STUDY OF THE DISEASE CITRUS CANKER AND FIELD TRIAL TO FIND ITS EFFECTIVE CONTROL MEASURE IN “KAVRE” NEPAL

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ABSTRACT

Of all the agricultural pests and diseases that threaten citrus crops, citrus canker is one of the most devastating. The disease, caused by the bacterium *Xanthomonas campestris* pv. *citri,* occurs in large areas of the world's citrus growing countries including Nepal. Severe infection of the disease produces a variety of effects including defoliation, dieback, severely blemished fruit, reduced fruit quality and premature fruit drop.

The causative agent of the disease *Xanthomonas campestris* pv. *citri* was isolated from the diseased plants and pure culture was isolated. The isolated culture was subjected to Gram staining, Catalase test, Oxidase test, O-F test, Starch hydrolysis, Nitrate reduction test, Methyl-red test, Voges-prausker test, Indole production test, Urease test. To re-confirm it, pathogenicity test was conducted on host plant and after the appearance of the typical citrus canker lesion on host, the bacteria was re-isolated, thus proving the Koch's postulates.

Different controlling chemicals, Copperoxychloride (2.5%), Copperoxychloride + Kasugamycin (1000X), Bordeaux mixture 1% and 2% were sprayed to the plants in citrus orchard at Dhulikhel and the decrease in disease severity after spraying of the chemicals were calculated with reference to the plants that were not sprayed with the chemicals. It was observed that spraying of the chemicals helps in decreasing the disease severity and Bordeaux mixture was superb in controlling the disease. The chemical spray however was not able to eradicate the disease.

It is concluded that *Xanthomonas campestris* pv. *citri* is the causative agent of the disease Citrus canker and the spray of Bordeaux mixture very early with the appearance of first symptoms of the disease can eliminate the disease in citrus fruits to minimum level.

Key words: Lime, Citrus Canker, *Xanthomonas campestris* pv. *citri*, Kavre

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LIST OF ABBREVIATIONS

C.	: Citrus
°C	: Degree Celsius
CBS	: Citrus Bacterial Spot
CC	: Citrus Canker
CFDD	: Citrus Food Development Division
cfu	: colony forming unit
EPS	: Exopolysaccharide
Gr.	: Greek
Ha	: Hectare
LPS	: Lipopolysaccharide
mt.ton	: Metric Ton
p.v.	: Pathovar
spp.	: Species
USDA APHIS	: United States Department of Agriculture, Animal and Plant Health service

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CHAPTER-I

1.1 INTRODUCTION

In an agricultural country like Nepal, horticulture is of great significance and it is one of the major sources of cash generation. The geographical and climatic conditions of mid-hill region of Nepal are favorable for the cultivation of different fruit crops like citrus fruit which occupies an important place.

The center of origin of Citrus species is the Asia, extending from the Himalayan foot hills of Nepal, Bhutan, North- Eastern India to North Central China and in South East from Philippines, East Burma, Thailand, Indonesia and New Caledonia (Lama and Kayastha, 1999).

Different species of Citrus are reported from Nepal (including exotic species). Among them, Citrus species generally cultivated in Nepal are *Citrus reticulate* Blanco, *C. sinensis* Osbek, *C. aurantifolia* Swingle, *C. pseudolemon* Tanaka, *C. limon* L, *C. grandis* Osbeck, *C. maxima*, *C. aurantium* Linn, *C. jambhiri* Lush, *C. medica* Linn, *C. limettiodes* Tanaka, *C. nobilis* XC, *C. unishiu* M., *Poncirus trifoliata* L., *Fortunella japonica* Swingle and possible hybrid's (locally known as Chaku and Narayani) (C.F.D.D., 2001).

Citrus is rich in Vitamin C, organic acids, inorganic constituents, nitrogen compounds, enzymes, pigments, lipids, volatile flavoring constituents, minerals, sugar and thus is highly recognized for the nutritional and medicinal value. Per capita consumption of citrus fruit in developed countries is about 10 kg per year where as in Asian countries it is about 4 kg per year (Aubert *et al*, 1990). This demand is increasing with growing urban population.

Of total area of Nepal, 61,345 sq. kilometer is covered by mid hill region. The mid hill region (1000 meter to 1500 meters altitude) has a comparative advantage in the cultivation of citrus fruits, especially 'mandarin' and sweet oranges. The area under citrus cultivation constituted about 26.7% of the total area covered by all types of fruits in Nepal in 1998/99 (Regmi, 2000).

In the fiscal year 2004/2005, the total area of 25,909 hectare has been cultivated with citrus fruits, out of which 14,606 hectare is described as productive area with the total production of 15,956 metric ton with the yield of 10.75 (mt./ha). Lime is one of the citrus fruit cultivated in Nepal. In the year 2004/2005, out of total area cultivated with lime, 2,390 hectare has been described as productive area with the total production of 19,132 metric ton (Statistical information on Nepalese Agriculture, 2004/2005).

Bacteria cause a wide variety of plant diseases characterized by such host reactions as galls (tumors composed of undifferentiated cells), wilts (loss of turgor), cankers (localized wounds or lesions resulting from necrosis of stems and bark, rots, deformed fruits, leaf spots, change in the color of plant parts, dwarfing, and retarded ripening of fruit (Pelczar *et al*, 1993).

Studies on bacterial disease in Nepal began in 1963. There are 28 bacterial plant pathogens recorded from 29 host plants of Nepal (Thapa and Manandhar, 1991). In Nepal the disease associated with citrus fruits includes Citrus Greening, Tristeza Viral Disease, Foot rot, Powdery mild dew, Sooty mould, Citrus Canker, Citrus Scab, Pink Disease, Citrus Melanose, Twig Blight, Greasy Spot, Felt Disease, Damping Off, Blue Mould and Green Mould (Deoju, 2005).

Of all the agricultural pests and disease that threaten citrus crops, citrus canker is one of the most devastating. The disease, caused by bacterium *Xanthomonas citri* occurs in large areas of the world. The disease is endemic in India, Japan and other south-East Asian countries, from where it has spread to all other citrus producing continents except

Europe. Citrus canker caused by *Xanthomonas citri* occurs world-wide, primarily in regions where rainfall increases and temperatures rise simultaneously. Occurrence of lesions is seasonal, coinciding with periods of heavy rainfall, high temperatures and growth flushes. These factors generally coincide with early summer in citrus growing regions where rainfall increases as temperatures increase. Citrus canker is unlikely to be found in regions where rainfall decreases as temperatures increase. Citrus canker is spread locally primarily by wind driven rain, overhead irrigation and contaminated equipment. Seed borne dissemination has not been reported (Graham *et al*, 2004).

All young plants above-ground tissues of citrus are susceptible to *Xanthomonas citri.* The bacterial pathogen enters plant tissues through natural openings (stomatas) and wounds. The earliest symptoms on leaves appear as slightly raised tiny blister-like lesions about 4-7 days after inoculation under optimum conditions, *i.e.*, a water film present and temperature between 20-30^{0}C (Koizumi 1985). Under less than optimum conditions, symptoms may take 60+ days to appear (Goto 1992: Loucks 1934).

Environmental conditions are very important in the intensity of canker in a given place. Studies should be conducted at different places to find the variations through the seasons and the years that will determine the expected severity (Canteros, 2004). Important factors are temperatures, relative humidity, and, most important, wind driven rain. The importance of the environment was well studied in Japan (Stall *et al*, 1993). Since the main inoculum come from the lesions in an infected tree, the elimination of symptoms will help in decreasing the inoculum to avoid reinfectation with the rain.

The principal methods of controlling plant disease are avoidance, exclusion, eradication, protection and immunization (Mehrotra, 1980).

CHAPTER-II

2. OBJECTIVES OF STUDY

2.1 GENERAL OBJECTIVE

To study the different aspects of the disease citrus canker in Kavre and to reveal effective control measure of the disease.

2.2 SPECIFIC OBJECTIVES

1. To perform a survey to reveal the status of the disease Citrus canker in different citrus species in different parts of 'Kavre'.
2. To identify and differentiate 'Citrus Canker Disease' from other diseases on the basis of Specific Symptoms shown of the Disease.
3. To find the exact cause of disease spread from one place to another.
4. To isolate the causative agent of the Disease and identify it on the basis of morphological, physiological and biochemical tests.
5. To conduct a pathogenicity test of the isolated Bacteria.
6. To reveal effective chemical control measures, its required dose and frequency of treatment for the control of the disease.

CHAPTER-III

3. LITERATURE REVIEW

The term pathology (Gr. Pathos= suffering + logos= the study or discourse) means the "study of suffering". Thus plant pathology is the study of the suffering plants. The main objectives of plant pathology are etiology, pathogenesis, epidemiology and control. The etiology deals with the living and non living entities including environmental conditions that cause diseases in plants. The pathogenesis deals with the process of infection and colonization of the host by the pathogen, in other words, mechanism of disease development. The study of how and when disease occurs is called epidemiology. The control of a disease concerns with the development of suitable methods for controlling diseases with the objective of reducing the loss in the yield of the crop to its minimum. In simple words plant pathology is concerned with all aspects of plant disease (Aneja, 1993).

3.1 CAUSES OF PLANT DISEASE

When a parasitic micro-organism enters into the causal complex of a disease, it is commonly considered to be the cause of the disease. However, microorganisms are not always the cause of disease as the latter may be sometime caused by the environmental variation (Mehrotra, 1980). The cause of plant disease can be divided into two main groups; those that are noninfectious in nature and those that are biological or infectious in nature. There are two broad categories of the noninfectious pathogens- the chemical agents and the physical agents. Chemical causes of the plant diseases may be excesses or deficiencies of plant nutrients, toxic or misused pesticides, air pollution and harmful chemicals etc. Physical agents causing plant diseases include unfavorable temperatures for growth of plants, excess or deficient moisture conditions, harmful aspects of radiations from different sources. The infectious pathogens are also of varied nature and

include the viruses, bacteria, fungi, algae, higher plants such as mistletoes and animals ranging from nematodes, insects and higher animals including man (Strobel and Mathre, 1970).

Bacteria are important group of plant disease causing organisms. The American plant pathologist J. Burrill (1880) was the first to prove that bacteria could cause plant disease. In contrast to viruses and some obligate fungal pathogens, all bacteria, pathogenic or non pathogenic, are saprophytes and can be cultured on artificial media. According to Mehrotra (1980) all the plant pathogenic bacteria are rod shaped and majority are flagellated. Only two genera viz. *Corynebacterium* and *Streptomyces* are Gram positive and the rest are Gram negative. In contrast to fungal pathogens, bacteria are incapable of mechanically penetrating the cutinized plant tissues, cuticle, periderm etc. the only way they can gain entry into the plants are through non cutinized areas, natural openings, incidental wounds etc. (Mehrotra, 1980).

3.2 XANTHOMONAS

All *Xanthomonas* cells are gram negative rods, sometimes slightly curved with rounded ends, measuring 0.4-0.7 micrometer and predominantly single. Exceptionally, cells up to 1.5 micrometer wide and 4 micrometer long were found, e.g. in the strains *X. campestris* A 647 (LMG 945) (Swings *et al*, 1993). The occurrence of pairs of cells is quite typical for *X. maltophila* and *X. campestris* but it is not clear whether it is a pair of cells or an elongated single cell. Most cells have intracellular granules. No spores or other resting stages occur; no pili or fimbriae have been reported. Cells are surrounded by xanthan gum (extracellular polysaccharide, EPS), not recognizable as capsules (Swings *et al*, 1993). They don’t have sheaths or prosthecae (Holt *et al*, 1994).

Cell Envelope

Based on electron micrographs, *Xanthomonas* has typical inner and outer membranre structure of Gram negative bacteria (dos. santos and Dianese, 1985). The outer membrane is a unique structure of Gram- negative bacteria, composed of mosaic of six to eight major proteins and 50 or more minor ones, one or two heterogenous lipopolysaccharide (LPS) molecules and several lipids. The outer membrane functions as protection from environment, selective permeability and export, receptor and interaction functions and an anchoring function of external structures (Swings *et al*, 1993).

Motility

Cells are motile, sometimes sluggish (Swings *et al*, 1993). Motility occurs by single polar flagellum except *X. maltophilia,* which has multitrichous flagella (Holt *et al*, 1994).

Phages

Two similar filamentous phages have been studied; Ct and Cflt (Kuo *et al*, 1987a). They behave as symbiont. While the host continues to grow and divide, the phage chromosome replicates as an episome. Filamentous phages are continuously extruded from the growing host cell and normally do not cause lysis or lysogeny of the bacterium.

Pigments

Xanthomonas strains form yellow water insoluble pigments, which are characteristic brominated aryl polyenes, known as 'xanthomonadins', except *X. maltophilia,* which does not produce xanthomonadins (Holt *et al*, 1994). The color intensity changes upon ageing (Swings *et al*, 1993). The yellow pigment is probably bound to the

cellular membrane (Dianese and Schaad, 1982). Xanthomonadins only occur in the genus *Xanthomonas* and can be used as a taxonomic marker, particularly to differentiate the genus *Xanthomonas* from other yellow pigmented plant- associated bacteria, e.g. *Pseudomonas*, *Erwinia*, *Flavobacterium* (Starr *et al*, 1977).
However, Basnyat and Kulkarni (1979) have reported pigment less white Xanthomonads from *Centella asiatica*, Linn. Urban, recorded as albino *Xanthomonas spp* in "Bergey's Manual of Systematic Bacteriology".

DNA

The average genome size of *Xanthomonas* is 2.5 x 10^9 Dalton. The percent of G + C content of DNA ranges from 63 to 73% (Swings *et al*, 1993).

Colony Morphology

Xanthomonas strains have convex, round, yellow, mucoid colonies in general agar medium. With even colony margin 'Mature' *Xanthomonas* colonies have a diameter of more than 2mm. sometimes up to 10mm (Swings *et al*, 1993).

Growth Temperature

Most species grow optimal at 28^0C except *X. populi* which do not grow above 23^0C. *Xanthomonas* is unable to grow at 4^0C (Swings *et al*, 1993).

pH Sensitivity

Xanthomonas is not able to grow at an initial pH of 4.5 or lower. At pH 6.5, all *Xanthomonas* strains grow well, except those of *X. fragariae,* which require a pH value of 7.5 (Swings *et al*, 1993).

