker of citrus along with information on the effect of salt stress and induction of resistance with phytohormones in relation to the disease and pathogen are reviewed here as under.

2.1. Symptomology

Balestra *et al.* (2008) proclaimed that canker lesions are hyperplasia type, 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.

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

Brunings and Gabriel (2003) noticed on citrus 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.

Graham *et al.* (2004) reported that the earliest symptoms on leaves appear as tiny, slightly raised blister-like lesions beginning around 9 days post-infection. As the lesions age, they first turn light tan, then tan to brown, and a water-soaked margin

appears, often surrounded by a chlorotic halo. The water soaked margin may disappear as the lesions age, and is not as prominent on resistant cultivars. The centre of the lesion becomes raised and spongy or corky. These raised lesions from stomatal infection are typically visible on both sides of a leaf. Eventually, the centres of the leaf lesions become crater-like. Defoliation becomes a problem as the disease intensifies. On twigs and fruit, citrus canker symptoms are similar: raised corky lesions surrounded by an oily or water-soaked margin. No chlorosis surrounds twig lesions but may be present on fruit lesions. Twig lesions on angular young shoots perpetuate the inoculum and prolong survival of *X. axonopodis* pv. *citri* in areas where citrus canker is endemic. If twigs are not killed back by girdling infections, the lesions can persist for many years, causing raised corky patches in the otherwise smooth bark.

Pruvost *et al.* (2002) expressed that the bacterium multiplies in lesions in leaves, stems and fruit. When there is free moisture on the lesion surface, bacteria are released from an extracellular polysaccharide matrix and dispersed to new growth by rain splash.

Swarup *et al.* (1991) noted that citrus canker lesions were first appeared as pin-point spots that became small, slightly raised pustules or blister-like eruptions. Initially, those appear on the lower leaf surface 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, light colored at first and became tan or brown later.The epidermis ruptured and the lesions became spongy or corky at matured stage. The lesions finally became crater-like with a raised margin and sunken centre. The centre of large, old lesions cracked and/or dropped out.

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

2.2. Isolation and identification of the pathogen

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.

Braithwaite *et al.* (2002) detected 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.

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

Chand and 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.

Goto (1962) exposed that the minimal 10^5 cfu/ml dose of *Xanthomonas* was necessary for stomatal infection and that for wound infection, about 10^2 to 10^3 cells/ml were required.

Gottwald and Graham (1992) observed that the concentrations less than 10^4 cfu/ml of *Xanthomonas* were insufficient to cause infection on unwounded citrus leaves under an impact pressure of 8.05 kPa, however 10^6cfu/ml gave consistent and successful infection.

Jabeen *et al.* (2012) noticed that *Xanthomonas* gave yellow, circular, smooth, convex and viscous bacterial colonies on yeast dextrose calcium carbonate agar medium (YDCA) after 48-72 h of incubation at 28°C. On SX medium the bacteria gave light

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

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

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.

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.

2.3. Effect of salt stress on disease development

Blacker and Mcdonald (1986) revealed that root rot of citrus caused by *Phytophthora parasitica* increased with the increase of salinity. Seedling grown for nine week in

salinized soil and infested with pathogen had 30% root decay while plants in infested nonsaline soil had only 10% decay.

Chojak *et al.* (2012) reported that with the increase of salt concentration the disease severity also increased. When cucumber plant treated with 50 mM and 100 mM NaCl solution infection of *Pseudomonas syringae* pv. *lachrymans* increased from 50 mM to 100 mM treated plants. Abiotic stress compromised the defence response to pathogen and disease severity was the highest in 100 mM NaCl-treated plants. The reduced performance of salinized plants under biotic stress could be related to salt stressinduced plant growth inhibition. The response of NaCl-treated and control plants to bacterial infection differed in terms of H_2O_2 generation and lipid peroxidation.

Garavaglia *et al.* (2010) affirmed that the citrus pathogen *Xanthomonas axonopodis* pv. *citri* possesses a PNP-like peptide (XacPNP) uniquely present in this bacteria. This pathogen can use the plant-like hormone to modulate the host cellular environment and in particular host metabolism and that such modulations weaken host defence. Chlorophyll fluorescence parameters and water potential of citrus leaves infiltrated with recombinant purified XacPNP were measured and demonstrated that the peptide improves the physiological conditions of the tissue. Importantly, the proteomic analysis revealed that these responses are mirrored by rapid changes in the host proteome that include the up-regulation of Rubisco activase, ATP synthase CF1 a subunit, maturase K, and a- and b-tubulin.

Gottiga *et al.* (2008) observed that the bacterial citrus pathogen, *Xanthomonas axonopodis* pv. *citri,* also contains a gene encoding a PNP-like protein, XacPNP, that shares significant sequence similarity and identical domain organization with plant PNPs but has no homologues in other bacteria. XacPNP is not expressed under standard nutrient rich culture conditions; it is strongly induced under conditions that mimic the nutrient poor intercellular apoplastic environment of leaves, as well as in infected tissue, suggesting that XacPNP transcription can respond to the host environment. The lesions caused by this mutant that lacked XacPNP were more necrotic than those observed with the wild-type, and bacterial cell death occurred earlier in the mutant. Moreover, when XacPNP were introduced in *Xanthomonas axonopodis* pv. *citri,*,the transgenic bacteria caused less necrotic lesions in the host than the wild-type. They also reported that plant-like bacterial PNP can enable a plant

pathogen to modify host responses and alter the physiological response such as stomatal conductance, net assimilation rate and water uptake to create conditions favorable to its own survival.

Nembaware *et al.* (2004) reported that *Xanthomonas axonopodis* pv. *citri* contains a gene encoding a PNP-like protein. Bacterial protein can alter plant cell homeostasis and thus is likely to represent an example of molecular mimicry that enables the pathogen to manipulate plant responses in order to bring about conditions favourable to the pathogen such as the induced plant tissue hyper-hydration seen in the wet edged lesions associated with *Xanthomonas axonopodis* infection.

Pharmawati *et al.* (1998) found that PNP helps to stomatal opening and water uptake into Xylem tissue. PNP elevated cyclic guanosine-3′, 5′-monophosphate (cGMP) in guard cell protoplasts. Effect of cGMP on stomatal opening is linked to Ca^{2+} levels. PNP fractions rapidly and specifically increased cGMP levels in stele tissue isolated from maize (*Zea maydis*) roots within 30 s and increased radial water movements out of the xylem of shoots.

Rafudeen *et al.* (2003) observed that more amount of PNP was up regulated in Arabidiopsis thaliana when treated with 300 mM NaCl solution than 100 mM solution. PNP was rapidly up regulated in higher salt stress condition than lower salt stress condition.

Ruiz *et al.* (1995) found that relative growth rate (RGR), net assimilation rate on a leaf weight basis (NARw), leaf weight ratio (LWR), and nutrient uptake of citrus plant treated with 80 mM salt concentration were reduced than non treated plants. Salinity had a significant effect on leaf concentrations of Cl, Na, K, Ca, Mg, P, Fe, Mn and Zn ion.

Turek *et al.* (2014) observed that Plant natriuretic peptides (PNPs) are secreted into the apoplast, are systemically mobile and elicit a range of responses signaling via cGMP. The PNP-dependent responses include tissue specific modifications of cation transport and changes in stomatal conductance and the photosynthetic rate. PNP also has a critical role in host defense responses. Surprisingly, PNP-homologs are produced by several plant pathogens during host colonization suppressing host defense responses.

Wang *et al.* (2011) reported that higher plants contain biologically active plant natriuretic peptide (PNP) like proteins have a role in the regulation of homeostasis in abiotic and biotic stresses. PNP (PNP-A) is mainly expressed in leaf mesophyll cells, and it is secreted using AtPNP-A: green fluorescent protein (GFP) reporter constructs and flow cytometry and can enhance its own expression. PNP-A expression is enhanced by heat, osmotica and salt stress.

Willers and Holmden (1980) noted that nematode reduced salt tolerance of citrus root and increased Cl uptake. Soil salinity caused a breakdown in root chemical defenses and increased susceptibility of citrus root to attack by the citrus nematode. Moreover intermittent salinity stress increased the nematode population density more than continues irrigation with saline water.

Yassin *et al.* (2004) reported that primarily salt-stress lowers net CO_2 assimilation, stomatal conductance, and water potential of citrus tree leaves, in addition to accumulation of excessive concentration of Chloride or Sodium in leaves.

2.4. Management of citrus canker with phytohormone chemicals

Ahn *et al.* (2007) expressed that Thiamine-treated rice, Arabidopsis (*Arabidopsis thaliana*), and vegetable crop plants showed resistance to fungal, bacterial, and viral infections. Thiamine treatment induces the transient expression of pathogenesis-related (PR) genes in rice and other plants. In addition, thiamine treatment potentiates stronger and more rapid PR gene expression and the up-regulation of protein kinase C activity. The effects of thiamine on disease resistance and defense-related gene expression mobilize systemically throughout the plant and last for more than 15 d after treatment. Treatment of Arabidopsis ecotype Columbia-0 plants with thiamine resulted in the activation of PR-1 but not PDF1.2. Furthermore, thiamine prevented bacterial infection in Arabidopsis mutants insensitive to jasmonic acid or ethylene but not in mutants impaired in the SAR transduction pathway. They demonstrated that thiamine induces SAR in plants through the salicylic acid and Ca^{2+}-related signaling pathways.

Awang *et al.* (2015) found that double spray of jasmonic acid showed less stress symptoms in different antioxidant enzymes activities (GPX, APX and CAT), reduce

percentage of disease incidence and severity of leaf curl disease and chilli veinal mottle virus as well as improve growth and yielding characters of chilli plant.

Beheshti *et al.* (2011) reported that β-Aminobutyric Acid (BABA), green tea and salicylic acid had inhibitory effects on citrus canker disease development. They showed that application of this chemicals increased the mRNA levels of β-1,3-glucanase and chitinase, during disease development and also reduced lesion size.

Chen *et al.* (1999) reported that level of salicylic acid was increased in cucumber plant when treated with *Pseudomonas spp* against *Pythium aphanidermatum*. SA levels were significantly higher in plants treated with bacteria compared to control, from one to five days after bacterization. SA did not inhibit mycelial growth of *Pythium aphanidermatum* at 100–200 μgml^{-1} in vitro, but higher levels inhibited mycelial growth. Zoospore germination increased at concentrations of 10–500 μgml^{-1}, but decreased at 1000 μgml^{-1} compared to lower concentrations.

Droby *et al.* (1999) found that postharvest application of jasmonates reduced decay caused by the green mold *Penicillium digitatum* after either natural or artificial inoculation of grapefruit (*Citrus paradisi* 'Marsh Seedless'). These treatments also effectively reduced chilling injury incidence after cold storage. The most effective concentration of jasmonates for reducing decay in cold-stored fruit or after artificial inoculation of wounded fruit at 24 °C was 10 μmol·L–1. Higher and lower jasmonate concentrations were less effective at both temperatures. MJ at 10 μmol·L–1 also most effectively reduced the percentage of fruit displaying chilling injury symptoms after 6 weeks of storage at 2 °C and 4 additional d at 20 °C. When tested in vitro, neither JA nor MJ had any direct antifungal effect on P. digitatum spore germination or germ tube elongation.

Hoffland *et al.* (1995) reported that application of isonicotinic acid on radish plant could not increase disease resistance while salicylic acid could. Salicylic acid induced resistance in plant by activating pathogenesi related PR gene.

Samia and Khallal (2007) observed that Induction of plant defense against pathogen attack is regulated by a complex network of different signals. Results appeared that production of reactive oxygen species (ROS), mainly H_2O_2 and $O^{\cdot}$, and lipid peroxidation increased in tomato leaves by increasing the time of infection with

Fusarium oxysporum. Salicylic acid (SA) treated plants had the highest Malondialdehyde (MDA) level but jasmonic acid (JA) treated plants recorded the highest LOX activity. SA especially when applied alone markedly decreased H_2O_2 scavenging enzymes (APX and CAT) and greatly increased SOD activity. Thus, imbalance between H_2O_2 generation and scavenging enzymes in leaves may reflect a defense mechanism in tomato or a pathogenicity strategy of the fungus. Levels of certain phenolic acids greatly changed in tomato leaves in response to Fusarium oxysporum. Benzoic and Galleic acids contents markedly decreased, however, contents of coumaric, cinnamic, chlorogenic and ferulic acids increased in leaves when treated with SA and JA. Also, activity of lignification enzymes POX, PPX and PAL significantly increased in leaves of infected tomato plants. JA-treated plants caused the highest POX and PPX activities, while SA-treated plants having the highest PAL activities.

Kim *et al.* (2011) found that exogenous 3-indolylacetonitrile markedly inhibits the heat resistance of *Paenibacillus alvei* without affecting cell growth. Observation of cell morphology with electron microscopy showed that indole inhibited the development of spore coats and cortex in *P.alvei*. 3-indolylacetonitrile also decreased *P. alvei* survival.

Kogel *et al.* (1994) found that treatment of susceptible barley (*Hordeum vulgare*) seedlings with 2, 6-dichloroisonicotinic acid (DCINA) induces disease resistance against the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*). A cytological analysis of the interaction reveals the hypersensitive cell collapse in attacked, short epidermal cells, along with the accumulation of fluorescent material in papillae that appear at the time of fungal arrest. The cell-type-specific hypersensitive reaction occurs prior to formation of haustoria, reminiscent of the mechanism identified in genetically resistant barley plants containing the functionally active Mlg gene.

Konan *et al.* (2014) reported that the total phenolic content significantly increased after MeJA 5.0 mM treatments compared to the other tested concentrations (0; 2.5; 10; 15; 20 mM). Among the eleven phenolic compounds which were found except for ferulic acid, gossypetin, gossypol, 3-p-coumaroylquinic acid, and piceatannol were identified as major phenolic constituents of cotton. Their content also significantly increased after the MeJA treatment. In addition, gossypol increased 64 times

compared to the control, in the 5.0 mM MeJA treatment. Furthermore, cichoric acid, chlorogenic acid, and pterostilbene are synthesized de novo in leaves of MeJA-treated plant. Treatment of cotton leaves with MeJA 5.0 mM followed 72 h of incubation hampered the expression of Fusarium wilt caused by *Fusarium oxysporium* f. sp. *vasinfectum* (FOV). Disease severity on MeJA-treated leaves was significantly lower as compared to the control.

Lee *et al.* (2011) reported that intercellular signal indole and its derivative hydroxyindoles inhibit *Escherichia coli* biofilm and diminish *Pseudomonas aeruginosa* virulence. However, indole and bacterial indole derivatives are unstable in the microbial community because they are quickly degraded by diverse bacterial oxygenases. Hence, this work sought to identify novel, non-toxic, stable and potent indole derivatives from plant sources for inhibiting the biofilm formation of *E. coli* O157:H7 and *P. aeruginosa*. Here, plant auxin 3-indolylacetonitrile (IAN) was found to inhibit the biofilm formation of both *E. coli* O157:H7 and *P. aeruginosa* without affecting its growth. IAN more effectively inhibited biofilms than indole for the two pathogenic bacteria. Additionally, IAN decreased the production of virulence factors including 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), pyocyanin and pyoverdine in *P. aeruginosa*. DNA microarray analysis indicated that IAN repressed genes involved in curli formation and glycerol metabolism, whereas IAN induced indole-related genes and prophage genes in *E. coli* O157:H7. It appeared that IAN inhibited the biofilm formation of *E. coli* by reducing curli formation and inducing indole production. Also, corroborating phenotypic results of *P. aeruginosa*, whole-transcriptomic data showed that IAN repressed virulence-related genes and motility-related genes, while IAN induced several small molecule transport genes. Furthermore, unlike bacterial indole derivatives, plant-originated IAN was stable in the presence of either *E. coli* or *P. aeruginosa*.

Li and Wang (2013) reported that small molecules inhibiting biofilm formation reduce *Xanthomonas citri* pv. *citri* infection and enhance the control of citrus canker disease. D-leucine and 3-indolylacetonitrile (IAN) were found to prevent biofilm formation by *X. citri* subsp. citri on different abiotic surfaces and host leaves at a concentration lower than the minimum inhibitory concentration (MIC). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis indicated that

IAN repressed expression of chemotaxis/motility related genes in *X. citri* subsp. *citri.* D-leucine and IAN applied alone or combined with copper reduced both the number of canker lesions and bacterial populations of *X. citri* subsp. *citri* on citrus host leaves.

Mandal *et al.* (2009) reported that exogenous application of 200 microM salicylic acid through root feeding and foliar spray could induce resistance against *Fusarium oxysporum* f. sp. *lycopersici* (Fol) in tomato. Endogenous accumulation of free salicylic acid in tomato roots was detected by HPLC and identification was confirmed by LC-MS/MS analysis. At 168 hr of salicylic acid treatment through roots, the endogenous salicylic acid level in the roots increased to 1477 ng/g FW which was 10 times higher than control plants. Similarly, the salicylic acid content was 1001ng/g FW at 168 hr of treatment by foliar spray, which was 8.7 times higher than control plants. The activities of phenylalanine ammonia lyase (PAL) and peroxidase (POD) were 5.9 and 4.7 times higher, respectively than the control plants at 168h of salicylic acid feeding through the roots. The increase in PAL and POD activities was 3.7 and 3.3 times higher, respectively at 168h of salicylic acid treatments through foliar spray than control plants. The salicylic acid-treated tomato plants challenged with Fol exhibited significantly reduced vascular browning and leaf yellowing wilting. The mycelial growth of Fol was not significantly affected by salicylic acid.

Ojha and Chatterjee (2012) reported that application of salicylic acid (SA) and a biocontrol agent, *Trichoderma harzianum* (TH) on tomato plant reduce the infection of *Fusarium oxysporum* f. sp. *lycopersici.* Tomato plants treated with different concentrations of SA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) showed significant increases in the activities of both peroxidase and polyphenol oxidase where a prominent enhancement was observed at a 1.5 mM concentration of SA. *F. oxysporum* infection resulted in the induction of both of the enzyme activities but at a lower level. The activities of both peroxidase and polyphenol oxidase reached their maximum on the 28th day.

Rabab *et al.* (2013) reported that rice plant treated with nicotinic acid at a concentration of 8 mM showed optimum resistance against blast pathogen *Magnaporthe grisea.* Some antioxidants and organic compounds; Bion (BTH, benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester), at concentrations of 0.1, 0.3 and 1 mM; Salicylic acid 8 mM (SA), Benzoic acid 8 mM, Nicotinic acid 8 mM,

H_2O_2 30%, and Compost tea 100% were directly applied as foliar spray of 21-days old seedlings prior to challenge inoculation with Magnaporthe grisea to promote blast resistance in rice leaves. Among this chemicals SA showed the best performance and it recorded the lowest area under disease progress curve (AUDPC), 409.48 compared by the control 1304.24, followed by Bion at 0.3 and 1.0.

Rasmussen *et al.* (1991) found that salicylic acid acts as an endogenous inducer of resistance in cucumber plant when infected with *Pseudomonas syringae* pv. *syringae*. Inoculation of one true leaf of cucumber (*Cucumis sativus* L.) plants with *Pseudomonas syringae* pv. *syringae* results in the systemic appearance of salicylic acid in the phloem exudates from petioles above, below, and at the site of inoculation.

Saikia *et al.* (2003) revealed that chickpea plant showed more resistance against Fusarium wilt when treated with salicylic acid along with *Pseudomonas fluorescens* (Pf4-92). Varied degree of protection against Fusarium wilt was recorded with SA. The reduction in disease was more pronounced when SA was applied with P. fluorescens. SA showed the highest protection of chickpea seedlings against wilting. 52- to 64% reduction of wilting was observed in soil treated with isolate Pf4-92 along with SA. Exogenously supplied SA also stimulated systemic resistance against wilt and reduced the disease severity by 23% and 43% in the plants treated with 40 and 80 microg $ml^{-1}$of SA through root application.

Al-Saleh *et al.* (2016) observed that mexican lime treated with Salicylic acid (SA) at 10 mM, *Pseudomonas fluorescens* (Pf) and distilled water controlled canker more effectively compared to separately applying Pf or SA. The application of Pf in combination with SA significantly reduced lesion number per leaf (72%) and disease severity (84%). Significant changes in the activities of peroxidase and catalase were found.

Shang *et al.* (2011) observed that Applying 0.06 mM jasminic acid (JA) and then 0.1 mM salicylic acid (SA) 24 h later, enhanced resistance to Cucumber mosaic virus (CMV), Tobacco mosaic virus (TMV) and Turnip crinkle virus (TCV) in Arabidopsis, tobacco, tomato and hot pepper. The inhibition efficiency to virus replication usually achieved up to 80–90%.

Song *et al.* (2013) found that para-aminobenzoic acid (PABA) a precursor of folic acid when applied in pepper plant at 1 mM concentration can induced Systemic acquired resistance (SAR) against the pathogen *Xanthomonas axonopodis* pv. *vesicatoria* .

Thaler *et al.* (2004) revealed that jasmonate response reduces damage by a wide range of pathogens from different lifestyles (necrotroph versus biotroph). Tomato plant susceptibility to five of the eight pathogens was reduced by the jasmonate response, including two bacteria (*Pseudomonas syringae* and *Xanthomonas campestris*), two fungi (*Verticillium dahliae* and *Fusarium oxysporum* f. sp. *lycopersici*), and an oomycete (*Phytophthora infestans*). Susceptibility to three fungi was unaffected (*Cladosporium fulvum*, *Oidium neolycopersici*, and *Septoria lycopersici*).

Thomma *et al.* (2000) observed that Pretreatment of Arabidopsis plants with gaseous methyl jasmonate (MeJA) caused efficient reduction of disease development by either the necrotrophic fungi *Alternaria brassicicola*, *Botrytis cinerea* or *Plectosphaerella cucumerina*. Protection by gaseous MeJA was also observed on the salicylic acid (SA)-degrading transformant NahG and the ethylene non-responsive mutant ein2-1, but not on the jasmonate non-responsive mutant coi1-1. In general, protection conferred by spraying with a MeJA solution was much lower compared to exposure to gaseous MeJA. 2, 6-Dichloroisonicotinic acid (INA), a compound that activates SA- but not JA-dependent defence responses, failed to cause protection against either *A. brassicicola* or *B. cinerea*. On the other hand, INA treatment was efficient in reducing disease development by *P. cucumerina*.

