"POLYMERASE CHAIN REACTION DETECTION OF *CANDIDATUS LIBERIBACTER ASIATICUS* ASSOCIATED WITH CITRUS HUANGLONGBING (GREENING) OF SWEET ORANGE"

BY

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B. Sc. (Agri.)

DISSERTATION

Submitted to the

Marathwada Krishi Vidyapeeth, Parbhani

in partial fulfillment of the requirement

for the award of the Degree of

MASTER OF SCIENCE

(Agriculture)

IN

PLANT PATHOLOGY

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE, PARBHANI

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PARBHANI - 431 402 (M.S.) INDIA

2011

CANDIDATE'S DECLARATION

I hereby declare that this dissertation or part thereof has not been Previously submitted by me for a degree of any other institution or University.

Place: PARBHANI.

Date : 8 / 8 / 2011

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ACKNOWLEDGEMENT

"Achievement is of no mean, without the sense of gratefulness and the recognition of this is the beginning of wisdom"

I think it is the matter of pleasure to glance back and recall the way one traverse, the days of hard work and perseverance. It is still great at the juncture to recall all the faces and spirits in the form of teachers, friends, near and dear once. In my opinion, this work is nothing more than incomplete, without attending to the task acknowledging, to overwhelming help I received during this endeavour of mine.

Inspiration is the best medicine can make it possible to run for crippled one and it is veritable gold of mine to get the talented and inspiring willful guidance by a man with enthusiasm and devotion Dr. G.P. Jagtap, Assistant Professor, M.K.V., Parbhani and chairman of my Advisory committee, who in his unique way, provided me with constant encouragement inspiring, scholastic guidance, love and affection offered to me during the course of my study and research. Words would be a poor vehicle to communicate him my sense of gratitude.

I sincerely express my deep sense of gratitude and great indebtedness to the advisory committee members, Dr. G.D. Deshpande, Ex-Head, Department of Plant Pathology, Dr. B.B. Bhosale, Head, Department of Agricultural Entomology, Dr. D.W. Wadnerkar, Associate Professor, Dept. of Agricultural Entomology, Marathwada Krishi Vidyapeeth, Parbhani for their valuable suggestions and constant encouragement.

My sincere thanks to Dr. K.P. Gore, Hon. Vice-Chancellor, Marathwada Krishi Vidyapeeth, Parbhani Dr. V.S. Shinde, Director of instruction and Dean, Faculty of Agriculture, Dr. N.D. Pawar, Associate Dean and Principal, College of Agriculture, Marathwada Krishi Vidyapeeth, Parbhani, Dr. A.P. Suryawanshi, Head, Department of Plant Pathology, M.K.V., Parbhani.

I wish to place on record my sincere thanks to Dr. K.T. Apet, Dr. O.D. Kohire, Dr. V.M. Gholve, Dr. Badgujar, Dr. Deshmukh, and other non-teaching

staff of Department of Plant Pathology for their kind co-operation during the completion of my P.G. education.

Everything in my life is nothing if I ignore the all time encouragement, ever green affection and latent love of my father Somnath S. Jagtap and mother Sunanda Somnath Jagtap which elevate my morals during the whole educational career. Words are insufficient to take account of encouraging and evergreen love of my broter Sachin and Kishor and Sister Vandana and Roopkala.

One needs sincere friends of major junctures in life to bear strains and fatigue I have been luckier in this respect that Tushar, Deepak, Popat, Vishal, Pramod, Pankaj and Suyog are the stars of my friend galaxy. Who proved the phrase "friend in need is a friend in deed" did not to say about their love and affection.

Possibly words may limit my expression in whose company, I never felt the burden because I could see the every helping hands in the dark of analgam of tension and anxiety from my colleuges friends Vijaysingh, Darshan, Suhas, Ranjeet, Amol, Nanasaheb, Navnath, Mangesh, Yogiraj, Miss Minauati, Miss. Ratna and Miss. Sudarshana.

I am especially grateful to Bablu Joshi (Shrey Xerox) who carried out the difficult task of typing the revised manuscript not only swiftly and accurately but cheerfully.

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CERTIFICATE - I

This is to certify that dissertation entitled **"POLYMERASE CHAIN REACTION DETECTION OF *CANDIDATUS LIBERIBACTER ASIATICUS* ASSOCIATED WITH CITRUS HUANGLONGBING (GREENING) OF SWEET ORANGE"** submitted by **Mr. JAGTAP AMOL SOMNATH** to the Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **PLANT PATHOLOGY** is record of original and bonafide research work carried out by him under my guidance and supervision. It is of sufficiently high standard to warrant its presentation for the award of the said degree. I also certify that the dissertation or part thereof has not been previously submitted by him for a degree of any university.

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Date : 8 /8 /2011

(G. P. Jagtap)
Research Guide

CERTIFICATE - II

This is to certify that the dissertation entitled **"POLYMERASE CHAIN REACTION DETECTION OF *CANDIDATUS LIBERIBACTER ASIATICUS* ASSOCIATED WITH CITRUS HUANGLONGBING (GREENING) OF SWEET ORANGE"** submitted by **Mr. JAGTAP AMOL SOMNATH** to the Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **PLANT PATHOLOGY** has been approved by the student's advisory committee after *viva-voce* examination in collaboration with the external examiner.

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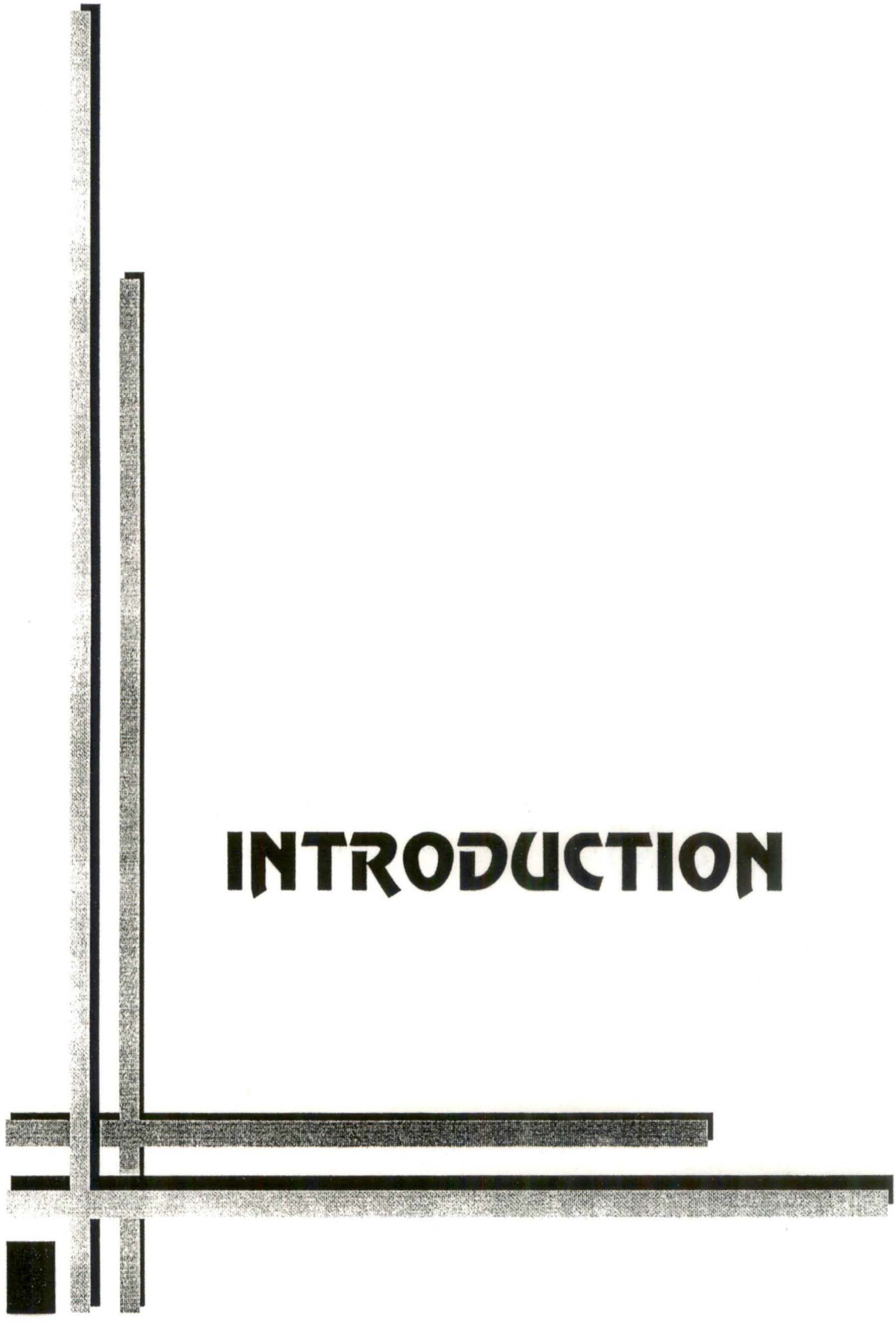
ABBREVIATIONS

PCR	:	Polymerase Chain Reaction
DNA	:	Deoxy Ribonucleic Acid
µl	:	Micro litre
M	:	Molarity
rpm	:	Rotation per minute
bp	:	Base pair
%	:	Per cent
TAE	:	Tris acetate EDTA
$MgCl_2$	:	Magnesium Chloride
LB	:	Larix Broth
SDX	:	Sterile distilled water
IPTG	:	Isopropropyl-β-D-tthiogalactopyromide
NaCl	:	Sodium Chloride
SDS	:	Sodium Dodecyl Sulfate
EDTA	:	Ethylene Diamine Tetra Acid
NADAc	:	Sodium acetate
ng	:	Nanogram
Pg	:	Picogram
KL	:	Kilobase Pair
M	:	Marker
H	:	Healthy
g	:	Gram
mg	:	Miligram
hr.	:	Hours
Min.	:	Minutes



INTRODUCTION

CHAPTER I

INTRODUCTION

Citrus belongs to family Rutaceae and has approximately 150 genera and 1600 species, which are found in tropical, temperate and arid region of the world. The Rutaceae is divided into seven sub-family comprising 11 tribes and 93 genera (Engler, 1931) of these, Auromtoidaeae, the subfamily of orange is classified into two tribes clauseneae and citrineae and six subtribes. Which contain over 215 species and 65 varieties. The genus citrus and its close relative such as fortunella, eremocitrus, clymerria, poncirus and microcitrus are the citrus fruit tree in group of the subtribe citrinae (*Tribe citrieae*) out of them only fortunella (Kumaquats), Poncirus (trifoliate orange) and citrus (eight species) are commercially important.

The following eight citrus species are commercially cultivated

Citrus reticulata Blanco (Mandarin)

C. grandis (L) osb (Shaddok or Pummelo)

C. Paradisi maet (grape fruit)

C. aurantium (L.) (sour orange)

C. sinensis (L.) Osb (Sweet orange)

C. aurantifolia (Christm) Swing (acid lime),

C. limon (L.) Burmf. (Lemon)

C. limon (L.) Burmf. (lemon),

C. Medica (L.) (Citron)

In India, total area under cultivation is 165.5 million ha, of which 15.3 million ha is under fruits and vegetables. The citrus is third largest fruit crop in India which occupies an area of around 0.56 million ha with production of 4.58 million tonnes. The Indian productivity of citrus is 8-9 tonnes per hectare, which is very low when compared with world average productivity of 25-30 tonnes per hectare (Economic survey, 2009-10). Disease are known as one of the important factors in low productivity of citrus fruits in India (Ahlawat *et al.*, 1996).

Among the diseases of citrus, viral diseases cause heavy economic losses in varying proportion. Around 30 viral diseases are known to infect citrus worldwide. In India, the major pathogens of economic importance in citrus are *Citrus tristeza* (CTV), Citrus yellow mosaic badna virus (CYMV), Indian citrus ring spot virus (ICRSV), viroids disease like citrus exocortis viroid and a fastidious prokaryote causing citrus greening disease (Ahlawat, 1997).

Citrus greening disease is an important disease of citrus which greatly affects the production of citrus fruits in several parts of India (Ahlawat, 1997). In India, the greening disease was first identified by Fisher in 1965 and its wide spread occurrence was confirmed by Varma *et al.* (1993), Ahlawat and Pant (2003).

The disease produces foliar symptoms like interveinal chlorosis and motting. Trees affected are generally stunted, have sparse yellow foliage and show die back. It is transmitted by an Asian citrus psylla (*Diaphorina citri* Kuwayama) in India (Capoor *et al.*, 1967). The graft and insect transmission of greening disease initially led to the conclusion that the causal agent was probably a virus. The electron microscopy finally proved that a microorganism, not a virus, was present in phloem sieve tubes of greening affected trees (Lafleche and Bove, 1970).