Osmo- tolerance

X. campestris and *X. maltophila* are the only species which tolerate 10% glucose, a few strains even tolerate 20%. *Xanthomonas* cannot grow in 30% glucose. They are not very tolerant of NaCl within each individual species. NaCl tolerance may vary between 0.5 and 5% among *Xanthomonas* (Swings *et al*, 1993).

Oxygen Requirements

Xanthomonas is an obligate aerobe, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor (Holt *et al*, 1994).

3.2.1 PHYSIOLOGY AND METABOLISM

Carbon Utilization

In presence of *Xanthomonas*, carbohydrate are oxidized, but never fermented. In O-F test *Xanthomonas* oxidize glucose only in open tubes. Being chemoorganotrophic, *Xanthomonas* are able to use a variety of carbohydrates and salts of organic acids as sole sources (Holt *et al*, 1994).

Nitrogen and Growth Factor Requirements

Nitrogen fixation does not occur in *Xanthomonas*. It is determined that most *X. campestris* strains can grow on a simple ammonium – glucose – salt medium (Swings *et al,* 1993). Growth factors usually required include methionine, glutamic acid, nicotinic acid or combination of these. However, *X. citri* does not require methionine or cystine for growth (Holt *et al*, 1994).

3.2.2 METABOLIC PATHWAYS

Enzymatic activities

Based on an examination of the enzymatic activities of over 200 Xanthomonas strains, it was found that the following enzymes were always present in all cultures; acid and alkaline phosphatase, esterase lipase (caprylate), leucine arylamidase and napthol - AS-BI-P\phosphohydrase. The following enzymes were never present; cystine arylamidase, -galactosidase, beta glucuronidase, mannosidase and alpha fucosidase (Swings *et al*, 1993).

a) -glucosidase: This constitutive enzyme is mainly extracellular, although activity is also in the periplasm (Swings *et al*, 1993).

b) D-xylose Catabolizing enzymes: D-xylose isomerase, D-xylose permease and xylulose kinase generase localized on DNA fragment of Xanthomonas XA1-1 of approximately 15 kg (Liu *et al*, 1987).

c) -galactosidase: Frank and Somkuti (1970) determined the general enzymatic properties of -galactosidase from *X. campestris* VB 1459 and concluded that a negative feedback control together with a low substrate affinity determine the inability of *X. campestris* to synthesize large amounts of extracellular polysaccharide in lactose- based media.

d) -mannanase: *Xanthomonoas* produce extracellular, as well as cell bound - mannanase constitutively (Swings *et al*, 1993).

e) Esculin hydrolysis: All Xanthomonas spp are able to hydrolyse esculin (Swings *et al*, 1993).

f) Starch hydrolysis: This feature is present in *Xanthomonas citri* (Swings *et al*, 1993).

g) Lignin breakdowns: Lignolytic Xanthomonas strains grow aerobically in a mineral medium of lignin as the sole energy and carbon source (Swings *et al*, 1993).

h) Cellulolytic activity: Cellulolytic activity has been demonstrated in several *Xanthomonas* cultures (Knosel and Garber, 1968).

i) Pectinolytic activity: Many *Xanthomonas* strains are able to degrade pectin gels (Burkholder and Starr, 1948; Hildebrand, 1971). *Xanthomonas citri* is capable of utilizing pectin (Holt *et al*, 1994).

j) Proteolytic activity: Most strains from the Xanthomonas campestris hydrolyze gelatin (Swings *et al*, 1993).

k) DNAse & RNAse: Over 80% of the strains display some DNAse activity (Swings *et al*, 1993).

l) Methyl red, Nitrate and Indole test: The bacterium is negative for nitrate reduction, indole production and for methyl red test (Chand and Pal, 1982; Goto, 1992).

m) Acetoin production: Acetoin is generally not formed by *Xanthomonas*, the only exception being *X. oryzae* pv. *oryzicola* (Swings *et al*, 1993).

3.2.3 BACTERIAL DETERMINANTS FOR VIRULENCE AND PATHOGENICITY

Membrane active substance:

All *Xanthomonas* species and pathovars, which induce local lesions on their host plants have the capacity to induce the hypersensitive response on non-host plants (Klement,

1982). The HR is characterized by a fast collapse of plant tissue (Within 7-8 hours) after infiltration of living bacteria in high concentrations (> 10^7 cells/ml) (Rudolph, 1993). The capability to induce the HR is predicative of pathogenicity (Stead, 1990), and it has therefore been used for many years as a diagnostic criterion (Rudolph, 1993).

Extracellular polysaccharide

All phytopathogenic xanthomonads synthesize the hetero-polysaccharide, Xanthan., the predominant component of bacterial slime. The typical xanthan consists essentially of a cellulose backbone, substituted on alternate residues containing trisaccharide side chains (Mannose, glucoronic acid), so that the repeating unit is a pentasaccharide composed of D- glucose, D- mannose and D- glucorinic acid in the ratio of 2:2:1 and of varying amounts of pyruvic and acetic acid (Rudolph, 1993).

EPS from Xanthomonads are regarded as determinants of disease due to several effects (Rudolph *et al*, 1989). Morris *et al* (1977) reported from rheological studies that ordered form of xanthan can bind co-operately to certain plant cell wall polysaccharide in away that suggests a role in the colonization of the plant host by bacterial pathogen. Xanthan is unique in showing a marked preference for interaction with b-1,4- glucose containing polysaccharides (including derivatized cellulose) rather than mannose (Morris *et al*, 1977). Joshi (2000) showed that there is statistically significant positive correlation (r = 0.994) between concentration of crude EPS and mean size of the leaf spot caused by *Xanthomonas campestris* pv. *campestris*, thus indicating definite role of EPS in pathogenesis.

Hypersensitive Reaction

The same "gene cluster" is responsible for the pathogenicity on the susceptible plant and for HR on the resistant plant. Schulte *et al* (1991) reported that DNA sequences

homologous to the amplify. Pectolytic enzymes are less common within *Xanthomonas* than cellulases (Rudolph, 1993).

Lipopolysaccharides

The lipopolysaccharide of Xanthomonads consists of a complex polysaccharide portion, to which a lipid component termed lipid 'A' is bound covalently. Lipid A represents the endotoxic center of LPS molecule (Rudolph, 1993).

Motility

Motility seems to play a role in penetrating the plant tissue but not in multiplication and spread in plant (Rudolph, 1993).

3.2.4 HOST RESISTANCE

Resistance, in broader sense, is the act or capacity to escape or exclude infection or efficiently slow down or suppress either intrusion of an agent into the host or its spread within the host or both (Neergaard, 1979). Plant resistant reactions to *Xanthomonas* can be described as following reaction types:

a. General activation or metabolism: During incompatible interaction with xanthomonads, a general activation of metabolism occurs. Especially increase in respiration has been reported (Rudolph, 1993).

b. Wound healing and cutinization: The formation of Physiological barriers has rarely been reported for xanthomonads (Rudolph, 1993). Delay of wound healing can also favour infection by Xanthomonads. For instance, infection of wounded citrus leaves by *X. c.* pv. *citri* was completely inhibited after the development of meristem tissue composed of their dividing cells (Koizumi, 1983). The latter did not appear during incubation in a moist environment, so

that the leaves remained susceptible until the cell walls in the outer tissue were lignified.

c. Hypersensative reaction: The hypersensitive reaction (HR) of plant tissue to inoculation with phytopathogenic bacteria is defined as a rapid and irreversible increase of membrane permeability (membrane leakage) leading to loss of structural integrity and confluent tissue necrosis within 24 hours. The HR is induced by Xanthomonads on non host plants and on certain resistant cultivars. In case of phytopathogenic bacteria, several aspects of HR indicate that this reaction type may not be of general importance as a resistance mechanism (Rudolph, 1993).

d. Accumulation of phenolics and related antibacterial substances: Many phytophysiological studies revealed that the level of phenolics and other secondary substances in plants increase after invasion by pathogens, especially in the resistance reaction (Rudolph, 1993).

e. Agglutination of bacteria: During the resistance response interactions between bacterial and plant polymers result in densely packed bacterial cells which are therefore unable to colonize in the intercellular spaces (Rudolph, 1993).

f. Degradation or inactivation of bacterial virulence factor: Several bacterial determinants for virulence and/or pathogenicity may be inhibited or degraded by the host plant. This resistance mechanism may be decesive for the phenolics, which have long been known as enzyme inhibitor (Rudolph, 1993). Non-synergistic interaction between bacterial extracellular polysaccharides and plant polymers (agglutinins) is another example of this type of resistance mechanism (Rudolph, 1993).

g. Induced resistance: Pretreatment with inducing agents such as living or killed bacteria, subcellular components of bacteria or abiotic agents and stress may protect plants against disease incited by pathogen (Rudolph, 1993). Xanthomonads appear to be inhibited by this type of induced resistance.

h. Adult resistance: It may appear as the plant matures.

i. Anatomic resistance: Anatomic structure of plant some time provides resistance to the disease. The mandarin variety "Szinkum" is resistant to the citrus canker a because it possess a broad cuticular ridge projecting over the stomata, which is otherwise infected if inoculated artificially (McLeon, 1921).

Structural and cellular aspects of host resistance

Two fundamental host determinants for citrus canker are the stage of leaf expansion and the resistance of mesophyll tissue (Gottwald and Graham, 1992; Graham *et al*, 1992a). The susceptibility of tissues to bacterial ingress is greatest when leaves are one-half to two-thirds expanded, a stage of leaf development at which stomates open, but the leaf cuticle is not fully developed. At this stage, leaves are most prone to water soaking. As leaves continue to expand, the cuticle rapidly thickens and the forces required for water to infiltrate tissue increases dramatically (Graham *et al*, 1992a).

3.2.5 TAXONOMY OF XANTHOMONAS

Based on DNA-rRNA hybridization studies, the genus *Xanthomonas* constitutes a distinct branch in the gamma group of Proteobacteria. As described in Bergey's Manual of Determinative Bacteriology, 9th edition, the following species are now recognized within the genus Xanthomonas.

X. albilineans (Ashby Dowson 1943)

X. axonopodis Starr and Graces 1950

X. campestris (Pammel) Dowson 1939

X. citri (Hasse, 1915)

X. fragariae Kennedy & King 1962

X. populi (Ride and Ride) 1978.

X. maltophilia (Hugh) Swings *et al*, 1983

X. oryzae (Ishiyama) Swings *et al*

X. citri and *X. phaseoli* were not included in Bergey's Manual of Systematic Bacteriology, Vol. 1. The two species were revived in 1989 by Gabriel *et al*.
The concerned pathovar citri (*Xanthomonas campestris* pv. *citri* (Hasse) appears to be pathogenic to all cultivated species of Rutaceae, at least when artificially inoculated (Gottwald *et al*, 1993).

3.3 CITRUS CANKER

Origin and history

The geographical origin of citrus canker is a matter of controversy. Lee (1918) reported that it may have arisen in southern China, and assumed *Fortunella hindsii* to be the wild host plant. However, Fawcett and Jenkins (1933) reported that citrus canker originated in India and Java, rather than in other region of orient, because they detected canker lesions on the oldest citrus herbaria kept at the Royal Botanic Gardens in Kew, England (i.e., *Citrus medica* collected from India in 1842-1831 and Indonesia in 1842-1844).

Of all the agricultural pests and disease that threaten citrus crops, citrus canker is one of the most devastating. The disease, caused by bacterium *Xanthomonas citri* occurs in large areas of the world. The disease is endemic in India, Japan and other south-East Asian countries, from where it has spread to all other citrus producing continents except Europe. Generally canker does not occur in arid citrus growing areas and has been eradicated from some areas. According to Das (2003) citrus canker presently occurs in

over thirty countries in Asia, the Pacific and Indian Ocean islands, South America, and the Southeastern USA.

Citrus canker caused by *Xanthomonas campestris* pv. *citri* (Hasse) is a major disease in Nepal. This disease is present throughout the citrus growing areas of the country. The incidence of disease is higher in lime (*Citrus aurantifolia*) than in other *Citrus spp*.

In India, citrus occupies third position among fruits after mango and banana and canker is one of the major constraints of its cultivation. Citrus canker was first reported from Punjab (Luthra and Sattar, 1942; Bedi, 1961). Its occurrence was further recorded in Tamil Nadu (Ramakrishnan, 1954), Andhra Pradesh (Govinda Rao, 1954), Karnataka (Venkatakrishnaiah, 1957; Aiyappa, 1958), Rajasthan (Prasad, 1959), Madhya Pradesh (Parsai, 1959), Assam (Chowdhury, 1951) and Uttar Pradesh (Nirvan, 1960). Several others have reported the incidence of canker on the acid lime and other species of citrus.

3.3.1 PATHOGEN BIOLOGY

Based on currently available information, at least three pathovars (sometimes called strains) of *Xanthomonas citri* have been recognized. These pathovars are distinguished from one another by geographical distribution and by different pathogenicity to members of genus *Citrus.* The pathogen for canker A was first identified and described as *Pseudomonas citri* by Hasse (1915). Bacterial nomenclature has undergone many changes since then and the causal bacterium is now known as *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin [Syns. *X. citri* (Hasse) Dowson and *X. campestris* pv. *citri* (Hasse) Dye] (Dye *et al,* 1980; Vauterin *et al,* 1995).

The taxonomy and classification in the genus *Xanthomonas* is constantly undergoing revision because of phytopathogenic diversity and continues to be controversial (Schaad *et al*, 2000; Vauterin *et al*, 2000). Genetic studies of xanthomonads causing citrus canker and citrus bacterial spot support the distinct symptomatology and histopathology

of these diseases. Most techniques reveal that citrus bacterial spot is caused by a heterogeneous group of strains, while groups of canker strains are quite uniform genetically (Graham and Gottwald, 1991; Stall and Civerolo, 1991).

In the late 1980s, strains associated with canker A were proposed as a new species, *Xanthomonas citri*, whereas types B and C, as well as strains causing citrus bacterial spot remained within *X. campestris* as pathovars *aurantifolii* and *citrumelo*, respectively (Gabriel *et al*, 1989). Schaad *et al* (2000) proposed a reclassification that places citrus canker and citrus bacterial spot strains within *Xanthomonas* as species citri (A strains), aurantifolii (B and C strains) and citrumelo (citrus bacterial spot strains). However, other authors rejected this new proposal, citing insufficient data to justify the removal of these strains from the species axonopodis (Vauterin *et al*, 2000; Young *et al*, 2001). Most recently, Brunings and Gabriel (2003) proposed the retention of *X. citri* as the species that includes only citrus canker strains (A and B–C).