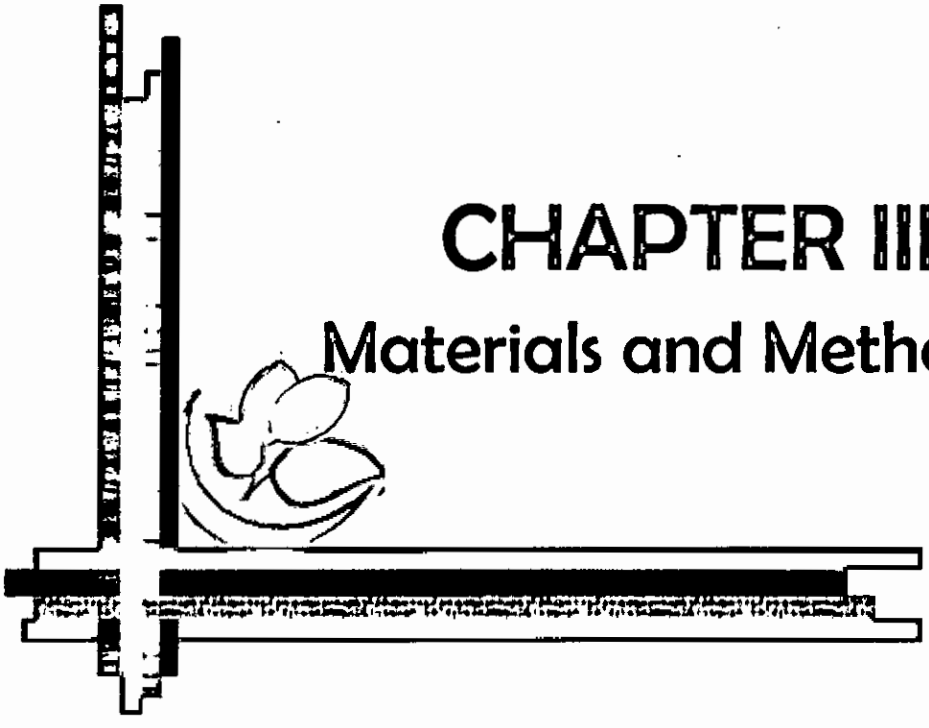
Wang and Liu (2012) found that exogenously applied salicylic acid at 2 mM significantly enhanced the endogenous free and bound SA. Upon exposure to Xac, lower disease incidence rate and smaller lesion sites were observed in the samples pre-treated with SA, accompanied by repression of bacterial growth at the lesion sites. Concurrent with the augmented disease resistance, SA-treated leaves had higher H_2O_2 level and smaller stomata apertures before or after Xac infection when compared with their counterparts pre-treated with water (control). SA treatment elevated the activities of phenylalanine ammonia-lyase and β-1, 3-glucanase, but only the latter was higher in the SA-treated samples after Xac infection. In addition, mRNA levels of two

pathogenesis-related genes, CsCHI and CsPR4A, were higher in the SA-treated samples relative to the control.

Wang *et al.* (2007) found that SA causes global repression of auxin-related genes, including the TIR1 receptor gene, resulting in stabilization of the Aux/IAA repressor proteins and inhibition of auxin responses in Arabidopsis thaliana plant. This inhibitory effect on auxin signaling is a part of the SA-mediated disease-resistance mechanism.

Wittek *et al.* (2015) reported that Folates are essential for one-carbon transfer reactions in all organisms and contribute, for example, to de novo DNA synthesis. Application of folic acid or the DHP precursor 7, 8-dihydroneopterin (DHN) enhanced resistance in Arabidopsis to *Pseudomonas syringae* and elevated the transcript accumulation of the salicylic acid (SA) marker gene pathogenesis-related1 in both the treated and systemic untreated leaves. DHN- and folic acid-induced systemic resistance was dependent on SA biosynthesis and signalling. Similar to SA, folic acid application locally enhanced Arabidopsis susceptibility to the necrotrophic fungus *Alternaria brassicicola.*

Yao and Tian (2005) found that Pre-harvest treatments with 2 mM salicylic acid (SA) and 0.2 mM methyl jasmonate (MeJA) significantly reduced lesion diameters on sweet cherry fruit caused by *Monilinia fructicola* compared with control post-harvest treatments. Pre-harvest treatment of sweet cherry with SA or MeJA induced β-1,3-glucanase, phenylalanine ammonia-lyase (PAL) and peroxidase (POD) activities during the early storage time. The efficacy of inducing resistance in sweet cherry fruit pre-harvest-treated with SA or MeJA to *M. fructicola* was better than that for fruit with post-harvest treatments, especially, at 25 ^{0}C. Activities of β-1,3-glucanase and PAL in SA- or MeJA-treated cherry fruit stored at 25^{0}C for both pre- and post-harvest treatments were significantly higher than those in fruit stored at 0 ^{0}C. SA with a concentration of 2 mM showed direct fungitoxicity on *M. fructicola* and significantly inhibited mycelial growth and spore germination of the pathogen in vitro. MeJA at 0.2 mM had little inhibitory effect on mycelial growth and spore germination of M. fructicola. The fruit treated with MeJA pre-harvest expressed higher activity of β-1, 3-glucanase and PAL than fruit treated with SA and the control during the early storage time.



CHAPTER III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

Round the research period three experiments were carried out with a view to study the canker disease of citrus. The experiments were as follows:

1. Isolation and identification of causal organism of citrus canker.
2. Effect of salt stress on citrus canker disease.
3. Assessment of the competence of some phytohormones in inducing resistance against citrus canker.

3.1. Isolation and identification of causal organism of citrus canker

3.1.1. Disease specimen collection

Diseased leaves with typical symptoms were collected from the infected citrus (*Citrus aurantifolia*) plants (Plate 1A). The specimens were kept in the refrigerator at 4 ^{0}C by following standard procedure of preservation of disease specimens until isolation was made.

3.1.2. Nutrient Broth (NB) Preparation

Nutrient broth (Appendix-I) was prepared according to the method followed by Schaad (1988). Five gram peptone and Three gram beef extract were taken in the Erlenmeyer flask containing 1000 ml distilled water and mixed well. The mixture was then autoclaved at 121^{0}C under 15 PSI pressure for 15 minutes.

3.1.3. Nutrient Agar (NA) Preparation

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

3.1.4. SX Agar media preparation

SX agar (Appendix-I) was prepared according to the method followed by Goszczynska *et al.* (2000). 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 for the preparation of 1 liter SX agar medium. The mixture was autoclaved at 121^0C under 15 PSI pressure for 15 minutes. Then it was cooled to 50^0C and 2 ml cycloheximide (100 mg/ml in ethanol) was added to it and mixed thoroughly.

3.1.5. Isolation and purification of canker pathogen of citrus

The diseased citrus leaves were washed with sterilized distilled water. Then the young lesions with green healthy portion of diseased leaves were cut into small pieces. It was then surface sterilized by dipping them in 0.1% mercuric chloride solution for 20-30 seconds. After that it was washed three times with sterile water. At the end of surface sterilization the cut pieces were kept in a Petri dish and chopped with a sharp sterile blade. Then it was taken in a test tube containing 3-4 ml of sterile water and kept for 30 minutes for bacterial streaming and getting stock. With the help of sterile pipette one ml of this stock solution was transferred into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-1} dilution (Plate 1B). Similarly, final dilution was made up to 10^{-4}. After finishing the preparation of different dilution, 0.1 ml of each dilution was spread over NA plate previously dried (to remove excess surface moisture) at three replications as described by Goszczynska and Serfontein (1998). The solution was spread with the help of alcohol flame sterilized glass-rod. The inoculated NA plates were kept in incubation chamber at 30^0C. It was observed after 24 hrs and 48 hrs. In order to get pure colony, single unmerged colony grown over NA plate was restreaked on another plate with the help of a sterile loop.

3.1.6. Preservation of canker pathogen of citrus

A slant culture of purified bacteria was done on NA slant in small screw-cap test tubes in order to preserve the bacteria for future use and kept it in refrigerator at 4^0C.

3.1.7. Identification of the pathogen

Citrus canker pathogen was identified on the basis of studying morphological, biochemical and cultural features of the pathogen as per standard microbiological procedures.

3.1.7. i. 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).

3.1.7. i.a. Gram's staining

A single drop of sterile water was placed on a clean microscope slide. Small amount of a young colony (18-24 hrs old) was taken with a cold, sterile loop from the nutrient agar medium and the bacteria were smeared on to the slide very thinly. The thinly spread 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. After that the slide was flooded with crystal violet solution for 1 minute. The slide 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.

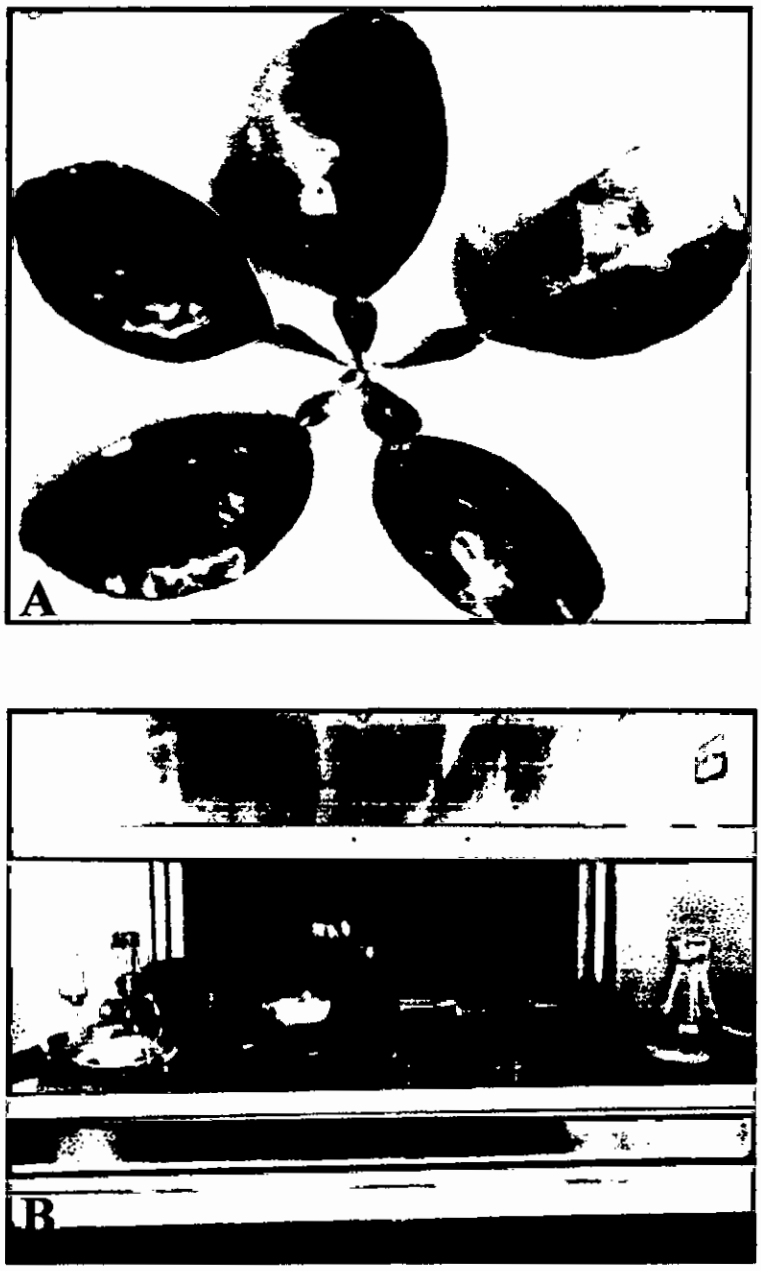


Plate 1: Infected sample and isolation of bacteria by dilution plate method

A. Disease infected citrus leaves collected from nursery for isolation of pathogen.

B. Isolation procedure in laminar air flow.

3.1.7.i.b. KOH solubility test

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

3.1.7.ii. Biochemical characters

Biochemical tests such as oxidase test, gelatine liquefaction test, starch hydrolysis test, catalase test, asculine hydrolysis test, milk proteolysis test, tween 80 lypolysis test, citrate utilization test, and salt tolerant test were studied as per the methods described by Schaad (1992) and Salle (1961).

3.1.7.ii.a. Oxidase test

1ml 1% aqueous (w/v) solution of NNN'Ntetramethyl-p-phenylene-diamine-dihydrochloride solution was spread on the middle of filter paper and the paper placed on a petri dish. Then some colony part of the bacteria was picked with a sterile toothpick and smeared onto the moistened filter paper and observed up to 60 seconds whether it changed color to dark purple or not.

3.1.7.ii.b. Gelatin liquefaction test

A tube containing 12% (w/v) gelatin was stub inoculated with one loop-full bacterial culture with the help of a sterile transfer loop. It was incubated at 30 ^{0}C for 24 hours. By the formation of liquid culture after keeping it at 5 ^{0}C in refrigerator for 15 minutes, gelatin liquefied microorganism was determined.

3.1.7.ii.c. Starch hydrolysis test

Nutrient agar plate containing 0.2% soluble starch was spot inoculated with pure colony of bacterium. It was then incubated at 30^{0}C for at least 48 hours in incubation chamber. Then the plates were flooded with lugol's iodine solution and observed whether a clear zone appeared around the colony or not.

3.1.7.ii.d. Catalase test

Some 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.

3.1.7.ii.e. Aesculin hydrolysis test

Petri dish containing aesculin media was streak inoculated by pure colony of bacterium with the help of a sterile transfer loop. The plate was incubated at 30^0C for 2-5 days in incubation chamber and observed the color developed by bacteria.

3.1.7.ii.f. Milk proteolysis

Petri dish containing milk media was streak inoculated by pure colony of bacterium with the help of a sterile transfer loop. The plate was incubated at 30^0C for 3-5 days in incubation chamber and observed the clear zone around the bacterial growth.

3.1.7.ii.g. Citrate utilization test

Petri dish containing simmon's citrate agar media was slant inoculated by Pure colony of bacterium with the help of a sterile transfer loop. Then it was incubated at 30^0C for 24 hours in incubation chamber. After incubation it was observed to determine color changed from green to bright blue.

3.1.7.ii.h. Tween 80 lypolysis test

Petri dish containing nutrient media with tween 80 was streak inoculated by pure colony of bacterium with the help of a sterile transfer loop. The plate was incubated at 30 ^{0}C for 7 days in incubation chamber and observed the clear zone around the bacterial growth.

3.1.7.ii.i. Salt tolerant test

1%, 2%, 3%, 4%, 5%, 6% and 7% NaCl solution were prepared and taken in 7 test tubes. Then 10 ml nutrient broth was added in each test tube. 1% NaCl solution was prepared by mixing 0.1 g NaCl in 10 ml NA broth. Alike 2%, 3%, 4%, 5%, 6% and 7% NaCl was prepared by mixing 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. Later the test tubes 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 collected after every 24 hours for 7 days.

3.1.7.iii. Cultural characters

Pathovars of *Xanthomonas* can be comprehended by growth and colony morphology on different media (Schaad, 1992). Growth characteristics of the pathogen were studied by using various differential, selective media.

3.1.7.iii.a. Growth on nutrient agar (NA) media

Freshly prepared Nutrient agar (NA) medium was poured into a sterile petri dish and cooled. Pure colony of bacterium was streak inoculated on the plate with the help of a sterile transfer loop. It was incubated at 30^0C for at least 24 hours in incubation chamber and observed the colony characters.

3.1.7.iii.b. Growth on selective SX agar media

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

3.1.8. Pathogenicity Test

Citrus plant (*Citrus aurantifolia*) grown on earthen pot under net house condition was used for examining the pathogenicity of *Xanthomonas axonopodis* pv. *citri* as per method described by Lin *et al.* (2008). Inoculum was prepared by growing bacterial cells overnight in NA broth and resuspending in sterile distilled water to a concentration of approximately 10^8 cfu/ml (OD:0.5_{650nm}). After that an aliquot of the inoculums suspension was injected forcedly into the lower surface of citrus leaf between two epidermal layers using a sterile syringe. Distilled water was used as a negative control. It was observed for 15 days. Visual symptoms were recorded and examined. Koch's postulates were confirmed by reisolating bacteria from the artificially infected leaves.

3.2. Effect of salt stress on citrus canker disease development

3.2.1. Selection of experimental site

This portion of experiment was piloted in the defined experimental place of Plant Pathology Department at Sher-e-Bangla Agricultural University (SAU), Dhaka during April to July, 2015. The location was good with no water lodging condition. Land elevation was high.

3.2.2. Preparation of potting media

Clay loam soil was used as potting media. The soil was collected from Agronomy field of Sher-e-Bangla Agricultural University (SAU), Dhaka. Then it was dried in sun for seven days. Later the soil was packed in different small jute bags and sterilized in autoclave at 120^0C and 15 PSI for one hour. Autoclaved materials were cooled at room temperature. No additional nutrient material was added in the soil.

3.2.3. Planting materials used for experiment

Eight months old lemon (*Citrus aurantifolia*) seedlings were collected from Krishibid nursery Agargaon, Dhaka. The seedlings were vigor, healthy, disease and insect free. Collected lemon seedlings were potted in twenty inch earthen pot and kept in shade for two days (Figure 1).

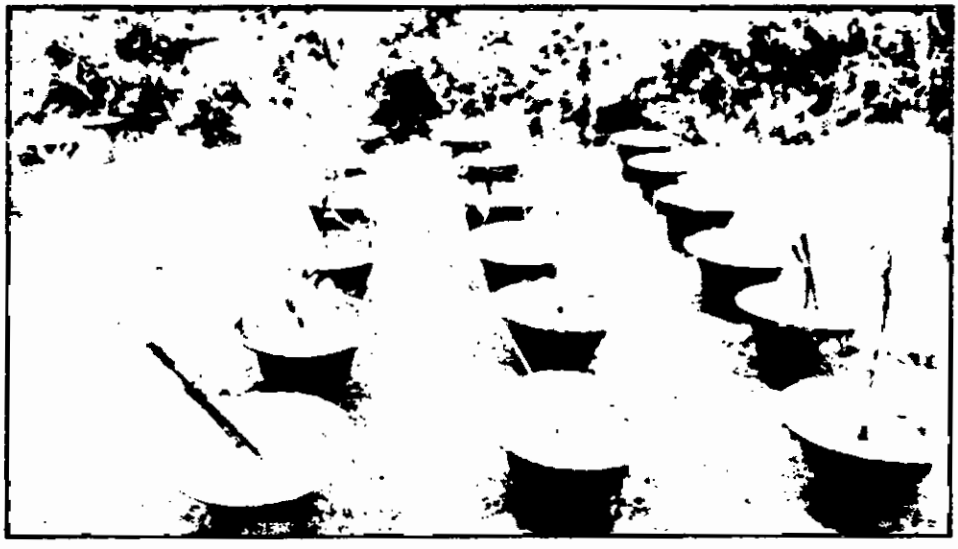


Figure 1: Planting materials used to study the effect of salt stress on canker disease.

3.2.4. Intercultural operation

Potted seedlings were irrigated everyday early in the morning with running tape water. Hand weeding was done occasionally to remove weeds from the pot. No pesticides were sprayed during the experiment period.

3.2.5. Treatments

The following treatments were used in the first experiment

Treatments	Description
T_1	Healthy plants
T_2	Bacteria + water inoculated plants
T_3	50 mM salt + Bacteria inoculated plants
T_4	100 mM salt + Bacteria inoculated plants
T_5	150 mM salt + Bacteria inoculated plants
T_6	200 mM salt + Bacteria inoculated plants

3.2.6. Preparation of salt solution

2.9 g, 5.8 g, 8.7 g and 11.6 g NaCl were weighted and diluted to 1000 ml sterilized distilled water separately to obtain 50 mM, 100 mM, 150 mM and 200 mM respectively (Banuls and Millo, 1992). The plants were irrigated with salt solution on weekly basis using 1litre solution per pot and treatments were maintained for 12 weeks. The plants were first irrigated with salt solution after one month of transplanting in pot.

3.2.7. Experimental design

This experiment was carried out in a complete randomized design (CRD) with three replications. There were 6 treatments combinations. The total numbers of unit pots were 18. Each treatment contains 3 pots with their individual plants.

3.2.8. Inoculums preparation and inoculation

Bacteria for inoculation were cultured in Nutrient broth (NB) and maintained in 30^0c temperature for 24 hours. Thereafter it was resuspended in sterile distilled water to a concentration of approximately 10^8 cfu/ml (OD: 0.5_{650nm}). Prepared inoculums were

injected in to the lower surface of the healthy leaf with syringe. Five leaves were inoculated from each plant and each leaf was inoculated with one ml bacterial solution. The plants were inoculated with bacteria after seven days of salt water irrigation.

3.2.9. Data collection

Data were collected on 15 plants on the following morphological and physiological parameters

1. Number of lesions per leaf.
2. Size of lesion (mm)
3. Disease incidence (%)
4. Disease severity (%)
5. Chlorophyll content ($\mu molm^{-2}s^{-1}$)
6. Net CO_2 assimilation rate ($gm^{-2}d^{-1}$)
7. Stomatal conductance (ppm)
8. Transpiration rate ($mmolH_2Om^{-2}s^{-1}$)
9. Intercellular CO_2 concentration ($molH_2Om^{-2}s^{-1}$)

Data over the parameters were taken in the following ways-

3.2.9.1. Number of lesions per leaf per inoculated plant

Number of lesions per leaf from inoculated plants was recorded at 10, 20, 30 and 40 days after inoculation (DAI). Total number of lesions were calculated and the average number were recorded

3.2.9.2. Size of lesion (mm) per leaf per plant

Size of lesion per leaf from inoculated plants was recorded at 10, 20, 30 and 40 days after inoculation (DAI). Total size of lesion was calculated and the average number was recorded.

3.2.9.3. Disease incidence (%)

Each inoculated leaf infected with canker disease was counted to calculate disease incidence and was expressed in percentage. Percent disease incidence (PDI) of foliar diseases was determined by the following formula (Rai and Mamatha, 2005)

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

3.2.9.4. Disease severity (%)

The disease severity was recorded by using the following scale developed by Rai and Mamatha (2005).

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

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of individual disease rating}}{\text{Total number of leaf examined} \times \text{maximum grade}} \times 100$$

3.2.9.5. Chlorophyll content in leaves per plant ($\mu molm^{-2}s^{-1}$)

The average Chlorophyll content was recorded from five leaves per plant by using "S-PAD" meter at 10, 20, 30 and 40 days after inoculation (DAI). (Maria *et al.*, 2008)

3.2.9.6. Net assimilation rate per plant ($gm^{-2}d^{-1}$)

The average net assimilation rate was recorded from five leaves per plant by using LCpro+ portable infrared gas analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) under ambient CO_2 and humidity machine at 10, 20, 30 and 40 days after inoculation (DAI). Light was provided by a photosynthetically active radiation lamp at $1000molm^{-2}s^{-1}$photon flux density. Air flow was set at $150mol^{-1}$and all measurements were performed between 8 and 11h (a.m). Inside the chamber, average temperature was 23.0±0.5°C and leaf-to-air vapor pressure deficit was 1.5±0.2kPa. (Maria *et al.*, 2008).