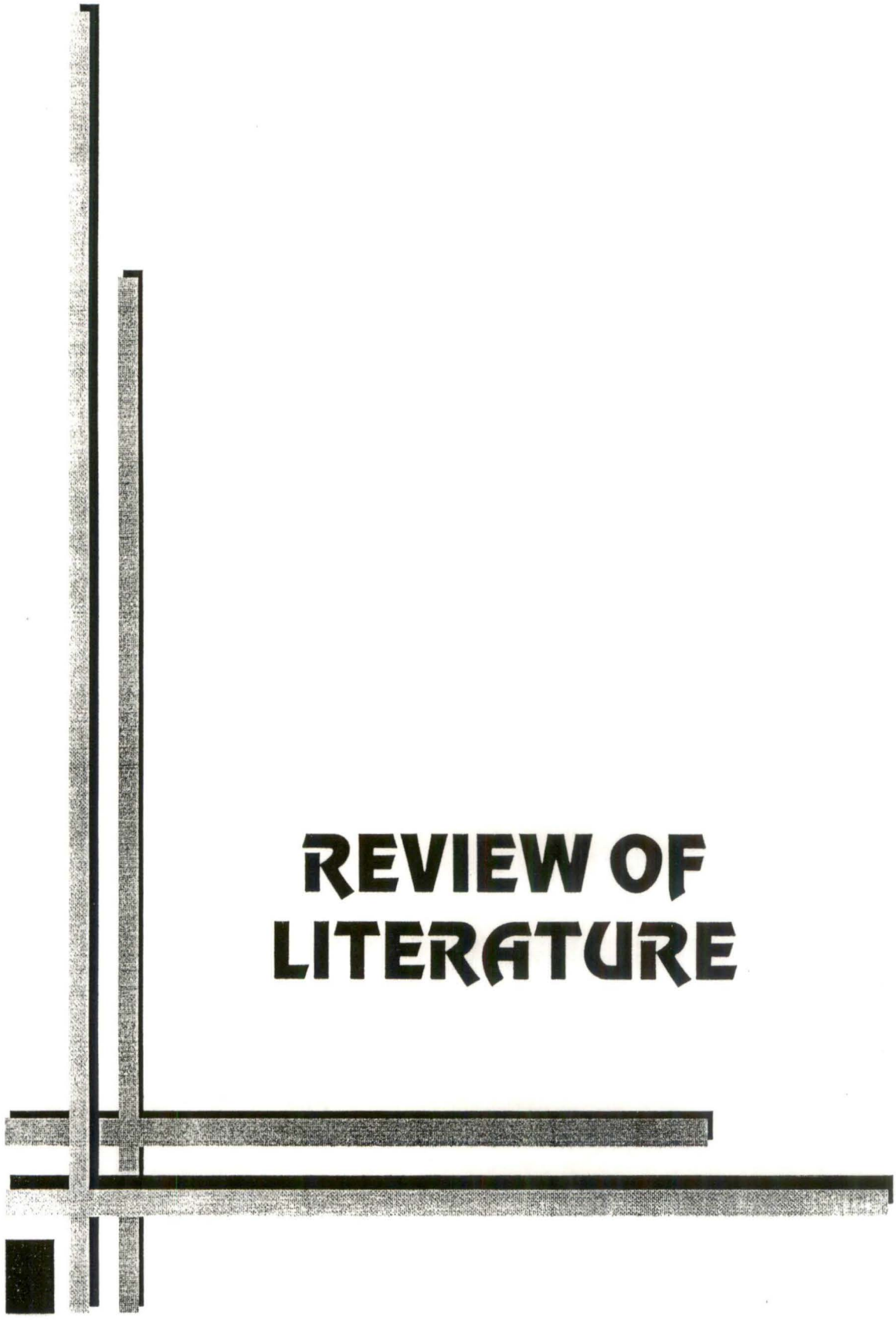
With the discovery of mycoplasma like organisms (MLOs), the microorganism was later thought to be mycoplasma like organism (MLO) but subsequent studies provided it to be bacterial in nature (Garnier and Bove, 1993) and called as bacteria like organisms (BLO). Sequencing of 16S ribosomal DNA confirmed that the greening BLO is an eubacterium (Villechanoux *et al.*, 1993). The sequence analysis also revealed that the greening bacterium belongs to the α subdivision of the proteobacteria having arthropod vectors. In 1994, the uncultured phloem-restricted bacteria of citrus greening disease in Asia and South Africa were characterized as *Liberibacter asiaticus* and *Liberibacter africanum* respectively. Following the rules of the international code of nomenclature of bacteria, the two bacterial species have now been renamed as *Candidatus liberibacter* asiaticus and *Candidatus liberibacter* africanus. The south African form of bacterium was found to be heat sensitive while Asian form of bacterium was heat tolerant.

Confirmation that a citrus trees is affected by greening has up to now relied on the electron microscopial identification of the bacterium, but due to erratic distribution of the pathogen in sieve tubes, it was cumbersome process to cut the right tissue having greening bacterium. However, several indirect approaches such as biological indexing, immunofluorescence tests with monoclonal antibodies and DNA-DNA hybridization with radioprobes have been used for greening diagnosis (Ahlawat and Pant, 2003). Biological indexing is a time consuming procedure and temperature dependent. It requires a well-equipped glass house and long term maintenance of indicator hosts. The use of monoclonal antibodies for field diagnosis has proven unsatisfactory (Korsten *et al.*, 1993). Detection by DNA probes though an accurate method for detection but requires handling of radioactive elements and is being discouraged now a days. Moreover, these are not

practically feasible methods for handling a large sampling unit. The recent study by Hocquellet *et al.* (2000) and Ahlawat *et al.* (2003) have shown that gene amplification of β operon ribosomal protein is a sensitive and promising technique for detection and differentiation of greening bacterium. However, more work is required for standardization of detection of greening organism in Polymerase Chain Reaction (PCR) and its application on large scale. In view of this, the present study has been planned with the following objectives :

Objectives

1. Occurrence and distribution of disease in Marathwada.
2. Symptomatology of disease
3. Detection of pathogen by template preparation using PCR technique.
4. Different method of DNA extraction from citrus tissue (sweet orange leaf midrib tissue)
5. Evaluation of cost of different DNA extraction protocol.
6. Validation of improved PCR detection technique of citrus greening bacteria and to reduce the cost of the technology for viable commercialization.



REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 History and geographical distribution

The presence of greening disease in India was suggested by Lillian Fraser, a scientist from Australia during the her visit to India in 1965. Following the suggestion Dr. S.P. Kapoor and his team at the Regional Station of Indian Agricultural Research Institute, Pune, experimentally demonstrated that the greening disease was present in India and was transmitted by an oriental Psyllid (*Diaphorina citri* Kuwayama) (Capoor *et al.*, 1967) suggested that possibly a virus was involved with the greening disease of citrus as BLOs and MLOs were not known at that time. Occurrence of greening disease was later reported from various parts of the country mostly based on field symptoms and limited transmission tests. Most of these reports were based on Zinc deficiency type symptoms on the leaves of affected trees but leaf, mottle a typical symptom of greening has been overlooked by subsequent workers.

Although citrus dieback was documented in India in the eighteenth century (Raychaudhari *et al.*, 1969) this disease may not have been greening induced decline. Indian citrus dieback was first accurately described in 1929 and attributed to poor drainage (Raychaudhari *et al.*, 1969). Yellow shoot disease was well known in south China even in the 1890 and likubin was identified in Taiwan 60 years ago as a nematode associated problem (Otake, 1990). The first mention of it in English was 1919 when Reinking (Reinking *et al.*, 1919) described a yellowing and a leaf mottle of citrus in southern China and by 1935 it had become a serious problem there. In 1921, Lee described mottle leaf disease in the Philippines, which he thought to be similar to a stubborn disease found

in California, probably stubborn. In Indonesia the diseases was noticed as early as in the 1940 (Aubert *et al.*, 1985).

2.2 Economic importance

Losses due to greening disease are difficult to assess due to uneven distribution of disease tree. Sometimes only a few sectors of a tree are affected and losses are not much, but in other cases the entire tree is infected resulting in the total loss (McClean, 1970). Although no detailed loss studies in terms of money have been published, but the severity of the disease is substantiated in the literature. In India, Fraser *et al.*, (1966) commented on catastrophic losses. In the Philippines, greening affected an estimated of 7 million trees in 1962 (Martinez and Wallace, 1969). It was largely responsible for reducing the area under citrus by over 60 per cent between 1961 and 1970 (Altamirano *et al.*, 1976). In India greening disease killed over 1 million trees in one province (Reddy, 1971). In Thailand up to 95 per cent of the trees in the northern and eastern provinces were severally affected (Bhavakul *et al.*, 1981).

In South Africa the incidence was severe from 1932 to 1936, 1939 to 1946 and again after 1958 (Obrholzer *et al.*, 1965). Crop losses of 30-100 per cent were recorded in some areas. Incidence in the late 1960s and early 1970s declined slight, but its overally effect is still marked, with citrus production eliminated in three major production areas (Moll and Van-Vuuren, 1982).

2.3 Symptomology

Symptoms can occur throughout the tree especially if the infection occurs during or soon after propagation (McClean, 1970). If infection occurs at a later stage, the symptoms and the causal organism are often partially confined. Earlier reports suggested that the organism's movement is restricted to new growth with very little

downward movement but recent findings show that it can move 30-50 cm downwards in 12 months. Infected trees or branches suffer heavy leaf drop followed by out-of-season flushing and blossoming, with dieback occurring in severe cases (Martinez, 1972).

In general, leaf symptoms are of two types : primary symptoms are characterized by yellowing of normal – sized leaves along the veins and sometimes by the development of a blotchy-mottle (Schneider, 1968). With secondary symptoms the leaves are small, upright, and show a variety of chlorotic patterns resembling those induced by zinc and iron deficiencies. Analysis of symptomatic leaves shows a higher potassium content and lower calcium, magnesium and zinc concentrations (Koen and Langenggar, 1970). In India, using the diagnostic techniques of electron microscopy, ELISA and DNA-DNA hybridization it has been established the mottling of leaves is the authentic symptoms of greening disease (Varma *et al.*, 1993).

Infected fruit are small lopsided and have a bitter taste (McClean, 1970). Probably because of higher acidity and lower sugars (Kapur *et al.*, 1978). Many fall prematurely, while those that remain on the tree do not color properly remaining green on the shaded side (McClean, 1970). Seeds in severally affected fruit are abortive.

2.4 Varietal susceptibility and host range

Greening in South Africa is primarily a disease of sweet orange (*Citrus sinensis*), with valencias showing more pronounced leaf symptoms than navels (Oberholzer and Hofmeyr, 1955). It is also particularly serve on mandarins (*C. reticulata*) and tangelos (*C. sinensis* x *C. reticulata*), but less on lemon (*C. limon*). The least affected is the acid lime (*C. aurantifolia*). In Taiwan, India and the Phillippines sweet orange and mandarin are the most susceptible, while lime, lemon, sour orange (*C. aurantium*) and grapefruit (*C. paradisi*) are more tolerant. In

India, rough lemon (*C. jambhiri*), sweet lime (*C. limettoides*) and pummelo (*C. grandis*) are tolerant and the trifoliate orange (*Poncirus trifoliata*) is fairly tolerant (Nariani et al., 1967) but it was found to be severely infected with greening disease in Coorg region (Ahlawat and Pant, 2003).

In India, the Kagzi lime (*C. auranrifolia*) to be used as an indicator for greening disease was suggested as both greening and *Tristeza* could be detected simultaneously (Nariani *et al.*, 1967). In Australia, the greening like disease is most severe on grapefruit and sour orange (Fraser *et al.*, 1966) and in Taiwan, the Wentan Pummelo, once regarded as tolerant, now known as susceptible to the likubin agent (Huang, 1979).

Manicom and Van-Vuuren (1990) have grouped the common citrus cultivars according to their general reaction to greening as follows, severe (sweet orange, tangelo, mandarin), moderate (grapefruit, sour orange) and tolerant (lime, pummelo, trifoliate orange). In some cases the rootstock can affect symptom expression. Five out of 23 rough lemon rootstock selections in India induced a degree of tolerance in the sweet orange scion in greenhouse trials (Cheema et al., 1982). In another study 100 per cent of trees on rough lemon were infected, compared to only 25 per cent of trees on "Blood red" sweet orange rootstock (Kapur *et al.*, 1984). In South Africa, the percentage of greening in valencias was higher in trifoliate orange rootstock than on Empress mandarin and Troyer citrange. Possibly the Trifoliate rootstock causes an extension of the flushing period and thus extends the feeding time of the insect vector, however, no differences were found in a Chinese study on the effects of 13 rootstocks in symptoms on Ponkan mandarin (Lin, 1963).

2.5 Transmission

In 1943, Chen suggested on the basis of graft inoculations that Huanglongbing or yellow shoot might be a viral disease. Similar opinions were soon expressed in South Africa, strengthened by the finding in grafting trials that greening was inconsistently transmitted to healthy plants. Meanwhile, a reported by Lin (1956) in China confirmed that yellow shoot was indeed graft transmissible. Graft-transmissibility of South African greening was confirmed in 1965 by McClean and Oberholzer. The pathogen does not readily pass to progeny trees propagated by buds from infected trees (McClean *et al.*, 1970), possibly because of necrosis of sieve tubes and uneven distribution of the pathogen, but more transmission occurs it stem pieces are used, no infection could be obtained when material from apparently healthy sectors of diseased trees were used. Schwarz (1970) reported a higher graft-transmissibility rate in winter.

In 1964, Schwarz reported that seedlings exposed to insects in a greening infected orchard developed yellowing symptoms similar to greening. McClean and Obertholzer (1965) noted that greening also spreads in the field. These investigators then placed insects from diseased trees to healthy seedlings, and found that only adults of the Citrus psylla species (*Trioza erytreae*), transmitted greening. Schwarz *et al.* (1970) later showed a positive correlation between the degree of greening infection, the number of psylla and the rate of transmission.

In 1966, Salibe and Cortez demonstrated graft transmission in the Philippines and an insect vector, soon identified as *Diaphorina citri*. In India, vector, citrus psylla, *Diaphorina citri* was also identified to transmit greening disease (Capoor *et al.*, 1967). The number of adult psyllids of either species in a population that carry the disease is relatively small (McClean *et al.*, 1970), but under

experimental conditions, a single adult of either species can transmit greening (Raychaudhuri *et al.*, 1972).

The pathogen can be acquired by *D. citri* in 15-30 min with a latent period of 8-12 days (Raychaudhuri *et al.*, 1972). One hour or more is required for 100 per cent transmission. *T. erytreae* acquires the organism after one day of feeding and transmits greening 7 days later and can infect with an exposure times of less than 1 hr. Longer feeding can render the psyllid more infective. Long over wintering feeding on old leaves makes adults highly infective on young flush in spring (Catling *et al.*, 1969). Psyllids are strongly attracted by yellow green of wavelength 550 nm (Samways *et al.*, 1987), making diseased trees attractive targets and thereby increasing the proportion of disease carrying insects.