3.3.2 HOST RANGE

The host range of *X. citri* A strain is broad, encompassing many citrus species and hybrids between citrus species and the citrus relative trifoliate orange *Poncirus trifoliate* (Gottwald *et al*, 1993; Graham *et al*, 1990a; Leite and Mohan, 1984). Among citrus cultivars and rootstocks, Asiatic citrus canker is most severe on grapefruit, some sweet oranges such as Hamlin, Pineapple and Navel, Mexican (Key) lime, and the hybrids of trifoliate orange used for rootstocks. These cultivars have proven very challenging or impossible to grow profitably in the presence of citrus canker in moist subtropical and tropical climates (Graham, 2001; Leite and Mohan, 1984). All other commercial cultivars of citrus, although varying in susceptibility, are susceptible enough that they must be removed in an eradication effort when they are diseased or exposed, especially where the citrus leaf miner occurs. Civerolo (1984) lists a number of plants in the Rutaceae other than Citrus and Poncirus that can serve as hosts of *X. citri* under experimental conditions. These plants would not be expected to play any significant

role in epidemiology where the disease is endemic and leafminer is absent, but serve as troublesome inoculum reservoirs in an eradication programme.

In India, citrus canker is reported to be relatively more on acid lime and less commonly on mandarin and sweet orange (Ramakrishnan, 1954). According to Aiyappa (1958) all the cultivated varieties of citrus and some wild species in Karnataka are suspectible to canker possibly due to high humidity and low temperature. Prasad (1959) from Rajasthan made similar observations. The descending order of susceptibility in citrus species is Kaghzi Lime, grape fruit, Karnakhata and sweet oranges (Nirvan, 1961). Mandarins and lemons are resistant and Kumquats are commercially immune under conditions existing in Uttar Pradesh. Jain (1959) reported that different varieties of sweet lime, grape fruit and sweet orange were infected to same extent in Himachal Pradesh. According to Naik (1949) acid limes, some varieties of lemon, sweet orange and grapefruit were very susceptible to canker, while Nepali oblong and round seedless lemons were highly resistant. Mundkur (1961) observed no infection in sweet orange and pummelo but Jambheri, sour orange and Kaghzi lime were very susceptible.

3.3.3 SYMPTOMS

An essential diagnostic symptom is citrus tissue hyperplasia (excessive mitotic cell divisions), resulting in cankers. In typical natural infestations, all young aerial parts of the Rutaceous host can be conspicuously affected. Cankers may be present on leaves, stems and fruit of mature trees; canker symptoms on leaves and fruit can be readily obtained in artificial inoculations. If cankers are not present on leaves, stems and fruit of mature trees, or if leaves and fruit of susceptible citrus species do not develop cankers following artificial inoculation, a diagnosis of citrus canker is not indicated. This may seem obvious, but a fungal disease that did not affect fruit was misdiagnosed in Mexico in 1982 as a "form" of citrus canker, and opportunistic leaf spotting xanthomonads that did not cause cankers or affect the fruit of mature trees were misdiagnosed in Florida in 1984 as yet another "form" of citrus canker (Swarup *et al*, 1991).

As the lesions age, they turn tan to brown, and a water-soaked margin appears surrounded by a chlorotic halo. The center of the lesion becomes raised and corky. Lesions are usually visible on both sides of a leaf. Eventually, the centers of leaf lesions become crater-like and may fall out, creating a shot-hole effect. Defoliation and twig dieback become a problem as the disease intensifies on a plant. On twigs and fruit, Citrus Canker symptoms are similar: raised corky lesions surrounded by an oily or water-soaked margin. No chlorosis typically surrounds twig lesions, but may be present on fruit lesions. Chlorosis symptoms can fade over time. It is the twig lesions on angular young shoots that provide much of the perpetuating *X. citri* inoculum in areas where Citrus Canker is endemic. Fruit blemishes and early fruit drop are major economic impacts of the disease. (Schubert *et al*, 2001).

Wounds become naturally infected at much lower inoculum concentrations than via the stomatal route (minimum dose of approx. 10^2 cells / ml for wounds vs. approx. 10^5 cells / ml for stomates) (Goto, 1992).

According to Das (2003) the economic importance of citrus canker can be analyzed from several different points of view. Loss assessment has not been determined clearly, as in the case of diseases of annual crops. When citrus infection occurs in the early growing stage, the fruits crack or become malformed as they grow, and the heavily infected ones fall prematurely. Light infection in later growth stages may cause only scattered canker lesions on the surface of fruits but makes fresh fruits unacceptable for market. The severity of fruit infection usually parallels that of foliage infection. Eighty to ninety percent of fruit infection is not uncommon in susceptible citrus trees that have already sustained severe foliage infection. Such heavy foliage infection often causes severe defoliation, leaving only bare twigs. In Argentina, for example, 83-97% of the fruit of grapefruit trees were diseased in unsprayed plots during 1979-1980 and in the same plots; up to 88% of the leaves were infected (Stall and Seymour, 1983).

Two fundamental host determinants for citrus canker are the stage of leaf expansion and the resistance of mesophyll tissue (Gottwald and Graham, 1992; Graham *et al*, 1992a). The susceptibility of tissues to bacterial ingress is greatest when leaves are one-half to two-thirds expanded, a stage of leaf development at which stomates open, but the leaf cuticle is not fully developed. At this stage, leaves are most prone to water soaking. As leaves continue to expand, the cuticle rapidly thickens and the forces required for water to infiltrate tissue increases dramatically (Graham *et al*, 1992a).

3.3.4 PATHOGENESIS

Epiphytic growth

Cells of Xanthomonads which are deposited on plant surfaces by water, splash aerosols or other means need to invade the plant tissue in order to build up high populations and to cause disease. Only when favorable conditions exist may the bacteria temporarily multiply on plant surfaces by a process similar to epiphytes.

Epiphytic populations of xanthomonads do not necessarily indicate epiphytic growth because they can also be created by bacterial exudation from lesions. As reported by Timmer *et al* (1991), when lesions of citrus canker were placed in water, 10^4 -10^5 cells/ml of *X. campestris* pv. *citri* exuded immediately, and within 30 min the bacterial concentration in the bathing solution increased 10-fold. These studies indicated that the bathing solution was the result of exudation of bacteria already existing in the lesions. Little or no multiplication appeared to occur on the surface of the leaves in water. Often, the development of external populations parallels internal populations, although on a lower level when expressed as bacterial cells per cm^2 leaf.

This dynamic exchange between internal (eg. substomatal cavities and intercellular spaces) and external bacterial populations probably depends on the prevailing conditions in and on the leaf. Thus, Egel *et al* (1991) determined a positive relationship between internal and external populations of *Xanthomonas campestris* pv. *citri* and bacteria associated with citrus bacterial spot (CBS) disease and concluded that external

populations represented bacteria exuded onto the moist leaf surface through the ruptured epidermis of the lesions.

Timmer *et al* (2000) reported that the population of epiphytic xanthomonads vary considerably during the day and are dependant on environmental conditions. Populations of *X. c.* pv. *citri* on symptomatic leaves varied from 10^4 to 10^6 per leaf in the early morning and declined to 10^2 to 10^5 , respectively, by mid afternoon.

Role of epiphytic phase for survival

Epiphytic populations of xanthomonads which survive from previous seasons are potential inoculum sources. The bacteria can remain dormant during the quiescent period in association with perennial plant hosts or parts, seeds, and leaf residues (Rudolph, 1993). Also, non-host plants, especially weeds, can be colonized by epiphytic populations of xanthomonads and, thus, serve as inoculum source for disease on susceptible hosts (Swings *et al*, 1993).

Penetration

Koizumi (1983) described that Xanthomonads lack active mechanisms for penetrating the protective barriers of plants. Therefore, the bacteria can enter the host plant only through wounds and through natural openings. The latter may be stomata, water pores of hydathodes, lenticels or nectaries. Most of the bacteria which are randomly dispersed over the leaf surface by water splash or aerosols soon disappear if they are not capable of epiphytic growth if they cannot gain entrance into the plant.

Conditions which favour Penetration

It has long been known that moisture and high temperature are necessary for bacteria to penetrate.

Disease cycle and epidemiology

Survival:

Xanthomonas citri survives primarily in naturally occurring lesions. Cankerous leaves, twigs and branches constitute the main source of inoculum. Since affected leaves drop early, they may not serve as the main source of inoculum (Nirvan, 1963), but Rao and Hingorani (1963) found that the bacterium survives up to 6 months in the infected leaves. The disease is carried from season to season mainly in the cankers on twigs and branches. The pathogen can survive in diseased twigs up to 76 months (Chakravarti *et al,* 1966). Vasudeva (1958) found that the organism survived in the infected leaves for more than six months, in the sterilized soils for 52 days and in the unsterilized soils for 9 days only. Under desiccation at 30^0C, he found the organism surviving for 11 or 12 days. Paracer (1961) observed that the bacterium was resistant to drying and was killed after 120 days in ordinary laboratory temperature. The bacterium also survives epiphytically at lower population levels on citrus hosts without symptom development, in association with non-citrus weed and grass hosts and also in soil (Goto, 1970, 1972, Leite and Mohan, 1984). But saprophytic survival of *X. citri* in soil in absence of plant tissue or debris has not been conclusively established (Goto, 1970). Graham (1989) reported that population of *X. citri* have very limited survival capability in subtropical soils. Attempts to detect surviving bacteria on various inanimate surfaces such as metal, plastics, cloth and processed wood in both shade and sun indicate the inoculum dies within 24-72 hours (Graham *et al*, 2000).

The bacteria easily persists season to season in old lesions, especially in warmer climates and in lesions formed late in the growing season (Pruvost *et al,* 2002). The pathogen is not systemic in the host plant. It can remain viable as long as host cells in the vicinity of the lesion remain viable, though the bacterial titer will drop considerably. Reports on inoculum longevity outside host tissue are inconsistent. On exposed, symptom less citrus, circumstantial evidence suggests some inoculum persistence for at least several months, since root sprouts from infected trees that have been removed months prior frequently become infected themselves. The bacteria may persist for

several weeks on non-host plant material, with some exceptional reports of longer persistence (about 8 months) in the root zone of certain grasses under infected trees in Japan (Goto *et al.* 1975). Once infected/exposed leaves or fruit drop to the ground, the bacterial population declines to a non-detectable level in 1-2 months because of antagonism and competition with saprophytic microorganisms (Goto, 1992). Reports of survival on inanimate surfaces vary from a few hours to several months. In general, when inoculum dries on nonporous surfaces, it dies. Concentrated inoculum, such as from natural lesions or from culture, survives longer than cells diluted in a water suspension. The intact polysaccharide slime coating on the bacterial cells is thought to be essential for longer survival.

Multiplication

After penetration through natural openings or wounds into intercellular space, phytopathogenic Xanthomonads begin to multiply. Even single bacterial cell may initiate infection as was shown for *X. c.* pv. *malvacearum* (Essenberg *et al* 1979) pointed out by Novacky & Ullrich- Eberins, the intercellular spaces of higher plants contain all the essential nutrients to enable the bacteria to multiply in the first phase of the infection process. Cell walls may be another nutrient source, since most of the Xanthomonads excrete cellulases.

In the intercellular spaces most Xanthomonads develop gummy masses due to secretion of the exopolysaccharide, xanthan that enlarges and pushes aside and kill the adjacent cells.
The stomata probably serve as the most common portal, since they are distributed universally over the leaves, and also occur on stems and fruits.

From the natural openings of plants, especially the hydathodes, water and nutrients may exude onto the plant surface. Motile bacterial cells with chemotactic response are

attracted to these sites. It has been assumed that chemotaxis plays a role only during the transitional infection phase in the life cycle of xanthomonads.

After the bacteria have reached the substomatal cavity actively or passively they multiply rapidly before infection is visible to the unaided eye. This initial proliferation appears necessary for the penetration into the leaf tissue, so the bacterial masses emerging from the substomatal cavities serve as inocula for the secondary infection (Swing *et al*, 1993).

3.3.5 DISSEMINATION OF PATHOGEN

Dissemination implies the spread of a plant pathogen into new geographical area. There are essentially two types of dissemination of plant pathogen, i.e. direct transmission and indirect transmission. Direct transmission is the dispersal which takes place along with seeds and vegetative parts while indirect transmission may be autonomous that is by means of wind, water, animals or human being. Water is an important agent of local dissemination, but it is not as important as wind for long distance dissemination (Mehrotra, 1980).

Since Xanthomonads have mucilaginous coat, they easily suspend in water and are dispersed in droplets. Spread of canker bacteria by wind and rain is mostly over short distances,i.e.in trees or to neighboring trees. Cankers develop more severely on the side of the tree exposed to wind-driven rain. Rainwater collected from foliage with lesions contains bacterial population between 10^5-10^8 cfu/ml (Goto, 1962; Stall *et al*, 1980).

If the average wind speed during rains exceeds 8 m/sec (18 mph), the disease may be very severe (Kuhara, 1978). Wind blown inoculum was detected up to 32 meters from infected trees in Argentina (Stall *et al*, 1982). Spread over longer distances, up to7 miles, can occur during severe tropical storms, hurricanes, and tornadoes (Gottwald *et al*, 2001). Long-distance spread more often occurs with the movement of diseased propagating material, such as budwood, rootstock seedlings, or budded trees. Insects

have also been found involved in the transmission of xanthomonads. *X. c*. pv *citri* have been reported to be transmitted by leaf miners (Cook, 1988).

3.3.6 DISEASE DIAGNOSIS

The disease is diagnosed on the basis of specific symptoms. 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 (Goto and Yaguchi, 1979; Gottwald *et al*, 1988). 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.

3.4 ISOLATION AND IDENTIFICATION OF XANTHOMONAS

Xanthomonas campestris pv. *citri* grows readily on most laboratory media and can be easily isolated from diseased tissue by standard techniques. Colonies appear on agar media after 2-3 days incubation at 28^0C. Colonies have distinctive yellow color of xanthomonads and xanthomonadin pigment can be extracted from the cells. On high carbohydrate media cultures are mucoid. Swing (1993) prescribed GYCA for the isolation of *Xanthomonas campestris* pv *citri* for the plant tissue. As prescribed by Schaad (1988), a differential media Glucose Yeast Chalk Agar (GYCA) can be used for examination of pigmentation.

Identification of the bacteria includes serological methods, morphological and biochemical characters, pathogenicity tests, electron microscopy, bacteriophases and others.