3.2.9.7. Stomatal conductance per plant (ppm)

The average stomatal conductance was recorded from five leaves per plant by using "LC-Pro+" machine as described in 3.2.9.5 at 10, 20, 30 and 40 days after inoculation (DAI).

3.2.9.8. Transpiration rate per plant ($mmolH_2Om^{-2}s^{-1}$)

The average Transpiration rate was recorded from five leaves per plant by using "LC-Pro+" machine as described in 3.2.9.5 at 10, 20, 30 and 40 days after inoculation (DAI).

3.2.9.9. Intercellular CO_2 concentration rate per plant ($molH_2Om^{-2}s^{-1}$)

The average Intercellular CO_2 concentration rate was recorded from five leaves per plant by using "LC-Pro+" machine as described in 3.2.9.5 at 10, 20, 30 and 40 days after inoculation (DAI).

3.3. Assessment of the competence of some phytohormones in inducing resistance against citrus canker.

3.3.1. Selection of experimental site

The experimental site was as described in 3.2.1. Sub-heading.

3.3.2. Preparation of potting media

The potting media was prepared as described in 3.2.2. Sub-heading

3.3.3. Planting materials used for experiment

One year old lemon (*Citrus aurantifolia*) seedlings were collected from Krishibid nursery Agargaon, Dhaka. The seedlings were vigor, healthy, disease and insect free. Collected lemon seedlings were potted in twenty inch earthen pot and kept in shade for two days (Figure 2).

Figure 2: Planting materials used for application of phytohormones

3.3.4. Intercultural operation

Intercultural operation was same as described in 3.2.4. Sub-heading.

3.3.5. Treatments

In this experiment the following treatments were used

Treatments	Description
T_1	Control (water)
T_2	Salicylic acid (3 mM)
T_3	Jasmonic acid (0.3 mM)
T_4	3-indolacetonitrile (0.6 mM)
T_5	Nicotinic acid (5 mM)
T_6	Folic acid (0.5 mM)

3.3.6. Experimental design

This experiment was carried out in a complete randomized design (CRD) with three replications. There were 6 treatments combinations. The total numbers of unit pots were 18. Each treatment contains 3 pots with their individual plants.

3.3.7. Preparation and application of chemicals

3.3.7.1. Preparation of salicylic acid solution

0.41g salicylic acid was weighted and dissolved in one liter sterile distilled water to get the concentration 3 mM (Wang and Liu, 2012). Prepared salicylic acid solution was taken in a Garden pump pressure sprayer and sprayed equally over the three plants. Each plant was sprayed with 330 ml solution approximately.

3.3.7.2. Preparation of jasmonic acid solution

0.06 g jasmonic acid was weighted and dissolved in one liter sterile distilled water to get the concentration 0.3 mM (Yao and Tian, 2005). Prepared jasmonic acid solution was taken in a Garden pump pressure sprayer and sprayed equally over the three plants. Each plant was sprayed with 330 ml solution approximately.

3.3.7.3. Preparation of 3-indolacetonitrile (IAN) solution

0.09 g 3-indolacetonitrile was weighted and dissolved in one liter sterile distilled water to get the concentration 0.6 mM (Li and Wang, 2013). Prepared 3-

indolacetonitrile solution was taken in a Garden pump pressure sprayer and sprayed equally over the three plants. Each plant was sprayed with 330 ml solution approximately.

3.3.7.4. Preparation of Nicotinic acid solution

0.61g Nicotinic acid was weighted and dissolved in one liter sterile distilled water to get the concentration 5 mM (Sundar *et al*, 2012). Prepared Nicotinic acid solution was taken in a Garden pump pressure sprayer and sprayed equally over the three plants. Each plant was sprayed with 330 ml solution approximately.

3.3.7.5. Preparation of Folic acid solution

0.22g Folic acid was weighted and dissolved in one liter sterile distilled water to get the concentration 0.5 mM (Song *et.al*, 2013). Prepared folic acid solution was taken in a Garden pump pressure sprayer and sprayed equally over the three plants. Each plant was sprayed with 330 ml solution approximately.

3.3.8. Inoculums preparation and inoculation

Inoculums preparation and inoculation was same as described previously in 3.2.8.

3.3.9. Data collection

Data was collected on the following parameters:

1. Number of lesions per leaf per plant
2. Size of lesion (mm) per leaf per plant
3. Disease incidence (%)
4. Disease severity (%)
5. Estimation of total phenolic compound (mg/g)
6. Estimation the amount of potassium (mg/g)

Data over the parameters were taken in the following ways-

3.3.9.1. Number of lesions per leaf per plant

Number of lesions per leaf from inoculated plants was recorded at 15, 30, and 45 days after inoculation (DAI).

3.3.9.2. Size of lesion per leaf per plant

Size of lesion per leaf from inoculated plants was recorded at 15, 30, and 45 days after inoculation (DAI).

3.3.9.3. Disease incidence

Disease incidence was taken as described previously in 3.2.9.3 at 15, 30, and 45 days after inoculation (DAI).

3.3.9.4. Disease severity

Disease severity was taken as described previously in 3.2.9.4 at 15, 30, and 45 days after inoculation (DAI).

3.3.9.5. Extraction and estimation of total phenolic compound

3.3.9.5.1. Preparation of standard curve by Catechol

Standard curve was prepared by the following method of Sharma and Kaur, (2015). O.5 g Catechol was dissolved in 10 ml 95% ethanol and made the volume 1lit with distilled water to get the concentration 500 ppm. This was the stock solution. 10. 20, 30, 40, 50, 60, 70, 80 ml from the above stock solution were taken in 100 ml volumetric flask and made the volume with water. Prepared solutions had the phenol concentration of 50, 100, 150, 200, 250, 300, 350, 400 mg/L catechol. From each solution 3 ml was taken in a test tube and 0.5 ml Folin-Ciocalteu reagent was added to it and waited for 5 min. Later 2ml 20% sodium carbonate was added and kept it at 20^0c for 1hour. After 1 hour color developed and absorbance reading were taken at 650 nm spectrophotometer (Table 1). A standard curve was prepared by plotting the absorbance reading on Y axis and concentration on X axis on a graph paper (Figure 3).

Table 1: Spectrophotometer reading at different catechol concentration

Catechol concentration (ppm)	Spectrophotometer reading (absorbance nm)
50	1.25
100	2.5
150	3.80
200	5.20
250	6.26
300	7.5
350	8.40
400	10.1

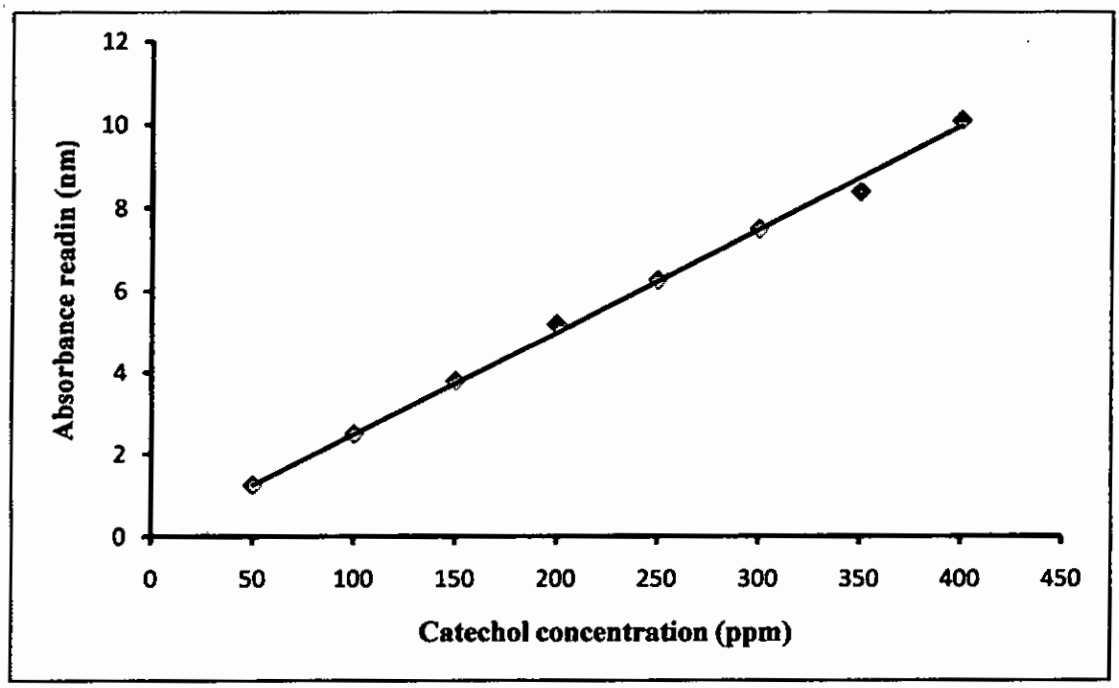


Figure 3: Standard curve prepared by different catechol conc (ppm) for estimation of total phenolic compound.

3.3.9.5.2. Extraction of total phenolic compounds from leaves

Phenolic compound was extracted by the following method of Biswas *et.al* (2012). At first 1g fully dried lemon leaves were ground in mortar and pestle along with 80% ethanol (1:10 w/v). It was then centrifuged at 10,000 rpm for 30 minutes at room

temperature in order to homogenate the suspension. Supernatant was separated and saved and re-extracted for 5 times with 80% ethanol, centrifuged and the supernatant was pooled. It was then evaporated to dryness and residues were dissolved in 5 ml of distilled water. Different aliquots were pipetted out into test tubes and the volume in each tube was made to 3 ml with distilled water. A test tube with 3 ml distilled water served as blank. Subsequently, 0.5 ml of FCR was added and after three minutes, 2 ml of 20% Na_2CO_3 solution was thoroughly mixed in each tube (Plate 2). After this, the tubes were placed in boiling water for 1 min and kept for three hours and then cooled at room temperature. Then absorbance at 650 nm against blank (water) was measured using Ultra Violet Visible (UV-VIS) spectrophotometer

3.3.9.5.3. Estimation of total phenolic compounds

From the standard curve, the concentration of phenols in the test sample was determined (Appendix II) and expressed as mg/g of fresh sample materials (Table 16).

3.3.9.6. Extraction and estimation of potassium from leaves

3.3.9.6.1. Extraction of potassium from leaves

Potassium was extracted by Di-acid mixer method (Sahrawat, 1980). Di- acid mixer was prepared by adding 60% $HClO_4$ to conc. HNO_3 in a 2:1 ratio. One gram oven dried leaves sample was taken in 250 ml conical flask. 20 ml Di-acid mixture was added to it (Plate 3). It was then heated at 200^0c until white fume evolved. Then it was allowed to cool 20-30 ml distilled water was added. This solution was filtered with whatman filter paper in 100 ml volumetric flask and the volume was made up to the mark with distilled water.

3.3.9.6.1. Preparation of standard curve

1.9818 g potassium chloride was taken in 1lit volumetric flask and volume the flask with distilled water. Thus 1000 ppm solution was prepared. From this 1000 ppm solution 10ml was taken in 100 ml volumetric flask and thus 100ppm solution was prepared. Series of standard solution containing 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm was prepared by taking 10 ml, 20 ml, 30 l, 40 ml, 50 ml from 100 ppm solution in to 100 ml flask. Then % emission reading was taken by flame emission

spectrophotometer (Table 2, Plate 4) and standard curve (Figure 4) was prepared (Cavell, 2006 and Worth, 1985).

3.3.9.6.2. Estimation of potassium compound in sample

From the standard curve, the concentration of potassium in the test sample was determined (Appendix II) and expressed as mg/g of fresh sample materials (Table 16)

3.3.9.7. Statistical analysis of data

The data were analyzed statistically by using the analysis of variance (ANOVA) and MSTAT-C software for proper interpretation. The mean value was compared according to Least Significant Difference Test (LSD) at 5% level of significance. Bar diagram and graphs were used to interpret the data as and when required.

Table 2: Flamephotometer reading at different potassium concentration for preparation of standard curve

Potassium concentration (ppm)	Flamephotometer reading (% emission)
10	2.30
20	4.6
30	7
40	9.5
50	11.5

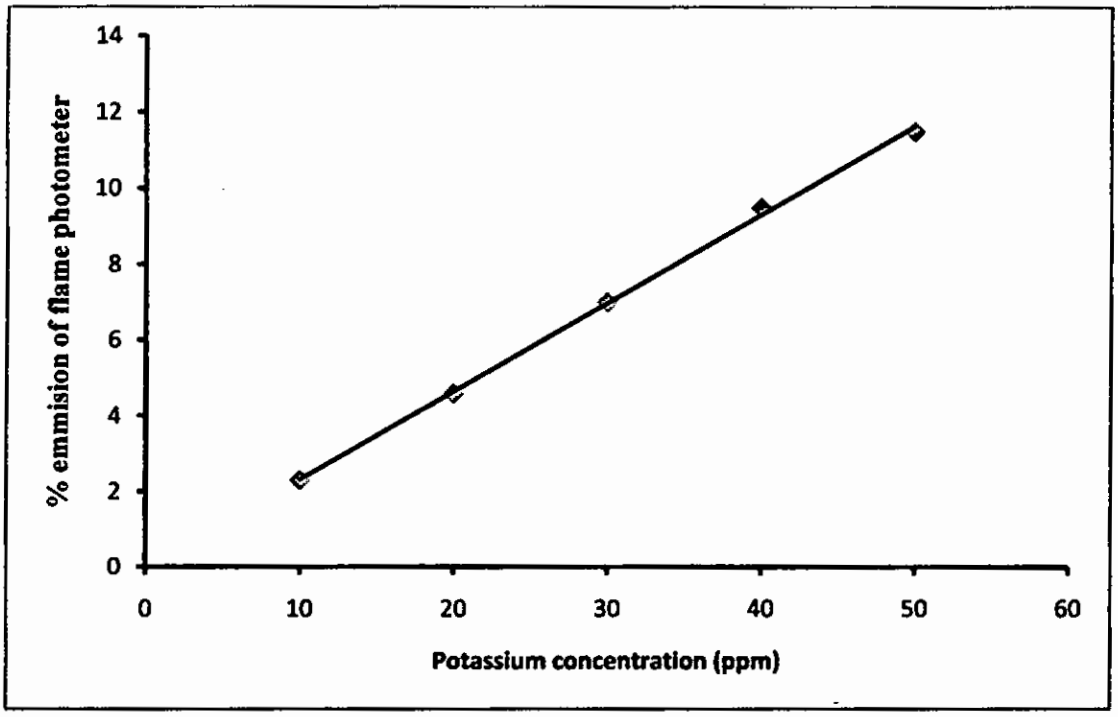


Figure 4: Standard curve prepared by different potassium chloride Conc (ppm)