Although nymphs are reportedly unable to transmit greening (Raychaudhuri *et al.*, 1972) the fourth and fifth instars of *D. citri* can acquire the pathogen and adults from such nymphs transmit the disease (Capoor *et al.*, 1974). Ahlawat and Pant (2003) reported that Psyllids can acquire CGB during feeding on diseased source but the percentage of the transmission is externally low. After acquisition the BLO passes through intestinal value and travels to acini in the salivary glands from where it is transferred to the plants. This is normally a long process (1-3 wk) for most of the prokaryotes. Except in few endemic regions for greening like Coorg in Karnataka and Hindpur in Andhra Pradesh, the field spread of CGB was extremely low in spite of the presence of the psyllid vectors (*D. citri*) in abundance which suggested the possibility of the presence of different biotypes in psyllid vector. Recent experiments showed that adult psyllids were unable to acquire CGB from known CGB infected pineapple sweet orange plants after variable acquisition feeding as determined in DNA-DNA hybridization tests. It appears that phyllids either acquire and transmit CGB at their

nymphal stages or there are biotypes in *D. citri* that alone act as efficient vector. Further comparative DNA-DNA hybridization results revealed that biotypes of Indian psyllids were less efficient vector than that of Malaysian psyllids. Different serotypes of the CGB were also determined with the help of monoclonal antibodies. The MAbs prepared from Pune strain of the CGB also recognized the Malaysian CGB showing similarities in the pathogen found in two countries but the presence of more number of viruliferous psyllids in citrus plantation in Malaysia further confirms the distinct biotypes in the vector, D. citri.

Greening has been experimentally transmitted to many *Citrus* spp., the trifoliate orange (McClean *et al.*, 1970) and to other rutaceous plants, causing leaf symptoms in kumquat (*Fortunella* sp.), stunting, small leaves and yellowing in *Murraya paniculata* and stunting in *Atalantia missionis* and *Swinglea glutinosa*. These and other species may be natural hosts as the insect vectors of greening feed on them (Chakraborty *et al.*, 1976). While the vector in South Africa prefers citrus possibly because of its softer flush leaves, but the vector in Asia displays a preference for *Murraya* spp. (Chakraborty *et al.*, 1976), which tend to flush all the year round, but insect survives well on *Clausena* and *Atlantia*. Moran (1968) suggests that vepris, unduluta. Clausena anisata and Zanthox-ylum (*f. Fagara*) capense are the original hosts of the vector in south Africa.

Greening has been experimentally transmitted by dodder to Periwinkle (*Cartharanthus roseus*) in which it induced marked yellowing (Garnier and Bove, 1983). As in its citrus hosts, South African greening in periwinkle required temperature below 27^0C for symptom development, whereas the Asian form was more heat tolerant. The dodder itself is a host for the greening pathogen with titers higher than has been observed in citrus (Ghosh *et al.*, 1977).

2.6 Nature of causal agent

The demonstrations that greening is a graft-and insect-transmissible disease led to the conclusion that a virus was involved in causing disease. In China, some researchers believed that tristeza virus could be the cause but Lin (1964) disagreed. In South Africa, it was shown that tristeza and greening could readily be distinguished since vectors of the two diseases were distinct. In 1970 Lafleche and Bove reported presence of mycoplasma like organisms (MLOs) in phloem tissue infected citrus plants with South African and Indian greening by electron microscopy. These organisms measured 100-200 nm in diameter, with pleomorphic forms upto 2 m long.

Apparently identical mycoplasm like organisms were soon observed in the haemolymph and salivary glands of *T. erytreae* and the phloem of yellow shoot and likubin-infected citrus and in infective *D. citri*. The identification of greening organism (GO) as MLOs was soon questioned as unlike MLOs greening organism surrounded by a true unit membrance of 10 nm thick, had an outer envelope of 20 nm thick, comparative electron microscopic studies on the structures of the greening organism and several other types of prokaryotes confirmed that it was not a MLO. Garnier *et al.* (1978) suggested that it should be classified as a true bacterium, possibly belonging to the gracilicute division of the prokaryotes, although no distinct R-layer of peptidoglycan (PG) had been observed the inner layer of the outer membrane was somewhat thicker in places, suggesting the presence of an R-layer to separate from the outer membrane of both *E. coli* and the greening organism. The R-layer disappeared after lysozyme treatment. They finally concluded that the greening organism is gram-negative bacterium.

In 1984, Garnier reported the isolation of a long rod-shaped gram negative organism from South African greening-infected

citrus leaf midribs. The ultra structure of this organism was described similar to that of the organisms observed in greening-infected citrus, periwinkle and insects vectors (Ariovich and Garnett, 1984).

Antibodies raised against GO gave positive ELISA results with greening-infected plants (Duncan and Grarnett, 1984). Gold-labeled antibodies also detected greening organism in infected citrus phloem and extracted sap.

Subsequent studies also proved it to be bacterial in nature (Garnier and Bove, 1993). Sequencing of 16S ribosomal DNA confirmed for the first time that the greening BLO is a Eubacterium (Villechanoux *et al.*, 1993). The sequence analysis also revealed that the greening bacterium belongs to the α subdivision of the proteobacteria having arthropod vectors. In 1994, the uncultured phloem restricted bacteria of citrus disease in Asia and South Africa were characterized as *Liberibacter asiaticum* and *Liberibacter africanum*, respectively. Following the rules of the international code of nomenclature of bacteria, the two bacterial species have now been renamed as *Candidatus Liberibacter asiaticus* and *Candidatus Liberbacter africanus*. The African form of bacteria was found to be heat sensitive while Asian form of bacteria was heat tolerant. However greening bacterium has not been cultured as yet.

2.7 Detection

The use of indicator plant for greening disease diagnosis have been reported by various workers. In India the Mosambi sweet orange or Darjeeling orange are used as indicator hosts (Nariani *et al.*, 1975; Ahlawat and Sardar, 1976). Symptoms normally appear in 3-4 months in greening house at 21-23^{0}C after grafting diseased scions on indicator hosts.

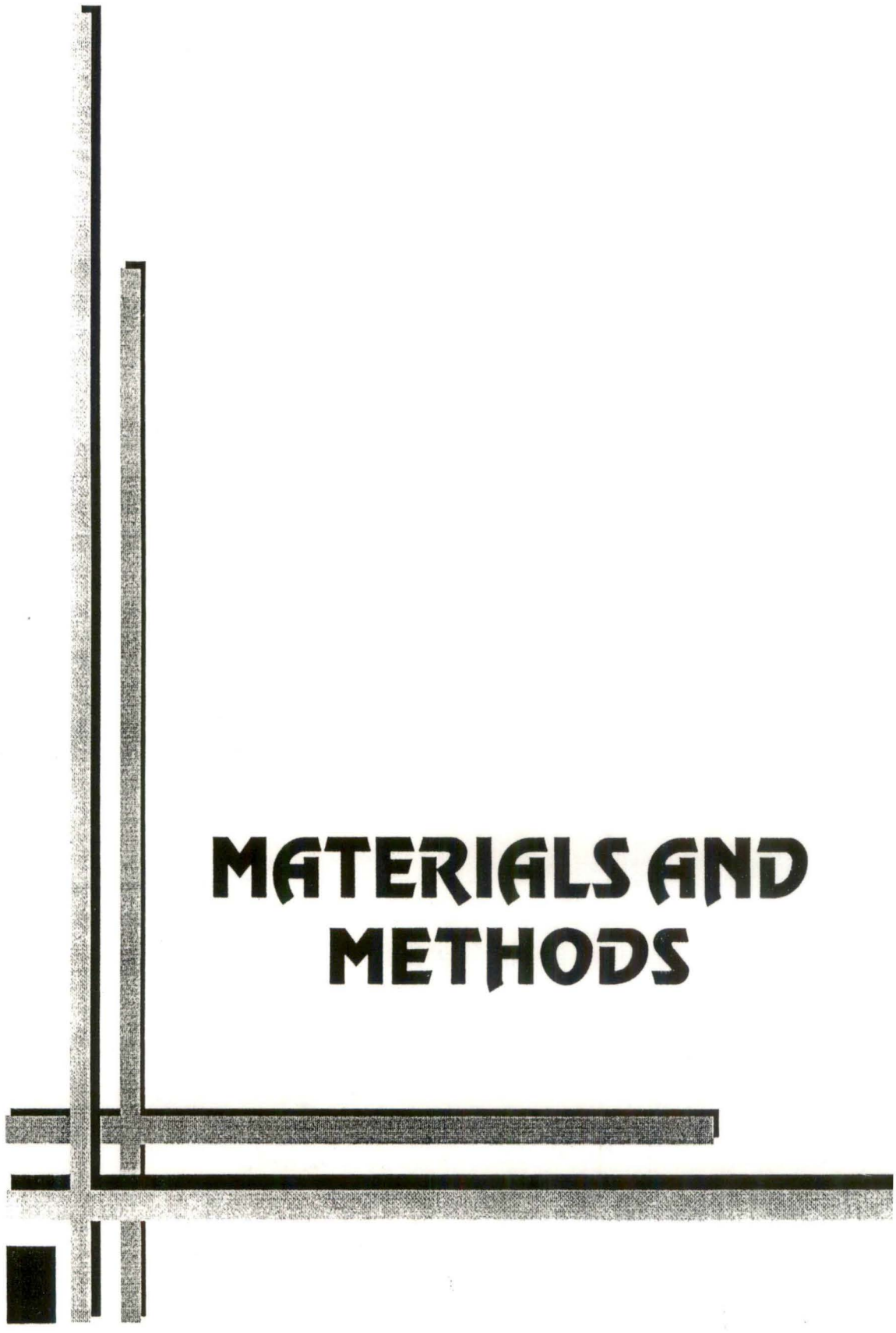
Schwarz (1970) reported the florescent substance later identified as gentisoyl glucoside was detectable in extract of greening infected bark but not in healthy one.

Field diagnosis of greening disease is very difficult because the symptoms on affected trees are often confused with that of zinc and other nutritional deficiencies. Varma *et al.* (1993) tested citrus trees showing variable symptoms with electron microscopy, ELISA and DNA-DNA hybridization and concluded that mottling of the leaves on affected trees was the main foliar symptom of the greening disease. Unusual coloring of the fruits and aborted seeds were its additional symptoms. Election microscopy of ultra thin sections is an important technique to detect the bacterium in sieve tubes from midribs of affected leaves. No polyclonal antibodies could be developed for this bacterium so far because of its pleomorphic nature and inability to culture in synthetic media. Monoclonal antibodies (MAbs) have however been prepared and used for strain-specific detection (Bove *et al.*, 1993, Varma *et al.*, 1993, Ahlawat *et al.*, 1995). The nucleic acid probes detected most of the strains of greening bacterium present in India. It is therefore, now possible to detect more strains of greening bacterium in ELISA, immuno-fluorescence and DNA-DNA hybridization (Varma *et al.*, 1993, Ahlawat *et al.*, 1995, Korsten *et al.*, 1993, Ahlawat and Pant, 2003).

In order to characterize the bacterium associated with citrus greening the highly conserved 16s ribosomal RNA gene obtained by PCR using universal primer has been utilized (Villechanoux *et al.*, 1992). Sequence analysis of 16s rDNA and β operon revealed that greening bacterium was a new bacterium was a new bacterial genus namely *Candidatus* liberibactor asiaticus and *Candidatus liberibacter* africanum. This work resulted in development of specific and sensitive detection technique by PCR (Hocquellet *et al.*, 2000).

Harakava *et al.* (2000) improved sensitivity in the detection of citrus greening bacterium from South Africa and Philippines by PCR using new PCR p rimers and Klen Taq sigma mixture of DNA polymerase.

With the availability of GO diagnosis by molecular technique Ahlawat *et al.*, (2003) reported greening disease for the first time from Bhutan based on its detection by PCR. Hung *et al.* (2004) developed PCR based assay for detection of citrus greening bacterium from psyllid, a vector of citrus greening disease in Asian region.



MATERIALS AND METHODS

CHAPTER III

MATERIAL AND METHODS

3.1 Survey for citrus greening disease

Occurrence and distribution disease in Marathwada. Citrus is grown in more than 26 state in the country. However, the commercial citrus cultivation is taken in central India including Vidarbha (Nagpur mandarin and acid lime and Marathwada Musambi, sweet orange, area of Marathwada and bordering Chindawada district of M.P. (Nagpur Mandarian), Coorg area of Karnataka (Coorg mandarian), Tirupati area of Andhra Pradesh (Sathgudi), Sweet orange and acid lime. Periyakulam district of Tamilnadu (acid lime). Srigangapur district of Rajastan and Abohar Batinda area of Punjab (Kinnow mandarin and Jaffa Malta Sweet-orange), Ranchi area of Jharkhand (Acid lime) of Kalimpong area of West-Bengal (Darjeeling Mandarin).

The citrus orchid located at different district of Marathwada region were surveyed for occurrence of citrus greening disease in Mosambi sweet orange. In total 200 trees were surveyed which were from 3-10 year of age. The affected trees were observed for greening symptom after every 2 months interval during year 2009-10. the incidence of disease based on visual symptom was recorded.

Among different district of Marathwada region. In Nanded district there were more incidence of greening disease on sweet orange as compared to other district and Marathwada region.

The index intensity calculated as follow

$$\text{Disease intensity} = \frac{\text{Number of tree affected by greening}}{\text{Total number of plant were surveyed}}$$

Plate 1 : Different farms surveyed for citrus greening disease

In disease intensity of greening disease is more in Nanded district followed by Jalna and Parbhani district in Marathwada region as compared to other district.

3.2 Confirmation of infection in trees

The tree showing typical symptom of greening were identified and labeled. The symptomatic leaves from individual tree were taken as source of material for extraction of DNA and confirmation of infection by polymerase chain reaction technique as described by Ahlawat *et al.* (2003).

3.3 Symptomology of disease

Symptoms can occur throughout the tree especially if the infection occur during or soon after propagation (McClean, 1970). If infection occur at later stage, the symptom and the causal organism are often partially confined. Earlier report suggested that the organism movement is restricted to new growth with very little downward movement but recent finding show that it can move 30-50 cm downward in 12 months. Infected tree or branch suffer heavy leaf drop followed by out of season flushing and blossoming with dieback occurring in severe case (Martinez, 1972).