Pathogen and strain identification

Because symptoms are generally similar, identification and separation of canker pathogens and strains are based on cultural and physiological characteristics (Schaad,1988), bacteriophage sensitivity (Goto *et al,* 1980; Civerolo, 1984), serology (Alvarez *et al*, 1991), plasmid fingerprints (Pruvost *et al*, 1992), DNA- DNA homology (Egel *et al*, 1991) and by various RFLP (restriction fragment length polymorphism) and PCR (polymerase chain reaction) analyses (Gabriel *et al*, 1988; Hartung and Civerolo, 1989; Gillings *et al,* 1995; Hartung *et al,* 1996; Miyoshi *et al,* 1998; Cubero and Graham, 2002). When the DNA-based assays are unavailable, strains of *X. citri* can be distinguished from other pathovars by infecting a panel of susceptible and resistant citrus hosts or as a bioassay on detached-leaves or leaf-disks (Gottwald *et al,* 1993). Such pathogenecity test is an essential component in diagnostic programmes for regulation of citrus canker diseases (Schubert *et al*, 2001).

3.5 DISEASE CONTROL

Worldwide, millions of dollars are spent annually on prevention, quarantines, eradication programs, and disease control. Undoubtedly, the most serious consequence of citrus canker infestation is the impact on commerce resulting from restrictions to interstate and international transport and sale of fruit originating from infested areas. The disease has been studied in greater detail in the U.S. where it caused very serious damage, so much so that millions of canker affected trees were cut and burnt. In Florida, for example, during the year 1915-33, nearly, 57,000 orchard trees and 3,000,000 nursery plants were destroyed at a cost of over $ 6 million and again during the year 1984-86, nearly 20 million citrus nursery plants were destroyed at a cost of over $ 25 million (Schoulties *et al*, 1987).

Copper-based bactericides are a standard control measure for citrus canker world-wide (Koizumi, 1985; Leite and Mohan, 1990). Copper reduces bacterial populations on leaf surfaces, and multiple applications are needed to achieve adequate control on

susceptible hosts (Stall *et al*, 1980). Copper-based spray programmes are effective when targeted to the spring leaf flush to protect leaves from the one-half to full expansion stage over a period of 2–4 weeks (Graham *et al*, 1992a; Stall *et al*, 1982b).

Fawcett (1936), Naik (1949), Cheema *et al* (1954), Ramakrishnan (1954), Govinda Rao (1954), Prasad (1959) and Paracer (1961) recommended pruning of infected twigs before the onset of monsoon and spraying of 1% Bordeaux mixture at periodic intervals for an effective control of the disease. Patel and Desai (1970) reported that pruning of affected twigs every year during Nov-Dec and 3 to 4 sprays of Bordeaux mixture (1%) in a year could reduce the disease. Two prunings alongwith 4 sprays of 5000 ppm copper oxychloride or 1% Bordeaux mixture is reported to be effective against the disease (Kishun and Chand, 1987).

Copper oxychloride (0.3%), streptocycline (100ppm) and neem cake suspension was found very effective in controlling the disease (Das and Singh, 2000). Canker incidence can also be reduced by periodic spraying of insecticides to control of leaf miner damage to newly unfolded leaves, as such damage facilitates citrus canker infection.

Spraying with copperoxychloride-kasugamycin during the growing season and immediately following strong wind and heavy rain gives protection against black rot. (Kenya Agricultureal Research Institute, 2006)

Copper hydroxide, basic copper chloride, copper oxychloride, and tribasic copper sulfate are the most effective bacterial sprays for protecting leaves and fruit. These materials can reduce the incidence of disease, but they will not eliminate established infections. Copperoxychloride plus streptomycin did not make significant different. Copperoxychloride and Kasugamycin have been used with satisfactory results. However, extensive use of copper may also cause phytotoxicity problems in treated groves (International citrus canker research workshop, 2000).

Use of kasugamycin + copper compound for effective controlling of Erwinia, Pseudomonas, Xanthomonas, Corynebacterium, Agrobacterium and Streptomyces has been proved by several researches.

Gottwald and Timmer (1995) reported the efficacy of wind- breaks in reducing the spread of citrus canker in Argentina. The windbreaks recommended are Eucalypt, Pine, Grevillea, Casuarina or any other tall trees. The effect should be to diminish the speed of wind, no to stop it completely (Canteros, 2004). Control measures developed in Japan include windbreaks (Koizumi *et al*, 1996) or pruning of diseased summer and autumn shoots, forecasting and chemical sprays.

Sanitation

Use of disinfectants is recommended in selected plots. All equipment used in the plot should be disinfected. Hands, clothing and gloves of laborers, collecting boxes and any other tools should be treated. Quaternary ammonium, phosphoric acid-iodine solutions, sodium hypochlorite or 70% ethanol can be used (Canteros, 2004).

Selective localized pruning.

A method used for years in Japan in canker infected plants is the pruning of affected tissue in fall and/or winter. This will decrease the available inoculum very sharply. Pruning of affected tissue is used in Argentina in new planting and in plots treated to get canker-free fruits. The objective is to eliminate all diseased tissue in selected plots. Herbicide defoliation is recommended in heavily infected plots to start a program toward the objective of keeping it free of canker symptoms (USDA, 1997).

Control of canker in the countries or the regions where the disease is not present include quarantine or regulatory programme to prohibit introduction of infected citrus plant

material and fruit, as well as continuous and strict surveying in the field and the immediate destruction of the trees. In countries where canker is present, integrated systems of compatible cultural practices and phytosanitary measures consisting of resistant hosts, removal of inoculum sources, properly designed windbreak systems, timely application of protective copper- containing and/or antibiotic sprays are generally the most effective means of disease management.

CHAPTER-IV

4. MATERIALS AND METHODS

A list of materials, chemicals, equipments, media and reagents required for the study is presented in Appendix 1.

4.1 METHODS

This study was carried out from 2005 to 2006. Lab works were conducted at Nepal Academy for Science and Technology (NAST), Khumaltar, Lalitpur, Nepal and field works were conducted at different Citrus orchards of Kavre.

4.1.1 SITE OF FIELD STUDY

Geographically, the 'Kavre' district is located between 27^0 20' N to 27^0 45' N latitude and 85^0 21' E to 85^0 59' E longitude. This is representative of mid hill district of Nepal. The elevation ranges from 200m to 3018m above the sea level. The average temperature ranges in high hills from 9-10^0C minimum and 17- 19^0C maximum and in low lands (below 900m) it is (14^0C-15^0C) minimum and 27^0C-28^0C maximum. The average rainfall of the district is about 1757mm. The district has privilege of having sub-tropical climate which is favorable for growing citrus in large areas of the district (Regmi, 2000).

According to statistical information of Nepalese agriculture (2004/2005), citrus fruits are extensively cultivated in 'Kavre' where the total productive area is 532 hectare with the total production of 5946 metric tons. Lime is one of the citrus fruits cultivated in 'Kavre', which has total productive area of 73 hectare with the production of 603 metric tons.

The study site, Dhulikhel, lies at 27^0 37' latitude and 85^0 33' Longitude. The elevation of the area from sea level is 1552m. The orchard lies in the slope of about 45 degree where narrow terraces were made for the cultivation of citrus plants.

The temperature of the area, its relative humidity and annual rainfall plays an important role in the increase of the disease in the area as these factors are essential for the survival and dissemination of the pathogen. The map of study areas and table showing average temperature, precipitation and relative humidity of 'Dhulikhel" is presented in the Annex II.

4.1.2 SURVEY OF THE DISEASE

Survey of the disease was carried out in different Citrus orchards of Kavre. The areas of Survey included were Saradha Batase, Thanal Thok and Dhulikhel. In the survey, Citrus plants were looked for the symptoms which include raised, corky, tan lesions with water-soaked margins and yellow halos on the leaves.

4.1.3 STATUS OF THE DISEASE

Status of the disease was found out by calculating disease frequency and severity.
Disease Frequency was calculated by using following formula (Johnston and Booth, 1983)

$$\text{Disease frequency} = \frac{\text{Number of infected plants}}{\text{Total Number of plants}} \times 100$$

Severity of the disease of each plant was calculated by counting the number of leaves showing more than 5 canker lesion out of total leaves. (Johnston and Booth, modified)

$$\text{Disease severity} = \frac{\text{No. of leaves with lesion}}{\text{Total no. of leaves}} \times 100$$

4.1.4 ISOLATION OF THE BACTERIA

The leaf sample was used for the isolation of the bacteria. The leaf sample taken was washed thoroughly in running water.

Using a sterile razor blade, younger portions of the lesion was cut from a recently collected material. Then after a lesion was taken on a clean microscopic slide and a drop of sterile water was added, it was observed under oil immersion, if streaming bacteria were seen, they were proceeded for isolation.

The cut portion was surface sterilized by dipping it in surface sterilant (0.1% Mercuric Chloride) for few seconds.. The leaf sample was then rinsed in 3 changes of sterile water to remove residual mercuric chloride. Then 1ml of sterile water was taken in Petri dish and the surface sterilized sample was transferred to it. The lesion was finely minced with a flamed razor blade. It was then allowed to stand for 10-15 minutes. The liquid minced with lesion was then serially diluted up to 10^{-3} dilution. Then after spread plate technique was done using 0.2 ml of each diluent on Glucose yeast Chalk Agar (GYCA). The plates were than incubated at 28^{0} C for 72 hours.

Then suspected mucoid colonies with typical yellow pigment were subjected to Gram staining and then for further biochemical and physiological tests.

GRAM STAINING

A thin smear of the bacteria sub-cultured on Nutrient Agar was made on a glass slide. The smear was air dried and heat fixed. The slide was then flooded with Crystal Violet for 1 minute. Then the slide was washed in tap water until no more stain could be removed from the smear. It was then flooded with Gram’s iodine solution for 30 seconds, and washed by water after this; it was decolorized by using 95% ethanol for few seconds to remove the stain. The slide was again washed with distilled water.

Finally it was counter stained by applying safranin for 1 minute, then washed with distilled water and blotted dried with absorbent paper. The dried slide was then observed under immersion oil of microscope. The bacteria if appeared pink red, are referred to as gram- negative and gram-positive if it retains the color of the primary stain, the crystal violet.

4.1.5 BIOCHEMICAL TEST

Catalase test

Catalase test was performed talking few ml of 3% hydrogen peroxide (H_2O_2) in the glass test tube and mixing pure bacterial cultures in it using sterile glass rod. Appearance of air-bubble was positive reaction otherwise negative.

Oxidase test

Oxidase test was performed on Whatmann filter paper No. 1. The filter paper was taken in a Petri dish and pure bacterial colony from fresh (24-48Hrs) culture was placed in it using sterile glass rod. Then, 1-2 drops of freshly prepared 1% aq. solution of tetramethyl p- phenylene diamine dihydrochloride was added to it. The change of the reagent to a purple color within 10 seconds of application of reagent was regarded as positive reaction test, otherwise not. In the test, *Pseudomonas aeroginosa* was taken as a positive control.

Oxidative- fermentative test

Oxidative- fermentative test was performed by using Oxidative- fermentative medium (Hugh and Leifson, 1953). The test organism was inoculated into two tubes of O-F medium. The inoculated medium in one tube was sealed with a layer of liquid paraffin

to exclude oxygen. Both the tubes were incubated at 28 C for 3-4 days. The observation was done on the basis of color change of the indicator bromothymol blue incorporated in medium. Oxidative organisms showed color change of only open tubes, whereas fermentative organisms showed color change to yellow in both sealed and open tubes. Further biochemical tests were performed only for those organisms which were Gram negative rods, catalase positive, oxidase negative and oxidative organisms having yellow pigmented mucoid, smooth colonies in GYCA agar.

Methyl Red and Voges-Proskauer tests

The bacterial suspension was inoculated to MR-VP medium and incubated at 27 C for 48 hours. After incubation, 5 drops of methyl red reagent were added to one tube while 12 drops of VP reagent I and 2-3 drops of VP reagent II were added to the other tube. Then after, the tubes were observed for color change. The change in color of the tube to red was indicative of the positive MR test and the turning of methyl red to yellow was the indicative of negative MR test.

The tube tested for VP was shaken gently with the cap off to expose the media to air. In case of VP test, the development of Crimson-to ruby pink (red) color was indicative of Positive VP test while no change in coloration was a negative test.

Indole test

The indole test was performed by inoculating a bacterial suspension to SIM medium and incubated for 27 C for 48 hours. After 48 hours of incubation, 1 ml of Kovac's reagent to both the test tube and control tube. The tubes were shaken gently and the tubes were observed. Development of a Cherry (deep) red color on the top layer of tube was indicated as positive test and the absence of red coloration was indicated as indole negative.

Urease test

Urease test was performed by inoculating the test organisms on urea broth. The development of deep pink colour of the tube was indicative of positive test while the failure to produce such colour was indicative of the negative urease test.

Starch hydrolysis test

The starch medium, as prescribed by Lelliot and Stead (1987) was used for starch hydrolysis test. Starch agar plates were inoculated by streaking the test bacterial colony (48 hour culture in Nutrient agar) and incubated at 28^0 C for 4-6 days.
Then the grown bacterial culture in agar plates was flooded with lugol's iodine. Appearance of clear zone, around and/or under the bacterial growth, was recorded positive test whereas medium turned to blue was recorded as negative test.

Nitrate reduction test

Nitrate broth was prepared as prescribed by Lelliot and Stead (1987). The test organism was inoculated in the medium and incubated at 28^0C for 48 hours. Then after few drops of Nitrate test reagent was added to the tubes. The appearance of red-pink color indicates the presence of nitrite, formed by the reduction of nitrate present in the medium where as no change in color indicates the absence of nitrite.

Utilization of Different Carbohydrates

Carbohydrate utilization test was carried on Dye's medium C (1962). The carbohydrate used for the test inclue Arabinose, Mannose, Galactose, Glucose, Sucrose, Fructose, and Sorbitol. The bacteria was inoculated to the medium and incubated at 27^0C for 21 days.

The organisms that were recorded as Gram negative rod, Catalase test positive, Oxidase test negative, Oxidative, starch hydrolysis test positive, nitrate reduction test negative, Indole test negative, Methyl red test negative, VP test negative, Urease test negative were identified as *Xanthomonas campestris* pv. *citri*

4.1.6 PATHOGENICITY TEST

Inoculation on Potted lime Sapling

Pathogenicity test was performed for the confirmation of host specific plant pathogenic bacteria, *Xanthomonas campestris* pv. *citri*.