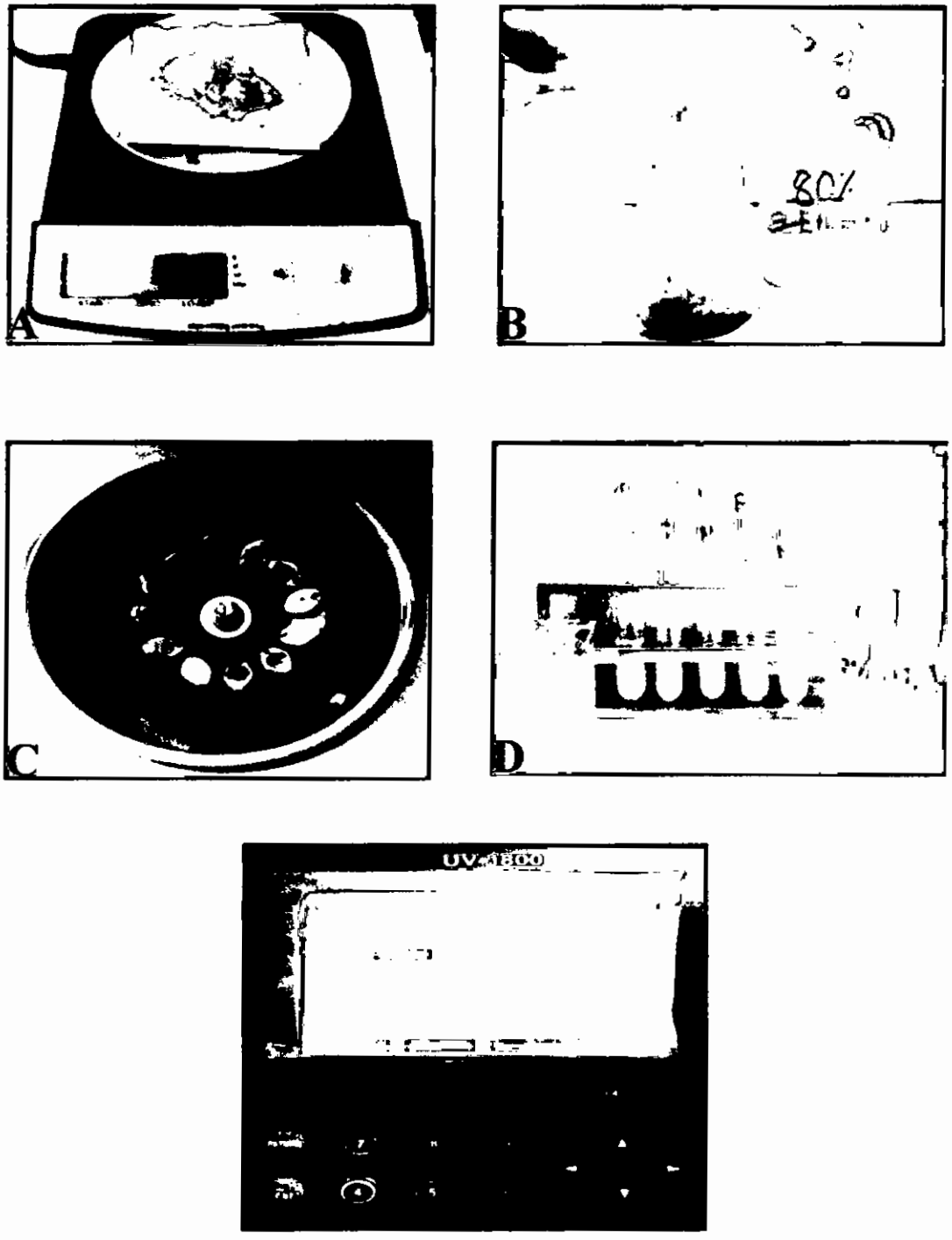


Plate 2: Extraction and estimation of total phenolic compound

A. Weighing of plant sample

B. Addition of ethanol with grounding leaf.

C. Centrifuging of sample materials.

D. Adding FCR and Na_2CO_3 with the extract

E. Spectrophotometer analysis

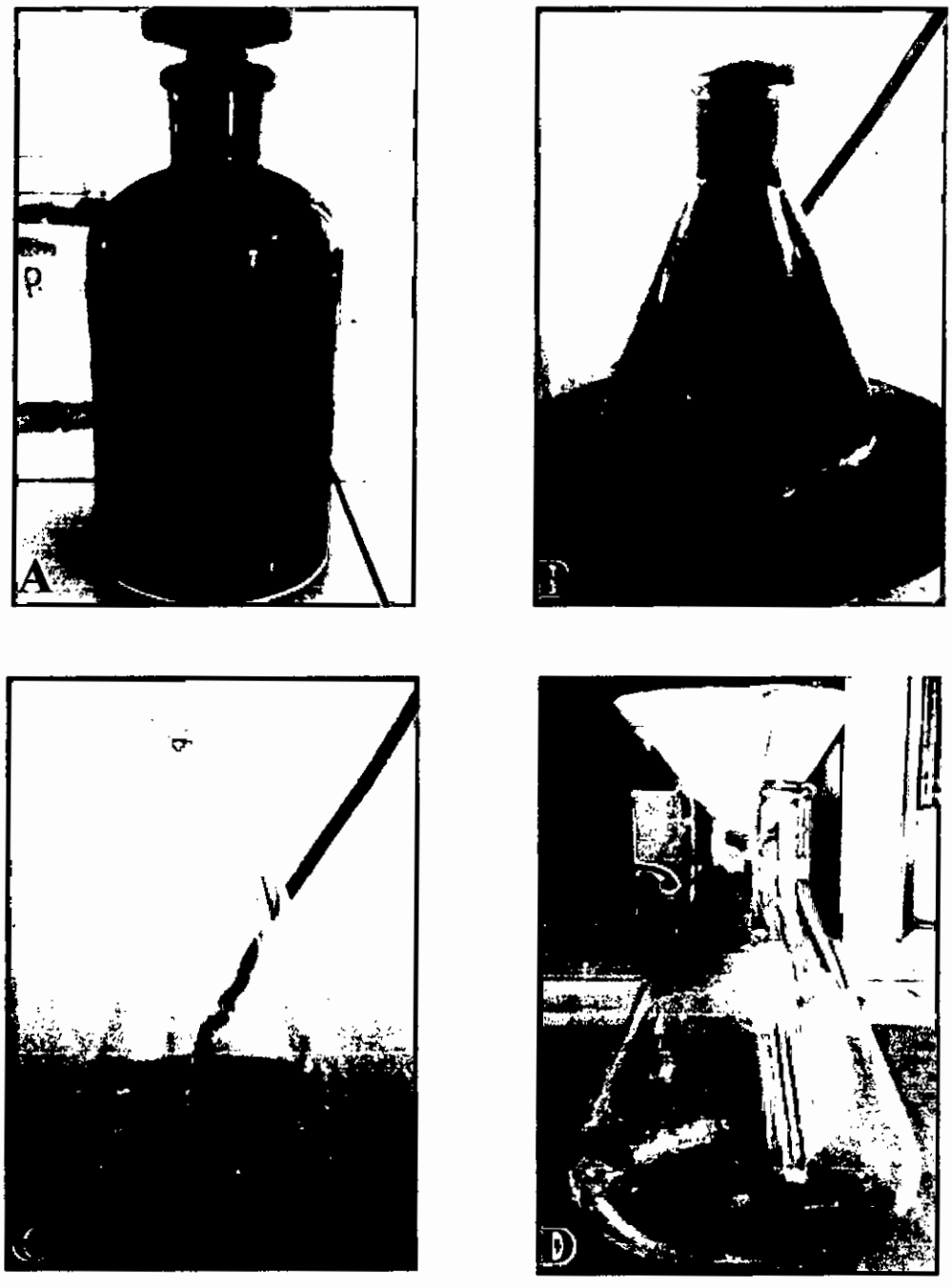


Plate 3: Extraction of potassium from leaves sample by di-acid mixture method

A. Di-acid solution

B. Colored fume produced at initial stage of extraction

C. White fume produced at the end of extraction

D. Filtering of extract with whatman filter paper

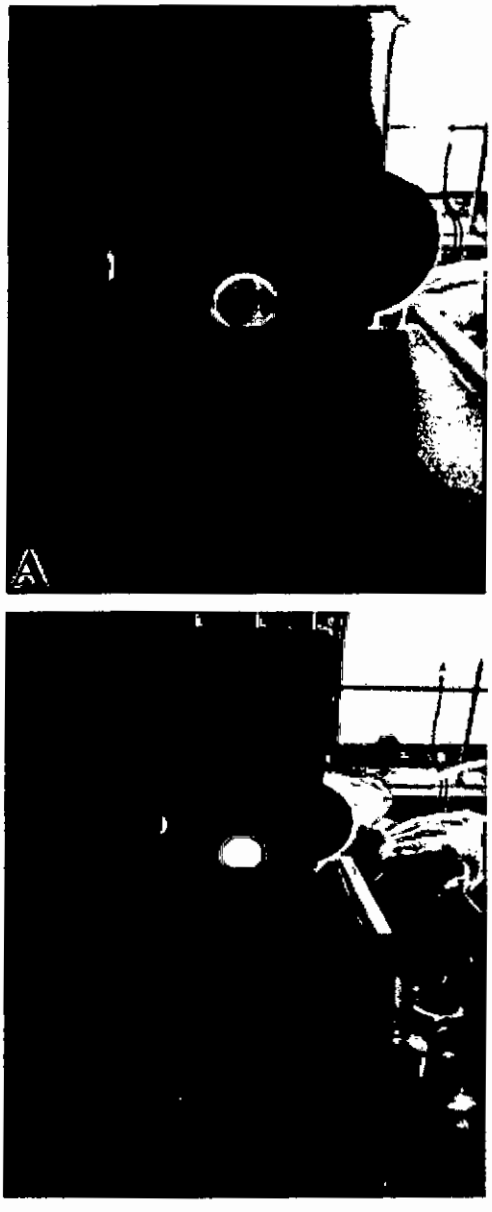
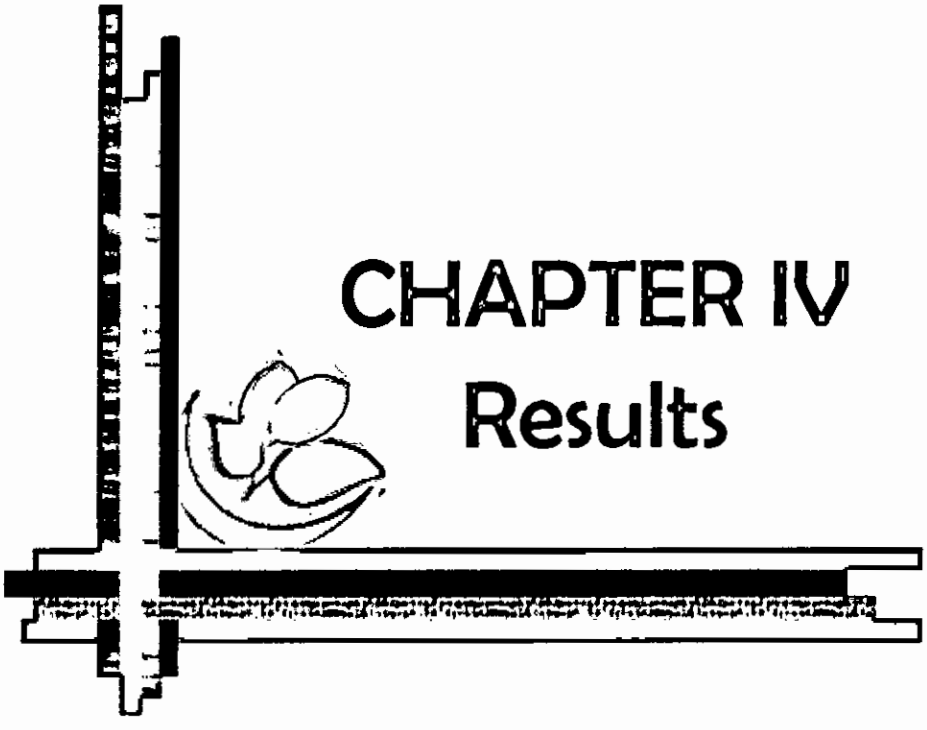


Plate 4: Estimation of potassium by flame photometer

A. Normal flame color at control(water)

B. Violet color flame produced at potassium solution



CHAPTER IV
Results

CHAPTER IV

RESULTS

4.1. Isolation and identification of canker pathogen of citrus

4.1.1. Isolation and purification of canker pathogen

The causal organism was isolated from the infected leaves of citrus showing typical symptoms of citrus canker. Dilution plate method was used to isolate causal organism. Typical, yellow, convex, mucoid, colonies of bacterium on nutrient agar medium were found after 48 hours of incubation at 30 ^{0}C (Figure 5). Colonies were purified by restreaking the isolated colony on nutrient agar plate.

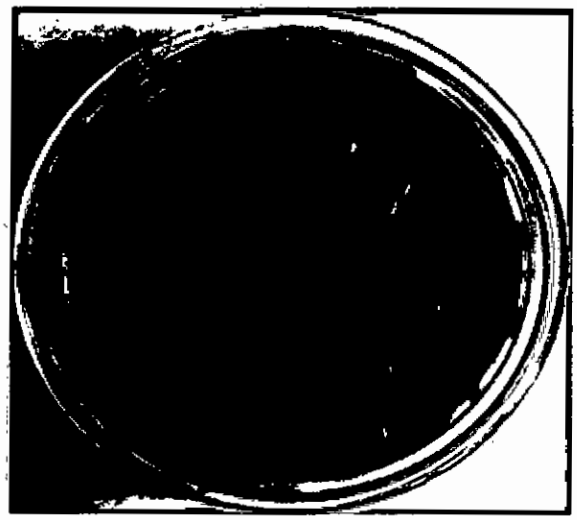


Figure 5: Yellow, convex, mucoid, colonies of pathogenic bacteria isolated from infected citrus leaves

4.1.2. Preservation pathogen

Purified bacterium on NA slant was kept in refrigerator at 4 ^{0}c in test tubes. It was served as a stock culture for further studies (Figure 6).

Figure 6: Slant culture of pathogenic bacteria.

4.1.3. Identification of the pathogen

The citrus canker pathogen was identified by studing morphological, biochemical and cultural characteristics of the pathogen as per standard microbiological procedures.

4.1.3.1. Morphological characters

The bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red color) and capsulated under the compound microscope at 100x magnification with oil immersion. The bacterium cells were stained with crystal violet (Figure 7).

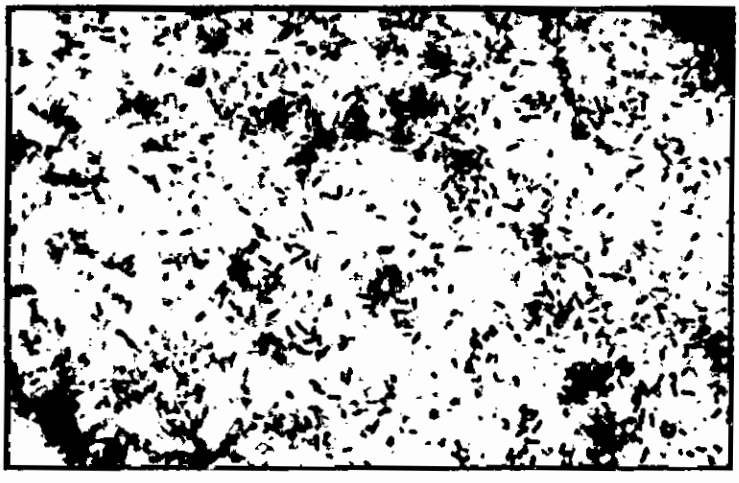


Figure 7: Microscopic view of pathogenic bacteria of citrus canker after gram's staining at 100x magnification.

Figure 6: Slant culture of pathogenic bacteria.

4.1.3. Identification of the pathogen

The citrus canker pathogen *was* identified by studing morphological, biochemical and cultural characteristics of the pathogen as per standard microbiological procedures.

4.1.3.1. Morphological character

The bacterium *was* rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red color) and capsulated under the compound microscope at 100x magnification *with* oil immersion. The bacterium cells *were* stained *with* crystal violet (Figure 7).

Figure 7: Microscopic view of pathogenic bacteria of citrus canker after gram's staining at 100x magnification.

4.1.3.2. Biochemical characters

Biochemical tests results for the pathogen are presented in Table 3.

Table 3: Biochemical characteristics of pathogenic bacteria of citrus canker

Biochemical tests	Results
KOH solubility test	Positive
Gelatine liquefaction test	Positive
Catalase test	Positive
Starch hydrolysis test	Positive
Oxidase test	Negative
Citrate utilization test	Positive
Aesculin hydrolysis	Positive
Tween 80 typolysis	Positive
Milk protolysis	Positive
Salt tolerance	Positive

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

In KOH solubility test, mucoid thread was produced by the bacterium (Plate 5B), that indicates the bacterium was gram negative.

In aesculin hydrolysis test, bacterium inoculated streak plate develop dark color (Plate 5C). Which indicated the present of β- glycosidase activity. The test was positive.

In tween 80 lypolysis test, clear zone was produced around the colony of bacteria (Plate 5D). Which revealed that the bacterium was able to produce esterase enzyme. The test was positive.

Gelatin was liquefied after 15 minutes of refrigeration at 4°C in gelatine liquefaction test by the causal organism (Plate 5E). Hence the bacterium showed the positive result.

4.1.3.2. Biochemical characters

Biochemical tests results for the pathogen are presented in Table 3.

Table 3: Biochemical characteristics of pathogenic bacteria of citrus canker

Biochemical tests	Results
KOH solubility test	Positive
Gelatine liquefaction test	Positive
Catalase test	Positive
Starch hydrolysis test	Positive
Oxidase test	Negative
Citrate utilization test	Positive
Aesculin hydrolysis	Positive
Tween 80 hydrolysis	Positive
Milk protolysis	Positive
Salt tolerance	Positive

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

In KOH solubility test, mucoid thread was produced by the bacterium (Plate 5B), that indicates the bacterium was gram negative.

In aesculin hydrolysis test, bacterium inoculated streak plate develop dark color (Plate 5C). Which indicated the present of β- glycosidase activity. The test was positive.

In tween 80 hydrolysis test, clear zone was produced around the colony of bacteria (Plate 5D). Which revealed that the bacterium was able to produce esterase enzyme. The test was positive.

Gelatin was liquefied after 15 minutes of refrigeration at 4°C in gelatine liquefaction test by the causal organism (Plate 5E). Hence the bacterium showed the positive result.

Green color of simmon's citrate agar slant changed into a bright blue color after 24 hours of incubation. That indicated the citrate utilization test was positive. The bacterium used citrate as a carbon source for their energy (Plate 5F).

In milk proteolysis test clear zone was produced around the colony of bacteria. That indicates the proteolysis of milk (Plate 6A).

In starch hydrolysis test, when lugol's iodine was added a clear zone was formed around the bacterial colony revealed starch hydrolysis (amylase activity) .The test was positive (Plate 6B).

In oxidase test, the bacterium when rubbed did not form any color in moistened oxidase disk (Plate 6C), which indicated that the test was negative.

Table 4. Salt tolerance test for pathogenic bacteria in nutrient broth (Plate 6D).

Time	Salt concentration			
	1%	3%	5%	7%
24 hr	+	+	-	-
48hr	+	+	-	-
72hr	+	+	+	-

Green color of simmon's citrate agar slant changed into a bright blue color after 24 hours of incubation. That indicated the citrate utilization test was positive. The bacterium used citrate as a carbon source for their energy (Plate 5F)

In milk proteolysis test clear zone was produced around the colony of bacteria. That indicates the proteolysis of milk (Plate 6A).

In starch hydrolysis test, when lugol's iodine was added a clear zone was formed around the bacterial colony revealed starch hydrolysis (amylase activity) .The test was positive (Plate 6B).

In oxidase test, the bacterium when rubbed did not form any color in moistened oxidase disk (Plate 6C), which indicated that the test was negative.

Table 4. Salt tolerance test for pathogenic bacteria in nutrient broth (Plate 6D).

Time	Salt concentration			
	1%	3%	5%	7%
24 hr	+	+	-	-
48hr	+	+	-	-
72hr	+	+	+	-

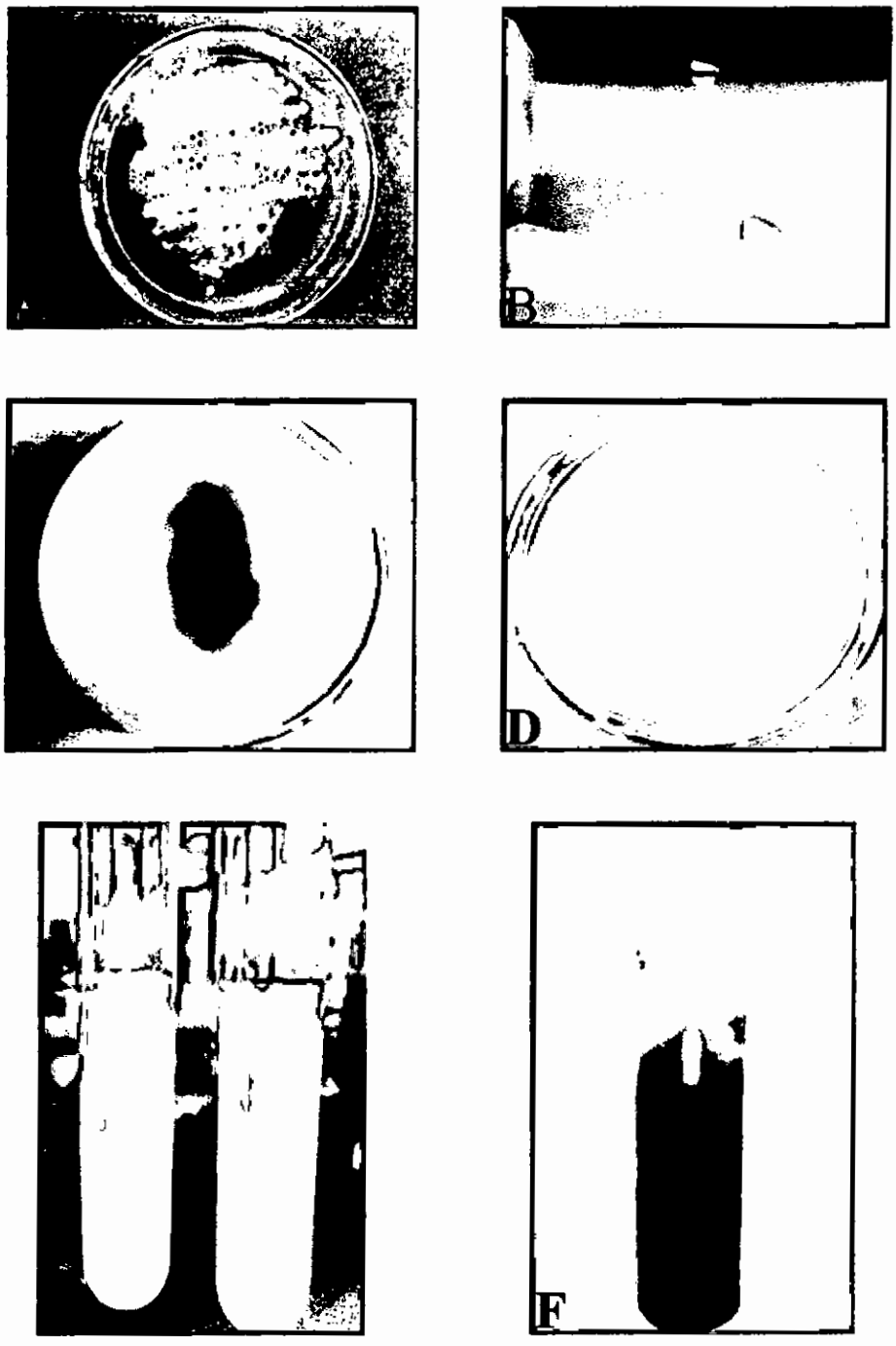


Plate 5: Biochemical characters of *Xanthomonas axonopodis* pv. *citri*

A. Catalase test.
B. KOH solubility test.
C. Aesculin hydrolysis test.
D. Tween 80 lypolysis test.
E. Gelatine liquefaction test.
F. Citrate utilization test.

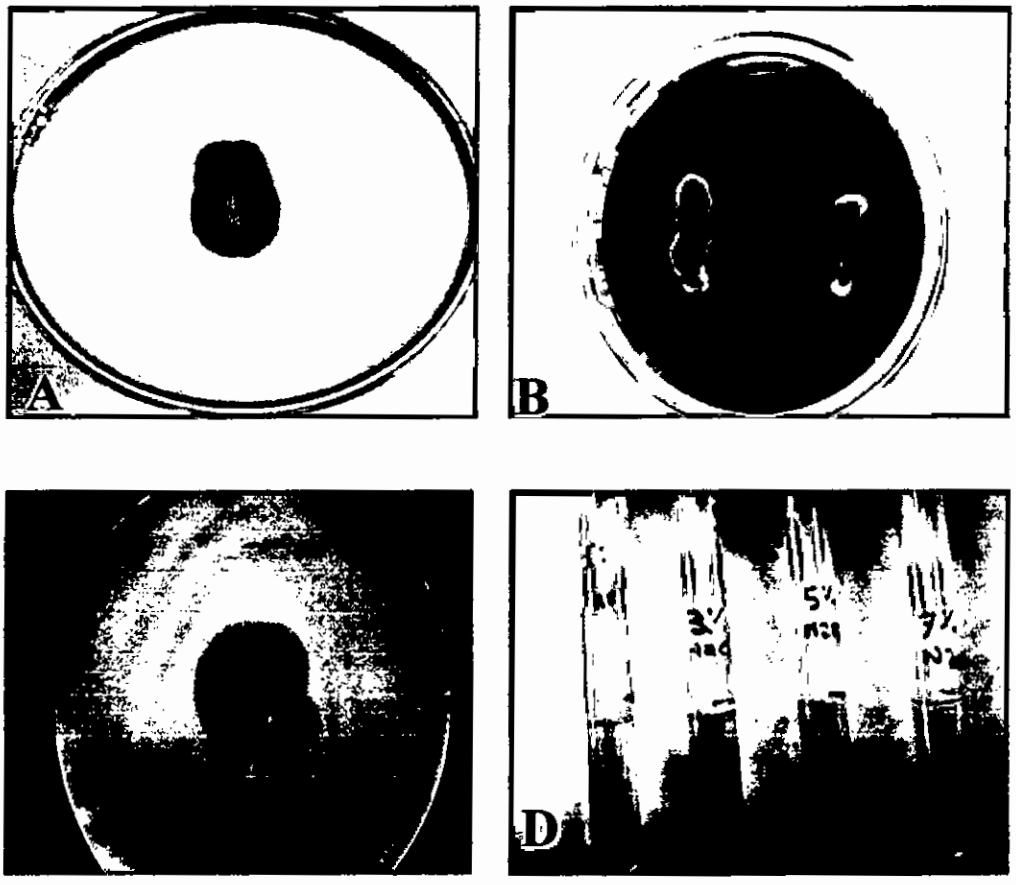


Plate 6: Biochemical characters of *Xanthomonas axonopodis* pv. *citri*

A. Milk proteolysis test.
B. Starch hydrolysis test.
C. Oxidase test.
D. Salt stress test.

4.1.3.3. Cultural characters

4.1.3.3.1. Colony morphology on different growth media

Colonies of pathogenic bacteria on NA medium shows as circular, mucoid, convex, orange color (Plate 7A). On SX medium bacterium showed very poor growth with light yellow to slightly blue, small, flattened, growth (Plate 7B).

4.1.4. Pathogenicity test

Bacterial cell suspension (10^8 cfu/ml, OD: 0.5_{650nm}) was injected into the lower surface of citrus leaf (Kagozi lemon) as described in "Materials and Methods" section. The inoculated leaves showed characteristic symptoms after fourteen days of inoculation as small, blister-like lesions, which later on turned gray to tan brown surrounded by a yellow halo (Plate 8). Bacteria were re-isolated from these lesions and comparisons were made with the original culture to confirm the identity of the pathogen. Both the colonies were similar.

On the basis of morphological, biochemical and cultural results it can be concluded that the pathogenic bacteria isolated from infected citrus leaf was *Xanthomonas axonopodis* pv. *citri*.

