MOLECULAR CHARACTERIZATION OF VIRUS CAUSING INFECTIOUS CHLOROSIS DISEASE OF BANANA

by

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(2014-21-115)

THESIS

Submitted in partial fulfillment of the requirements for the Degree of

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DECLARATION

I, hereby declare that the thesis entitled **"MOLECULAR CHARACTERIZATION OF VIRUS CAUSING INFECTIOUS CHLOROSIS DISEASE OF BANANA"** is a bonafide record of research work done by me during the course of research and this thesis has not been previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"MOLECULAR CHARACTERIZATION OF VIRUS CAUSING INFECTIOUS CHLOROSIS DISEASE OF BANANA"**is a bonafide record of research work done independently by Mr. Ahamed Mujtaba V. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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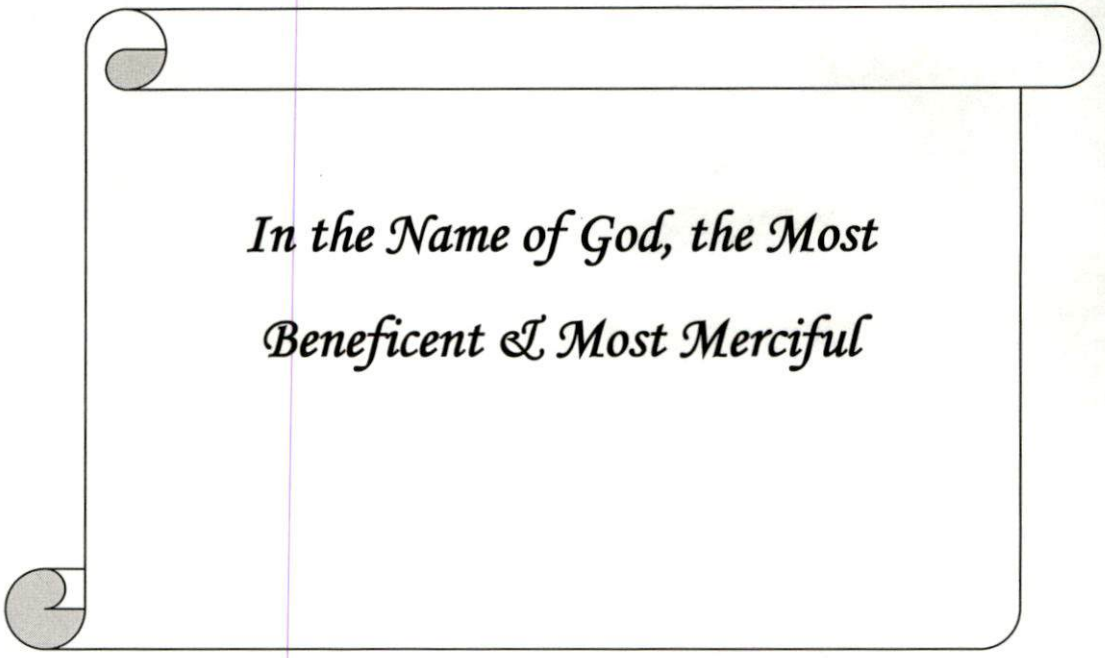
In the Name of God, the Most
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Abbreviations

%	:	Percentage
µg	:	Microgram
µl	:	Microliter
µM	:	Micromole
AFLP	:	Amplified Fragment Length Polymorphism
APS	:	Ammonium per sulphate
AR	:	Aspect ratio
BBrMV	:	*Banana bract mosaic virus*
BBTV	:	*Banana bunchy top virus*
bp	:	Base pair
BSA	:	*Bovine serum albumin*
BSV	:	*Banana streak virus*
CCB	:	Coomassie Brilliant Blue
C-DNA	:	Complementary Deoxyribonucleic Acid
cM	:	Centimorgan
cm	:	Centimeter
CMV	:	*Cucumber mosaic virus*
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DAC- ELISA	:	Direct antigen coating Enzyme linked immunosorbant assay
DIBA	:	Dot immuno binding assay
DNA	:	Deoxyribonucleic Acid
dNTP	:	Di-Nucleotide Triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
ELISA	:	Enzyme Linked Immunosorbant Assay
FAO	:	Food and agricultural organization
g	:	Gram
GNRs	:	Gold nanorods
ha	:	Hectare