For the pathogenicity test, Disease free Lime saplings were pot cultured. Five plants were used for the pathogenicity test and one plant was taken for the control.

Single isolated colony from the 3 days old culture in GYCA was taken and mixed with sterile distilled water to prepare bacterial suspension containing 10^6-10^7 CFU/ml. (compared with Mac Ferland's Scale)

0.1 to 0.2 ml of the suspension was inoculated on 3 to 4 points of each leaf. In each test plant 2 to 3 leaves were inoculated with bacterial suspension using 1ml syringe. Similarly sterile water was inoculated to the plant which served as a control.

Incubation

The inoculated seedlings in pot were covered with polythene bags to maintain the humidity. The bags were removed after 3 days.

Observation of the Symptoms

Symptoms were first observed beginning four days after inoculation as a raised margin surrounding a slightly chlorotic region. Over time, the raised margin becomes pronounced, roughened and corky, while the central region of the lesion becomes

necrotic and collapsed. After several weeks, the necrotic lesions may split and the leaves abscise.

Observation of anatomical change on infected leaves

The leaf showing disease symptom with characteristic lesion was taken. The thin section of the leaf was cut at the margin of the lesion. Then the cut section was dipped in safranine for few minutes and then observed under microscope at 10X and 40X. Similarly, the section was made for the leaf without disease symptoms and the difference in the morphology of the diseased tissue and disease free tissue were compared.

Measurement of the spot

The lesions formed on the inoculated sites were measured using centimeter scale and recorded.

Evaluation

As the symptoms were developed, the lesions were cut and observed for the bacterial oozing under microscope from cut surface. It was confirmed by isolating the organism in GYCA and by comparing its colony and morphological characteristics with reference.

Pathogenicity test on Detached leaves

Young disease free leaves were washed 10 minutes in running tap water, surface sterilized in 1% Sodium hypochlorite for 1-2 minutes and aseptically rinsed thoroughly with distilled water. Aseptically, two leaves were placed in two different Sterile Petri dishes containing water soaked filter paper to retain the moisture. Then, one of the leaf

in Petri dish was inoculated with the bacterial suspension and the other was inoculated with sterile distilled water. The Petri dishes were maintained in a lighted incubation at 25-30^0 C. After 5-7 days, the leaves were looked for the lesion characteristic of Citrus canker. The inoculated leaf showing characteristic lesion was taken, the portion showing the lesion was cut and taken in a sterile Petri dish. Two to three drops of sterile distilled water was added to it. The leaf sample was minced using sterile razor and the liquid was streaked on Glucose Yeast Chalk agar (GYCA) and incubated at 27^{0}C for 72 hours. The isolated colonies were gram stained and then further biochemical tests were performed to confirm the bacteria to *be Xanthomonas campestris* pv. *citri*.

4.1.7 CHEMICAL SPRAY FOR DISEASE CONTROL

The control of the diseased plant was carried out in the Citrus Orchard of Kavre. First of all a general survey was carried out to observe the disease status of the Orchard. The number of plants with different level of severity was recorded. The orchard was divided into five different plots in order to control the disease using different chemicals. From each plot five plants were selected on Randomized Complete Block Design (RCBD) where each plant was considered as one replication for the treatment. The fifth plot was taken as a control and water was sprayed in that plot.

TREATMENTS

For the control of the disease different controlling agents were chosen. The selected controlling agents were 2.5% Copperoxychloride, Copperoxychloride + Kasugamycin (1000X), Bordeaux mixture 1% and 2%.

Treatment 1

The first plot was spread with 2.5% Copperoxychloride for three times at the interval of 15 days.

Treatment 2

The second plot was sprayed with Copperoxychloride +Kasugamycin (1000X) for three times at the interval of 15 days.

Treatment 3

The third plot was sprayed with Bordeaux mixture (1%) for three times at the interval of 15 days.

Treatment 4

The forth plot was sprayed with Bordeaux mixture (2%) for three times at the interval of 15 days.

Treatment 5

The fifth plot was sprayed with water as a control for three times at the interval of 15 days.

After each spray the disease severity was observed on the basis of approximate number of leaf showing canker lesion and documented. All together 5 observations were made at 15 days intervals. Two of the observations were made prior to the chemical treatment and three after each of the treatment.

4.1.8 OBSERVATION FOR DISEASE SEVERITY

The disease severity was calculated two times in 15 days interval prior to spray and next three times after each chemical spray.

Dynamics of disease development

Dynamics of the disease development was studied on the basis of increase in disease severity of control plot sprayed with water in every 15 days interval.

CHAPTER-V

RESULTS

The study was conducted from October 2005 to August 2006 and during this period a survey was conducted in 3 orchards of Kavre i.e. Khanal Thok, Sharada Batase and Dhulikhel.

In the orchard of Dhulikhel, which was chosen as the field for the work, only *Citrus aurantifolia* was cultivated where out of total 55 plants, 48 were found infected with different level of severity and 7 were found uninfected. In Khanal thok, out of 24 citrus fruits cultivated which included *C. aurantifolia* and *C. reticulata*, only 9 of *C. aurantifolia* was found infected, though both the fruits were grown in close vicinity. In Saradha Batase, the total of 97 citrus plants were surveyed which included *C. reticulata* and *C. sinensis* and none of them were infected with the disease Citrus canker.

Table 1: Species of Citrus fruits infected with Citrus Canker in Survey Area

Species of Citrus fruit	No. of Plant Observed			No. of plants with Citrus canker		
	Dhulikhel	Khanal Thok	Saradha Batase	Dhulikhel	Khanal Thok	Saradha Batase
C. aurantifolia	55	9	X	48	9	X
C. reticulata	X	15	72	X	X	X
C. sinensis	X	X	25	X	X	X

It was observed that only *C. aurantifolia* was infected with the disease citrus canker and no other citrus species cultivated in the study area were infected. In Dhulikhel, *C. aurantifolia* were highly infected with the disease status of 87.27%. In Khanal Thok the disease status of the orchard was 40.9% and the disease status among cultivated lime alone was 100%. The disease was not observed in Saradha Batase.

THE DISEASE SYMPTOMS OBSERVED ON LEAF

The symptoms of the disease were observed as tan to brown, and a water-soaked margin that appears surrounded by a chlorotic halo. The center of the lesion becomes raised and corky. Lesions were usually visible on both sides of a leaf. (Photo No. 1)

MICROSCOPIC OBSERVATION OF CANKER LESION

The lesion showing typical canker lesion when cut in a drop of water in a microscopic slide showed bacteria oozing from the edges of cut lesions.

ANATOMY OF DISEASED LEAF

When observed under microscope, V. S of the leaf showed the swollen region surrounding a central region with brown color (Photo No.3). The region also showed degraded chloroplast when compared with V.S. of healthy leaf (Photo NO.3)

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS OF THE ISOLATED PATHOGEN

The isolated bacteria were found non sporing non capsulated, rods with round ends (Gram staining photo no.4). They formed mucoid yellow colonies on GYCA (Photo no.5) The bacteria was found catalase positive, oxidase negative, oxidative (Photo No.6), starch hydrolysis test positive (Photo No. 7), nitrate reduction test negative. Further more the bacteria were able to produce acid on Dye's medium C within 21 days of incubation from only Glucose, Sucrose, Mannose and Galactose and were not able to utilize Arabinose, and Fructose (Photo No.8).

Thus, most of the characteristics of *Xanthomonas campestris* pv. *citri* as described in Bergey's manual was shown positive by the isolated bacteria. Therefore the bacteria was identified as *Xanthomonas campestris* pv. *citri* (*Xanthomonas citri*)

Table 2: Biochemical tests performed for confirmation of *Xanthomonas citri*

S.No.	Test performed	Results
1.	Gram staining	Gram negative rod
2.	Catalase test	Catalase positive
3.	Oxidase test	Oxidase negative
4.	O-F test	Oxidative
5.	Methyl-Red test	Methyl-Red negative
6.	Voges- Proskeur test	Voges- Proskeur negative
7.	Indole test	Indole test negative
8.	Urease test	Urease test negative
9.	Starch hydrolysis test	Positive
10.	Nitrate Reduction test	Negative
11.	Growth on Nutrient agar incorporated with 0.1% Triphenyl tetrazolium Chloride	No Growth Observed
12.	Growth at 37^0C	Positive
13.	Acid production from Carbohydrates (Arabinose, Glucose, Sucrose, Mannose, Galactose Fructose, Sorbitol)	Acid production from Glucose, Sucrose, Mannose and Galactose

PATHOGENICITY TEST

The pathogenicity test conducted on potted lime sapling showed disease on all of the test plant (Photo no.12). Thus the bacterium was found to be pathogenic that was able to produce disease on inoculated saplings which were disease free prior to the bacterial inoculation. The symptoms were found from 9 to 13 days of inoculation to the test plants with the average size of lesion around 2mm, while the control inoculated with sterile distilled water showed no disease symptoms.

Table 3: Pathogenicity Test: Time taken (days) to appear typical canker lesion

S.No	Potted plants	Time taken to produce typical canker lesion
1.	T1	9 days
2.	T2	8 days
3.	T3	13 days
4.	T4	9 days
5.	T5	11 days

The pathogencity test of the isolated bacteria on potted plant showed the disease symptoms with the characteristics lesion of Citrus canker on all the plants and the time taken to produce symptoms for T1,T2, T3, T4, T5 were 9 days, 8 days, 13 days, 9 days and 11 days. The plant inoculated with sterile distilled water showed no symptoms (Photo no.9).

Graphical Representation showing the time taken to produce canker lesion on test plants

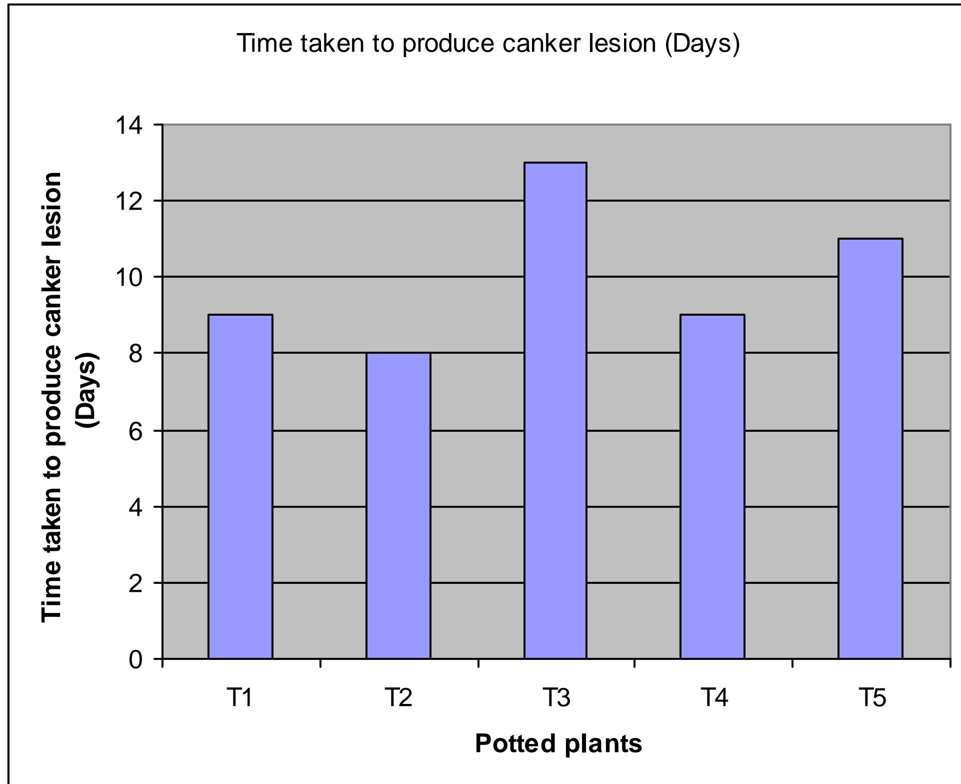

Fig.1: Time taken to produce the canker symptoms on potted test plants

Similarly the pathogenicity test conducted with detached leaf placed in a Petri plate also showed a lesion that resembled citrus canker on 4th day of inoculation (Photo No.10) while the leaf inoculated with distilled water showed no symptoms (Photo No.10)

Disease Status of the Orchard

$$\text{Disease Status of the Orchard} = \frac{\text{Number of infected plants}}{\text{Total Number of plants}} \times 100$$

$$= \frac{48}{55} \times 100\%$$

$$= 87.27\%$$

Table 4: **Disease severity on the orchard on first observation (2063/2/27)**

Plot number	P1	P2	P3	P4	P5	Average
1st plot	55%	60%	50%	58%	65%	57.6%
2nd plot	50%	70%	65%	65%	75%	65%
3rd plot	60%	60%	65%	70%	40%	59%
4th plot	70%	65%	60%	70%	60%	65%
5th plot	50%	70%	70%	65%	30%	57%

The first observation showed that the disease was in average status of 57.6%, 65%, 59%, 65% and 57% on 1st, 2nd, 3rd, 4th and 5th plot respectively.

Table 5: **Disease severity on second observation (2063/3/15)**

Plot Number	P1	P2	P3	P4	P5	Average
1st	59%	64%	58%	65%	70%	63.2%
2nd	57%	80%	72%	77%	79%	73%
3rd	65%	70%	70%	76%	54%	67%
4th	85%	73%	70%	79%	67%	74.8%
5th	64%	77%	75%	68%	45%	65.8%

The second observation done at the interval of 17 days interval showed the slight increase in average disease status as compared to 1st observation. The observation was done prior to chemical spray.

Table 6: Disease severity on third observation (Observation after 1st spray) (2063/3/31)

Plot Number	P1	P2	P3	P4	P5	Average
1st	61%	64%	58%	67%	72%	64.4%
2nd	59%	83%	73%	79%	79%	74.6%
3rd	65%	70%	71%	78%	54%	67.6%
4th	85%	73%	70%	81%	67%	75.2%
5th	67%	81%	79%	73%	54%	70.8%

This observation made after 1st chemical spray showed almost no increase in the disease severity except in the control plot in which the average severity increased from 65.8% to70.8%.