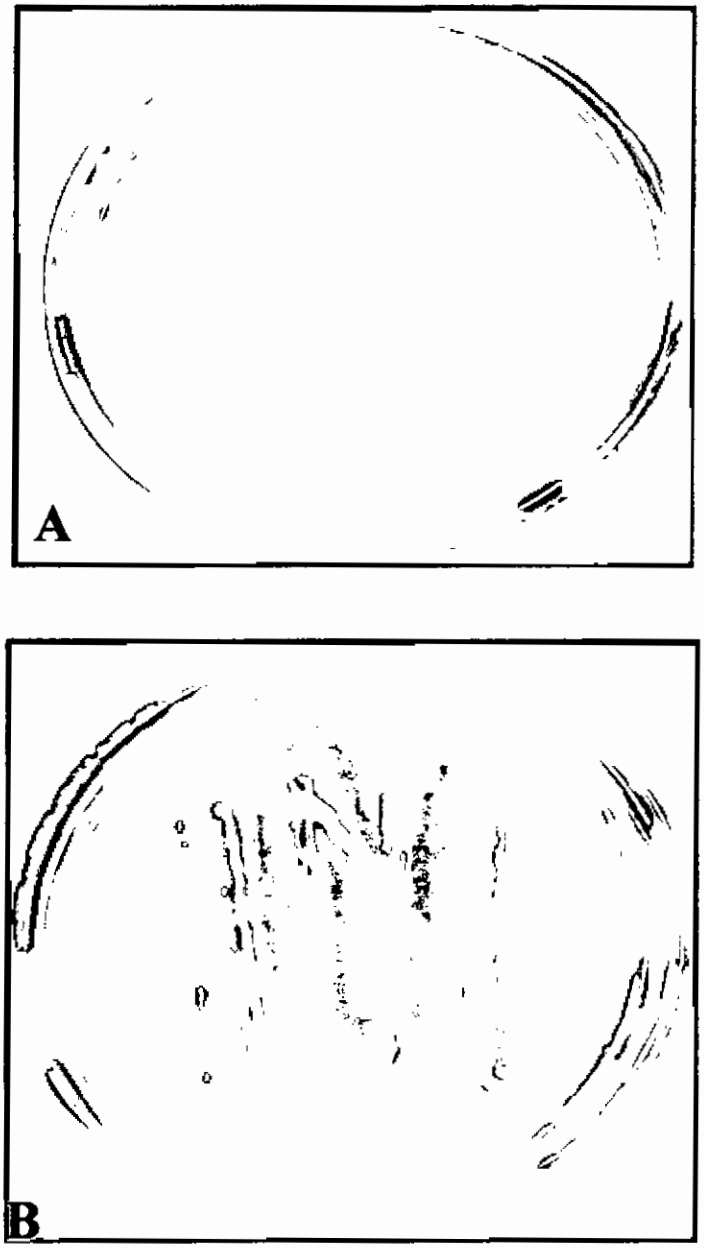


Plate 7: Cultural characteristics of *Xanthomonas axonopodis* pv. *citri* on different growth media

A. On Nutrient agar (NA) medium .
B. On SX agar medium .

Plate 8: Pathogenesity test

A. Inoculation of bacteria on lower surface of leaf.

B. Typical symptom develops at 20 days after inoculation.

4.2. Effect of salt stress on citrus canker disease development

4.2.1. Number of lesions and lesion size of citrus canker disease under different salt concentration

Data showed significant interaction between salt stress and development of citrus canker caused by *Xanthomonas axonopodis* pv. *citri*. Number of lesions varied from treatment to treatment and ranged from 0.0 to 29.47 (Table 5, Plate 9 and 10). At 40 days after inoculation (DAI), among the treatments the highest lesion number (29.47) occurred in T_5 (bacteria+150 mM salt) which was statistically similar with T_6 (bacteria+200 mM salt) followed by T_4 (bacteria+100 mM salt) and T_3 (bacteria+50 mM salt) treatment while T_1 (healthy) treatment showed lowest lesion number (0.0) as it was bacteria uninoculated. Among the bacteria and salt treated plants lowest lesion number (11.00) showed by T_2 (bacteria+water) treatment. Similar results pattern were found in case of 10 DAI, 20 DAI and 30 DAI. Number of lesion increased from 10 DAI to 40 DAI at different treatments.

Statistical significant difference on lesion size was found under different treatments. At 40 days after inoculation (DAI), among the treatments highest lesion size (10.33 mm) occurred in T_6 treatment which was statistically similar with T_2 (9.46 mm) and T_5 (9.33 mm) followed by T_4 (4.60mm) treatment. T_1 (healthy) treatment showed lowest lesion size (0.0) as it was bacteria uninoculated. Among the bacteria and salt treated plants lowest lesion size (3.33 mm) showed by T_3 which is satistically similar with T_4 treatment. Same results pattern were found at 10, 20 and 30 DAI. Lesion size increased from 10 DAI to 40 DAI at different treatments.

4.2.2. Disease incidence and severity of citrus canker under different salt concentration

Statistically significant difference on incidence was found under different treatments (Table 6, Plate 9 and 10). At 10 DAI highest incidence (66.67%) occurred in T_5 and T_6 treatment followed by T_4 (60.00 %) and T_3 (53.33%) treatments. All of these traeatments showed statistically similar result. At the same time lowest incidence (0.0%) occurred in T_1 and T_2 treatment. At 20 DAI highest and statistically similar incidence (100%) occurred in T_5 and T_6 treatment followed by T_4 (80.00 %), T_3 (73.33 %) and T_2 (73.33 %) treatments while lowest incidence (0.0%) occurred in T_1

treatment. At 30 DAI highest and statistically similar incidence (100%) occurred in T_5 and T_6 treatment followed by T_4 (93.33 %), T_3 (93.33 %) while lowest incidence (0.0%) occurred in T_1 treatment as it was bacteria uninoculated. At 40 DAI highest and statistically similar incidence (100%) occurred in T_3, T_4, T_5 and T_6 treatments while lowest incidence (0.0%) occurred in T_1treatment as it was bacteria uninoculated. Among the bacteria treated plants T_2 treatment showed lowest incidence (93.33 %). Disease incidence increased from 10 DAI to 40 DAI at different treatments.

Statistical significant difference on disease severity was found under different treatments. At 40 days after inoculation (DAI), among the treatments highest severity occurred in T_6 (52.33%) followed by T_5 (51.20%), T_4 (40.80%) and T_3 (32.60%) treatment while T_1 (healthy) treatment showed lowest lesion size (0.0) as it was bacteria uninoculated. Among the bacteria treated plants T_2 treatment showed lowest severity (24.93%). The treatments showed significant difference except T_5 and T_6. Same results pattern were found at 10, 20 and 30 DAI. Severity increased from 10 DAI to 40 DAI at different treatments.

Table 5: Lesion number and lesion size of citrus canker disease under different salt concentration.

Treatments	Lesion Number				Lesion size (mm)			
	10 DAI	20 DAI	30 DAI	40 DAI	10 DAI	20 DAI	30 DAI	40 DAI
T_1	0.00 c	0.00 e	0.00 e	0.00 e	0.00 d	0.00 d	0.00 d	0.00 c
T_2	0.00 c	2.06 d	5.93 d	11.00 d	0.00 c	2.60 b	5.60 a	9.46 a
T_3	4.06 b	9.53 c	12.67 c	16.73 c	0.69 b	1.06 c	1.66 c	3.33 b
T_4	7.20 b	11.60 b	17.33 b	22.40 b	1.03 b	2.40 b	3.00 b	4.60 b
T_5	13.73 a	20.00 a	23.67 a	29.47 a	1.93 a	4.40 a	6.26 a	9.33 a
T_6	13.47 a	19.87 a	23.87 a	28.57 a	2.13 a	4.73 a	6.13 a	10.33 a
LSD (0.05)	3.66	1.59	1.34	1.19	0.34	0.63	0.82	1.27
CV (%)	26.18	6.95	4.44	3.03	16.14	11.55	10.06	9.45
Level of Significance	**	**	**	**	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P= 0.01.

T_1 = Healthy plant

T_2= Bacteria + water

T_3= Bacteria+ 50 mM salt conc

T_4= Bacteria+ 100 mM salt conc

T_5= Bacteria+ 150 mM salt conc

T_6= Bacteria+ 200 mM salt conc

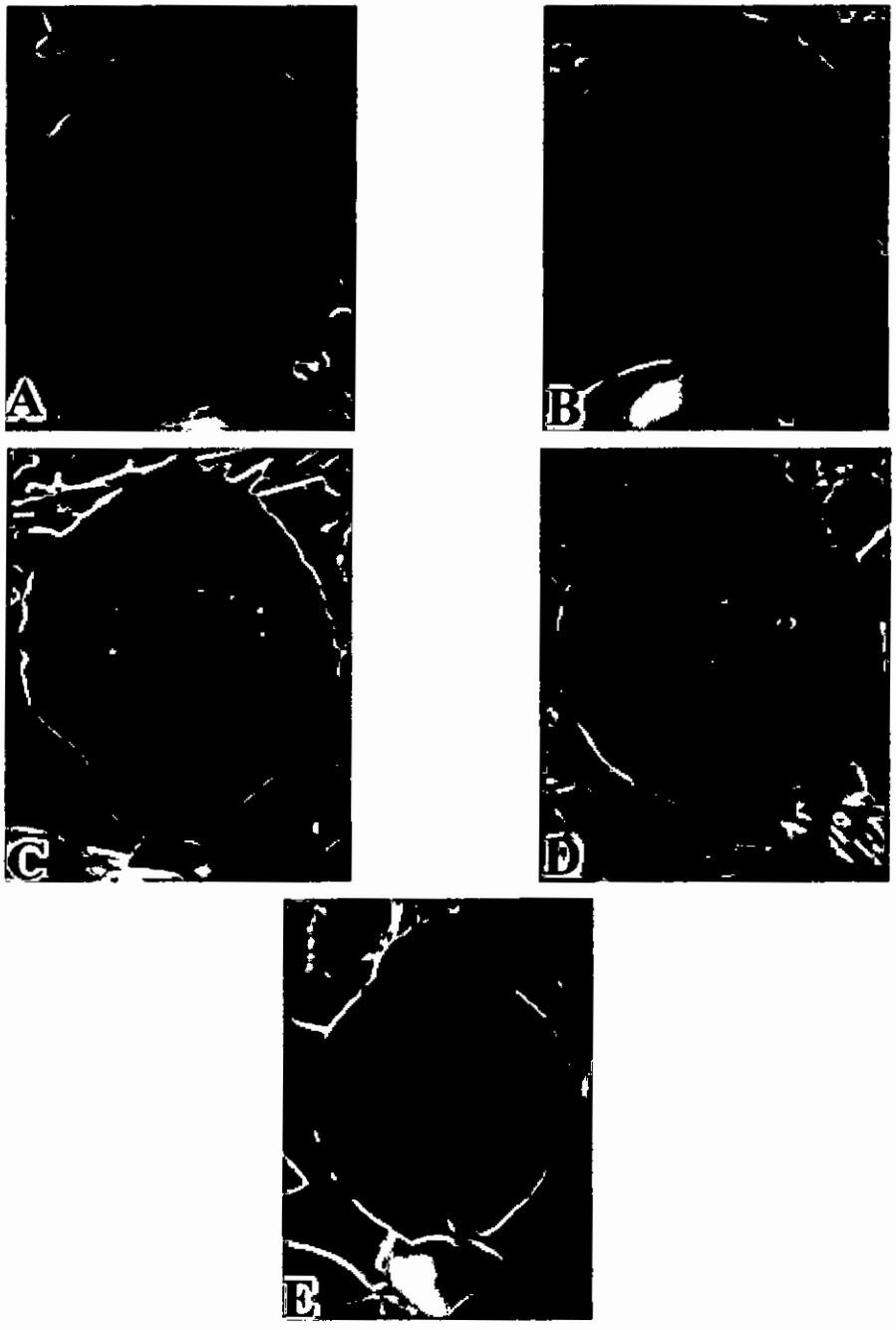


Plate 9: Disease symptoms at 10 days after inoculation (DAI)

A. Water + bacteria

B. 50 mM salt + bacteria

C. 100 mM salt + bacteria

D. 150 mM salt + bacteria

E. 200 mM salt + bacteria

4.2.3. Effect of salt stress and pathogenic stress on plant physiology

Data revealed significant interaction between pathogenic bacteria and plant physiology under salt strss. Data on different physiological parameter showed that salt and pathogenic stress have significant effect on plant physiology.

4.2.3.1. Net assimilation rate and intercellular CO_2 rate of citrus plant under salt and pathogenic stress

Statistical significant difference on net assimilation rate was found under different treatments (Table 7). At 40 DAI highest net assimilation rate (4.66 $gm^{-2}d^{-1}$) occurred in T_2 treatment followed by T_1 (3.20 $gm^{-2}d^{-1}$), T_4 (3.16 $gm^{-2}d^{-1}$) and T_3 (3.03 $gm^{-2}d^{-1}$) treatments while lowest and statistically similar net assimilation rate (0.23 and 0.26 $gm^{-2}d^{-1}$) occurred in T_6 and T_5 treatments respectively. T_1, T_3 and T_4 treatments showed statistically similar result. At 30 DAI highest net assimilation rate (5.33 and 5.30 $gm^{-2}d^{-1}$) occurred in T_4 and T_3 treatments followed by T_2 (4.63 $gm^{-2}d^{-1}$) and T_1 (3.26 $gm^{-2}d^{-1}$) while lowest and statistically similar net assimilation rate (1.00 and 1.03 gm^{-2} d^{-1}) occurred in T_6 and T_5 treatments respectively. T_3 and T_4 treatments showed statistically similar result. Same results pattern were found at 10 DAI and 20 DAI. Net assimilation rate increased from 10 DAI to 30 DAI at T_3 and T_4 treatments and then decreased at 40 DAI. In treatments T_5 and T_6 net assimilation rate were decreased from 10 DAI to 40 DAI. T_2 treatment gradually increased net assimilation rate from 10 DAI to 40 DAI. In case of T_1 treatment it remained stable from 10 DAI to 40DAI.

In case of intercellular CO_2 concentration statistical significant difference was found under different treatments (Table 7). At 40 DAI highest intercellular CO_2 concentration (14.67 ppm) occurred in T_6 treatment followed by T_5 (14.33 ppm), T_1 (12.00 ppm), T_4 (9.27 ppm) and T_3 (9.20ppm) treatments while lowest intercellular CO_2 concentration (7.73 ppm) occurred in T_2 treatment. At 30 DAI highest statistically similar intercellular CO_2 concentration occurred in T_6 (12.67 ppm) treatment followed by T_5 (12.33 ppm), T_1 (12.33 ppm) treatments while lowest and statistically similar intercellular CO_2 concentration occurred in T_3 (3.36 ppm) and T_4 (3.40 ppm) treatments. Same results pattern were found at 10 and 20 DAI. Intercellular CO_2 concentration decreased from 10 DAI to 30 DAI at T_3 and T_4 treatments and then increased at 40 DAI. In treatments T_5 and T_6 intercellular CO_2

concentration were increased from 10 DAI to 40 DAI. T_2 treatment gradually decreased intercellular CO_2 concentration rate from 10 DAI to 40 DAI. In case of T_1 treatment it remained stable from 10 DAI to 40 DAI.

Table 7: Net assimilation rate and intercellular CO_2 rate of citrus plant under salt and pathogenic stress

Treatments	Net assimilation rate (g m^{-2} d^{-1})				Intercellular CO_2 concentration (ppm)			
	10 DAI	20 DAI	30 DAI	40 DAI	10 DAI	20 DAI	30 DAI	40 DAI
T_1	3.23 b	3.00 c	3.26 c	3.20 b	12.33 a	11.67ab	12.33 a	12.00 b
T_2	3.16 b	3.83 b	4.63 b	4.66 a	9.00 ab	12.20 a	10.70 b	7.73 d
T_3	3.83 a	4.60 a	5.30 a	3.03 b	6.23 b	4.50 d	3.36 c	9.20 c
T_4	3.80 a	4.60 a	5.33 a	3.16 b	6.30 b	4.96 d	3.40 c	9.27 c
T_5	2.43 c	1.75 d	1.03 d	0.26 c	9.30 ab	10.43 c	12.33 a	14.33 a
T_6	2.33 c	1.66 d	1.00 d	0.23 c	8.86 ab	10.60bc	12.67 a	14.67 a
LSD (0.05)	0.11	0.12	0.14	0.23	4.60	1.23	1.32	1.38
CV (%)	2.13	2.15	2.48	5.49	29.87	7.63	8.12	7.79
Level of Significance	**	**	**	**	*	**	**	**

*- significant at P=0.05, **- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P=0.05 or 0.01.

T_1 = Healthy plant

T_2= Bacteria + water

T_3= Bacteria + 50 mM salt conc.

T_4= Bacteria + 100 mM salt conc

T_5= Bacteria + 150 mM salt conc

T_6= Bacteria + 200 mM salt conc

Table 7: Net assimilation rate and intercellular CO_2 rate of citrus plant under salt and pathogenic stress

Treatments	Net assimilation rate (g m^{-2} d^{-1})				Intercellular CO_2 concentration (ppm)			
	10 DAI	20 DAI	30 DAI	40 DAI	10 DAI	20 DAI	30 DAI	40 DAI
T_1	3.23 b	3.00 c	3.26 c	3.20 b	12.33 a	11.67ab	12.33 a	12.00 b
T_2	3.16 b	3.83 b	4.63 b	4.66 a	9.00 ab	12.20 a	10.70 b	7.73 d
T_3	3.83 a	4.60 a	5.30 a	3.03 b	6.23 b	4.50 d	3.36 c	9.20 c
T_4	3.80 a	4.60 a	5.33 a	3.16 b	6.30 b	4.96 d	3.40 c	9.27 c
T_5	2.43 c	1.75 d	1.03 d	0.26 c	9.30 ab	10.43 c	12.33 a	14.33 a
T_6	2.33 c	1.66 d	1.00 d	0.23 c	8.86 ab	10.60bc	12.67 a	14.67 a
LSD (0.05)	0.11	0.12	0.14	0.23	4.60	1.23	1.32	1.38
CV (%)	2.13	2.15	2.48	5.49	29.87	7.63	8.12	7.79
Level of Significance	**	**	**	**	*	**	**	**

*- significant at P=0.05, **- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P=0.05 or 0.01.

T_1 = Healthy plant

T_2= Bacteria + water

T_3= Bacteria + 50 mM salt conc

T_4= Bacteria + 100 mM salt conc

T_5= Bacteria + 150 mM salt conc

T_6= Bacteria + 200 mM salt conc

4.2.3.2. Correlation between Net assimilation rate ($gm^{-2}d^{-1}$) and intercellular CO_2 rate (ppm) of citrus plant under salt and pathogenic stress

Correlation study was conducted to establish the relationship between net assimilation rate ($gm^{-2}d^{-1}$) and intercellular CO_2 rate (ppm). Study has revealed significant correlation between the parameters (Figure 8). From the figure it is proved that the equation y = -0.486x + 14.1 gave a good fit to the data, and the correlation co-efficient (R^2 = 0.78) showed that, fitted correlation line had a significant correlation co-efficient. From these relations it can be concluded that net assimilation rate is strongly and negatively correlated with intercellular CO_2 concentration. The relation also showed that when the intercellular CO_2 concentration increased then the net assimilation rate decrease within the leaf cell.

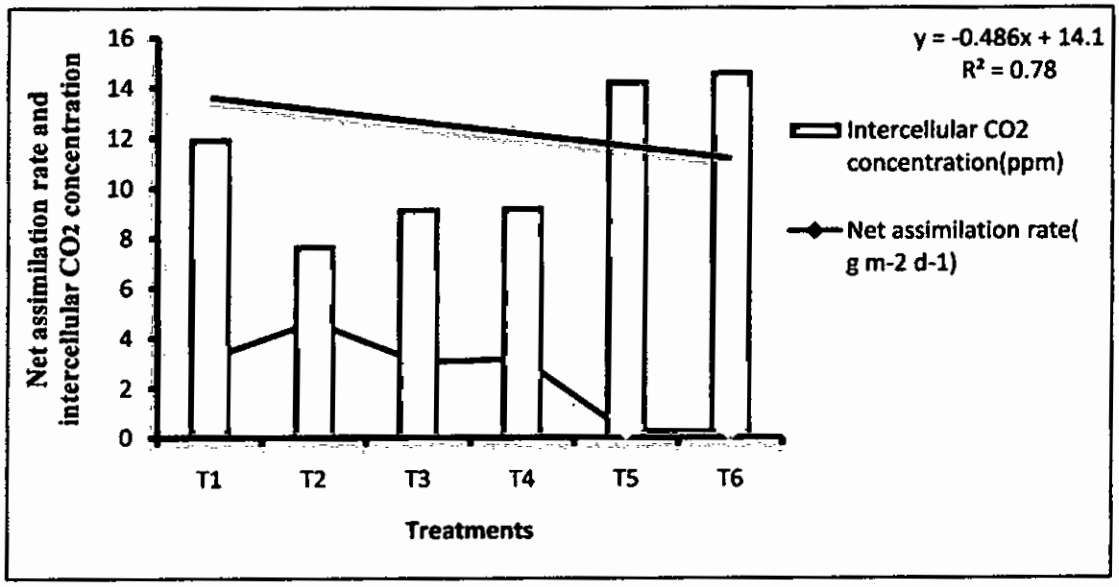


Figure 8: Correlation between net assimilation rate and intercellular CO_2 rate.

T_1= Healthy plant

T_2= Bacteria + water

T_3= Bacteria + 50 mM saltsolution

T_4= Bacteria + 100 mM saltsolution

T_5= Bacteria + 150 mM saltsolution

T_6= Bacteria + 200 mM saltsolution

4.2.3.3. Transpiration rate and stomatal conductance of citrus plant under salt and pathogenic stress

Statistical significant difference on transpiration rate was found under different treatments (Table 8). At 40 DAI highest transpiration rate (9.03 $mmolH_2Om^{-2}s^{-1}$) recorded in T_2 treatment followed by T_1 (7.06 $mmolH_2Om^{-2}s^{-1}$), T_3 (6.73 $mmolH_2Om^{-2}s^{-1}$) and T_4 (6.73 $mmolH_2Om^{-2}s^{-1}$) treatments while lowest and statistically similar transpiration rate (2.76 and 2.83 $mmolH_2Om^{-2}s^{-1}$) recorded in T_6 and T_5 treatments respectively. T_1, T_3 and T_4 treatments showed statistically similar result. At 30 DAI highest transpiration rate (10.00 $mmolH_2Om^{-2}s^{-1}$) occurred in T_3 and T_4 treatments followed by T_2 (8.76 $mmolH_2Om^{-2}s^{-1}$) and T_1 (7.06 $mmolH_2Om^{-2}s^{-1}$) while lowest and statistically similar transpiration rate (3.76 and 3.80 $mmolH_2Om^{-2}s^{-1}$) occurred in T_6 and T_5 treatments respectively. T_3 and T_4 treatments showed statistically similar result. Same result pattern were found in case of 10 DAI and 20 DAI. Transpiration rate increased from 10 DAI to 30 DAI at T_3 and T_4 treatments and then decreased at 40 DAI. In treatments T_5 and T_6 transpiration rate were decreased from 10 DAI to 40 DAI. T_2 treatment gradually increased transpiration rate from 10 DAI to 40 DAI. In case of T_1 treatment it remained stable from 10 DAI to 40 DAI.

Again statistical significant difference on stomatal conductance was found under different treatments (Table 8). At 40 DAI highest stomatal conductance (0.73 $molH_2Om^{-2}s^{-1}$) occurred in T_2 treatment followed by T_1 (0.40 $molH_2Om^{-2}s^{-1}$), T_3 (0.15 $molH_2Om^{-2}s^{-1}$) and T_4 (0.15 $molH_2Om^{-2}s^{-1}$) while lowest and statistically similar stomatal conductance (0.04 and 0.05 $molH_2Om^{-2}s^{-1}$) occurred in T_6 and T_5 treatments respectively. T_3 and T_4 treatments showed statistically similar result. At 30 DAI highest stomatal conductance (0.83 and 0.80 $molH_2Om^{-2}s^{-1}$) occurred in T_4 and T_3 treatments followed by T_2 (0.63 $molH_2Om^{-2}s^{-1}$) and T_1 (0.36 $molH_2Om^{-2}s^{-1}$) while lowest and statistically similar stomatal conductance (0.08 and 0.13 $molH_2Om^{-2}s^{-1}$) occurred in T_6 and T_5 treatments respectively. T_3 and T_4 treatments showed statistically similar result. Same result pattern were found in case of 10 DAI and 20 DAI. Stomatal conductance increased from 10 DAI to 30 DAI at T_3 and T_4 treatments and then decreased at 40 DAI. In treatments T_5 and T_6 stomatal conductance were decreased from 10 DAI to 40 DAI. T_2 treatment gradually increased stomatal

conductance from 10 DAI to 40 DAI. In case of T_1 treatment it remained stable from 10 DAI to 40 DAI.