HAuCl4	:	Hydrogen tetra-chloroauratehydrate
Ig	:	Immunoglobulins
KAU	:	Kerala Agricultural University
kb	:	Kilo base
Kb	:	Kilo basepairs
kDa	:	Kilo dalton
KP	:	Potassium phosphate
l	:	Liter
LSPR	:	Localized Plasmon Resonance
M	:	Molar
Mb	:	Mega base pair
mg	:	Mili gram
min	:	Minute
ml	:	Mililiter
mM	:	Mili molar
mm	:	Milimeter
mM	:	Milimole
MPA	:	3-Mercaptopropinic acid
MUA	:	Mercaptoundecanoic acid
ng	:	Nano gram
NIR	:	Near infra red
nm	:	Nanometer
°C	:	Degree Celsius
OD	:	Optical Density
PAGE	:	Polyacylamide gel electrophoresis
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
PEG	:	Poly ethylene glycol
pH	:	Hydrogen ion concentration
pM	:	Pico molar
pNPP	:	Para-Nitrophenylphosphate

PVP	:	Polyvinylpyrrolidone
QTL	:	Quantitative Trait Locus
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic Acid
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
SSR	:	Simple Sequence Repeat
TEM	:	Transmission electron microscope
TEMED	:	Tetramethylethylenediamine
V	:	Volt
β	:	Beta

Introduction

I. INTRODUCTION

Banana (*Musa* spp.) is known for its antiquity and is interwoven with Indian heritage and culture. The plants are considered as the symbol of 'prosperity and fertility'. Owing to its greater socio-economic significance and multifaceted uses, it is referred as 'Kalpatharu' (Plant of Virtues) and Kalpavriksh. It is one of the world's most important fruit crops and plays a critical role in the income security of many tropical and sub tropical countries. It is also referred as poor man's apple. It is grown in around 150 countries across the world in an area of 5,393,811 million ha producing 114,130,115 million tons. Among the countries, India is the largest producer of banana with an annual production of 29,724,550 million tons from 8,02,570 ha contributing 27.43 per cent followed by China, Philippines, Brazil and Ecuador (FAO, 2017). Within the country, it is cultivated in all the states and major banana growing states are Tamil Nadu, Karnataka, Andhra Pradesh, Maharastra, Gujarat, Kerala, Assam, West Bengal, Bihar, Madhya Pradesh, Odisha, Chhattisgarh and Uttar Pradesh. In Kerala, banana is grown in almost all districts with an area of 1.85 lakh ha and production of 11.6 lakh tones (DAC and FW, 2015).

However, banana cultivation is threatened by various biotic problems like pests and diseases. In the recent years, due to increase in area of banana cultivation, an increased occurrence of pests and diseases causing significant reduction in yield was reported. Diseases caused by fungal (Fusarium wilt, Leaf spot), viral (banana bunchy top, banana streak, banana bract mosaic, cucumber mosaic), bacterial (bacterial wilt, bacterial soft rot, moko disease) and nematodes (*Radopholus similis*) are the major production constraints (Ramesh, 2009). Diseases, particularly virus borne are considered as major threat due to abundance of insect vectors, easily available collateral hosts and vegetative method of propagation of planting material. The viral diseases known to infect banana are banana bunchy top, banana streak, banana bract mosaic and infectious chlorosis disease. Of these, infectious chlorosis disease caused by *Cucumber mosaic virus* is now emerging as a serious threat, affecting yield and is wide spread in Kerala (Estellita *et al.*, 1996). Intercropping banana with cucurbitaceous vegetables is a common practice in

Kerala which led to an increase in the incidence of this disease. The potential effect of climate change also aggravated the spread of viruses through the vector. This disease is also known as heart rot, virus sheath rot, cucumber mosaic and banana mosaic (Stover, 1972).

Banana viruses are primarily transmitted through vegetative planting materials such as suckers and have direct effect on production by decreasing plant growth and yield (Vishnoi *et al.*, 2013). Since viral diseases cannot be eliminated by chemicals or by any other treatments, the management of viral diseases is based on preventing infection rather than curing the disease (Hull, 2002). Successful management of viral diseases particularly in vegetatively propagated crops including banana is achieved through the use of virus free planting materials which are produced through tissue culture technique. In recent years, tissue culture serves as a tool to mass multiply virus free planting material. However, the important disadvantage of tissue culture are somaclonal variation (Vuylsteke *et al.*, 1998) and the possibility of multiplication of virus in infected planting materials (Thomas *et al.*, 1995) if proper indexing and multiplication procedures are not followed. Hence, development of quick, sensitive, rapid and cost effective techniques for the early detection of viruses in the planting material is necessary for large scale quality testing of planting materials.