Table 7: Disease severity on 4th observation (Observation after 2nd spray) (2063/4/15)

Plot Number	P1	P2	P3	P4	P5	Average
1st	59%	61%	57%	65%	68%	62%
New flush on 1st plot	0%	0%	0%	14%	29%	8.6%
2nd	56%	79%	69%	73%	75%	70.4%
New flush	0%	22%	12.5%	0%	20%	10.9%
3rd plot	61%	64%	66%	74%	48%	62.6%
New flush	0%	0%	0%	10%	0%	2%
4th plot	77%	67%	66%	77%	63%	70%
New flush	0%	0%	0%	0%	0%	0%
5th plot	70%	84%	83%	77%	57%	74.2%
New flush	20%	25%	27%	26%	12.5%	22.1%

This observation after 2nd spray showed decrease in the disease severity. The new flushes were almost free from the disease with average severity of 8.6%, 10.9%, 2% and 0% on 1st, 2nd , 3rd and 4th plot whereas in case of control plot new flushes had the disease severity of 22.1%.

Table 8: Disease severity on 5th observation (Observation after 3rd spray) (2063/5/15)

Plot Number	P1	P2	P3	P4	P5	Average
1st plot	53%	57%	52%	64%	68%	58.8%
New flush	0%	0%	0%	11%	29%	8%
2nd plot	51%	78%	67%	69%	75%	68%
New flush	0%	19%	10.6%	0%	22%	10.32%
3rd plot	53%	61%	61%	73%	44%	58.4%
New Flush	0%	0%	0%	11%	0%	2.2%
4th plot	72%	62%	61%	74%	57%	65.2%
New flush	0%	0%	0%	0%	0%	0%
5th plot	75%	85%	85%	82%	59%	77.2%
New flush	33%	30%	34%	28%	16%	28.2%

The table showed the disease status after 3rd spray. It was found that the disease severity was in decreasing phase after the spray of chemicals.

The tables 3 to 7 showed the status of the disease in the field where the first two tables, i.e. 3 and 4, showed the disease status before treatment of the chemicals whereas the Table 5, 6 and 7 showed the status of the disease after treatment of the plots with respective chemicals. The data from the table showed that there was increase in the disease severity from 1st observation to 2nd in all the plots, including the control plot. However after the first spray of the chemicals, the disease in the treated plot was in static condition or showed very slight increase when compared to 2nd observation table. Table 6 and 7 also showed the disease severity on new flushes. It was observed that very few of the new flushes on treated plot showed disease symptoms where as the new flushes in the control plot too were infected. The disease severity on new flushes on treated plot was 8%, 10.32%, 2.2% and 0% on plot treated with copper oxychloride

(2.5%), copper oxychloride + Kasugamycin (1000X), Bordeaux mixture (1%) and Bordeaux mixture (2%) respectively.

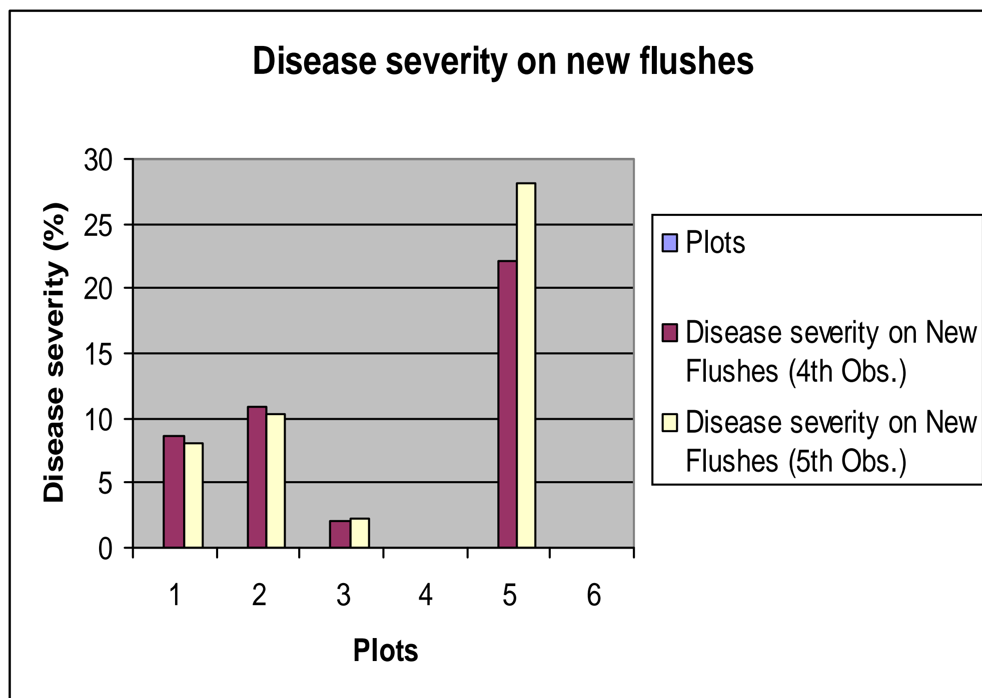

Figure 2: Comparison of Disease severity in few flushes of the treated plots with control plot

It was observed that the new flushes of all the sprayed plants showed the decrease or absence of canker lesion. However, the control plot sprayed with water showed increase in disease severity among new flushes. The graph was plotted on the basis of disease severity that was calculated using the data obtained after 4th and 5th observation.

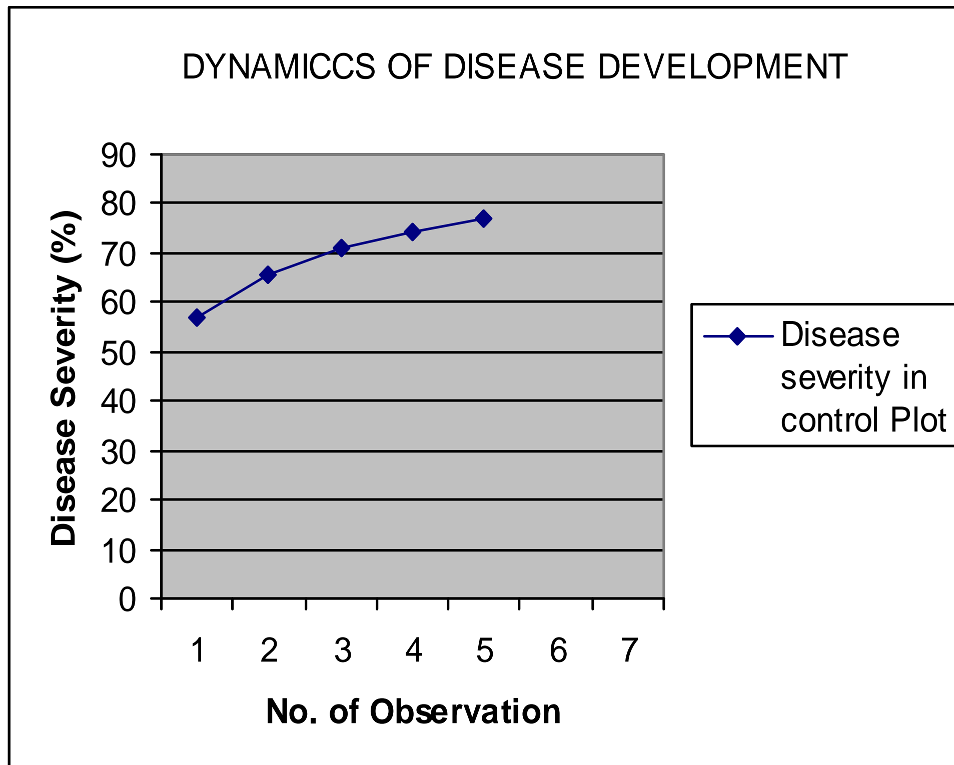

Figure 3: Disease severity in control plot from 1st to 5th observation at 15 days intervals

The graph was plotted using the Data obtained from the control plot, sprayed with water. The average of the disease severity of five plants when plotted against the number of observation, it was found that disease gradually increased to higher level then it was in the beginning.

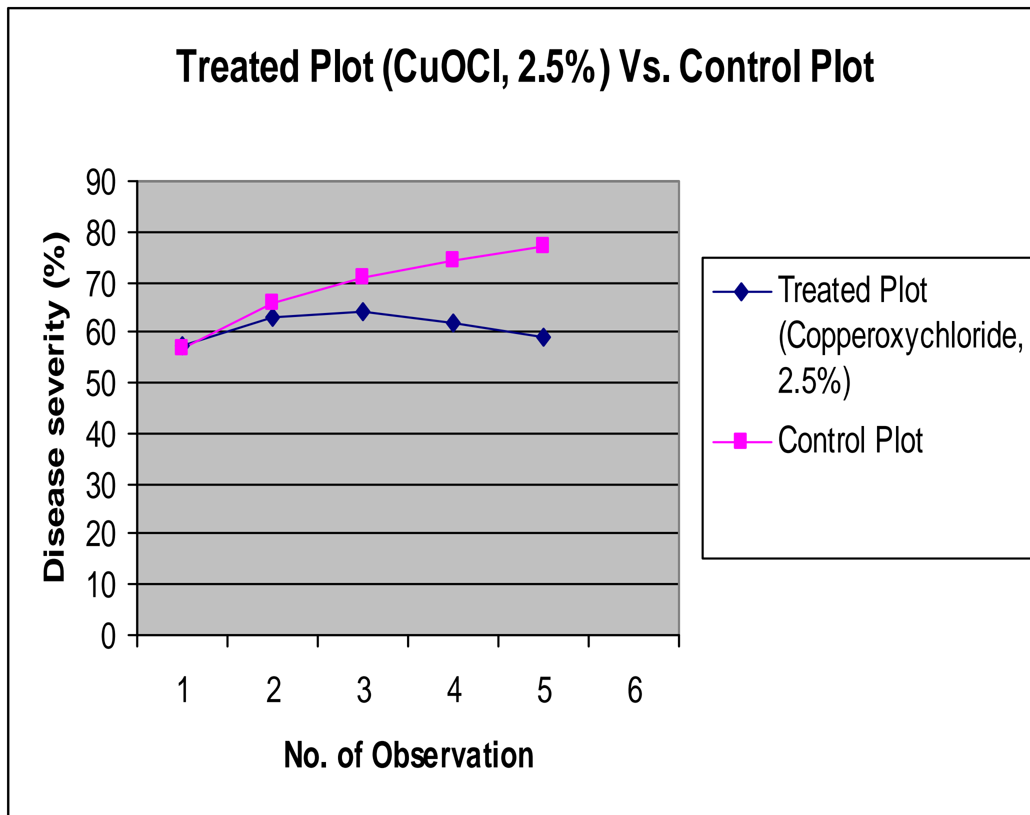

Figure 4: Comparision of Disease Severity of the plants treated with CuOCl (2.5%) and the plants of the control plot

The graph plotted showed the average severity of disease on control plot sprayed with water and that of treated plot (copperoxychloride, 2.5%). It was observed that the disease severity increased in the control plot where as treated plot showed gradual decrease in disease severity.

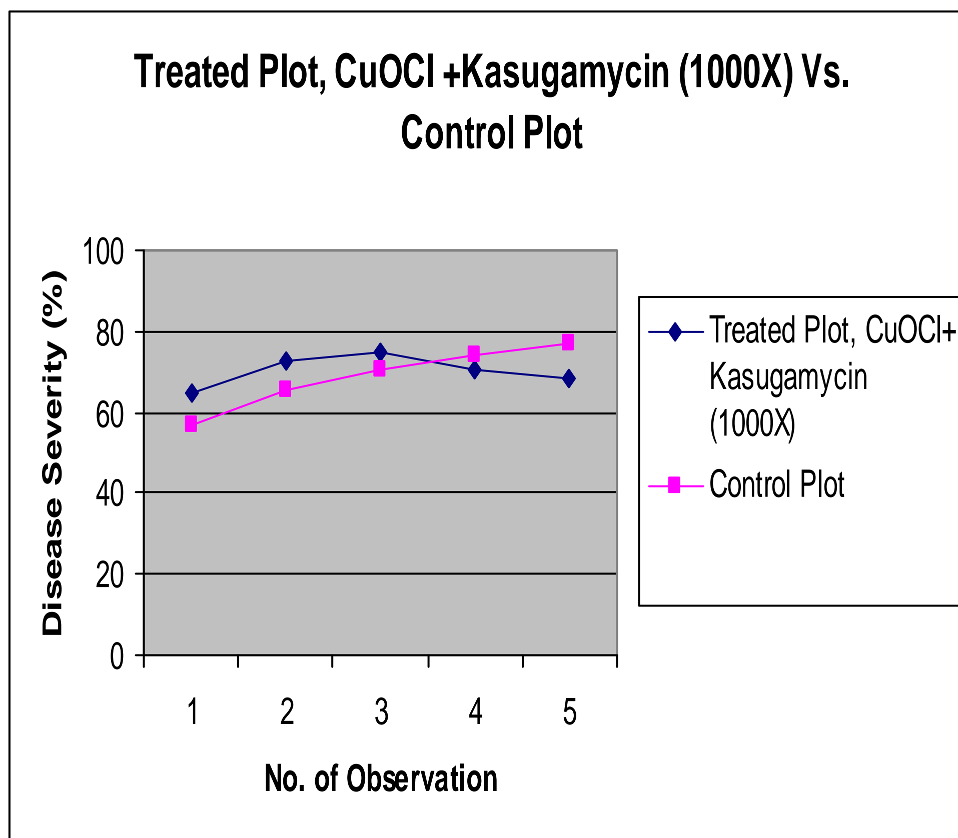

Figure 5: Comparision of Disease severity of the plants of the plot sprayed with Copper oxychloride + Kasugamycin (1000X) against the plants of the control plot

The graph showed the average of the disease severity of the treated plot (Copperoxychloride+ Kasugamycin (1000X) compared to the average of the disease severity of the control plot. The data obtained showed very slow decrease in disease severity in the treated plot.