In general, leaf symptoms are of two type. Primary symptom are characterized by yellowing of normal mixed leaves along the vein and sometime by the development of blotchy mottle (Schneider, 1968) with secondary symptom. The leaves are small, upright and show a variety of chloratic patterns resulting those induced by zinc and iron deficiencies. Analysis of symptomatic leaves shows a higher potassium content and lower calcium, magnesium and zinc concentration (Koen and Langenggar, 1970). In India using the diagnostic technique of electron microscopy ELISA and DNA-DNA hybridization it has been

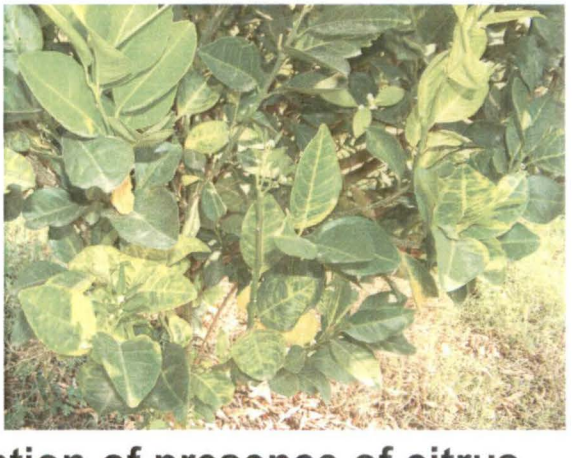


Plate 2 : Confirmation of presence of citrus greening in infected trees

Plate 3 : Symptomology of greening disease

established that molting of leaves is authentic symptoms of greening disease (Varma *et al.*, 1993).

Infected fruit are small lopsided and have a bitter taste (McClean, 1970) Probably because of higher acidity and lower sugar (Kapur *et al.*, 1978). Many fruit falls prematurely while those that remain on tree do not colour properly, remaining green on the shaded side (McClean, 1970). Seed in the severally affected fruit are abortive.

3.4 Detection of pathogen by template preparation using PCR technique

Material required in PCR (Polymerase Chain Reaction)

Principle	:	Thermal Cycling
DNA Template	:	15 µl
CGB 450 F primer	:	1. Forward : 2 µl
		2. Reverse : 2 µl
DNA polymerase enzyme i.e. Tag Polymerase enzyme	:	0.5 µl
10 M dNTP	:	1 µl
10X buffer solution	:	5µl
Divalent cation	:	Mg^{++} or Mn^{++}
Monovalent cation	:	K^{+}
Distilled water	:	24.5µl
Total master reaction mixture (for one sample)	:	50 µl

PCR steps	Temperature (^{0}C)	Time
Initiation step	94^{0}C	5 min
Denaturation step	94^{0}C	30 sec
Primer annelaling	60^{0}C	30 sec
Extension/elongation step	72^{0}C	1 min
Final elongation	72^{0}C	10 min
Holding temperature	4^{0}C	

3.5 DNA extraction from citrus tissue

The DNA was isolated from midrib and petiole of symptomatic leaves of infected field tree. Three method of DNA extraction *viz.* DNA-easy plant mini kit (QIAGEN, Germany), Sodium sulphide (Baranwal *et al.*, 2003) and membrane base nucleic acid technique (V.K. Barnwal and Gupta 2007).

3.6 DNA extraction protocol by commercial kit method (The protocol of QUAGEN was followed)

1. 150 mg tissue from leaves of greening infected midriff were ground in liquid nitrogen in sterilized pestle and mortar.
2. The tissue power was transferred in 1 ml eppendorf tube.
3. 400 µl of buffer AP1 and 4µl of RNAase a stock solution (100 mg 1 ml) to a maximum of 100 mg of ground plant tissue was added and vortexed vigoursly.
4. The mixture was incubated for 10 minute at 65^{0}C and was thoroughly mixed 2-3 times during incubation by inverting the tube.
5. 130µl of buffer AP2 was added to the lysate, mixed and incubated for 5 minute of ice.

6. The tube was centrifuged for 5 minutes at 12000 x g.

7. The lysate was applied to the QIA shredder spin column (Lilac) sitting in 2 ml collection tube and centrifuged for 2 minutes at 12000 rpm.

8. The flow through fraction was transferred to new tube without distributing the cell debris pellet.

9. 1.5 volume of buffer AP3 was added to the cleared lysate and mixed by pipetting.

10. 650µl of mixture from above tube was transferred to DNase mini spin column sitting in 2 ml collection tube.

11. It was centrifuged for 1 min at 6000 x g and flow through was discarded.

12. The above step was repeated with remaining sample and flow through was discarded.

13. The DNase column was placed in new 2 ml collection tube and 500 µl buffer AW was added to the DNeasy column and centrifuge for 1 min at 6000 x g.

14. 500 µl of buffer AW was added to the DNeasy column and centrifuged for 2 min at maximum speed to dry membrane.

15. DNeasy column was transferred to 1.5 ml micro centrifuge tube and 50 µl of preheated 65^0C buffer AE was directly added into the DNeasy membrane and it was incubated for 5 min at room temperature and centrifuge for 1 min at 6000 rpm.

16. The above step was repeated.

3.7 DNA extraction by sodium sulphite method (Baranwal *et al.*, 2003)

1. 150 mg midrib of leaves were ground in liquid nitrogen
2. 10 ml extraction buffer was prepared (Appendix)
3. 10 ml extraction buffer was heated to 65^0C before adding to powdered tissue.
4. The powdered tissue was taken into eppendorf.
5. 1 ml hot extraction buffer was added.
6. The eppendorf containing powdered tissue in extraction buffer was kept at 95^0C heating block for 10 min.
7. Regular vortexing after every 2 min was done.
8. Kept on ice for 2 min.
9. The tube was centrifuged at 12000 rpm for 5 min.
10. Approximately 800 µl of supernatant was taken and transferred to new tube containing 5 µl of RNase.
11. Incubated at 37^0C for 20 min.
12. 480 µl of isoporpanol was added to it and mixed by gentle rocking
13. The tube was centrifuge for 5 min at 12000 rpm.
14. To the pellet, 30 µl of sterile distilled water was added.
15. To dissolve DNA pellet with distilled water was heated briefly at 50^0C and flicking was done.

16. The DNA was precipitated with 30µl of 3 M sodium acetate and $1/10^{th}$ volume of 95 per cent of ethanol.
17. The tube was kept on ice for 10-20 min.
18. The tube was centrifuged for 5 min with 12000 rpm
19. The ethyl alcohol was poured off.
20. 400 µl of 70 per cent ethanol was added and vortexing was done
21. Centrifugation for 3 min at 12000 rpm was carried out
22. The supernatant was poured off
23. The pellet was dried for 40 min at 37^0C.
24. The pellet was dissolved in 70-100 µl of double distilled water, pre heated 45-50^0C.

3.8 DNA extraction by using membrane based nucleic acid technique (Baranwal, Gupta and Singh, 2007)

1. Take 100 mg of petiole and midrib of leaves tissue from cla infected plant were homogenized in 1 ml of alkaline solution of NaOH ELISA (Turtoetal, 1998).
2. The resulted extract were incubated at room temperature (24-32^0C) for 15 min or centrifuged at 1200 g for 10 min.
3. 5 µl of sap were spotted on untreated NCM5 (BAS 85, poresize 0.45 µm Scwicher and Schuee, Kece, N.H.) that were dried for 30 min at 24-32^0C
4. Individual spot (4.0 mm) for each sample were cut out with paper hole punch (Kangaro industries, Ludiana, India) and eluted in 30 µl of sterile distilled water by incubation at 80^0C for 10 min on a heat block.

5. The liquid was collected by centrifugation (termed NCM eluted extract).

6. Volume of 2.5, 5, 10 and 20 µl were used for detection of cla ANAs by PCR.

3.9 Quantification of DNA

The purified total DNA was diluted in double distilled water 5 µl DNA of each sample was diluted in 495 µl of double distilled water for recording uv absorption at 260 nm and 280 nm. Recording were recorded and average value of sample was calculated. The yield of DNA per µl was calculated using formula.

$$\text{ng of DNA per } \mu l = \frac{\text{UV absorption at 260 x dilution factor x 50}}{1000} \times 100$$

Quality of DNA was evaluated based on A260/A280 ratio for each sample and average value was calculated.

3.10 Approximately cost evaluation of each method of DNA extract

The cost per sample was calculated of all the three method based on the chemical used by each method except the commercial kit. The cost per sample from the kit was calculated on the basis of total cost of the kit.

3.10.1 Primer synthesis

Pair of primer from conserved region of ribosomal β-operon gene and ribosomal DNA was synthesized and used to study the detection of citrus greening bacterium in PCR system (CG3450F) primer.

Table 1 : Specific primers used for amplification of ribosomal β-operon gene and 16s ribosomal DNA of citrus greening bacterium

Primers	Primer sequence	Annealing temperature	Amplicon size
A	5'GCGCGTATCCAATACGAGCGGCA3' 5'GCCTCGCGACTTCGCAACCCAT3'	62^0C	1160bp
B	5'TATAAAGGTTGACCTTTCGAGTTT3' 5'ACAAAAGCAGAAATAGCAAACA3'	58^0C	703bp
C	5'TGGGTGGTTTACCATTCAGTG3' 5'CGCGACTTTCGCAACCCATTG'	58^0C	450bp

3.10.2 Ploymerase enzyme

Polymerase enzyme (Tag polymerase) were evaluated for their efficiency to amplify and it was used with primer to compare their efficacy.

3.10.3 PCR amplification

The amplification was performed in thermal cycle using primer and polymerase enzyme (Tag) for comparing their efficiency in amplification of DNA of greening bacterium based on the number of sample amplified and intensity of amplified DNA band. The composition of various request and their volume used in PCR with their thermo cycling profile.

Table 2 : Composition of amplification mixture used for PCR with Taq enzyme

Reagents	Volume required (µl)
1X PCR buffer	2.5
Template DNA	2
*Primer F (1µM) *Primer R (1µM)	0.5 0.5
$MgCl_2$ (1.5mM)	2
dNTP (200 µM each)	0.5
Taq polymerase (0.1/u µl)	0.25
Double distilled water	16.75
Total	25

* All the three sets of primers with same dilution were used

Table 3 : Composition of amplification mixture used for PCR

Reagent	Volume required	
	1 sample (µl)	40 samples (µl)
Distilled water	24.5	980
10x buffer	5	200
10 mm dNTPs	1	40
(CG 3450 F) Primer F	2	80
(CG 3450 R) primer R	2	80
Taq polymerase enzyme	0.5	20
DNA	15	140
Total	30	1400

1400/40 = 35

35 + 15 = 50 µl

For total 40 samples 50 µl/sample

A pair of primers with same dilution were used.

Table 4 : Thermal cycling profile chosen for PCR

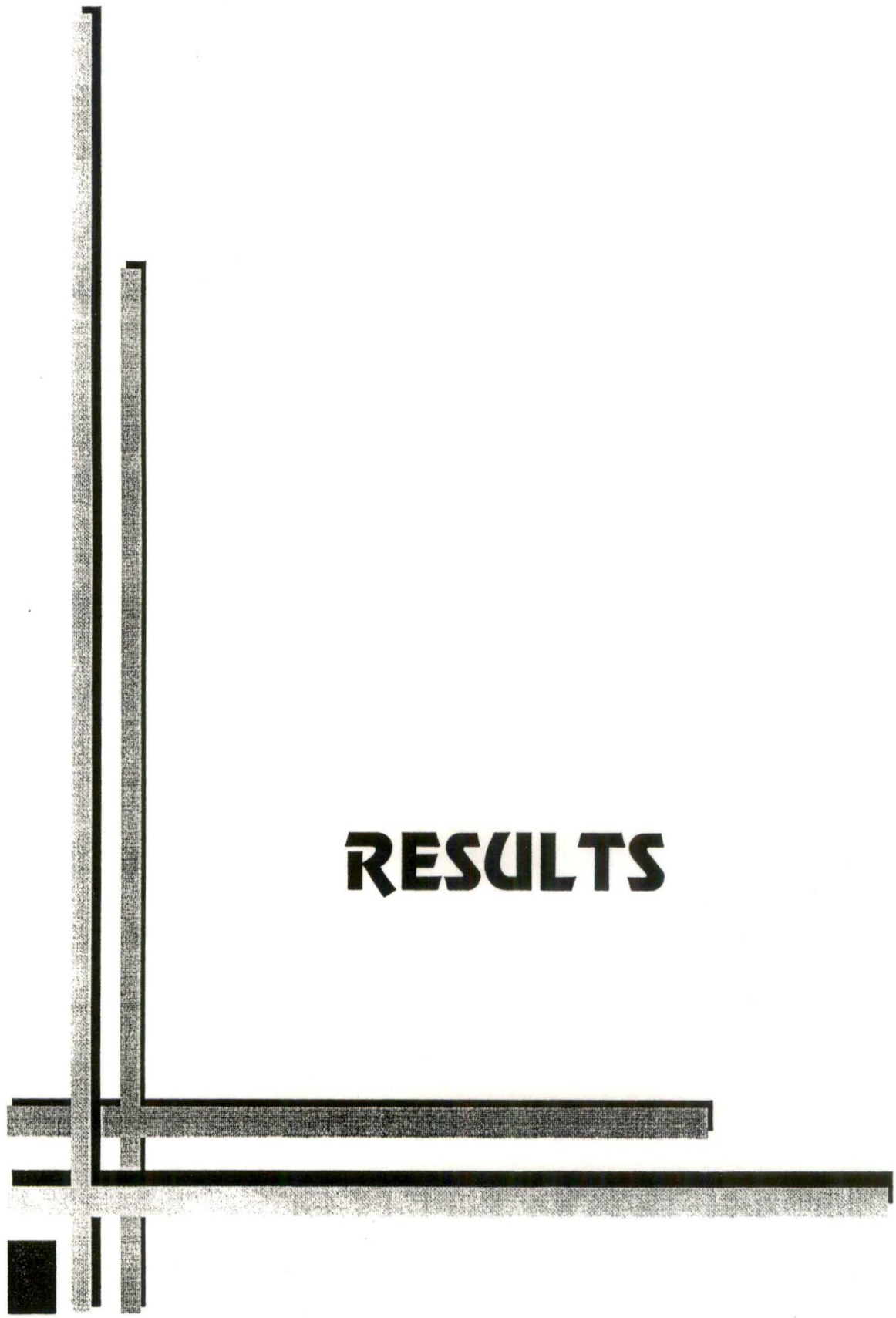
Step involved	Temperature (^{0}C)	Duration	No. of cycle
Denaturation	94	5 min	1
	94	30 sec	30
Primer annealing	60	30 sec	
Extension	72	1 min	
Final extension/ elongation	72	10 min	
Short range holding temperature	4	Hold	

3.10.4 Analysis of PCR product by electrophoresis

Following PCR, amplicon were analyzed in 1 per cent agarose and electrophoresis in Tris-acetate EDTA (TAE) buffer containing ethidium bromide 0.5 g agarose was melted in 50 ml 1x TAE running buffer and 2µl ethidium bromide was added to it after cooling to around 50^{0}C and poured into a casting tray for polymerization placing the 12 well comb. The comb was removed after polymerization and the gel was then placed on electrophoresis tray filled with 1 x PAE buffer 20 µl each of PCR product mixed with 2 µl of 6 x loading dye loaded into the well and was run at 60 volt for 30 min. A aliquot of 1 kb DNA ladder 4 µl was named with dye similarly and electrophoreses to serve as molecular weight marker. After the run the gel was observed under ultraviolet (UV) transillumina and photographed on thermal paper using gel documental system.