Crop improvement through development of resistant varieties using conventional breeding methods is very difficult in banana. Hence, biotechnological approaches like pathogen derived resistance mechanisms such as coat protein and movement protein mediated resistance have to be applied for the development of stable resistant varieties and for which, knowledge on the molecular characterization of the virus and its diversity analysis is very essential. A perusal of literature revealed that not much work was done on the characterization and detection of the virus causing infectious chlorosis disease in Kerala.

The present study on "Molecular characterization of virus causing Infectious chlorosis disease of banana" would give detailed inference on biological and molecular

characterization of the virus, sources of varietal resistance and standardization of methods to eliminate the virus from planting material through *in vitro* techniques. Recombinant clones were also developed which have immense application in the field of disease diagnostics and management. These clones could also be used for the production of antiserum through recombinant DNA technology and for coat protein mediated resistance in genetic engineering. These could also be used as disease diagnostic probes for more sensitive molecular techniques like Nucleic acid spot hybridization. Hence, this project was proposed with objectives like biological characterization of the virus, molecular characterization, immunological studies, varietal screening for disease resistance and Development of Nano-biosensor based detection of the virus.

Review of literature

II. REVIEW OF LITERATURE

The present investigation on "Molecular characterization of virus causing Infectious chlorosis disease of banana" was carried out during 2014-17 and studied various aspects of the disease *viz*., symptomatology, characterization of the virus, host range, disease resistant sources, molecular of immunodiagnostics and development of gold nano-biosensor for the early detection of the virus. The relevant literature on the work done on these aspects so far in India and elsewhere are briefly reviewed in this chapter.

Banana is one of the most important fruit crops of the country which is the world's fourth most important commodity and is grown in around 150 countries across the world in an area of 5,393,811 million ha producing 114,130,115 million tons (FAO, 2017). Among the major producers, India alone accounts for 27.43 per cent and is the largest producer of banana with a total annual production of 29,724,550 million tons from 8,02,570 ha (FAO, 2017) followed by China, Philippines, Brazil and Ecuador. Being a tropical crop, this could be grown in humid tropics to humid sub-tropics and semi arid tropics up to an elevation of 2000 m above MSL and our country is endowed with diverse varieties catering to various local needs. Inspite of its wide cultivation, the crop is prone to many biotic stress caused by different pathogens. Among the diseases, viral disease is the major threat for successful cultivation of banana (Magnaye and Valmayor, 1995).

Banana bunchy top, Infectious chlorosis, Banana streak and Banana bract mosaic are the four most important viral diseases affecting banana in our country (Singh *et al.,* 2012).

2.1 CHARACTERIZATION OF VIRUS

2.1.1 Occurrence

The disease was first described in New South Wales, Australia during 1930. Due to this disease individual growers in the Richmond River district had experienced considerable loss in banana and in some cases led to the abandonment of the entire

plantations (Magee, 1930). Since then, several sporadic outbreaks of this disease were reported from most of the banana growing countries. The global distribution and occurrence of this disease have been reviewed and enlisted in Table 1. This disease was variously named as nimbin disease, heart rot, banana mosaic, cucumber mosaic and sheath rot is now commonly called as infectious chlorosis disease of banana (Lockhart and Jones, 2000).

The disease is caused by *Cucumber mosaic Virus* (CMV) (Magee, 1940). Wardlaw (1961) reported severe incidence of CMV on banana variety Gaint Cavendish in Hondurus where, about 150 acres had to be totally eradicated due to disease. CMV was prevalent on banana grown in Srilanka and caused considerable yield reduction even when symptoms were mild (Ariyaranthe and Liyanage, 2002). Yot-Dauthy and Bove (1966) reported about 30 per cent disease incidence in the banana plantations of Ivory Coast and in the French Antilles and later the disease had developed to considerable proportion over the years and in some young plantations, all the plants were infected and hence complete eradication was advocated.

In India, CMV was first reported during 1943 in Jalagaon district in Maharastra and the cause of which was then attributed to improper manuring and irrigation (Kamat and Patel, 1951). They reported that Dwarf Cavendish variety of banana which was commercially grown in eastern Khandesh, Surat, Thane, Nasik and Pune districts was severely infected with infectious chlorosis disease causing serious loss to banana growers. Since then, the disease was reported from most of the banana growing states of the country (Table 2).