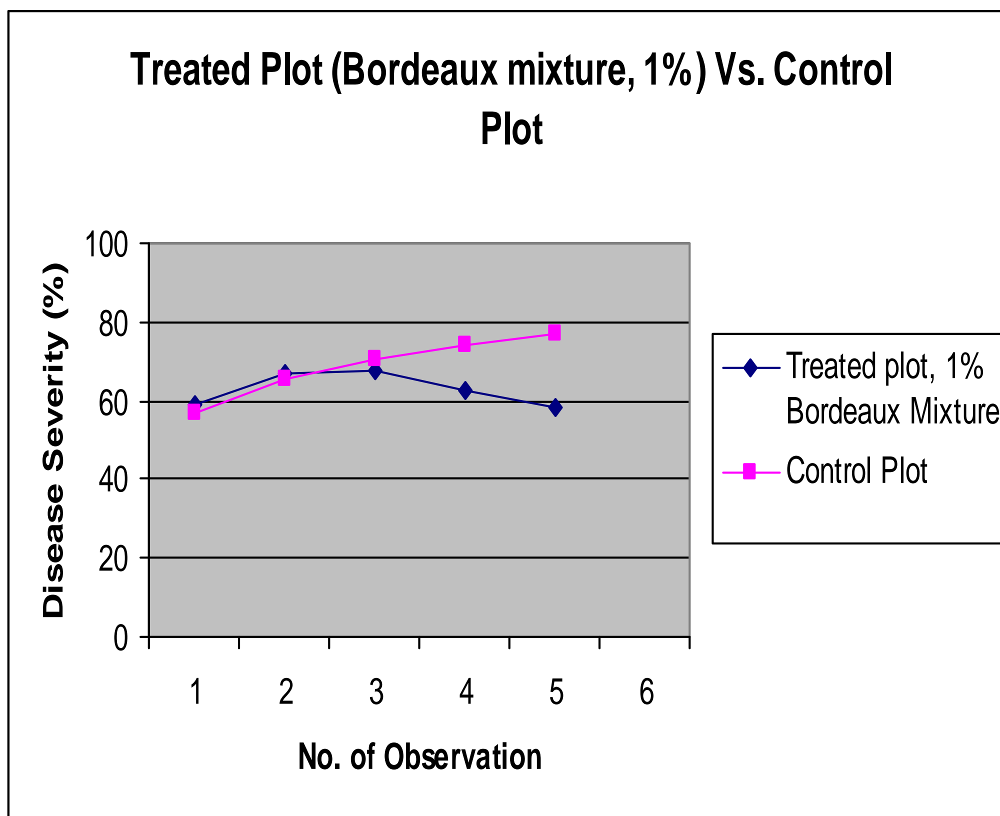

Figure 6: Comparision of Disease Severity of Plants of the plot treated with Bordeaux Mixture (1%) and the plants of the plot plot

The graph plotted on the basis of data showed the decrease in disease severity in the plot treated with Bordeaux mixture, 1%. The disease showed the decrease in disease status immediately after the chemical was sprayed.

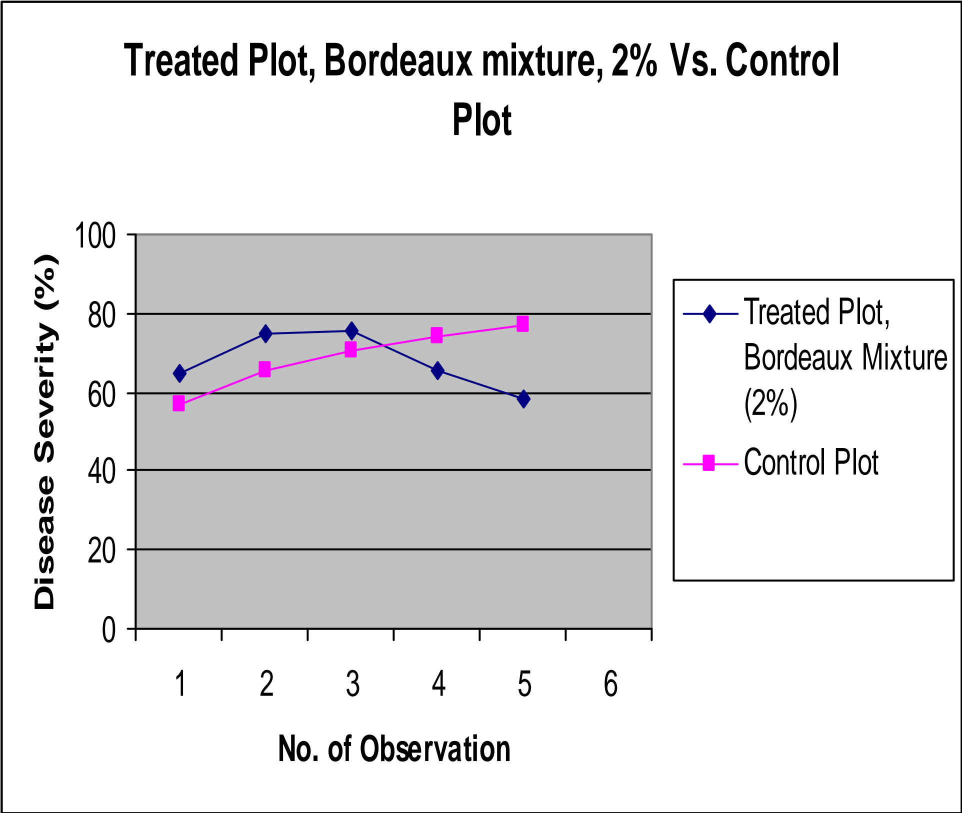

Figure 7: Comparision of Disease Severity of the plants treated with Bordeaux Mixture (2%) and the plants of control plot

Photograph 1: Leaf of *C. aurantifolia* (Left: Disease free, Right: Infected)

Photograph 2: Leaf showing Disease Development

Photograph 3: V.S. of leaf (40X) with citrus canker (Top) and without Citrus canker (Bottom)

Photograph 4: Gram staining micrograph (X100, oil immersion) of *X. citri*

Photograph 5: Colonies of *Xanthomonas citri* on GYCA after 3 days of incubation

Photograph 6: O-F test of *X. citri*

Photograph 7: Starch hydrolysis test of *X. citri*

Photograph 8: Carbohydrate utilization test of *X. citri*
Yellow color (Utilization of Carbohydrates)

Photograph 9: Pathogenicity test on potted plants.

Photograph 10: Pathogenicity test on detached leaves (Left: Test, Right: Control)

Photograph 11: Diseased plant in Field

Photograph 12: Investigator working in laboratory

Photograph 13: New flushes in plant free of disease

CHAPTER-VI

7. DISCUSSION AND CONCLUSION

7.1 DISCUSSION

Out of different citrus diseases found in Nepal, Citrus canker was reported as one of the important bacterial diseases. Though the disease was detected in Nepal in early 1968, no research had ever been carried out in the isolation of the causative agent of the disease *Xanthomonas campestris* pv. *citri*. Hence this was a first research carried out for the thorough study of the disease including the cause of infection of the plants with the disease, isolation of the pathogen and testing its pathogenicity and finding a proper and economic chemical control that can be used with ease.

In order to find the disease status of Citrus canker in Kavre district, a general survey was conducted. The areas included in the survey were citrus orchards of Dhulikhel, Sharadha Batase and Khanal Thok. In the survey, *Citrus aurantifolia* was found principally infected with citrus canker while other citrus fruits were infected with different diseases but not with citrus canker. Thus our observation comply with the finding of Civerolo (1984) who described that lime served as a principal host of the disease citrus canker and the disease is very severe because of their high susceptibility.

In Khanal Thok, 9 lime trees were found infected with high severity but the orange trees planted in the vicinity were found uninfected. This was probably due to host specificity of the bacteria or resistance shown by other varieties of citrus plants towards the disease. Ramakrishnan (1954), Graham (1992b) too described that the disease is more severe on acid lime and less common on mandarin and sweet orange.

In Sharadha Batase, no lime trees were found and the sweet orange showed the disease foot rot. Foot rot was a principle disease found in orange in Sharadha- Batase.

In an orchard of Dhulikhel, lime plants that were planted three years back showed very high status of the disease. The plants were all brought from the private nursery at Gotikhel, Lalitpur and planted there. As no disease was found in nearby areas, the disease in the area must have been transmitted from the place from where the plants were brought.

In Dhulikhel, out of total 55 plants, 48 were found infected with citrus canker with different level of disease severity. The orchard was divided into 5 plots and 5 plants from each plot were chosen. The plants were indicated as P1, P2, P3, P4 & P5 in each plot and the severity of disease of each plants were calculated. The high status of the disease in Kavre signifies that the area is favorable for the disease regarding the survival of the pathogen, its dissemination and other factors. Furthermore, the survey was conducted from June to August, and the time was pretty favorable for the disease to spread by means of rain drops. The spreads of disease by means of rain drop has been reported and proved by Graham *et al* (2004), Peltier and Frederich (1926), Ramakrishnan (1954) who reported that the disease occurs primarily in region where rainfall increases and temperatures rise simultaneously. Occurrence of lesions is seasonal, coinciding with periods of heavy rainfall, high temperatures and growth flushes. These factors generally coincide with early summer in citrus growing regions where rainfall increases as temperatures increase. Aiyappa (1958) also found that all the cultivated varieties of citrus and some wild species in Karnataka are susceptible to canker possibly due to heavy rainfall, high humidity and temperature.

Out of 55 plants in the orchard of Dhulikhel, 48 were infected with different levels of disease severity. The disease was clearly diagnosed on the basis of symptoms present on leaves and stem. An essential diagnostic symptom was citrus tissue hyperplasia (excessive mitotic cell divisions), resulting in cankers. The aged lesions were found tan

to brown in color and there was a water-soaked margin surrounded by a chlorotic halo. The center of the lesion was raised and corky and the lesions are visible on both sides of a leaf. Such diagnostic symptoms were described by Goto (1992), Swings *et al* (1993), Gabriel *et al* (2000), Das (2003) and our observations also do not differ from them.

The leaf samples were taken to the laboratory and proceeded for the isolation of the bacteria. First of all, one of the leaves showing the canker lesion was taken. The lesion was cut in a drop of water in a microscopic slide and observed under microscope under oil immersion. It was observed that bacteria were streaming from the edges of cut lesions as reported by Schaad (1988).

Then a ventral section of the lesion was cut and observed under microscope which showed the showed the swollen region surrounding a central region with brown color. As suggested by Kishore and Chand (1972) the brownish region was due to production of phenolics and other antimicrobials produced by the plant. The region also showed degraded chloroplast when compared with V.S. of healthy leaf which was due to decrease in Chlorophyll a, b as reported by Padmanabhan (1973).

The leaf sample was first of all washed in running water for few minutes to remove any debris and bacterial and fungal population present on the leaf surface. Then a small portion of the leaf was and the cut portion was surface sterilized to kill the surface contaminants and well rinsed to remove the residual sterilizing agent if present in the sample Then after the lesion was minced using a sterile razor in a Petri plate and incubated for about 15-30 minutes. This time stand allows the bacteria to ooze out from the cut tissue to the water in the Petri plate. Then the liquid in from the Petri plate was serially diluted up to 10^{-3} dilution. From each of the diluents 0.2 ml of the dilutions was used for spread plate on Glucose Yeast Chalk agar. As no selective medium have ever been proposed for *Xanthomonas citri*, GYCA was used as this media was proposed by Swing (1993) and Schaad (1988) as well. The agar provides adequate nutritional requirements for *Xanthomonas*. There is a good balance on agar between the acidity

resulting from sugar metabolism and the alkalinity resulting from protein breakdown, any net increase in hydrogen ion concentration is handled by the excess calcium carbonate. The media incubated after 3 days at 27^0C was then observed and yellow, mucoid colonies were sub cultured on Nutrient agar. In fact many colonies with yellow colonies were obtained in case of GYCA because of the presence of large number of fermentator and other bacteria present in the environment. In fact, other yellow pigmented bacteria were also isolated from citrus tissue, some of which might belong to genus *Xanthomonas*, hence pathogenicity test was an important tool for the confirmation of the bacteria.

The isolated bacteria on GYCA were sub cultured on Nutrient agar and the bacteria were then subjected to different tests to identify the disease causing bacteria, *Xanthomonas citri*. Gram staining was the first step to differentiate the bacteria. After gram staining, the bacteria that were gram negative were further subjected to tests like catalase, oxidase, and oxidative- fermentative test. The bacteria that were found gram negative rods, catalase positive, oxidase negative and oxidative were then further subjected to different biochemical test such as nitrate reduction test, starch hydrolysis test in which the bacteria was unable to reduce nitrate to nitrite but capable of hydrolyzing starch as described in Bergey's manual of determinative bacteriology. As the bacteria gave positive results as described in Bergey,s manual of Determinative bacteriology as well as test described by Swings *et al* (1993), Goto (1992), Chand and Pal (1982), the bacteria was then subjected to pathogenicity test.

Pathogenicity test was performed for the confirmation of the citrus canker causing bacteria, *Xanthomonas citri* this process, disease free lime seedlings that were pot cultured were inoculated with the isolated bacteria. As described by Graham *et al* (2004) symptoms are generally first observed beginning four days after inoculation as a raised margin surrounding a slighly chlorotic region the disease symptoms on the inoculated test plants were observed. The plats showed the characteristics symptoms of the disease after 9 to 13 days. The time required in this case was slightly more which

was probably due to low level of virulence, inappropriate humidity or temperature that was actually required for the bacteria to develop the disease on host plant. The lesions were of brown color surrounded by yellow zone and water soaked margin, a characteristic of citrus canker as described. In case of natural infection it takes lot more time, even a month or more to develop symptoms of the disease because of the various reasons like the size of innoculum, the climatic conditions etc, but during artificial inoculation, high inoculum of around 10^7 was used and the humidity and temperature conditions were maintained making favorable for the disease development. However the difference of 9 to 13 days was probably due to some resistance shown by individual plants and some other factors.

After observation of the symptoms, the leaf inoculated was again used to re-isolate the bacteria because only the re-isolation of the bacteria from the inoculated plant can establish that a particular organism is the causative agent of a particular Disease. During the re-isolation process, similar processes were utilized and the bacteria isolated were tested with the previously identified culture.

For the control of the disease, chemical measures were used and chemicals like copperoxychloride, copperoxychloride in combination with Kasugamycin and Bordeux mixture in two concentration, 1% and 2% were sprayed to the infected plants.

Das and Singh, (1999, 2000) too suggested the periodic spraying of suitable copper-based bactericides (to reduce inoculum build-up on new flushes and to protect expanding fruit surfaces from infection) along with an insecticide (to control insect injury). Similarly, USDA APHIS (1997) reported that when environmental conditions are favorable for the spread of the disease, chemical control measures are not entirely effective. However, materials containing copper (Bordeaux mixture, copper hydroxide, basic copper chloride, copper oxychloride, and tribasic copper sulfate) are the most effective bacterial sprays for protecting leaves and fruit.