3.11 Validation of PCR for detection of greening bacterium

Following the standardization of DNA extract method primer and enzyme. The technology was validated by taking 40 random sample collection from the apparently infected mosambi tree in orchard.



RESULTS

CHAPTER IV

RESULTS

4.1 Survey for citrus greening disease

4.1.1 Occurrence of greening of sweet orange severity in different districts

The survey were aimed at assessing the disease scenario in Marathwada region, with special emphasis on greening of sweet orange. Greening was the most prevalent disease and had its presence in mild to severe form in Marathwada. Hence, an extensive survey was conducted during the year 2009 to 2010 in Parbhani, Hingoli, Nanded, Jalna, Aurangabad, Latur and Osmanabad districts of Marathwada region. Total one hundred and three mandarin orange orchards were observed out of which 25, 18, 15, 20, 16, 5 and 4 were observed in Nanded, Jalna, Hingoli, Parbhani, Aurangabad, Osmanabad and Latur districts, respectively.

Table 5 : Status of greening of sweet orange in Marathwada region in 2009-10

Sr. No.	Districts	Talukas	No. of orchard	Per cent orchards affected with greening of mandarin orange			
				Several (Incidence >40%)	Moderatel (Incidence > 10 to 40%)	Traces (Incidence upto 10%)	Greening free
1	Nanded	6	25	26.65	14.45	25.38	33.52
2	Jalna	4	18	17.65	17.65	22.99	41.71
3	Hingoli	3	15	11.55	04.71	19.29	64.45
4	Parbhani	4	20	08.45	13.68	25.00	52.87
5	Aurangabad	5	16	00.00	09.78	26.86	63.36
6	Osmanabad	2	05	00.00	00.00	10.00	90.00
7	Latur	2	04	00.00	07.20	08.50	84.30
	Total		103				

The data presented in Table 5 showed that the severity of disease was maximum in Nanded district followed by Jalna, Parbhani, Hingoli, Aurangabad, Latur and Osmanabad district. In Nanded during 2009-10 about 68 per cent of surveyed orchards were affected by greening. Sever attack of greening was recorded in less than 20% orchards of Latur and Osmanabad districts. The disease severity was moderate or in traces in Aurangabad district orchards. Jalna, Hingoli, Parbhani and Nanded were found affected with greening in varying intensities. The diseases severity observed minimum in Osmanabad district.

4.1.2 Occurrence of and distribution of incidence of greening disease in Marathwada region

Table 6 : Occurrence and distribution of incidence of greening disease in Marathwada region

Location (district)	Number of trees tested	Condition of tree tested	Number of tree with greening
Jalna	18	S	11
Parbhani	20	S	10
Nanded	25	S	17
Hingoli	15	S	6
Latur	04	As	1
Aurangabad	16	As	5
Osmanabad	05	As	1
Total	103		51

S = Symptomatic, As = A symptomatic G = Greening

Highest greening disease incidence occur in Nanded district as compared to other district in Marathwada region.

4.1.3 Occurrence and distribution of greening disease intensity in Marathwada region

Table 7 : Occurrence and distribution of greening disease intensity in Marathwada region

Location (district)	Number of trees tested	Number of tree with greening	Disease intensity (%)
Jalna	18	11	61
Parbhani	20	10	50
Nanded	25	17	68
Hingoli	15	6	40
Latur	04	1	25
Aurangabad	16	5	31
Osmanabad	05	1	20
Total	103	51	

Highest disease intensity occurred in Nanded district followed by Jalna and Parbhani as compared to other district in Marathwada region.

Disease intensity calculated by using following formula

$$\text{Disease intensity} = \frac{\text{Number of tree with greening disease}}{\text{Number of tree tested}}$$

4.1.4 Weather parameter and disease development

Weather data were recorded by manual observatory at different places under Marathwada Krishi Vidyapeeth, Parbhani and disease development was correlated with weather parameters.

Table 8 : Meteorological observations and greening development at Nanded district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	19.4	37.3	13.0	45.0	00.00	02.70
May, 2009	20.4	41.9	17.5	57.2	19.40(4)	01.00
June, 2009	18.5	39.9	35.4	68.9	26.15(5)	04.90
July, 2009	18.4	36.5	60.3	85.3	50.10(11)	14.20
August, 2009	18.8	35.6	55.7	88.9	39.55(10)	07.00
September, 2009	18.3	33.7	52.3	87.1	46.53(7)	07.50
October, 2009	16.5	35.2	48.6	82.2	15.00(3)	06.60
November, 2009	14.4	32.2	48.9	80.5	52.15(5)	20.60
December, 2009	11.0	31.1	41.5	72.2	02.00	15.50
January, 2010	12.3	31.6	33.3	73.3	00.00	42.80
February, 2010	13.8	32.6	22.4	58.0	00.00	62.20
March, 2010	15.7	32.5	18.2	48.1	12.00(1)	53.70

Table 9 : Meteorological observations and greening development at Jalna district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	18.5	38.5	11.7	41.7	1.8	02.00
May, 2009	19.9	41.5	17.5	52.3	7.1(2)	01.70
June, 2009	23.3	39.7	25.7	59.1	14.5(3)	05.40
July, 2009	21.5	34.8	32.5	84.2	20.4(7)	18.90
August, 2009	21.7	33.1	47.3	89.5	74.9(10)	03.20
September, 2009	20.8	32.6	38.4	82.8	87.5(10)	02.80
October, 2009	18.1	31.4	41.7	75.4	92.7(5)	01.00
November, 2009	14.5	32.2	37.8	78.2	21.2(4)	19.70
December, 2009	12.1	28.5	44.3	85.5	3.5(2)	13.50
January, 2010	13.9	34.9	28.5	72.3	0.0	39.40
February, 2010	15.3	37.2	21.0	62	0.0	57.50
March, 2010	17.5	39.3	25.1	59.2	3.7(1)	40.10

Table 10 : Meteorological observations and greening development at Hingoli district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	18.2	38.7	18.4	42.3	1.3	02.40
May, 2009	19.1	42.5	19.5	52.7	8.1(2)	01.00
June, 2009	18.0	37.9	30.3	68.4	17.8(3)	04.20
July, 2009	20.1	35.8	57.5	88.9	20.13(9)	12.40
August, 2009	19.4	36.2	59.5	92.0	66.7(10)	07.00
September, 2009	18.9	34.3	57.5	85.4	57.4(8)	07.50
October, 2009	17.3	33.74	52.0	82.3	39.3(6)	06.20
November, 2009	14.7	32.5	49.1	79.5	27.8(5)	17.90
December, 2009	10.3	28.7	45.6	77.2	3.7(1)	10.70
January, 2010	11.4	31.3	32.5	70.1	0.00	33.50
February, 2010	14.2	32.5	20.8	61.2	0.00	40.20
March, 2010	16.1	32.7	19.3	51.9	3.2(1)	35.60

Table 11 : Meteorological observations and greening development at Parbhani district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	19.0	40.9	12.2	40.8	2.4	03.00
May, 2009	22.4	42.3	18.6	50	8.3(2)	01.20
June, 2009	24.3	40.5	28.8	66.3	13.2(3)	02.70
July, 2009	22.8	33.3	54.0	80	22.4(8)	19.20
August, 2009	21.2	32.15	57.8	82.5	79.6(10)	08.00
September, 2009	21.5	32.7	57.8	82.1	41.6(7)	06.00
October, 2009	17.2	32.7	43.5	78.3	100.1(3)	03.20
November, 2009	14.4	31.0	45.8	78.00	23.9(5)	20.70
December, 2009	10.5	29.7	38.6	78.2	4.9(1)	14.50
January, 2010	11.7	31.4	29.5	76.5	0.0	47.50
February, 2010	13.7	34.6	21.8	60	0.0	52.50
March, 2010	15.7	37.5	20.6	52.7	3.4(1)	38.30

Table 12 : Meteorological observations and greening development at Aurangabad district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	18	43	14	76	21(2)	02.00
May, 2009	18	41	18	76	14.4(2)	02.40
June, 2009	23	38.5	31	78	45.6(6)	03.70
July, 2009	21.8	35	34	92	137.6(5)	10.00
August, 2009	21	32	42	96	80(1)	09.00
September, 2009	20.2	33.5	36	88	198(17)	08.00
October, 2009	18.0	33.6	19	91	35.5(6)	32.00
November, 2009	14.2	34.0	21	93	55.7(4)	14.50
December, 2009	13.0	31.9	18	90	2.5(2)	13.20
January, 2010	13.8	35.5	18	91	0.0	38.00
February, 2010	16.2	38.6	20	63	0.0	38.00
March, 2010	19	40.2	24	69	4.1(1)	32.00

Table 13 : Meteorological observations and greening development at Latur district (2009-10)

Month/year	Temperature (°C)		Humidity (%)		Total rainfall in mm (No. of rainy days)	Per cent greening incidence
	Min.	Max.	Min	Max.		
April, 2009	20.0	37.9	11.0	38.2	2.1	02.50
May, 2009	21.6	38.7	19.2	55	32(2)	01.00
June, 2009	21.5	34.2	29.9	67.2	47(5)	05.10
July, 2009	20.9	30.5	58.0	65.3	19(9)	10.00
August, 2009	19.7	28.9	54.0	87.5	55(5)	09.00
September, 2009	20.9	30.9	57	89.2	71.5(7)	06.70
October, 2009	17.6	30.5	44.2	83.2	92.5(3)	04.80
November, 2009	22.2	25.4	47.8	79.3	10.75(4)	18.40
December, 2009	13.5	28.4	39.5	76.2	0.00	12.90
January, 2010	13.9	27.5	31.3	74.1	15.5(1)	24.30
February, 2010	13.5	30.5	25.7	60	9(3)	27.50
March, 2010	14.4	33.2	20.1	51.3	2.8(1)	22.70

Perusal data in Table 8, 9, 10, 11, 12 and 13 shows that the greening and brown rot severity started increasing in the month of January 2010 when the temperatures ranged between 23-38.5°C, humidity between 31-78% and intermittent rainfall with 3-6 rainy days rain ranging between 1.5 to 27.5 mm. The maximum severity reached in February 2010, when the temperatures ranged between 18-33.6°C, humidity between 19-71% and conductive temperatures and humidity conditions stimulated the development of greening and hence, had increased severity. The disease severity suddenly fell down in September, probably because of dipping down of minimum temperatures of to 14°C though day temperatures and humidity remained conductive. The severity of greening continued to decrease till November 2010 due to low night temperatures and no rainfall, However, increase in night temperature to 19°C or more with slight drizzle in the month of March resulted in sudden increase in disease severity. This showed that though day temperatures remain conductive for most part of the year in different districts of Marathwada. Night temperature and slight drizzles play important role in disease development, whereas, more number of rainy days play role in the spread of pathogen slight drizzles provide free water for entry of pathogen through natural openings or injuries.

4.2 Confirmation of the presence of greening in infected tree

The tree identified during survey and tested for the presence of greening by PCR. It was found that the pathogen was amplified from all infected tested tree. The leaves from there infected tree were collected and used in further experiment.

4.3 DNA isolation from citrus tissue

As mentioned in material and method total DNA was isolated from the leaves of individual infected trees. Three method of DNA extraction were used commercial kit method, sodium sulphite method. Nucleic acid membrane method. The total DNA was successfully extracted by all the three method. But quality of DNA was different by each method. The quantification of isolated DNA was done by formula mentioned in the material and method. The average yield of all three method of DNA extraction was taken into consideration.

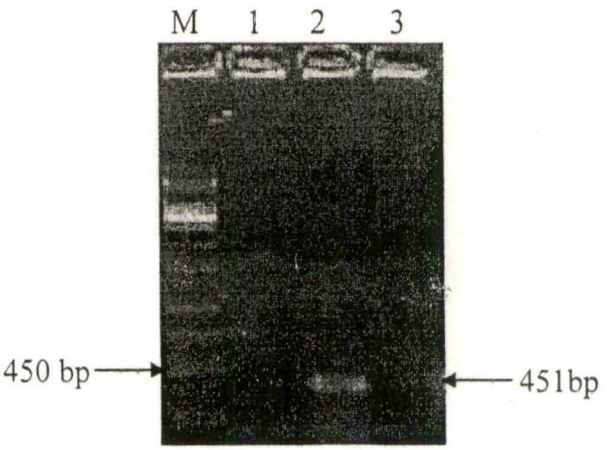
Table 14 : Quantity of DNA obtained from infected sample by 3 method of DNA isolation

Sample	Sodium sulphite (ng/μl)	Commercial Kit (ng/μl)	Nucleic acid membrane method
1	1531.0	123.25	535.0
2	2864.0	201.00	1056.0
3	986.5	165.00	2113
4	796.0	124.75	1053
5	552.0	207.50	2312
6	568.0	136.50	1608
Total	12165.16	159.60	1429.16

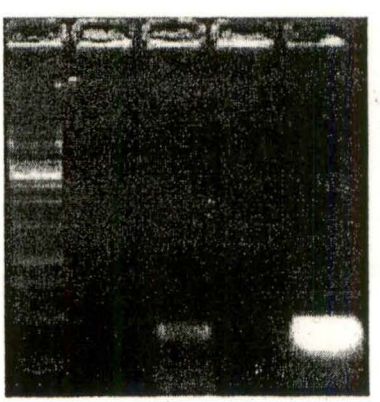
4.4 Determination of quality of DNA isolated by three method

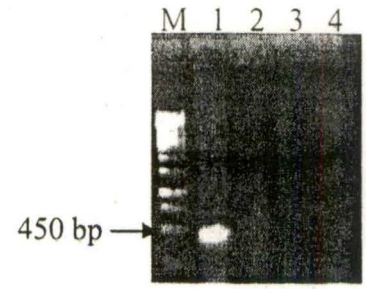
The quality of DNA extracted by each method was tested by calculating A260/A280 ratio of each sample. The average value of DNA extracted by sodium sulphite method was 1.50 and by commercial kit was 1.54 while 1.14 with DNA extracted by nucleic acid membrane method.