Mohan and Lakshimanan (1988) conducted survey in different districts of Tamil Nadu and reported CMV infection on banana variety Robusta, Dwarf Cavendish and Poovan (Mysore). The survey carried out during 1990 in 19 districts of Tamil Nadu revealed an incidence 13.4 per cent (Elangovan *et al.*, 1990).

Table 1: Geographical distribution of infectious chlorosis disease of banana

Sl. No.	Year of report	Region	Reference
1	1930	Australia	Magee, 1930
2	1933	Brazil	Wardlaw and McGuire, 1933
3	1934	Caribbean (Trinidad and Haiti)	Wardlaw, 1934
4	1935	British (Bermuda)	Wardlaw, 1935
5	1935	France (Guadeloupe)	Wardlaw, 1935
6	1937	Philippines	Reinking, 1937
7	1938	Caribbean (Haiti)	Wardlaw, 1940
8	1938	France (Guadeloupe)	Wardlaw, 1938
9	1943	India	Kamat and Patel, 1951
10	1948	Central Africa	Wardlaw, 1948
11	1949	South America (Colombia)	Ciferri, 1949
12	1950	Asia: British North Borneo	Reinking, 1950
13	1953	South West Pacific region	Magee, 1953
14	1955	Africa: Italian Somaliland	Castellani, 1955
15	1957	Central America (Costa Rica)	Allen, 1957
16	1960	Israel	Nitzany, 1960
17	1961	South America (Venezuela)	Herold and Dao, 1961
18	1962	Carribian Island (Puerto Rico)	Bird and Wellman, 1962
19	1962	South America (Surinam)	Van-Hoof, 1962
20	1962	Africa (Egypt)	Bird and Wellman, 1962
21	1963	Brazil	Medeiros, 1963
22	1969	Middle East (Lebanon)	Nienhaus *et al.*, 1969
23	1974	Middle East Turkey	Salih and Nas, 1974
24	1990	North Africa (Morocco)	Bouhida and Lockhart, 1990
25	1992	USA (California)	Daniels and Campbell, 1992
26	1995	USA (Hawaii)	Hu *et al.*, 1995
27	1995	West Africa (Ghana)	Osei, 1995
28	1998	South Africa	Magnaya and Valmayor, 1995
29	2001	Central America (Costa Rica)	Hord *et al.*, 2001
30	2002	Srilanka	Ariyaranthe and Liyanage, 2002
31	2004	China	Zhou *et al.*, 1999
32	2005	Africa (Southern Nigeria)	Dongo, 2005

Table 2: Distribution of infectious chlorosis disease of banana in India

Sl. No.	Region	Reference
1	Maharashtra	Kamat and Patel, 1951; Kamat, 1955; Mali and Deshpande, 1975; Mali and Despande, 1976; Mali and Rajegore, 1979; Mali and Rajegore, 1980; Patil, 1982; Aglave *et al.*, 2007; Khan *et al.*, 2011
2	Southern India	Capoor and Varma, 1968; Capoor and Varma, 1970; Rao, 1980; Srivastava *et al.*, 1995; Singh, 1999
3	Gujarat	Joshi and Joshi, 1976
4	Tamil Nadu	Mohan and Lakshimanan, 1988; Elangovan *et al.*, 1990; Thiribhuvanamala and Sabitha 2001; Khan *et al.*, 2011
5	Kerala	Estelitta *et al.*, 1996; Cherian *et al.*, 2004
6	Karnataka	Thammaiah *et al.*, 2004; Khan *et al.*, 2011; Prema *et al.*, 2012; Khan *et al.*, 2012; Preeti., 2015; Khan, 2015
7	Andhra Pradesh	Ramesh, 2009
8	Uttar Pradesh	Khan *et al.*, 2011; Ali *et al.*, 2012; Vishnoi *et al.*, 2013; Khan, 2015
9	North East	Lepcha *et al.*, 2017

Estelitta *et al.* (1996) conducted a survey in Thiruvanthapuram, Kollam, Kottayam, Ernakulam, Thrissur, Palakkad and Malappuram districts of Kerala and reported that the infectious chlorosis disease was prevalent in different banana varieties and in all the districts surveyed.