The orchard was divided into 5 plots and from each plot 5 plants with different level of severity were selected. It was done to compare the efficacy of each of the chemical sprayed. The 5th plot was spread with water and considered as control so that the efficiency of the chemicals could be compared with the control plot. After each spray, it was observed that the plants sprayed showed the decrease in the disease severity where as in case of control plot the disease showed more rapid increase.

The increase in the disease severity in the control plot was also due to climatic condition as rainfall and temperature rise was high during the period. It was so because bacteria exude from lesions during wet weather and are disseminated by splash dispersal at short range, windblown rain at medium to long range and human assisted movement at all ranges. Bacteria dispersed by wind blown rain were detected up to 32 m from infected trees in Argentina (Stall *et al*, 1980).

The dynamics of the disease was studied on the basis of 5th plot which was sprayed with water alone. The graph plotted showed that there is regular increase in the disease severity from 1st observation to 5th observation. Thus it was revealed that in absence of any control agent the disease goes on increasing.

The plot sprayed with copperoxychloride showed gradual decrease in the severity of disease after spraying. It was observed that after the 1st spray there was very slight decrease in the disease severity but after 2nd and 3rd treatment the disease showed gradual decrease in severity. Furthermore, the graph representing the severity of new flushes compared to control plot was low, hence copperoxychloride was effective in controlling the disease.

In case of second plot, it was observed that even after spraying, the disease was increasing; however it showed the decrease in disease status after second and third spray. The disease severity on new flushes was low when compared to new flushes of control plot but the disease severity on new flushes was high when compared to other

plots sprayed with other chemicals. Though several research suggested that the combination of copper compound and Kasugamycin as effective method for the treatment, it was observed that this combination was least effective among the chemicals used. The reason might be probably that the antibiotic was of low grade or other reasons which could not be understood.

The 3rd plot sprayed with 1% Bordeux mixture showed that there was a sharp decline in disease status after the spray. The graph showed that the disease severity goes on decreasing after the spray. Furthermore, the disease severity on new flushes was also low as compared to control, hence Bordeux 1% is an effective control measure for the control of the disease citrus canker.

In case of the plot sprayed with 2% Bordeux mixture, the decline in disease severity was much better. No new flushes showed any lesion after 3rd spray and it continued up to 1 month of the last spray. The plant sprayed with 2% Bordeaux mixture were those plants which were at the highest severity in the orchard, and hence the much satisfactory result was achieved in controlling the severity of the disease. Though the mixture was good in the control of the disease, extensive use of copper has been reported to be phyto-toxic by USDA APHIS (1997). It was later observed that there was slight discoloration of the leaves sprayed with 2% Bordeaux mixture. Furthermore, the cost of 2% is twice as that of 1% Bordeaux mixture. Again it was observed that there is only slight difference in the disease decline in case of 1% and 2% Bordeaux mixture, 1% Bordeaux mixture is beneficial.

When canker occurred in the USA, the emphasis was on eradication, and other measures for control of canker were not adequately researched (Stall and Civerolo, 1991) but in Nepal such measures are seldom used as state could not support the farmer for the loss that occurs during eradication program.

The spraying of chemicals three times in the interval of 15 days has been recommended by several researchers. However for the eradication of the disease, the spraying alone is not effective. Different sanitary measures as well as quarantine are essential steps to eradicate the disease.

7.2 CONCLUSION

Hence, a survey was conducted in different parts of citrus growing regions of Kavre to determine the status of the disease citrus canker in the area. It was found that the disease was prevalent in high status in Lime. The pathogen of the disease was isolated in the lab and confirmed by morphological, physiological and pathogenicity test. Furthermore, field study was conducted to find the effective control measure of the disease and it was found that Bordeaux mixture 1% was effective in controlling the disease.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

Citrus fruits are major cash crops of Nepal. The mid-hill regions which occupy 58% of total area of Nepal have been found favorable for cultivation of citrus fruits. The disease citrus canker is a major disease of citrus fruits and has devastating effect on it. Although the disease does not directly reduce fruit quality and yield, the impact is worsened because the presence of citrus canker in an area triggers immediate quarantine restrictions, disrupting the movement of fresh fruit as well.

The study was focused on the survey of the disease on the study area, isolation of the casual agent of the disease, its pathogenicity test and control measures that can be applied for the control of the disease.

Since, the study of disease was essential, the study carried out was an important milestone in the study of plant pathology in Nepal which was carried out in Kavre, Nepal.

The different aspects of the study are summarized as follows:

SURVEY OF THE DISEASE

Survey of the disease was an essential step to predict the actual status of the disease in the study area. The survey was conducted not only to confirm the status of the disease but also to find the disease status among other Citrus species if present. The survey conducted on different orchards of ‘Kavre’ suggested that the disease was principally confined to Lime.

ISOLATION OF THE PATHOGEN

Isolation of the pathogen was done from the leaf sample infected with the disease Citrus canker. The sample for the isolation was collected from the Orchard of Dhulikhel, 'Kavre'. The isolated bacteria were confirmed on the basis of possible and available biochemical tests.

PATHOGENICITY TEST

Pathogenicity test was done to re confirm the pathogen as an agent of the disease. The test was conducted using test plants and the production of similar symptoms as citrus canker in the field was observed. The pathogen was re-isolated from the test plants.

CONTROL

The control of the disease was an important part of the study. Different chemicals were used for the control of the disease and the most effective chemical for the control of the disease was discovered. It was found that 1% and 2% Bordeux mixture if applied at 15 day's interval for three times will be effective in the disease control.

RECOMMENDATIONS

1. This research is a basic and a preliminary research conducted as a partial fulfillment of Masters Degree in Microbiology. The research is therefore limited not only in terms of budget but also in terms of time that is required to conduct a thorough research of the real citrus canker problem of Nepal.

2. In this research, sample was collected from only one location and pathogenicity was performed only on lime, thus we could not make sure the host resistance of different Citrus fruits that are planted in Nepal.

3. During the research, there was a need to compromise on the availability of different chemicals and media required to conduct every test for the confirmation of the bacteria, therefore further tests should be done with different samples to assign the pathovar that are present in Nepal.

4. At present, all the tests are conducted in molecular levels but we are limited to only certain biochemical tests and pathogenicity tests, hence DNA profiling, plasmid profiling should be carried out.

5. Furthermore, Citrus canker continues to be the cause of worldwide concern as a potentially hazardous threat to citriculture. There is a wide range of physiological, biochemical, serological, molecular and pathogenic variation among strains of bacteria associated with citrus canker. Moreover new strains are originating regularly as a result of mutation. A better understanding of the pathogenic specialization and proper identification of *Xanthomonas* is needed.

6. For the control of the Disease, Bordeaux mixture 1% is best recommended on the basis of results obtained in the research.

CHAPTER-VIII

8. REFERENCES

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APPENDIX-1

MATERIALS

Bacteriological Media

a. Nutrient agar
b. Nutrient broth
c. Glucose Yeast Chalk agar
d. Muller Hinton Agar
e. Hugh- Leifson's Media
f. Starch agar
g. Gelatin Medium
h. Triphenyl tetrazolium Agar
i. Nitrate medium
j. Dye's C medium

Chemicals/Reagent

a. Sodium hypochlorite (NaOCl 1%)
b. Hydrogen peroxide (H_2O_2 3%)
c. Oxidase Reagent
d. Hucker's Crystal Voilet
e. Lugol's iodine
f. Ethanol (70%)
g. Safranin
h. Glucose
i. Sucrose
j. Arabinose
k. Mannose
l. Galactose

m. Mannose
n. Fructose
o. Ammonium dihydrogen phosphate
p. Di-potassium hydrogen Phosphate
q. Magnesium Sulphate
r. Sodium Chloride
s. Yeast extract
t. Bromocresol purple
u. Agar

Glass wares

a. Petridishes
b. Test-tubes
c. Beakers
d. Pipettes
e. Slides
f. Cover slips
g. Glass rod
h. Graduate Cylinder

Equipments

a. Incubator
b. Laminar flow
c. Autoclave
d. Oven
e. Heater
f. Bunsen Burner

Other equipments

a. Bacteriological loop

b. Test tube Stands
c. Syringes (1ml)
d. Blotting paper
e. Plastic Bags
f. Pot

Recipes of the Media

Compositions of Media

A. Culture Media

1. Glucose Yeast Chalk Agar (GYCA)

Yeast extracts	5.0 g
Glucose	5.0 g
$CaCO_3$	40.0 g
Agar	15.0 g
Distilled water	1000 ml

Before setting mix well on Vortex mixer and set quickly in cold water to avoid settling of $CaCO_3$

1. Nutrient Agar

Peptone	5.0 g
Beef Extract	3.0 g
Agar	20.0 g
Distilled Water	1000ml

2. Nutrient Broth

Peptone	5.0 g
Beef-Extract	3.0 g
Distilled Water	1000 ml

B. Biochemical Media

1. Starch Medium (Lelliot and Stead, 1987)

Soluble starch	2.0 g
Nutrient agar	28.0 g

Distilled Water	1000 ml

Dissolve the nutrient agar powder in the water by heating. Dissolve the starch in 10ml distilled water and add to molten agar.

2. Nitrate Broth (Lelliot and Stead, 1987)

KNO_3	10.0 g
Peptone	5.0 g
Yeast Extract	3.0 g
Distilled Water	1000 ml

Adjust to pH, 7-7.2, if Necessary. Sterilize by autoclaving at 121^0C for 15 min.

3. Oxidation- Fermentation (O-F) Medium (Hugh and Leifson, 1953)

Tryptone or peptone	2.0 g
Sodium Chloride	5.0 g
di- potassium hydrogen phosphate anhydrous (K_2HPO_4)	0.3 g
Agar	2.5 g
Distilled Water	900 ml

Mix, and dissolve by heating, adjust pH to 7.1, cool and Add Bromothymol blue, 10 g/l (1% W/V) 3ml

Dispense in test tubes and sterilize by autoclaving at 1210C for 15 minutes, and after cooling add sterilized Glucose (10% W/V)

Methyl-Red and Voges- Proskauer Medium

Peptone	7.0 g
Dextrose/ Glucose	5.0 g
Potassium Phosphate	5.0 g
Distilled Water	1000 ml

Pour 5ml broth in each tube and sterilize by autoclaving at 15 lb pressure for 15 minutes.

4. SIM Media

Peptone	30.0 g
Beef extract	3.0 g
Ferrous ammonium sulphate	0.2 g
Sodium thiosulphate	0.025 g
Agar	3.0 g
Distilled Water	1000 ml
pH	7.3

Pour the media in 5 ml tube and sterilize by autoclaving at 15 lb pressure for 15 minutes.

5. Carbohydrate utilization Test (Dye's Medium C)

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	5.0 g
Yeast Extract	1.0 g
Carbon Source	5.0 g
Bromocresol purple	1.5% (alcoholic solution)
Agar	12.0 g

The media is then pourd to test tubes and autoclaved at 15 lb pressure for 15 minutes.

Appendix 1.2 Chemicals and reagent

1. Gram Stain (Hucker Modification) Lelliot and Stead, 1987)

Hucker's Crystal Violet

Solution A

Crystal Violet	2.0 g
Ethanol, 95%	20.0 ml

Solution B

Ammonium Oxalate	0.8 g
Distilled Water	80.0 ml

Mix the two solutions

Lugol's iodine (Gram modification)

Iodine	1.0 g
KI	2.0 g

Distilled Water	300.0 ml

Grind the solids in the pestle and mortar and then dissolve in the water and stir in a closed container for several hours to complete dissolution.

Decolourizer

Ethanol	95%

Safranin (Counter Stain)

Safranin O	2.5 g
Ethanol (95%)	100. 0 ml

Dilute the stock solution 1: 10 in Distilled water for use.

Catalase Reagent

Hydrogen Peroxide (H2O2)	3.0 ml
Distilled Water	100.0 ml

Oxidase Reagent

Tetramethyl- p- phenylenediamine Dihydrogen chloride	0.1 g
Distilled Water	10.0 ml

Starch Hydrolysis test Reagent

Lugol's iodine (Lelliot and Stead, 1987)

Iodine	5.0 g
KI	10.0 g
Distilled Water	500.0 ml

Disslove it by stirring in a closed container for several hours to complete dissolution.

Nitrate Test Reagent (Lelliot and Stead, 1987)

Glacial acetic acid	50.0 ml
Distilled Water	360.0 ml
Sulphanilic acid	0.25 g
- napthol	0.20 g
10% (V/V) aq. 0.88 ammonia solution	90.0 ml

Add the acetic acid to the water, mix warm to 50 C and pour into a dark, glass bottle containing the sulphanilic acid. When this has dissolved, and - napthol ammonia solution and allow to dissolve. Cool the mixture to room temperature and then add ammonia solution. Store in the Dark at 4^0C.

Kovac's Reagent (for detection of Indole)

P- Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (conc.)	25.0 ml

Dissolve the dimethylaminobenzaldehyde in the amyl alcohol. Then add the hydrochloric acid to the above preparation. Store the reagent in a glass- stoppered bottle in refrigerator.

Alpha- napthol solution (Voges- Proskauer test reagent)

- napthol	5.0 g
Ethyl alcohol (absolute)	95.0 ml

Dissolve the - napthol in the ethyl alcohol with constant stirring.

Preparation of Chemical Spray

Bordeaux mixture (1%)

$CuSO_4$	25.0 g (In 1.0 liter of Water)
CaO	25.0 g (In 1.5 liter of Water)

The two solutions are prepared separately in wooden or earthern vessel and then the solution of $CuSO_4$ is slowly poured into lime solution with constant stirring. The mixture is used immediately after preparation.

Bordeaux mixture (2%)

$CuSO_4$	50.0 g (In 1.0 liter of Water)
CaO	50.0 g (In 1.5 liter of Water)

Similar procedure is applied as used in case of 1% Bordeaux mixture.

APPENDIX-2

Temperature, Relative humidity and annual rain fall of 'Dhulikhel', Kavre during the year of Study.

Months	Temperature in C		Relative Humidity		Rainfall
	T Max	T min	At 8:45	At 17:45	
Jan,06	16.8	4.5	74.0	48.6	0.0
Feb,06	NA	NA	NA	NA	0.0
Mar,06	21.8	9.0	64.8	49.8	20.2
Apr,06	23.8	12.1	69.3	56.0	118.6
May,06	24.7	15.9	86.9	77.3	148.0
Jun,06	26.3	18.1	90.7	81.3	224.8
Jul,06	NA	NA	NA	NA	NA (Not Available)

Source: Department of Hydrology and Meteorology (Preliminary Data, 2006)