4.2.3.4. Chlorophyll content of citrus plant under salt and pathogenic stress

Varied range of chlorophyll content was found in different treatment from 10 DAI to 40 DAI (Table 9). At 40 DAI highest chlorophyll content (65.53 $\mu molm^{-2}s^{-1}$) recorded in T_2 treatment followed by T_4 (54.37 $\mu molm^{-2}s^{-1}$), T_3 (53.77 $\mu molm^{-2}s^{-1}$) and T_1 (53 $\mu molm^{-2}s^{-1}$) treatments while lowest and statistically similar chlorophyll content (38.33 and 38.60 $\mu molm^{-2}s^{-1}$) occurred in T_6 and T_5 treatments respectively. T_1, T_3 and T_4 treatments showed statistically similar result. At 30 DAI highest chlorophyll content (66.10) occurred in T_4 treatments followed by T_3 (65.73 $\mu molm^{-2}s^{-1}$), T_2 (61.53 $\mu molm^{-2}s^{-1}$) and T_1 (54.47 $\mu molm^{-2}s^{-1}$) while lowest and statistically similar chlorophyll content (42.23 and 42.43 $\mu molm^{-2}s^{-1}$) occurred in T_6 and T_5 treatments respectively. Same result pattern were found in case of 10 DAI and 20 DAI. Chlorophyll content increased from 10 DAI to 30 DAI at T_3 and T_4 treatments and then decreased at 40 DAI. In treatments T_5 and T_6 chlorophyll content were decreased from 10 DAI to 40 DAI. T_2 treatment gradually increased chlorophyll content from 10 DAI to 40 DAI. In case of T_1 treatment it remained stable from 10 DAI to 40 DAI.

Table 8: Transpiration rate and Stomatal conductance of citrus plant under salt and pathogenic stress

Treatments	Transpiration rate ($mmolH_2Om^{-2}s^{-1}$)				Stomatal conductance ($molH_2Om^{-2}s^{-1}$)			
	10DAI	20DAI	30DAI	40DAI	10DAI	20DAI	30DAI	40DAI
T_1	7.03 b	7.06 c	7.06 c	7.06 b	0.43 b	0.43 bc	0.36 c	0.40 b
T_2	6.93 b	7.90 b	8.76 b	9.03 a	0.43 b	0.56 ab	0.63 b	0.73 a
T_3	7.96 a	8.96 a	10.00 a	6.73 b	0.56 a	0.70 a	0.80a	0.15 c
T_4	7.93 a	8.96 a	10.00 a	6.73 b	0.60 a	0.73a	0.83 a	0.15 c
T_5	5.90 c	4.83 d	3.80 d	2.83 c	0.33 bc	0.26 cd	0.13 d	0.05 d
T_6	5.90 c	4.83 d	3.76 d	2.76 c	0.26 c	0.20 d	0.08 d	0.04 d
LSD (0.05)	0.73	0.54	0.64	0.83	0.11	0.16	0.13	0.24
CV (%)	5.92	4.33	4.98	8.03	15.19	19.51	15.89	21.64
Level of Significance	**	**	**	**	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P=0.05 or 0.01.

T_1= Healthy plant
T_2= Bacteria + water
T_3= Bacteria + 50 mM saltsolution
T_4= Bacteria + 100 mM saltsolution
T_5= Bacteria + 150 mM saltsolution
T_6= Bacteria + 200 mM saltsolution

Table 9: Chlorophyll content of citrus plant under salt and pathogenic stress

Treatment	Chlorophyll content($\mu molm^{-2}s^{-1}$)			
	10DAI	20DAI	30DAI	40DAI
T_1	54.20 b	53.47 c	54.47 c	53.70 b
T_2	53.83 b	57.73 b	61.53 b	65.53 a
T_3	57.70 a	61.77 a	65.73 a	53.77 b
T_4	58.37 a	62.17 a	66.10 a	54.37 b
T_5	50.03 c	46.23 d	42.43 d	38.60 c
T_6	49.67 c	45.87 d	42.23 d	38.33 c
LSD	1.01	0.98	1.19	1.14
CV (%)	1.06	1.02	1.22	1.27
Level of Significance	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P= 0.01.

T_1= Healthy plant

T_2= Bacteria + water

T_3= Bacteria + 50 mM saltsolution

T_4= Bacteria + 100 mM saltsolution

T_5= Bacteria + 150 mM saltsolution

T_6= Bacteria + 200 mM saltsolution

4.2.3.5. Correlation between Net assimilation rate ($gm^{-2}d^{-1}$) and Chlorophyll content ($\mu molm^{-2}s^{-1}$) of citrus plant under salt and pathogenic stress

Correlation study was conducted to establish the relationship between net assimilation rate (g m^{-2} d^{-1}) and chlorophyll content (μ mol m^{-2} s^{-1}). Study has revealed significant correlation between the parameters (Figure 9). From the figure it is proved that the equation y = 2.390x + 36.62 gave a good fit to the data, and the correlation co-efficient (R^2 = 0.99) showed that, fitted correlation line had a significant correlation co-efficient. From these relations it can be concluded that net assimilation rate is strongly and positively correlated with chlorophyll content. The figure also revealed that augmentation of chlorophyll content helps to increase net assimilation rate.

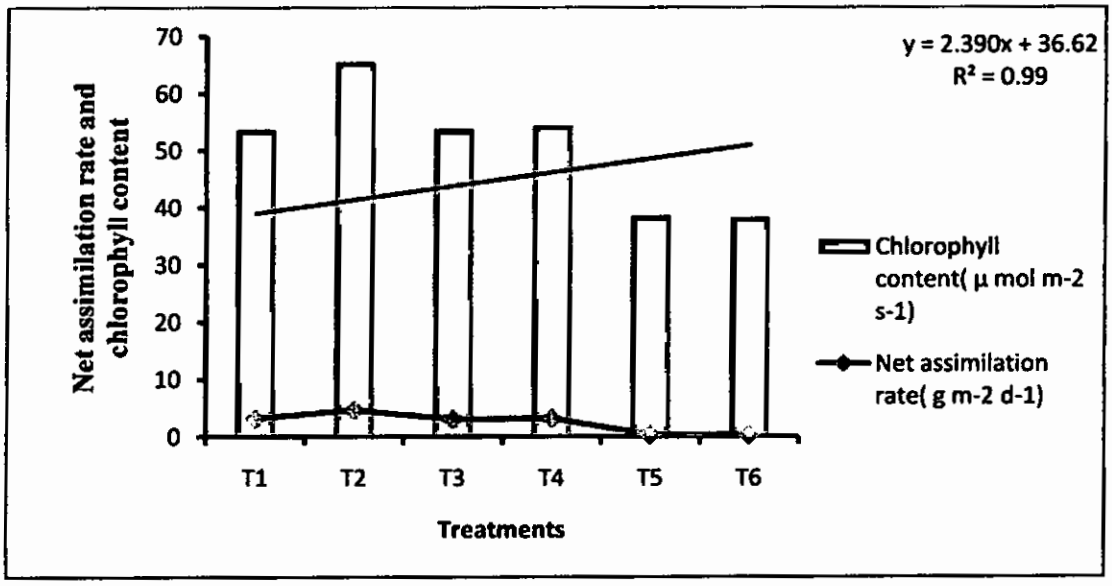


Figure 9: Correlation between Net assimilation rate and chlorophyll content.

T_1= Healthy plant

T_2= Bacteria + water

T_3= Bacteria + 50 mM saltsolution

T_4= Bacteria + 100 mM saltsolution

T_5= Bacteria + 150 mM saltsolution

T_6= Bacteria + 200 mM saltsolution

4.3. Assessment of the competence of some phytohormones in case of induced resistance against citrus canker

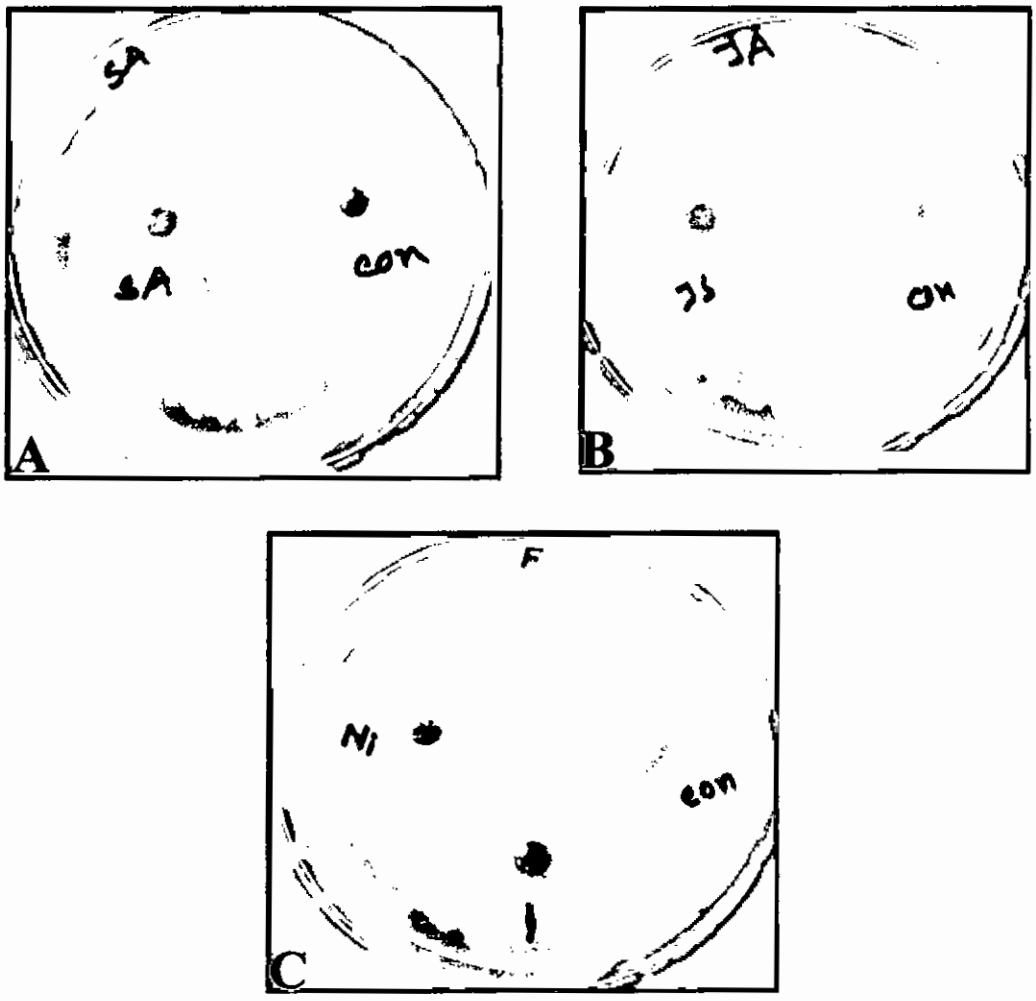
Plant elicitors such as salicylic acid (SA), jasmonic acid (JA), 3-indoleacetonitrile (IAN), folic acid (FA) and nicotinic acid (NA) were applied in the plant and challenged with *Xanthomonas axonopodis.* Data indicates that all the elicitors were able to reduce the disease severity and to increase the total phenolic compounds and potassium in leaf cell. The chemicals did not show any antibiotic properties at *In vitro* test

4.3.1. *In vitro* measurement of antibacterial properties of the selected chemicals against *Xanthomonas axonopodis*

In vitro analysis showed that no one chemical was able to produce inhibition zone on NA plate against *Xanthomonas axonopodis* (Table 10). That means the chemicals did not possess any antibacterial properties against the bacteria (Plate 11)

Table 10: Efficacy of chemicals as antibiotic against the growth of *Xanthomonas axonopodis* pv. *citri*

Treatments	Chemicals	Inhibition zone (mm)
T_1	Control(water)	0.0
T_2	Salicylic acid	0.0
T_3	Jasmonic acid	0.0
T_4	3-indoleacetonitrile	0.0
T_5	Folic acid	0.0
T_6	Nicotinic acid	0.0



Plates 11: Bioassay of chemicals against the bacteria

A. Salicylic acid (SA) and control (water)
B. Jasmonic acid (JA) and control (water)
C. 3-indoleacetonitrile (I), folic acid (F), nicotinic acid (Ni) and control (water)

4.3.2. Effect of some selected phytohormones on lesion number and lesion size of canker disease

All the treatments significantly reduced the lesion number of canker on seedlings of citrus over control (Table 11, Plate 12 and 13). At 45 DAI, highest lesion number (20.80) recorded in T_1 (control) treatment followed by T_6 (18.40) and T_5 (15.60) treatments while lowest lesion number (6.10) found in T_2 treatment. T_3 and T_4 treatments showed moderate and statistically similar lesion number (9.66) and (9.06) respectively. Similar result pattern were found in case of 15 DAI and 30 DAI. Lesion number increased from 15 DAI to 45 DAI under all treatments.

Statistical significant difference on lesion size was found under different treatments (Table 11, Plate 15 and 16). At 45 DAI, highest lesion size (9.40 mm) occurred in T_1 (control) treatment followed by T_6 (9.06 mm) and T_5 (8.13 mm) treatments while lowest lesion size (3.56 mm) found in T_2 treatment. T_3 and T_4 treatments showed moderate and statistically similar lesion size (6.90 mm) and (7.00 mm) respectively. Similar result pattern were found in case of 15 DAI and 30 DAI. Lesion size increased from 15 DAI to 45 DAI under all treatments.

Table11: Effect of some selected phytohormone on lesion number and lesion size of citrus canker

Treatment	Lesion number			Lesion size (mm)		
	15 DAI	30 DAI	45 DAI	15 DAI	30 DAI	45 DAI
T_1	15.66 a	18.27 a	20.80 a	8.13 a	8.80 a	9.40 a
T_2	0.00 d	5.26 e	6.10 e	0.00 c	2.60 d	3.56 c
T_3	0.00 d	9.00 d	9.66 d	0.00 c	6.33 c	6.90 b
T_4	0.00 d	8.86 d	9.06 d	0.00 c	6.26 c	7.00 b
T_5	10.27 c	12.30 c	15.60 c	6.90 b	7.50 bc	8.13 ab
T_6	12.80 b	15.50 b	18.40 b	7.56 ab	8.13 ab	9.06 a
LSD	0.67	0.43	0.96	0.91	1.28	1.38
CV (%)	5.89	2.12	4.10	13.65	10.95	10.62
Level of Significance	**	**	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P= 0.01.

T_1= Water (control) + bacteria
T_2= Salicylic acid + bacteria
T_3= Jasmonic acid + bacteria
T_4= 3-indoleacetonitrile + bacteria
T_5= Folic acid + bacteria
T_6= Nicotinic acid + bacteria

4.3.3. Effect of some selected phytohormones on disease incidence and severity of citrus canker

Disease incidece varied from treatment to treatment and ranged from 0.0 to 100% (Table 12, Plate 12 and 13). At 45 DAI, highest incidence (100%) recorded in T_1 (control) treatment followed by T_6 (86.67%) and T_5 (80%) treatments while lowest incidence (60%) occurred in T_2 treatment. T_3 and T_4 treatments showed moderate and statistically similar incidence (73.33%) and (73.33%) respectively. Similar result pattern were found in case of 15 DAI and 30 DAI. Disease incidence increased from 15 DAI to 45 DAI under all treatments.

Statistical significant difference on disease severity was found under different treatments (Table 12, Plate 12 and 13). At 45 DAI, highest severity (54.20 %) found in T_1 (control) treatment followed by T_6 (38.90 %) and T_5 (32.13 %) treatments while lowest severity (15.87 %) recorded in T_2 treatment. T_3 and T_4 treatments showed moderate and statistically similar severity (23.80 %) and (24.50 %) respectively. Similar result pattern were found in case of 15 DAI and 30 DAI. Disease severity increased from 15 DAI to 45 DAI under all treatments.

Table 12: Effect of some selected phytohormone on disease incidence and severity of citrus canker

Treatments	Disease incidence (%)			Disease severity (%)		
	15DAI	30DAI	45DAI	15DAI	30DAI	45DAI
T_1	66.67 a	80.00 a	100.0 a	21.87 a	37.33 a	54.20 a
T_2	0.00 c	46.67 c	60.00 c	0.00 d	7.43 e	15.87 e
T_3	0.00 c	60.00 bc	73.33 bc	0.00 d	12.80 d	23.80 d
T_4	0.00 c	66.67 ab	73.33 bc	0.00 d	13.07 d	24.50 d
T_5	46.67 b	73.33 ab	80.00 b	8.50 c	23.70 c	32.13 c
T_6	53.33 ab	80.00 a	86.67 ab	13.80 b	28.13 b	38.90 b
LSD (0.05)	14.53	14.53	14.53	2.35	2.80	2.10
CV (%)	29.39	12.05	10.35	7.20	5.11	3.23
Level of Significance	**	**	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P= 0.01.

T_1= Water (control) + bacteria
T_2= Salicylic acid + bacteria
T_3= Jasmonic acid + bacteria
T_4= 3-indoleacetonitrile + bacteria
T_5= Folic acid + bacteria
T_6= Nicotinic acid + bacteria

4.3.4. Effect of some selected phytohormones on the length of latent period of citrus canker

Latent period of citrus canker pathogen was significantly varied from treatment to treatment (Table 13). T_2 treatment showed the highest latent period (19.00 days) followed by T_3 (16.33 days) and T_4 (16.67 days). Lowest latent period was showed by T_1 treatment (14.00days) followed by T_6 and T_5 treatments. T_6 and T_5 treatments showed statistically similar latent period (14.67 and 15.00 days) with T_1 treatment.

Table 13: Effect of some selected phytohormones on the length of latent period of *Xanthomonas axonopodis*

Treatments	Latent period (days)
T_1	14.00 c
T_2	19.00 a
T_3	16.33 b
T_4	16.67 b
T_5	15.00 c
T_6	14.67 c
LSD	1.02
CV (%)	5.12
Level of Significance	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P= 0.01.

T_1= Water (control) + bacteria
T_2= Salicylic acid + bacteria
T_3= Jasmonic acid + bacteria
T_4= 3-indoleacetonitrile + bacteria
T_5= Folic acid + bacteria
T_6= Nicotinic acid + bacteria

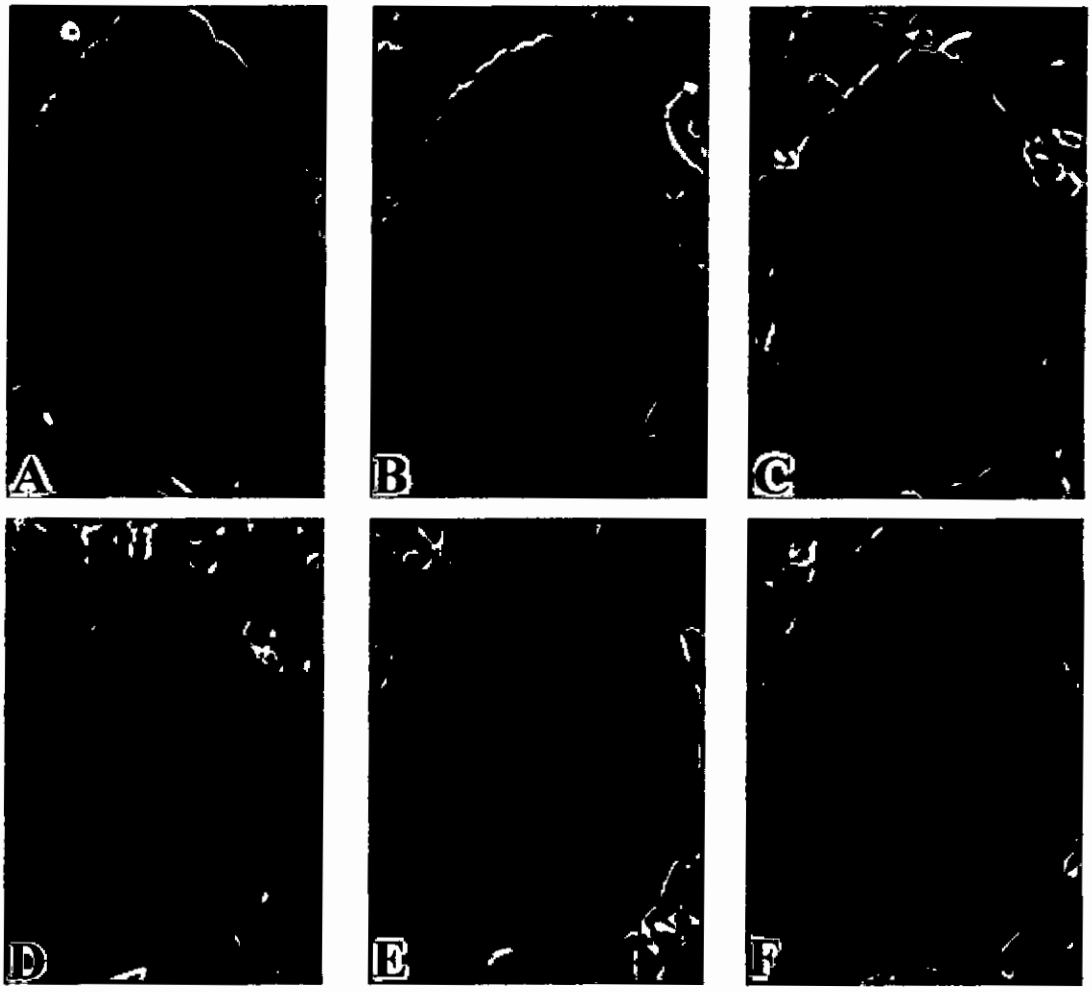


Plate 12: Disease symptoms at 15 DAI

A. Control (water) + bacteria
B. Salicylic acid + bacteria
C. Jasmonic acid + bacteria
D. 3-indoleacetonitrile + bacteria
E. Folic acid + bacteria
F. Nicotinic acid + bacteria