Result of PCR detection of CGB by kit in citrus leaf (Sweet orange, midrib)

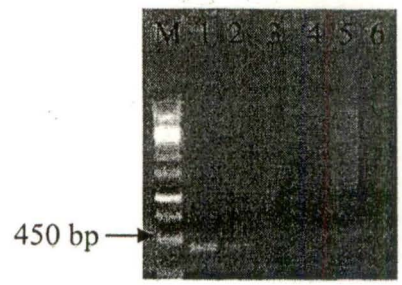


Lane M: Marker 1kb
Lane1: sample KBJ1 (-ve)
Lane 2: Sample BKN3 (+ve)
Lane 3: Sample PB13 (+ve)

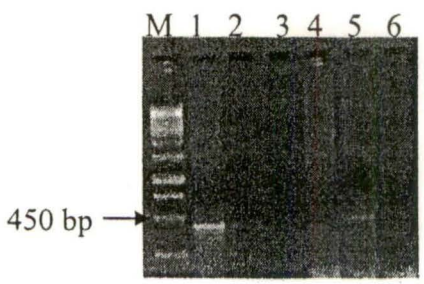




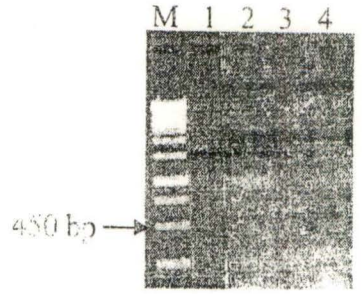
Lane M: Marker 1kb
Lane1: sample SH1(+ve)
Lane 2: Sample SP4 (-ve)
Lane 3: Sample SH12 (-ve)
Lane 4: Sample SH15 (-ve)



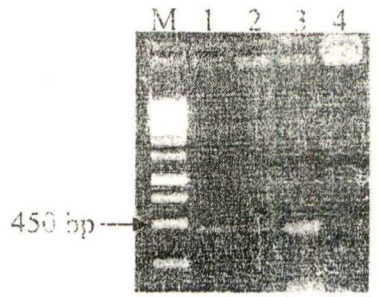
Lane M: Marker 1kb
Lane1: sample SH14 (+ve)
Lane 2: Sample AP1 (+ve)
Lane 3: Sample Ap2 (+ve)
Lane 4: Sample AP3 (-ve)
Lane 5: Sample AP4 (-ve)
Lane 6: Sample AP5 (-ve)



Lane M: Marker 1kb
Lane1: sample APII (+ve)
Lane 2: Sample AP7 (+ve)
Lane 3: Sample Ap8 (-ve)
Lane 4: Sample AP9 bark (-ve)
Lane 5: Sample AP10 (+ve)
Lane 6: Sample AP11(-ve)



Lane M: Marker 1kb
Lane1: sample PI 6 (-ve) (kit)
Lane 2: Sample SH2(+ve) (kit)
Lane 3: Sample SP2 (+ve) (kit)
Lane 4: Sample BKN3 (by Hughes protocol)



Lane M: Marker 1kb
Lane1: sample AP8(+ve)
Lane 2: Sample JB13(+ve)
Lane 3: Sample PST14(+ve)
Lane 4: Sample PB19 (-ve)

Table 15 : Quality comparison of three method (A260/A280)

Sample	Sodium sulphite (ng/μl)	Commercial Kit (ng/μl)	Nucleic acid membrane method
1	1.06	1.05	1.14
2	1.53	2.86	1.02
3	1.13	1.05	1.01
4	2.54	1.44	0.99
5	1.76	1.60	1.55
6	1.01	1.27	1.55
Total	1.50	1.54	1.14

4.5 Cost comparison

The cost was approximately calculated as shown in Table that the cost of isolation of DNA per sample was only Rs. 0.78 by sodium sulphite, Rs. 1.25 per sample by nucleic acid membrane method and Rs. 140 per sample by commercial kit.

Table 16 : Approximately cost involved in DNA isolation per sample by 3 method

Sodium sulphite method		Nucleic acid membrane method	Commercial kit method
Chemical	Cost		
Extension buffer	0.14	Untreated NCM NaOH EDTa	
Isopropanol	0.10		
3 m NaoAc	0.14		
Ethanol (600 μl)	0.20		
RNase (5 μl)	0.20		
Total	0.78	1.25	140

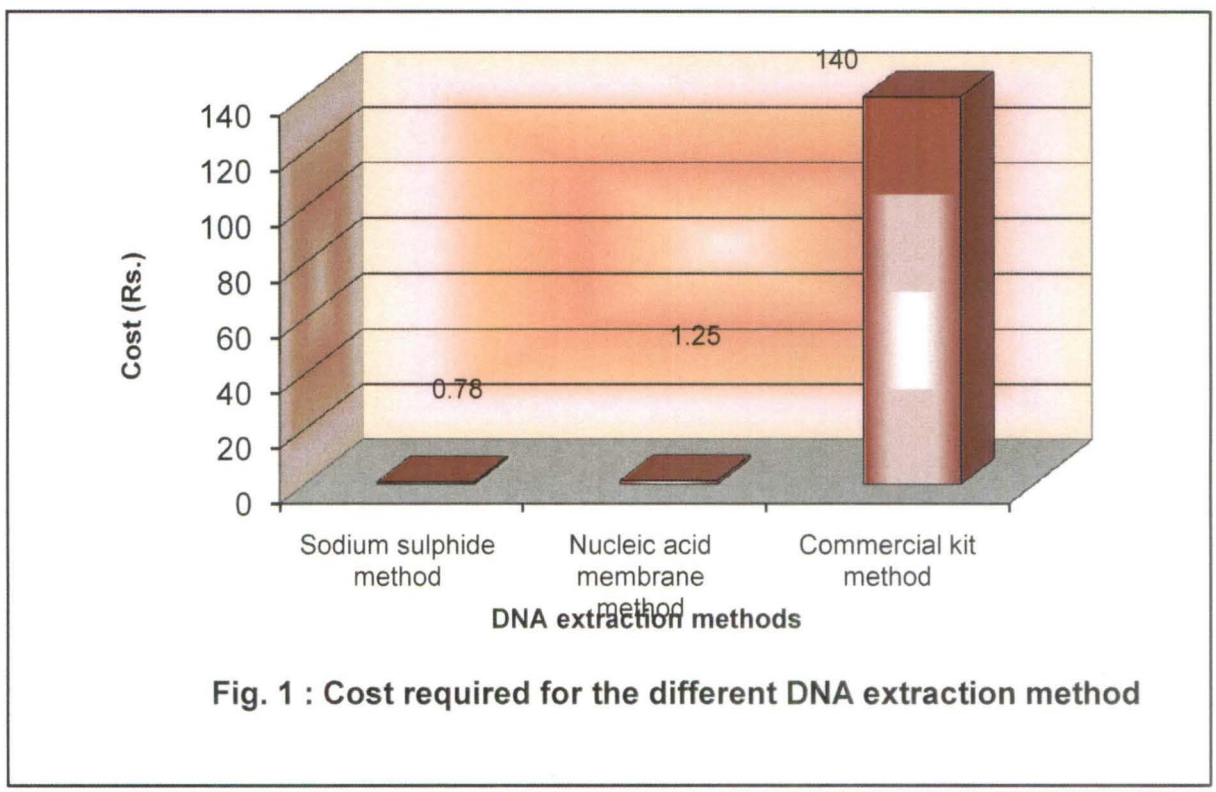
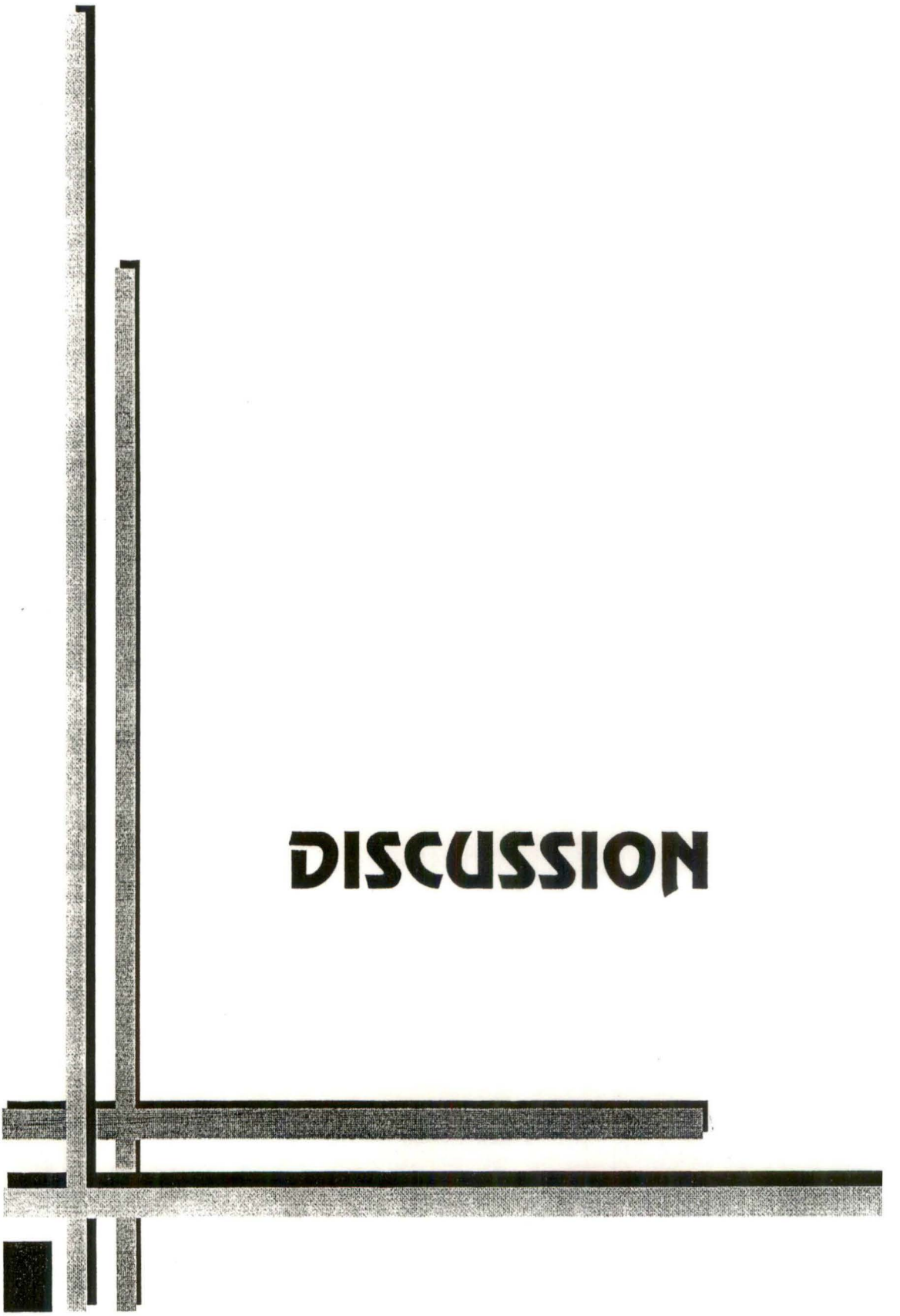


Fig. 1 : Cost required for the different DNA extraction method

4.6 Validation of standardized PCR amplification technique for greening disease

It has been established through earlier experiment that following combination did the best diagnosis of citrus greening disease.

- 2 µl of template DNA extracted by sodium sulphate method and nucleic acid membrane.
- Use of Tag enzyme at rate of 0.25 µl/25 µl reaction mixture.
- Primer (CG 3450 F) at the rate of 0.5 µl per 25 µl reaction mixture.



DISCUSSION

CHAPTER V

DISCUSSION

In India, citrus is the largest fruit industry after banana and mango. Citrus fruits are grown all over the country but its commercial cultivation is done in Maharashtra, Andhra Pradesh, West Bengal, Sikkim, Punjab and Assam. The most preferred species grown in India are Mandarin, Sweet Oranges and acid lime. The productivity of citrus fruits in India is comparatively low owing to many biotic stresses of which some fungi, bacteria, viruses and viroids and photoplasmal diseases play a very significant role. More than 30 viral diseases, 2 photoplasmal diseases, one spiroplasmal disease, 3 viroid diseases, 11 fungal diseases, 3 bacterial diseases are known to occur in citrus through out the world (Ahlawat and Pant, 2003).

Among viral diseases, *citrus tristeza*, Indian citrus ringspot, citrus mosaic and citrus yellow vein clearing are important in India whereas others are of minor importance and endemic in nature. Citrus greening disease now known to be caused by a bacterium, *Candidatus* Liberibacter asiaticus is very important and wiped out citrus cultivation in several parts of the country (Ahlawat, 1997).