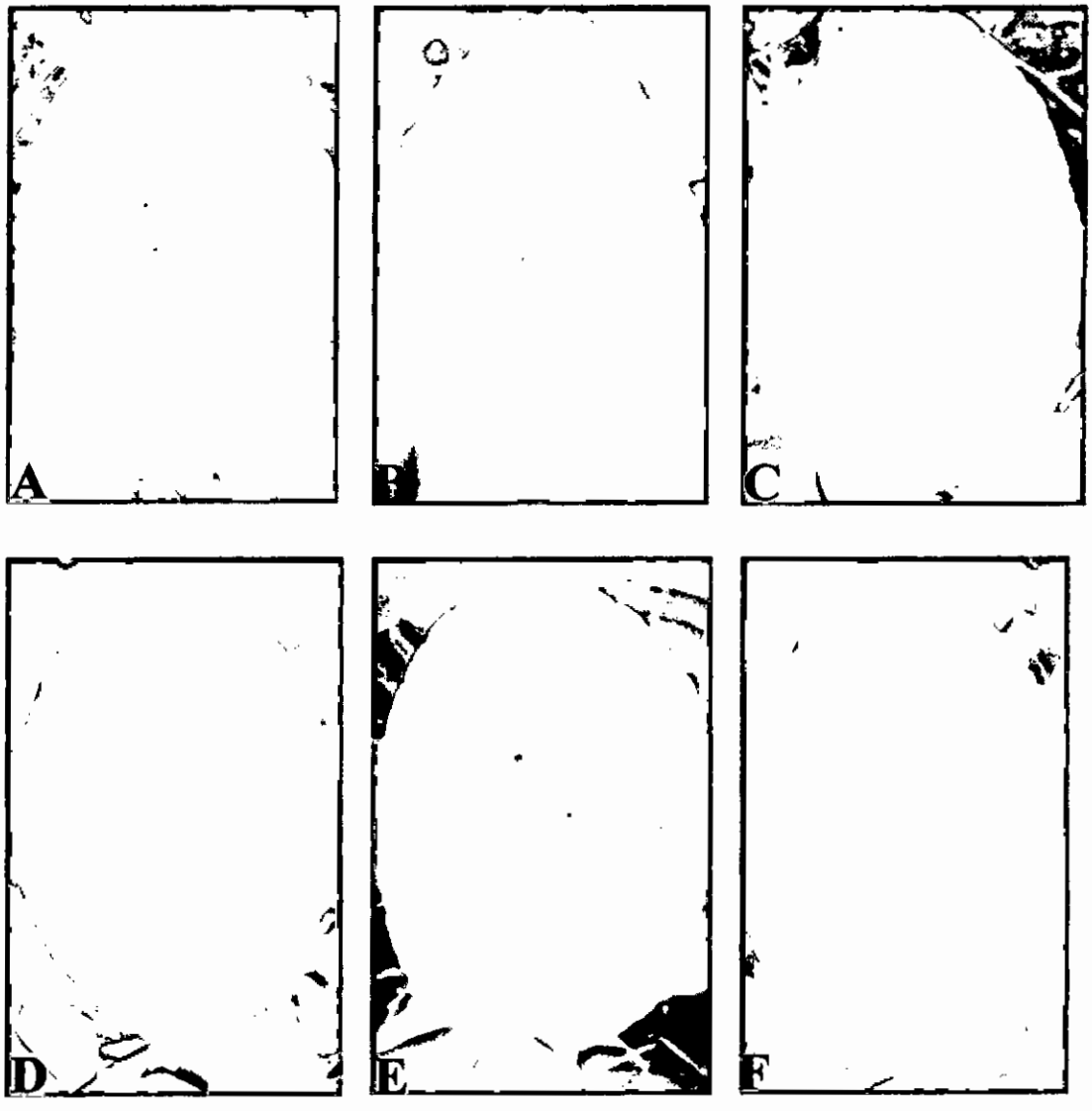


Plate 13: Disease symptoms at 45 DAI

A. Control (water) + bacteria
B. Salicylic acid + bacteria
C. Jasmonic acid + bacteria
D. 3-indoleacetonitrile + bacteria
E. Folic acid + bacteria
F. Nicotinic acid + bacteria

4.3.5. Amount of total phenolic compound (mg/g) on leaves at different treatments

All the applied phytohormone significantly increased the total plenolic acompounds and potassium concentration in leaves compare to control treatment (Table 14, Plate 14). At 45 DAI, the highest amount of total phenolic compound (12.23 mg/g) found in T_1 (control) treatment followed by T_3 (10.47 mg/g) and T_4 (8.70 mg/g) treatments while lowest amount of total phenolic compound (4.03 mg/g) recorded in T_1 treatment. T_5 and T_6 treatments showed moderate amount of total phenolic compounds (7.43 mg/g) and (6.56 mg/g) respectively. Similar results pattern were found at 15 DAI and 30 DAI. Total phenolic compounds increased from 15 DAI to 45 DAI at all treatments except T_1. It remained stable from 15 DAI to 45 DAI.

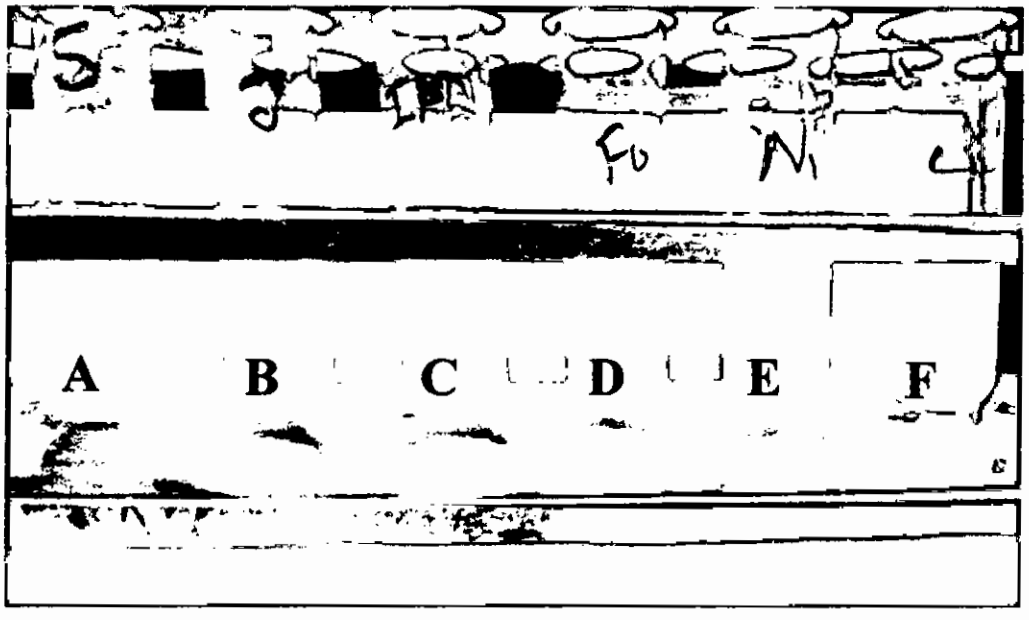


Plate 14: Amount of total phenolic compounds on leaves at different treatments

A. Salicylic acid
B. Jasmoic acid
C. 3-indoleacetonitrile
D. Folic acid
E. Nicotinic acid
F. Control (water)

4.3.6. Amount of potassium contents (mg/g) on leaves at different treatments

Again statistical significant difference on potassium content was found under different treatments (Table 14, Plate 15). At 45 DAI, highest amount of potassium content (11.33 mg/g) found in T_1 (control) treatment followed by T_3 (10.33 mg/g) and T_4 (10.47 mg/g) treatments while lowest amount of potassium content (7.16 mg/g) recorded in T_1 treatment. T_5 and T_6 treatments showed moderate amount of potassium content (9.12 mg/g) and (8.96 mg/g) respectively. Similar result patterns were found at 15 DAI and 30 DAI. Amount of potassium content increased from 15 DAI to 45 DAI at all treatments

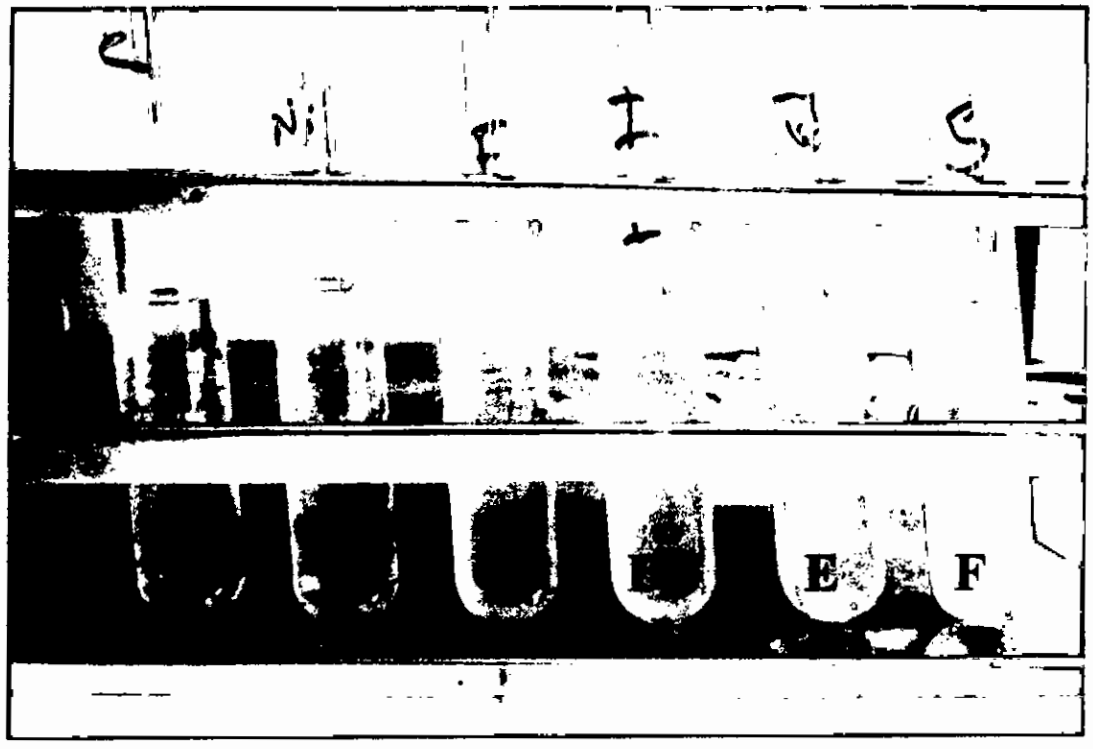


Plate 15: Amount of potassium contents on leaves at different treatments

A. Control(water)
B. Nicotinic acid
C. Folic acid
D. 3-indoleacetonitrile
E. Jasmoic acid
F. Salicylic acid

Table 14 : Amount of phenolic compounds and potassium on leaves at different treatments

Treatments	Phenolic compound (mg/g)			Potassium concentration (mg/g)		
	15 DAI	30 DAI	45 DAI	15 DAI	30 DAI	45 DAI
T_1	4.10 f	4.16 f	4.03 f	6.50 d	6.66 e	7.16 e
T_2	9.30 a	10.67 a	12.23 a	9.60 a	10.40 a	11.33 a
T_3	8.33 b	9.40 b	10.47 b	8.70 ab	9.63 b	10.33 b
T_4	6.73 c	7.83 c	8.70 c	8.83 ab	9.70 b	10.47 b
T_5	5.83 d	6.66 d	7.43 d	8.10 bc	8.93 c	9.12 c
T_6	4.90 e	5.70 e	6.56 e	7.33 cd	8.16 d	8.96 d
LSD (0.05)	0.50	0.58	0.61	0.91	0.64	0.77
CV (%)	4.33	4.46	4.22	6.29	7.01	6.00
Level of Significance	**	**	**	**	**	**

**- significant at P=0.01. Different lowercase letters beside the mean value indicate significant at P=0.05 or 0.01.

T_1= Water (control) + bacteria
T_2= Salicylic acid + bacteria
T_3= Jasmonic acid + bacteria
T_4= 3-indoleacetonitrile + bacteria
T_5= Folic acid + bacteria
T_6= Nicotinic acid + bacteria

4.3.7. Correlation between disease severity (%) and phenolic compound (mg/g) of citrus plant under different treatment

Correlation study was conducted to establish the relationship between disease severity (%) and phenolic compound (mg/g). Study has revealed significant correlation between the parameters (Figure 10). From the figure it is proved that the equation y = -9.533x + 77.23 gave a good fit to the data, and the correlation co-efficient (R^2 = 0.955) showed that, fitted correlation line had a significant correlation co-efficient. From these relations it can be concluded that disease severity is strongly and negatively correlated with the concentration of phenolic compound. The figure also revealed that accumulation of phenolic compounds in leaves has reduced disease severity.

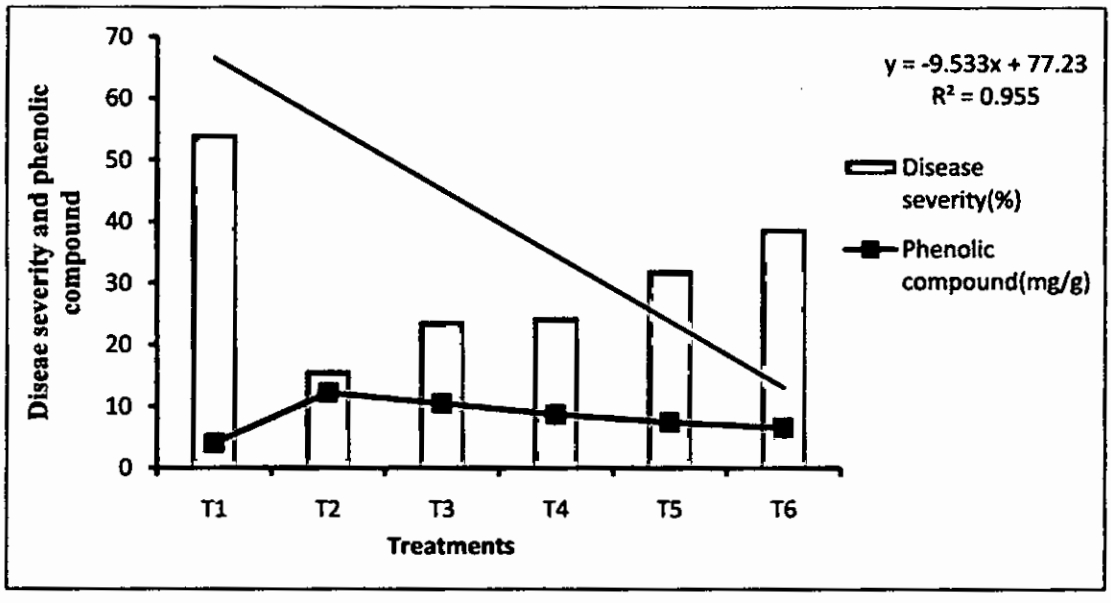


Figure 10: Correlation between disease severity and phenolic compounds.

T_1= water + bacteria

T_2= Salicylic acid + bacteria

T_3= Jasmonic acid + bacteria

T_4= 3-indoleactonitrile + bacteria

T_5= Folic acid + bacteria

T_6= Nicotinic acid + bacteria

4.3.8. Correlation between disease severity (%) and amount of potassium (mg/g) of citrus plant under different treatment

Correlation study was conducted to establish the relationship between disease severity (%) and amount of potassium (mg/g). Study has revealed significant correlation between the parameters (Figure 11). From the figure it is proved that the equation y =20.54x + 94.67 gave a good fit to the data, and the correlation co-efficient (R^2 =0.937) showed that, fitted correlation line had a significant correlation co-efficient. From these relations it can be concluded that disease severity is strongly and negatively correlated with amount of potassium. The figure also revealed that accumulation of potassium in leaves has reduced disease severity.

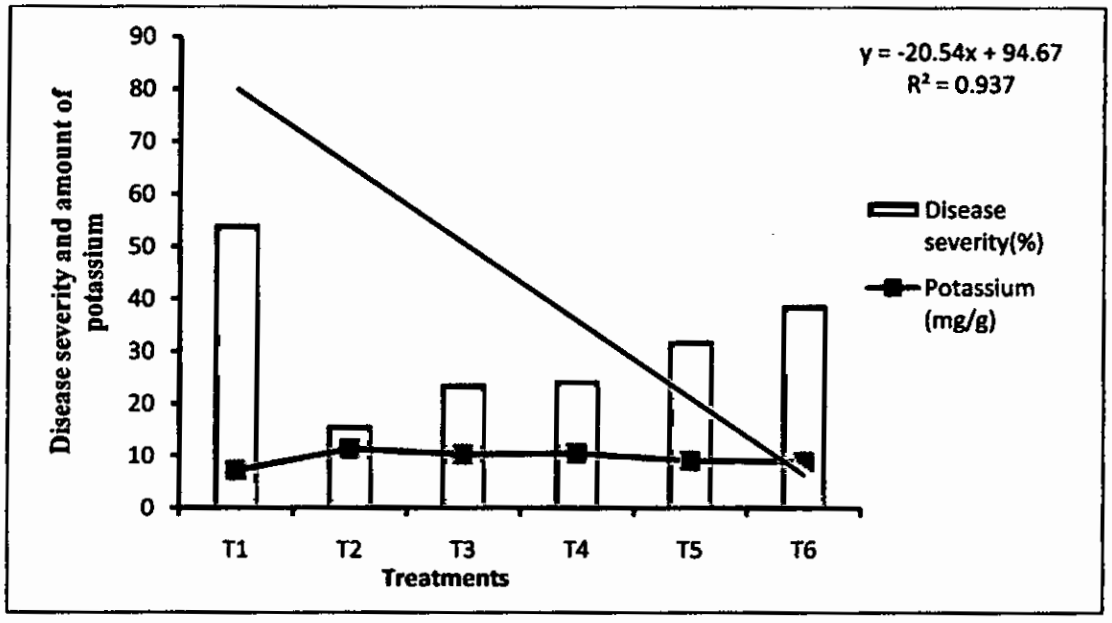


Figure 11: Correlation between disease severity and amount of potassium.

T_1= water + bacteria

T_2= Salicylic acid + bacteria

T_3= Jasmonic acid + bacteria

T_4= 3-indoleactonitrile + bacteria

T_5= Folic acid + bacteria

T_6= Nicotinic acid + bacteria

4.3.9. Correlation between phenolic compounds (mg/g) and amount of potassium (mg/g) of citrus plant under different treatment

Correlation study was conducted to establish the relationship between phenolic compounds (mg/g) and amount of potassium (mg/g). Study has revealed significant correlation between the parameters (Figure 12). From the figure it is proved that the equation $y = 1.726x + 2.14$ gave a good fit to the data, and the correlation co-efficient ($R^2 = 0.895$) showed that, fitted correlation line had a significant correlation co-efficient. From these relations it can be concluded that phenolic compounds are strongly and positively correlated with amount of potassium. The figure also revealed that accumulation of phenolic compounds in leaves help to accumulate potassium within cellular level.

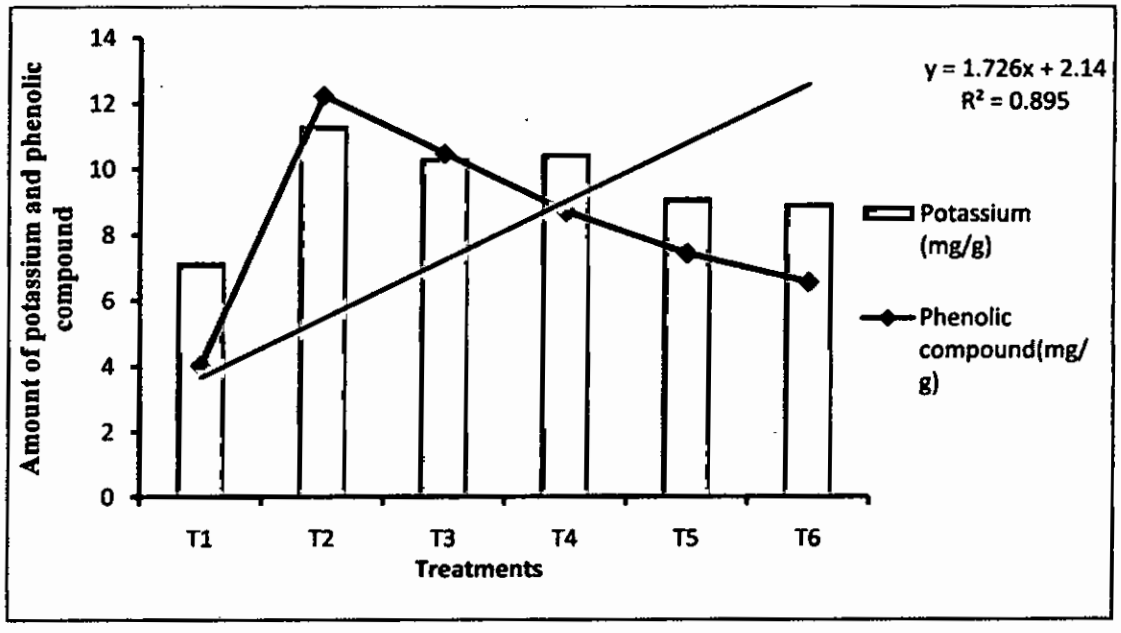


Figure 12: Correlation between disease severity and amount of potassium

T_1= water + bacteria

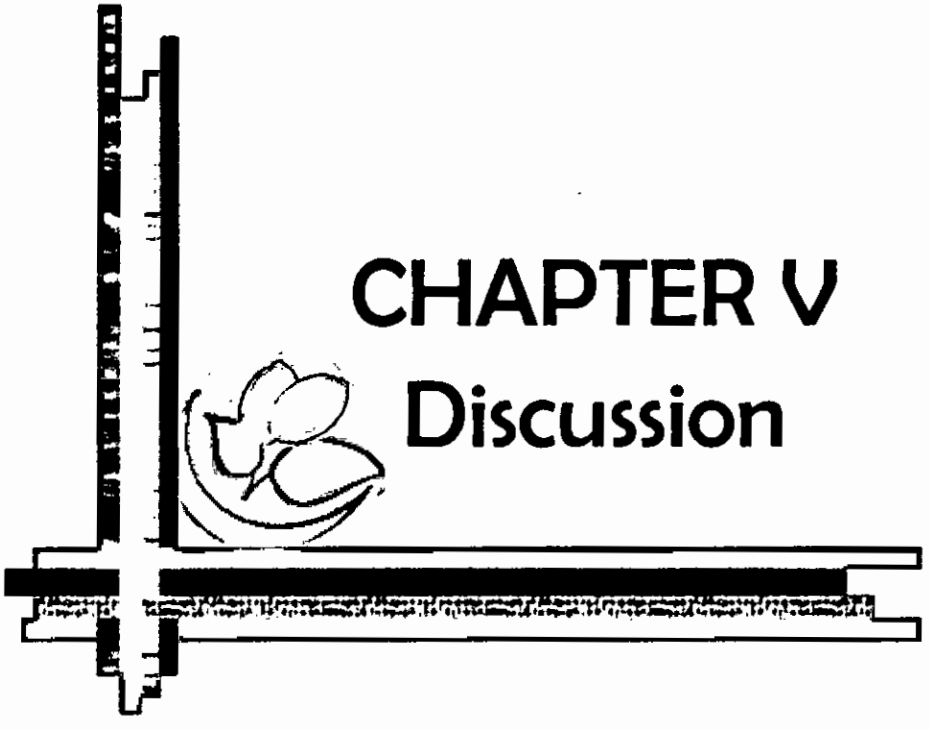
T_2= Salicylic acid + bacteria

T_3= Jasmonic acid + bacteria

T_4= 3-indoleactonitrile + bacteria

T_5= Folic acid + bacteria

T_6= Nicotinic acid + bacteria



CHAPTER V
Discussion

CHAPTER V

DISCUSSION

Xanthomonas axonopodis pv. *citri*, the putative causal organism of citrus canker was isolated from infected leaves of citrus which had also been reported from different countries of the world (Burhan *et al.*, 2007; Eshetu and Sijam, 2007; Awasthi *et al.*, 2005; Bal and Dhiman, 2005; Graham *et al.*, 2004; Gottwald *et al.*, 2002; Schubert *et al.*, 2001 and Koizumi, 1985). This devastating disease had been reported by Hossain (2011) and Chowdhury (2009) in the citrus growing areas of Bangladesh. Yellow, convex, mucoid, colonies of bacterium were found on nutrient agar medium after 48 hours of incubation at 30^0C. This characteristic of the bacterium has also been proclaimed by other researchers around the world (Lin *et al.*, 2008; Vudhivanich, 2003; Kale *et al.*, 1994; Chand and Kishun, 1991 and Qui and Ni, 1988). The bacterium, *Xanthomonas axonopodis* was identified by studing morphological, biochemical and cultural features following procedures stated by Braithwaite *et al.* (2002) and Schaad (1992). The bacterium was proofed as gram negative after gram staining test that was supported by KOH solubility test which are in aggrement with the findings of Kishun and Chand (1991), Gerhardt (1981), Bradbury (1970) and Starr and Stephens (1964). In the current study the bacterium *Xanthomonas axonopodis* pv. *citri* showed positive results in asculine hydrolysis test, milk proteolysis test, tween 80 lypolysis test, starch hydrolysis test, gelatine liquefaction test catalase test, citrate utilization test, and and negative result in oxidase test which corroborates with results of Yenjerappa (2009), Chand and Pal (1982) and Kishun and Chand (1991). The bacterium *Xanthomonas axonopodis* grew on selective SX medium and produced light yellow to slightly blue, mostly circular, small, flattened, mucoid colonies. Balestra *et al.* (2008), Vudhivanich (2003) and Braithwaite *et al.* (2002) have also found similar result on SX medium. In stress tolerance test the bacterium tolerated salt concentration up to 5% after 72 hours of incubation and it is supported by Verniere *et al.* (1998).

Present study revealed that citrus canker disease was significantly influenced by salt stress. Lesion number, size, disease incidence and severity were increased with the increase of salt concentration and duration of exposure in to salt. These findings are consistant with the result of Afek (1993), Blaker (1986), Dann *et al.* (1998) and

Willers and Holmden (1980). The highest lesion number (29.47), lesion size (10.33mm), disease incidence (100%) and severity (52.33%) were found in 150 mM and 200 mM salt treated plants at 40 days after inoculation. At the same time combined effect of biotic (*Xanthomonas axonopodis*) and abiotic (salt) stress on citrus plant physilogy were observed. Net assimilation rate (NAR), chlorophyll content (CC), Intercellular CO_2 concentration (ICC), Stomatal conductance (SC) and Transpiration rate (TR) were significantly influenced by the combined effect of biotic and abiotic stress. The present study showed that the bacterium *Xanthomonas axonopodis* regulated physiological performances of citrus plants under salt stress. The Xac bacterium enhanced NAR, CC, SC and TR and downturned ICC in citrus plant up to a certain period of time and certain salt concentration. The bacterium modulated physiological performance of the plants up to 30 days after inoculation at 100 mM salt concentration. Beyond this range the bacterial performance to modulate plant homeostasis was reduced. The highest NAR (5.33 gm^{-2} d^{-1}), CC (66.10$\mu molm^{-2}s^{-1}$), SC (0.8 $molH_2Om^{-2}s^{-1}$) and TR (10.0 $mmolH_2Om^{-2}s^{-1}$) and the lowest ICC (3.36ppm) were found in 100 mM+bacteria treated plants at 30 days after inoculation. Higher disease development was probably due to lowering resistance power of plants under salt stress. As the plants were irrigated with salt water on weekly basis, they were exposed to salt stress for long duration. As a result more Na^+ and Cl^- ions may be accumulated in leaves. Salt stress reduces the resistance power of plants through the physiochemical modification within the cellular level (Banuls *et al.*, 1992 and Alva and Syvertsen, 1991). At the higher salt concentration more Na^+ and Cl^- ions accumulated in citrus leaves (Garcia-Sanchez *et al.*, 2002). In salt stress leaf Na^+ and Cl^- concentrations are significantly elevated while K^+ and Ca^+ concentration are significantly reduced. Lack of K^+ affects the cell permeability, division and cell wall thickening. Besides antioxidant activities are reduced in salt sensitive plants (Balal *et al.*, 2012; Almansa *et al.*, 2002 and Ashraf and Ahmad, 2000). Again salt stress diminishes the activity of nitrate and nitrite reductase enzyme which causes nitrogen imbalance in the plant. Moreover accumulation of Cl^- ion reduces the nitrogen uptake. Due to the imbalance of nitrogen within the plant, enzyme production and activities are impared that assist in lowering resistance in plants (Yassin, 2004). Meanwhile ionic imbalance and toxicity presumably reduced bacterial ability to enhance plant physiological performance under salt stress which is in consistant with Balal *et al.* (2012); Chozak *et al.* (2012); Garavaglia *et al.* (2010) and Yassin (2004). The

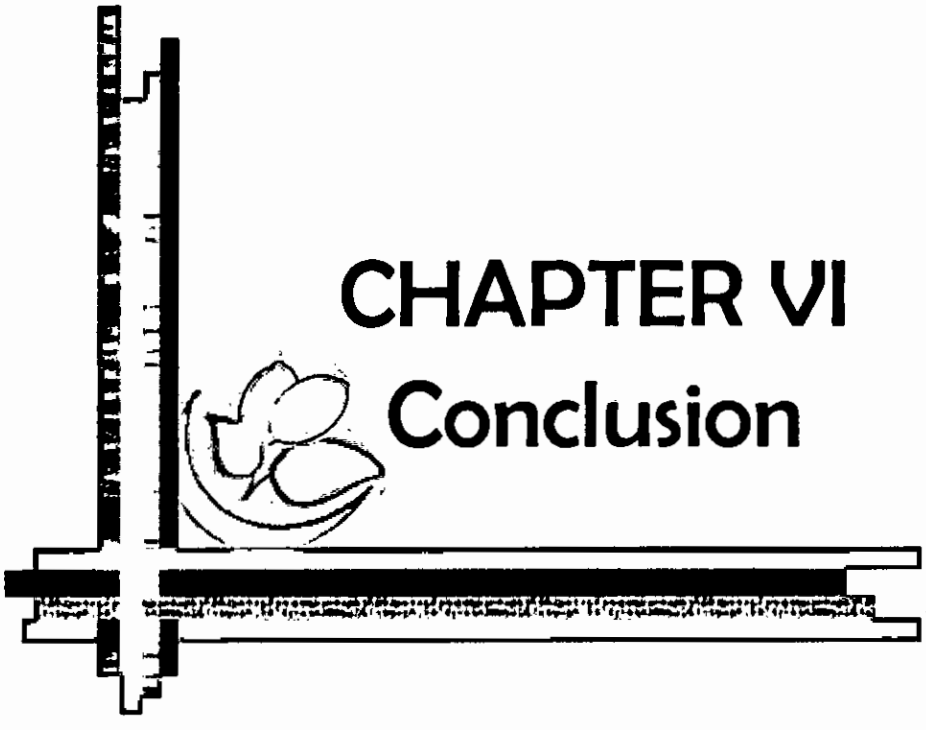
bacterium increased stomatal conductance which indicates the entry of more CO_2 in to leaf cell and decreased intercellular CO_2 concentration which indicates less accumulation of unused CO_2 and more photosynthetic reactions in cellular level. Besides increased chlorophyll content and net assimilation rate by the bacteria also indicates higher photosynthetic rate in the plant which corroborate the result of (Garavaglia *et al.*, 2010). Physiological performance of the plants was enhanced probably due to upregulation of plant natriuretic peptides (PNP) by the bacterium.The bacterium contains a gene (XacPNP) encoding a PNP-like protein within the plant cell. This protein can alter plant cell homeostasis and manipulate plant physiology in order to bring about conditions favorable to the pathogen such as increased photosynthesis rate, stomatal conductance, water uptake (Nembaware *et al.*, 2004). XacPNP are secreted at lower nutrient condition. XacPNP causes starch degradation in guard cells with a consequent rise in solute content, which, in guard cells, causes stomatal opening and can lead to increases in net water flux through the leaf (Gottig *et al.*, 2008). XacPNP promotes water uptake into protoplasts (Wang *et al.*, 2007). XacPNP can influence cell turgor and draw water to the infected tissue and reduces the damage to the host through the maintenance of photosynthesis and PNP dependent net H_2O flux and thus favors pathogen survival.

Comparative efficacy of five different phytohormones viz. salicylic acid (SA), jasmonic acid (JA), 3-indoleacetonitrile (IAN), folic acid (FA) and nicotinic acid (NA) were evaluated both *in vitro* and *in vibo* against the causal agent of citrus canker. In the present study none of this chemicals showed *in vitro* antibiotic properties against *Xanthomonas axonopodis*. In field evaluation significant effect of these chemicals on the latent period, lesion number, lesion size, incidence and severity of citrus canker were observed. Among the chemicals SA showed the best performance while JA and IAN showed moderate performance and at the same time FA and NA showed less performance against the bacterial pathogen of citrus. Highest latent period (19.00 days) and lowest lesion number (6.10), lesion size (3.56 mm), incidence (60%) and severity (15.8%) were found in SA treatment. Latent period is the time period between entry of pathogen and symptom expression. Longer latent period indicates the pathogen required enough time to generate infection. In this study the longer latent period by SA proved that SA prevented the pathogen to start infection for a longer period. Although complete suppression of pathogen was not

possible by the SA, it induced resistance in the plant to fight with the pathogen for a definite time period. This findings are in agreement with the findings of Ojha and Chatterjee, (2012); Fakhriya and Mohammed, (2011); Kolupaev *et al.*, (2011); Hadi and Balali (2010); Farouk *et al.* (2008); Nie (2006); Katoch *et al.* (2005); Yao and Tiana (2005); Dann *et al.* (1998); Raskin (1992) and Yalpani *et al.* (1991). Exogenous application of phytohormones increased endogenous total phenolic compounds and potassium content in cellular level which corroborate the result of Biswas *et al.* (2012) and Samia and Khallal (2007). The present study indicated that highest amount of total phenolic compound (12.23 mg/g) and potassium (11.33 mg/g) was accumulated in SA treatment while JA. INA, FA and NA showed moderate accretion of total phenolic compound and potassium but had significant difference between them. Correlation analysis of total phenolic compound and potassium along with disease incidence and severity revealed that disease incidence and severity was decreased due to increase of total phenolic compound and potassium content in cellular level which are in aggrement with Maddox *et al.* (2010); Nicholson and Hammerschmid (2003) and Matern and Kneuse (1988). Phenolic compounds acts as phytoalexins and antibiotics against pathogen. Phenolics are stored in plant cells as inactive bound forms but are readily converted into biologically active antibiotics by plant hydrolysing enzymes (glycosidases) in response to pathogen attack. Besides phenolic compounds produce reactive oxygen substances such as super oxide anion, hydrogen peroxide which deter pathogenic growth. Phenolic compounds trigger an increase in extra cellular pH and K^+, while eliciting an influx of calcium and hydrogen ions into the cell resulting in cell death and formation of local lesions. Moreover phenolic radical is formed by SA which is involved in lipid peroxidation that activates defense gene expression (Lattanzio *et al.*, 2006). Again Higher K^+ concentrations decreased the internal competition of pathogens for nutrient resources. This nutritional status enables plants to allocate more resources to developing stronger cell walls for preventing pathogen infection and insect attack and to obtain more nutrients to be used for plant defense and damage repair (Holzmueller *et al.*, 2007).

Correlation analysis showed positive relation between total phenolic compound and potassium content in cellular level that indicates there is no rivalry between total phenolic compound potassium content.

Basically these phytohormones increase disease resistance in plant by boosting enzymatic activity such as phenylalanine ammonia-lyase and β-1, 3-glucanase, phenolic compounds, reactive oxygen substances (ROS), Pathogenesis related (PR) protein, ethylene production, nutrient absorption and accumulation and biofilm inhibition etc (Song *et al.*, 2013; Wang and Liu, 2012; Vallad and Goodman 2004, Sing *et al.* 2003; Thoma *et al.* 2000; Van wees *et al.* 2000; Creelman and Mullet, 1997; Porat *et al.*, 1993).



CHAPTER VI
Conclusion

CHAPTER VI

SUMMARY AND CONCLUSION

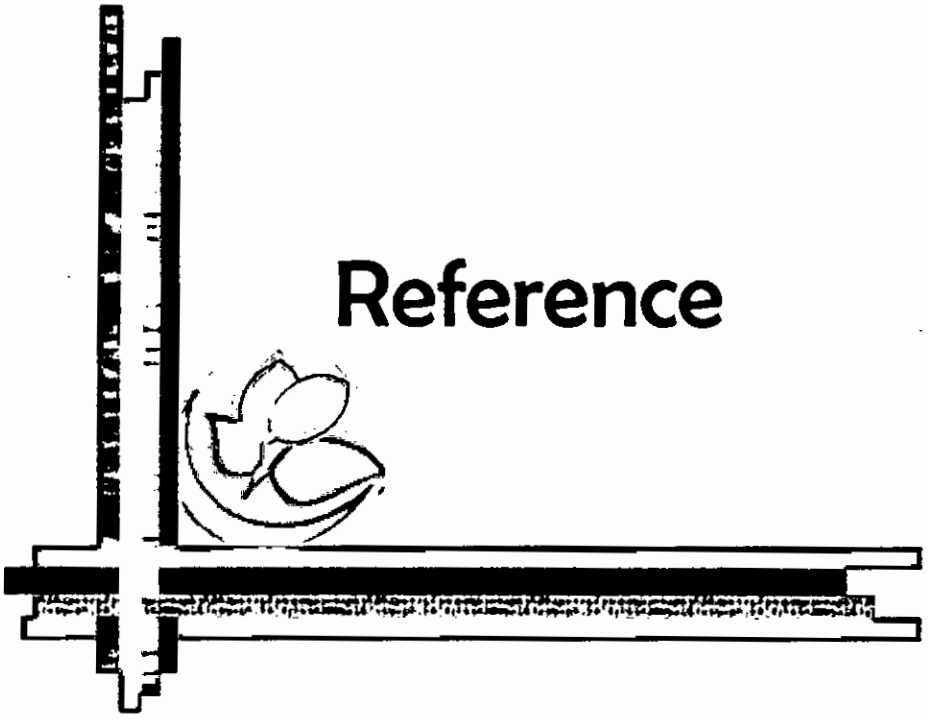
Citrus canker is generally occured on leaf surface and caused by the hemibiotrophic bacteria *Xanthomonas axonopodis* pv. *citri*. Citrus canker pathogen was isolated from the infected leaf by following dilution plating method using nutrient agar medium. It was purified by restreaking on nutrient agar medium with single colony and confirmation was done by pathogenicity test. The causal organism was gram negative, rod shaped with rounded ends. It showed positive result in KOH solubility test, asculine hydrolysis test, starch hydrolysis test, milk proteolysis test, catalase test, tween 80 lypolysis test citrate utilization test, gelatine liquefaction test, salt tolerant test and negative result in oxidase test. The bacterium appeared as circular, mucoid, convex, yellow to orange colour on NA medium. The bacterium grew on selective SX media and produced yellow to slightly blue, mostly circular, small, flattened, mucoid colonies. On the basis of morphological, biochemical and cultural features it can be concluded that the causal organism was *Xanthomonas axonopodis* pv. *citri*.

Salt stress and disease development showed significant interaction. Lesion number and size, disease incidence and severity were significantly increased with the increase of salt concentration. This may be due to loss of resistance power of host plant against pathogen under increasing salt stress. The study showed that pathogenic stress and salt stress had combined impact on plant physiological properties. Different physiological properties such as net assimilation rate, chlorophyll content, intercellular CO_2 concentration, stomatal conductance, transpiration rate were significantly influenced by the combined effect of pathogenic and salt stress. The bacterium *Xanthomonas axonopodis* has the genetic potentiality to improve and maintain plant homeostasis for its own survival in to the host cell. The result showed that the bacterium was able to regulate plant physiology up to 30 DAI at 100 mM salt concentration under salt stress condition. But in salt untreated plants the bacterium showed no fixed time limit for modulating physiological features. It was stable in salt untreated plants after 30 DAI rather than increase or decrease. Therefore the performance of the bacterium to modulate plant physiology was increased at moderate salt stress for certain period of time. But in case of higher salt stress the performance of the bacterium to enhance physiological properties was abated. A positive

correlation was found between net assimilation rate and chlorophyll content. Intercellular CO2 concentration and net assimilation rate showed negative correlation.

Phytohormones are plant elicitors that trigger the defense mechanism of plants against various pathogens. In these study phytohormones such as salicylic acid, jasmonic acid, 3-indoleacetonitrile, folic acid, nicotinic acid showed significant result against bacterial canker pathogen. All the elicitors reduced lesion number, size, incidence and severity of canker disease compared with control. Among the elicitors salicylic acid (SA) showed highest performance and folic acid (FA) and nicotinic acid (NA) showed lowest performance while jasmonic acid (JA) and 3-indoleacetonitrile (IAN) showed moderate performance against canker disease. The bacterium required prolonged time for symptom expression after inoculation in SA treated plants. Bacterium required less time in JA and INA treated plants than SA treated plants but higher time than FA and NA treated plants. Endogenous total phenolic compound and potassium contents were increased after application of the elicitors. SA treated plants showed highest accumulation of total phenolic compound and potassium content in leaves cell followed by JA and INA and then FA and NA compared with control. Negative correlations were found among disease incidence and severity along with total phenolic compound and potassium content. A positive correlation was found between total phenolic compound and potassium content. These phytohormones did not show *in-vitro* antibacterial properties against *Xanthomonas axonopodis*. Moreover they were able to accumulate endogenous total phenolic compound and potassium content in the leaves cell and thus inhibited citrus canker disease development. So it can be concluded that all the elicitors applied in this study were able to induce resistance against *Xanthomonas axonopodis*. Among the elicitors SA showed the highest induced resistance against bacterial canker pathogen followed by JA and INA while FA and NA showed the lowest induced resistance compared to control.

The present study ravealed that understanding the interaction between *Xanthomonas axonopodis* and salt stress helps to proper management of the disease in saline prone area. Further molecular study is suggested for proper understanding of the interaction between the bacteria and citrus plant under salt stress condition. Besides the study also revealed that salicylic acid, jasmonic acid and 3-indoleacetonitrile should be used for citrus canker management as eco-friendly alternatives to chemical compounds.



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APPENDICES

Appendix I. Preparation of culture media and reagents

The compositions of the media used in this thesis work are given below. All media were autoclaved at 1210C 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
NH4Cl	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

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

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

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

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

Tween 80 reagent

Peptone	10g
KBr	10g
$CaCl_2$	0.25g
Agar	15g
Tween 80	10ml
Cephalexin	3.5ml
5-fluorouracile	2ml
Cyclohexamide	0.5ml
Water	1000ml

Asculine reagent

Peptone	10g
Asculine	1g
Ferric citrate	0.5
Agar	15g
Water	1000ml

Milk proteolysis reagent

Beef extract (Difco)	3g
Peptone (Difco)	5g
Bacto agar	15
Yest extract	5g
Powder milk containing 0.0004% bromocreasol purple	4g
Water	1000ml

Appendix II. Spectrophotometer and flamephotometer reading at different catechol and potassium chloride concentration

Table 1: Average absorbance reading and total phenolic concentration in leaves at different treatments.

Treatments	15 DAI		45 DAI		30 DAI	
	Absorbance reading (nm)	Phenol conc.(ppm)	Absorbance reading (nm)	Phenol conc.(ppm)	Absorbance reading (nm)	Phenol conc.(ppm)
T_1	3.80	150	3.80	150	4.01	160
T_2	8.10	340	9.10	380	11.60	450
T_3	7.5	300	8.40	350	9.10	380
T_4	6.26	250	6.86	280	7.92	320
T_5	5.50	210	6.05	240	6.47	260
T_6	4.20	175	5.50	210	6.05	240

T_1= Water (control)
T_2= Salicylic acid
T_3= Jasmonic acid
T_4= 3-indoleacetonitrile
T_5= Folic acid
T_6= Nicotinic acid

Appendix II. Spectrophotometer and flamephotometer reading at different catechol and potassium chloride concentration

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T_2	8.10	340	9.10	380	11.60	450
T_3	7.5	300	8.40	350	9.10	380
T_4	6.70	250	6.80	280	7.92	320
T_5	5.50	210	6.05	240	6.47	260
T_6	4.20	175	5.50	210	6.05	240

T_1 - Water (control)
T_2 = Salicylic acid
T_3 - Jasmonic acid
T_4 - 3-indoleacetonitrile
T_5 - Folic acid
T_6 - Nicotinic acid

Table 2: Average % emission reading and potassium concentration in leaves at different treatments.

Treatments	15 DAI		30 DAI		45 DAI	
	% emission reading	potassium conc.(ppm)	% emission reading	potassium conc.(ppm)	% emission reading	potassium conc.(ppm)
T_1	2.99	13	2.99	13	3.45	15
T_2	4.44	19	4.6	20	5.30	23
T_3	3.91	17	4.4	19	4.60	20
T_4	3.91	17	4.4	19	4.60	20
T_5	3.68	16	3.91	17	4.14	18
T_6	2.76	14	3.68	16	3.91	17

T_1= Water (control)
T_2= Salicylic acid
T_3= Jasmonic acid
T_4= 3-indoleacetonitrile
T_5= Folic acid
T_6= Nicotinic acid