The presence of greening disease in India was suggested by Fraser *et al.*, (1966). Since, then, disease has been reported from various parts of the country (Ahlawat, 1997). In the absence of reliable diagnostic reagents and tools like electron microscope, the actual incidence and distribution of citrus greening bacterium (CGB) in India could not be achieved till 1991. With the establishment of Advanced Centre for Plant Virology at the Indian Agricultural Research Institute (IARI), New Delhi, the serological and molecular techniques for detection of greening bacterium were developed. During the period from 1991 to 1996. Presence of CGB was confirmed by electron microscopy,

immunoflorescence and DNA-DNA hybridization technique (Bove *et al.*, 1993, Ahlawat and Pant, 2003). Subsequently, the PCR technology was developed for detection of CGB (Harakava *et al.*, 2000; Hocquellet *et al.*, 2000, Tian *et al.*, 1996; Bove *et al.* 1993; Jagouiex *et al.*, 1996; Hung *et al.*, 2004). However this PCR technique was used for first time to detect greening disease in citrus in India by Ahlawat *et al.* (2003). Serodiagnosis was not found effective as it requires a panel of strain-specific monoclonal antibodies and due to sacrifice of animals in production of monoclonal antibody, it is now being discouraged all over the world. The nucleic acid hybridization technique required radioactive material and is not advisable if other efficient and reliable techniques like PCR are available. Therefore, during the present investigation, the information has been developed on suitability and reliability of PCR technique for detection of greening bacterium as a routine procedure of indexing. There was no information available on characterization of Asian greening bacterium by amplification, cloning and sequencing although reports are three for African greening bacterium (Planet *et al.*, 1995).

During present study trees of Mosambi sweet orange of the age of 3-10 years were periodically surveyed at the orchards of Marathwada region. A very high incidence of 65 per cent was observed. The high incidence of greening disease has also been reported by Ahlawat and Sardar (1975) from Darjeeling hills, Ahlawat (1997) and Verma *et al.* (1993) from other citrus growing states. The greening incidence information in these investigation, was determined on the basis of typical symptoms of greening diseases in field trees and the analysis of candidate trees by Polymerise Chain Reaction (PCR) using protocol of Ahlawat *et al.*, (2002). Trees were identified based on PCR reaction for these studies. The leaves from these trees were collected and preserved at 80^0C in deep freeze for the use in various experiments. However, only some plants showed positive amplification in PCR. It is

known that the distribution of greening bacterium is erratic in the plant tissue (Varma *et al.*, 1993; Bove *et al.*, 1993) and this could be the reason of non transmission in plants by grafting. Since the studies were planned with a view to standardize and validate PCR diagnostic in field trees, the materials collected from identified greening positive trees were used in these studies.

Three steps are important for detection of pathogen in PCR. They are : a) Nucleic acid isolation and its quantity and quality b) primer designing, synthesis and its evaluation c) evaluation of polymerase enzymes for PCR amplification.

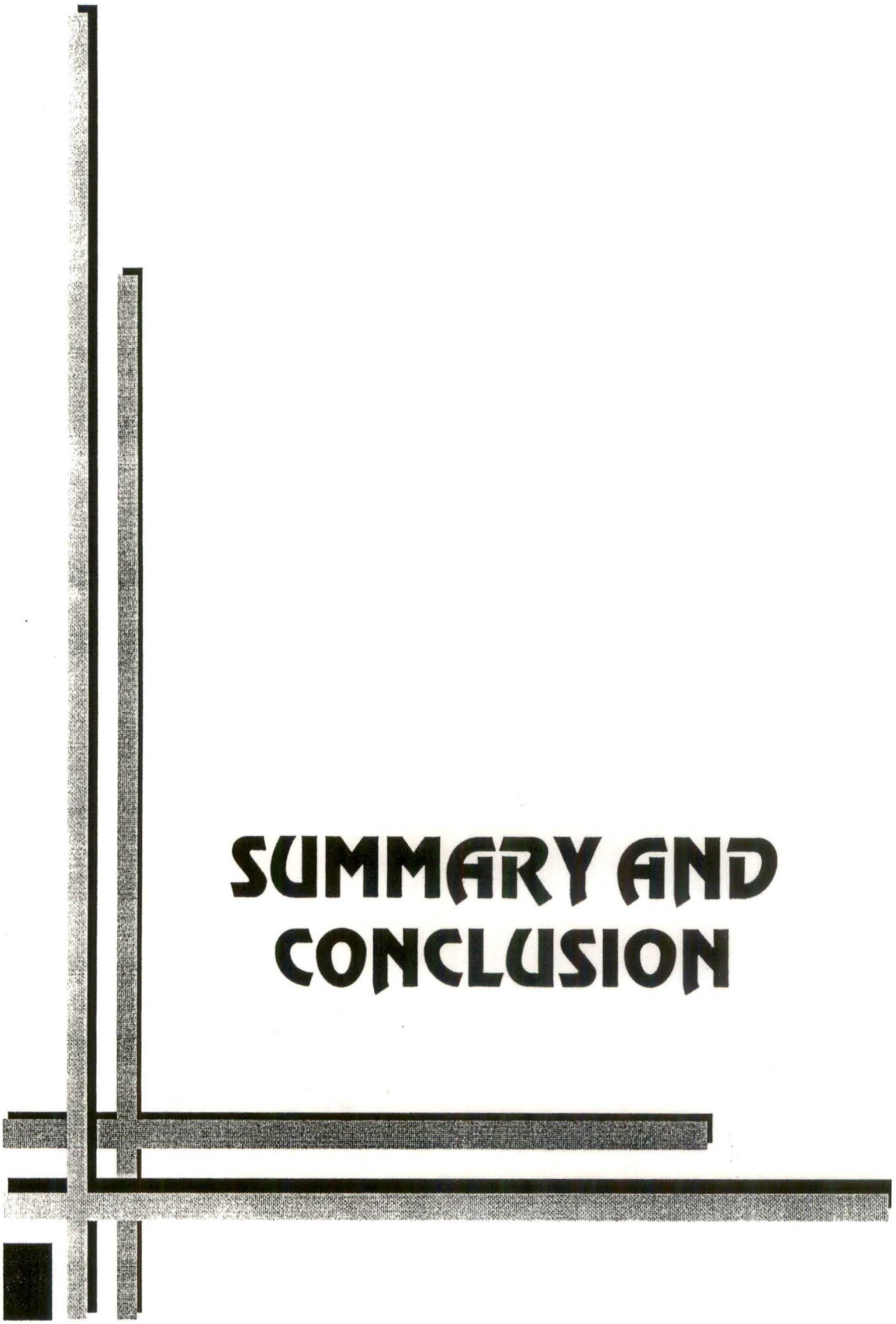
During the present studies all the three steps were standardized. The DNA was isolated by three methods, sodium sulphite (Baranwal *et al.*, 2003). Nucleic acid membrane method (Baranwal, 2007) and commercial kit obtained from QIAGEN Germany. The leaf material from PCR positive trees was used in most of the experiments. Similar results were obtained by Singh *et al.*, (2002) of commercial kit and while working with potato and a cherry virus and Baranwal *et al.* (2003) with citrus yellow mosaic virus. However, the yield of DNA by commercial it (159.60 ng/ml) was much less as against Sodium sulphite method (1216.16 ng/ml). The quality of DNA obtained by all the three methods was also assessed by calculating A260/A280 ratio and it was observed that quality obtained by Sodium sulphite method and commercial kit was almost at par (1.50 and 1.54, respectively). Although the best quality of DNA is known with the A_{260}/A_{280} of 1.8. However, this ratio has not been achieved with the any of the methods. But the satisfactory amplification was obtained even of the DNA extracted by sodium sulphite method with A_{260}/A_{280} ratio of 1.50. That is why this method was used in the experiment too. Since the quantity and quality of DNA isolated by sodium sulphite method was found to be better, this method was preferred over the methods of commercial kit

and membrane based nucleic acid extraction. Further, an approximate cost per sample was calculated of all the 3 methods of DNA isolation and it was interesting to know that the cost per sample by sodium sulphite method was only Rs. 0.75 as against Rs. 1.25 per sample by nucleic acid membrane method and Rs. 140 by commercial kit. Therefore, the protocol developed for DNA isolation by sodium sulphite method was not only superior but also very cost effective. Such comparative studies have not been reported as yet. Two polymerase enzymes, Tag and Klen Tag were also compared and evaluated using DNA template obtained by three methods described earlier and it was apparent from experiment. The per cent amplification was obtained by Tag as it is a mixture of two DNA polymerase with proof reading activity. Therefore, its sensitivity was more than Tag and this also does not require addition of $MgCl_2$ separately in the amplification reaction mixture like Tag polymerase.

During the present study, the DNA extraction method by sodium sulphite, Klen Tag enzyme and 450 bp set of primers were identified to give best amplification in PCR system of diagnosis. Using these protocols, samples from 40 more Mosambi tree collected once in January, 2010 and another in March, 2010 were analyzed in PCR using combination of all the three standardized steps. It was found that the amplification of DNA isolated from out of 40 samples, 32 and 38 were PCR positive during the month of January and March, respectively. During the survey of greening diseases, 65 per cent of incidence of disease was observed while testing 40 trees for the presence of green bacterium in PCR, 15 were positive during January and 19 in March indicating much higher incidence of disease with PCR testing. The results suggested that the PCR is reliable technique for detection of greening bacterium as against the diagnosis based on visible symptoms. It was therefore, evident that protocol developed during present studies can detect greening bacterium in field trees irrespective of the season

and the concentration of pathogen in the host. However, Since more samples showed the presence of greening bacterium during March, the samples for detection of the bacterium should be taken in March to get maximum detection.

Detection of viruses and virus like pathogens by PCR technique are gaining importance over the other techniques as it is comparatively sensitive and reliable and can detect the pathogen even at a very low concentration upto 10 pg. The concentration of greening bacterium is very low in sieve tubes and distribution is also uneven (Varma *et al.*, 1933) and it is important that the samples are taken from different direction of the tree. The technology of PCR detection of greening bacterium developed during present investigation is new and validated for the first time in India. It will be very useful for field diagnosis of greening disease in planting material. The technology developed is cost effective and highly reliable for indexing bud wood certification programmes in citrus in India and elsewhere. These studies will also be useful in plant quarantine for export and important of citrus germplasm.



SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

Citrus greening disease caused by Candidatus Liberibacter asiaticus, a fastidious bacterium, is one of the important disease of citrus in India and elsewhere. The polymerase Chain Reaction (PCR) diagnosis is more reliable and sensitive diagnostic tool for greening bacterium than other conventional approaches like Electron Microsocpy, DNA-DNA hybridization, and immunofluorescence (IF) for detection of citrus greening. During the present study, diagnostic procedure PCR was developed for detection of citrus greening bacterium and it was standardized for improving sensitivity and reliability than known before. The leaf material from trees showing typical greening symptoms and found positive in PCR were used as source of material for detection of greening bacterium. Three methods of DNA isolation *viz.* sodium sulphite, nucleic acid membrane DNeasy plant mini kit, A pair of primers and polymerase enzymes were used and their efficacy was compared during experiment it was observed that sodium sulphite method of DNA isolation provided higher yield and better quality DNA than other methods. Primer C 43450 F was more efficient in amplifying the DNA of greening bacterium even at a very lower concentration of 0.1pg. To confirm the reliability of PCR, the greening bacterium was also detected in graft-inoculated plants, which showed typical greening symptoms.

PCR protocol standardized during the study was further validated by testing samples from 40 field trees during month of January and March. 32 and 38 samples out of 20 tested showed amplification of 450 bp in PCR suggesting sampling in March is more suitable for PCR detection of greening bacterium.

Further to know sequence identity of greening isolate (present study) with other isolates of greening bacterium, 450 bp amplicon of ribosomel RNA gene of greening bacterium was cloned and sequenced. The sequence analysis and comparison revealed that, the isolate of greening bacterium of present study shared maximum sequence identify with Pune isolate, and it was distinct from China and Brazil isolates. Such study has been done for the first time in India. The study showed wide variability in the greening bacterium all over the world.

CONCLUSION

The following conclusion were generated during present study

1. Severity of disease was maximum in Nanded district followed by Jalna, Parbhani, Hingoli, Aurangabad, Latur and Osmanabad district.

2. In Nanded during 2009-10 about 68 per cent of surveyed orchard were affected by greening

3. Severe attack of greening was recorded in less than 20-25 per cent orchard of Osmanabad and Latur district.

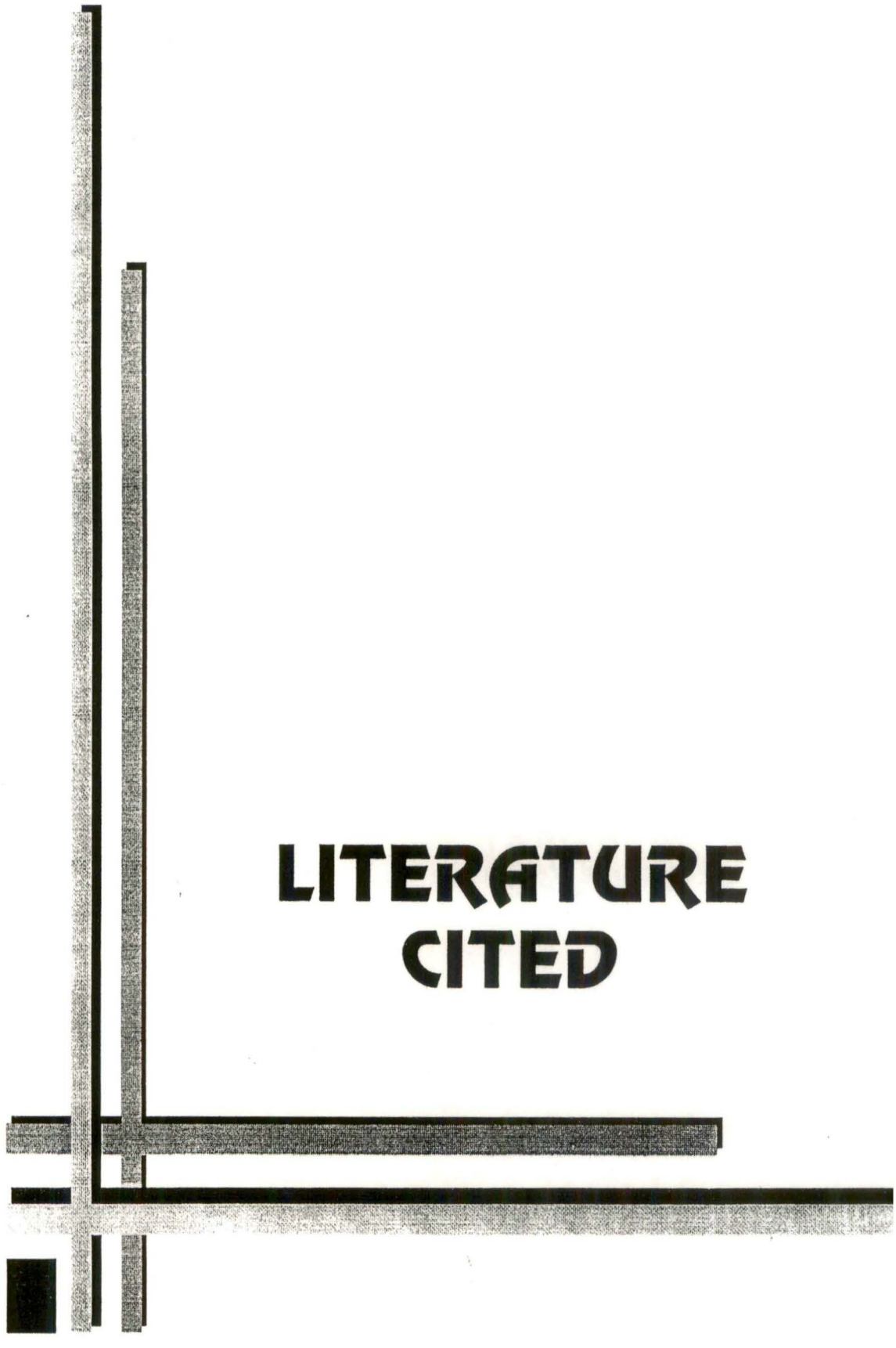
4. The disease severity observed minimum in Osmanabad district.

5. The maximum severity of disease reached in February 2010 when temperature range between 18-33.6^{0}C humidity between 19-71% and disease severity suddenly fell down in September.

6. Among the three method of DNA extraction protocol sodium sulphite method is less costly and effective for commercial use.

7. PCR based diagnostic system is developed for detection of greening bacterium.

8. The comparative cost of detection by various combinations of reagents and sampling time was determined and cost effective technology was standardized and validated.



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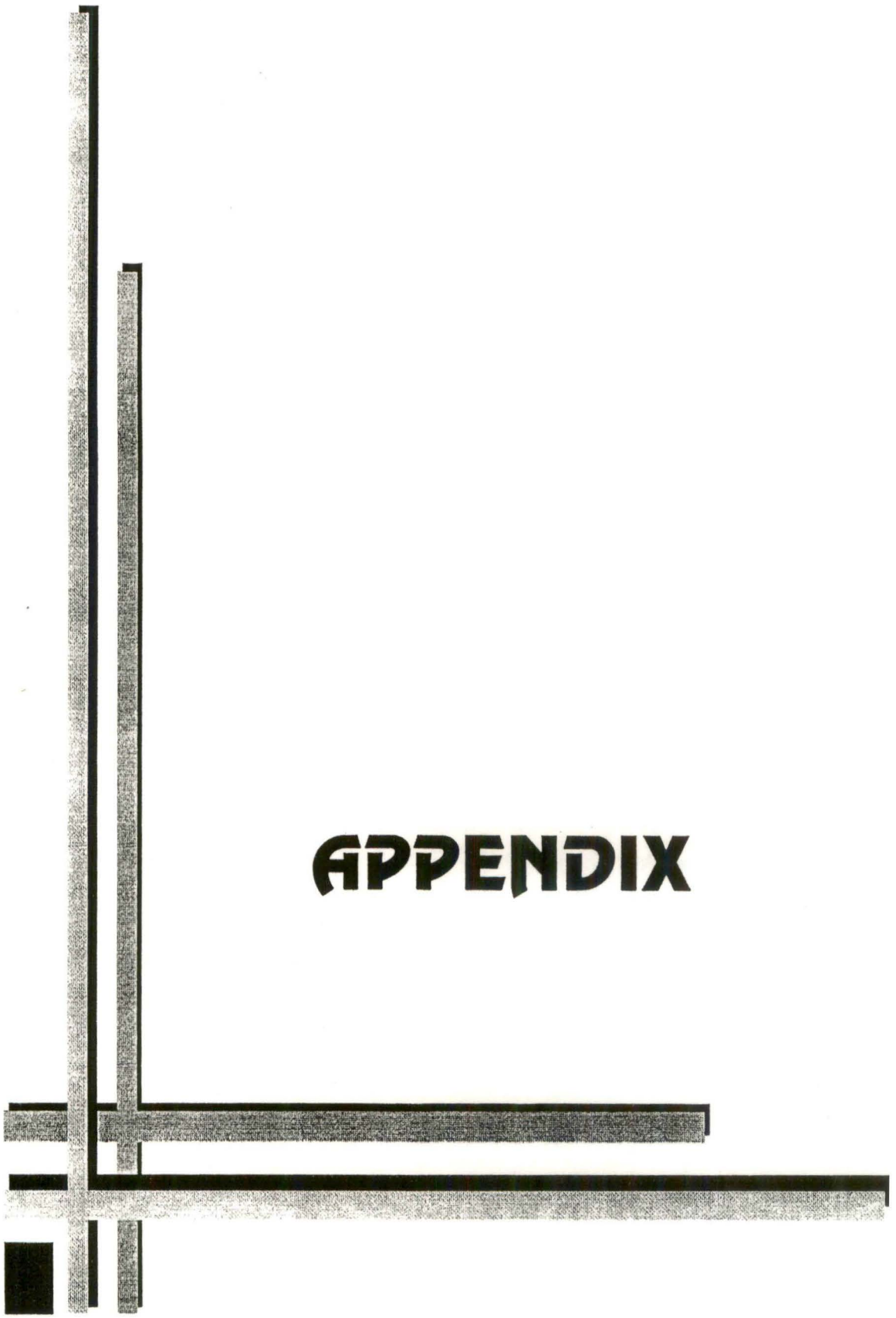


APPENDIX

APPENDIX

1. Buffers used for DNA extraction

A. DNA extraction Buffer for Sodium Sulphite method

1M Tris HCL (pH.8)	-	1ml
3M KCl	-	3.3 ml
0.5 EDTA	-	0.2 ml
Double distilled water	-	5.5 ml
Sodium sulphite	-	0.065 g
Total	-	10 ml

2. Electrophoresis Reagents

50 x TAE Tris base	-	242.09
Glacial acetic acid	-	57.1 ml
0.5 M (EDTA) pH 8.0	-	1.00 ml
Distilled water to 1 litre	-	

3. Loading dye

1% Bromophenol Blue	-	200 µl
Glycerol	-	200 µl
10% of SDS	-	60 µl
0.5 M EDTA	-	50 µl
10 x TAE	-	60 µl
Distilled water	-	30 µl

4. Preparation of commonly used stock solution

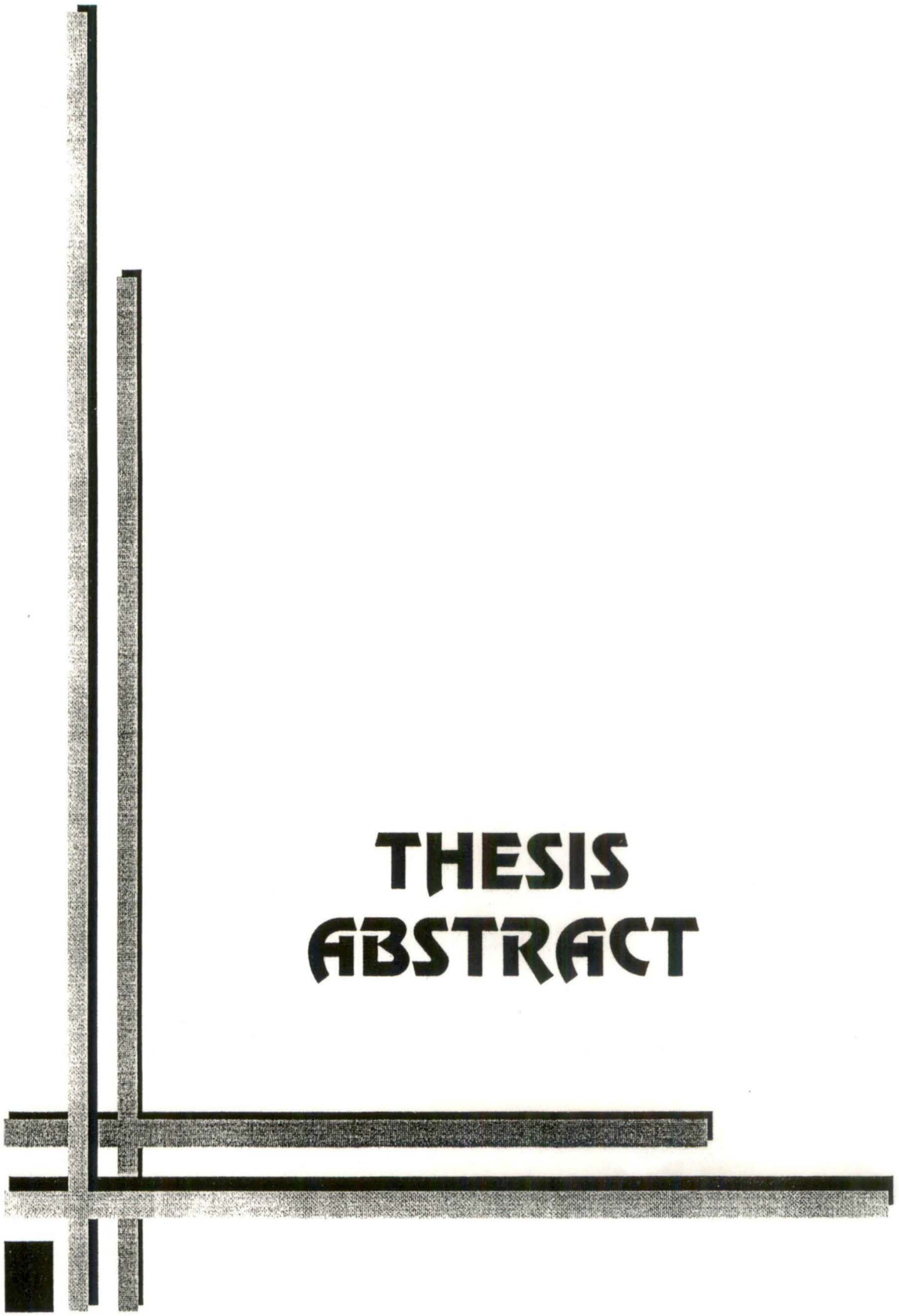
Solution		Method of preparation
0.5 M EDTA (pH 8.0)	-	186.1 g of ethylene diamine tetra acetic acid disodium salt, $2H_2O$ was added to 800 ml of distilled water, stirred vigorously on magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaoH pellets), volume made upto 1 l with distilled water, dispersed into aliquots and sterilized by autoclaving
Ethidium bromide (10 mg ml^{-1})	-	1.0 g of ethidium bromide was added to 100 ml of distilled water and sterilized on magnette stirred for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature.
1 M $MgCl_2$	-	203.3 g $MgCl_2$ $6H_2O$ was dissolved in 800 ml distilled water. The volume was made up to 1 litre, dispensed into aliquots and sterilized by autoclaving.
3M soldium Acetate (pH 4.8)	-	408.1 g of NaOAc $3H_2O$ was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acid. Volume made up to 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
5M NaCl	-	233.8 g of NaCl were dissolved in 800 ml of distilled water, volume made upto 1 L with distilled water, dispense of into

aliquots and sterilized by autoclaving.

1 M Tris HCl	-	121.1 g Tris base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated Hcl. The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made upto 1 litre with distilled water, dispensed into aliquots and sterilized by autoclaving.

5. Materials, Source, Catalogue number of chemicals reagents

Material	Source	Catalogue No.
Vector		
P-Drive	QIAGEN	
Cells		
+ Escherichia coli DH 5 α	Stratagene, USA	260233
Marker		
1 Kb DNA ladder	MBI Ferments	5 M 0311
Enzymes		
+RNase A	Sigma chemical Co.	R5000
+ T_4 ligase	Amersharm, USA	E2050Y
X gal	USB	12385
IPTG	USB	17886
Amplificillin	Signma Chemical Co.	A0104-10g
Kits		
DN Easy plant mini kit	QIAGEN, USA	69104
Gel Extraction Kit	QIAGEN, USA	28704



THESIS ABSTRACT

POLYMERASE CHAIN REACTION DETECTION OF *CANDIDATUS LIBERIBACTER ASIATICUS* ASSOCIATED WITH CITRUS HUANGLONGBING (GREENING) OF SWEET ORANGE

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THESIS ABSTRACT

Polymerase chain reaction diagnosis of disease is molecular technique which is used for detection of disease when pathogen present is very low concentration in disease sample. Before molecular technique use, first survey of the Marathwada region for greening disease, then confirm the presence of disease in infected samples. Use three different method for DNA isolation from infected sample i.e. commercial kit method, sodium sulphite method, nucleic acid membrane method. Among these three DNA isolation method is sodium sulphide is cost effective for commercial use. In nucleic acid membrane method for DNA extraction isolation there is no use of liquid nitrogen.

Polymerase chain reaction detection of disease is based on principal of thermal cycling in which PCR instrument allow to run generally 60-65 thermal cycle, during PCR operation it allow different stages of cycle at different temperatures for different period of time i.e. initiation (94^0C), denaturation (94^0C), primer annealing (60^0C), extension/elongation step (72^0C), final elongation (72^0C) and holding temperature (4^0C). After all completion of procedure and cost effective analysis of different DNA extension and isolation method. Final following conclusions are drawn 1) PCR based diagnosis system is developed for detection of greening bacteria 2) The comparative cost of detection by various combination of reagent and sampling time was determined and cost effective technology was standardized and validated.