Thiribhuvanamala and Sabitha (2001) surveyed banana growing districts of Tamil Nadu to evaluate the incidence of major viral diseases of banana and reported that the incidence of CMV in Coimbatore (14%) and Trichy (10.51%).

Thammaiah *et al.* (2004) surveyed northern districts of Karnataka and reported the incidence of the disease in Gokak (2.34%), Bagalkot (1.66%) and Raibagh (0.25%) taluks of North Karnataka.

Ambika *et al.* (2011) conducted roving surveys in Belgaum, Dharwad and Haveri districts of Karanataka to assess the incidence of viral diseases of banana and reported the disease incidence ranging from 0.02 to 11.11 per cent. Maximum incidence was recorded in Belgaum district followed by Dharwad (0.69%) and Haveri (0.56%) district of Karnataka. They also reported that among the different cultivars, Grand Naine was the most susceptible followed by banana var. Rajpuri with an incidence of 4.16 per cent. Vishnoi *et al.* (2013) observed severe mosaic accompanied by leaf and fruit deformation on banana plants grown in three farms of Uttar Pradesh and reported the disease incidence of 18-25 per cent.

A survey conducted on 21 orchards of three bananas growing states *viz.*, Karnataka, Maharashtra and Uttar Pradesh revealed that the lowest rate of infection was in Uttar Pradesh followed by Maharashtra while maximum infections was observed in samples collected from Karnataka (Khan, 2015). Preeti (2015) carried out a survey to find out the occurrence of banana viral diseases in Dharward, Bagalkot, Belagavi, Haveri and Uttar Kannada districts of Karnataka and reported that the highest mean incidence (2.8%) of the disease was recorded from Bagalkot district.

2.1.2 Causal Agent

Infectious chlorosis disease of banana, also named as leaf fall, nimbin disease, heart rot, banana mosaic and sheath rot is caused by *Cucumber mosaic virus* (Magee, 1940; Yot-Dhauthy and Bove, 1966 and Stover, 1972).

The virus is a member of cucumovirus group having spherical particles of size 28-30 nm diameter containing ssRNA (Francki *et al.*, 1979). They also reported that the isolates of CMV possessed three genomic and one subgenomic RNA species. Kaper and Waterworth (1977) mentioned about a subgenomic fifth RNA species which occurred in

some virus isolates and had been linked to modulation of symptom expression banana (Gafny *et al.*, 1996).

2.1.3 Symptomatology

The symptoms of the disease were first described as appearance of whitish or yellowish-white streaks on younger leaves which later expressed chlorosis turning light to yellow streaks or bands extending from the midrib which were the most specific symptoms produced by CMV in banana (Campbell, 1931 and Simmonds, 1931).

Simmonds (1935) in addition to symptoms described earlier also reported that during colder months, the funnel leaf or heart leaf or cigar leaf developed soft black rot and the rotting further extended down into the corms, causing death of infected plants. Similar symptoms were described by Mali and Rajegore (1979) and Patil (1982) and they also reported that the extent of rotting was depended on environmental conditions.

Yot-Dauthy and Bove (1966) while working in French Antilles and Ivory Coast described that banana mosaic symptoms consisted of appearance of small pale yellow broken streaks, spot or lines ('en tirets' - like a dotted line), sometimes anastomosing pale green strands or patterns, the affected leaf blade turned undulating, deformed and sometimes reduced in size with little more than the central mid-rib; Mosaic appearance was also observed on the fruit which got reduced in size and also variously deformed, flattened with bloated or turgid areas and lines or strands of mosaic discoloration.

This disease also produced heart rot (decay of the central portion of the pseudostem) during winter months and with severe chlorosis or mosaic during summer (Capoor and Varma, 1968; Capoor and Varma, 1970; Mali and Rajegore, 1979; Jones, 1991; Jones and Lockhart, 1993).

Stover (1972) correlated chlorosis of leaves to the age of the plant and environmental conditions and reported that when infected suckers were raised, the plants showed interveinal chlorosis at an early stage and later the symptoms were not pronounced and disappeared periodically. Mali and Deshpande (1975), Mali and

Rajegore, (1979) and Patil (1982) observed typical symptoms such as stunted growth, chlorotic bands or streaks on older leaves, sheath rot, linear necrotic patches on the pseudostem base and resulted in plant death.

The symptoms like severe yellowing and mottling of the young foliage, followed by rotting of heart leaf and central portion of the pseudostem were associated with the disease which was named as heart rot disease. The heart rot stage of the disease apparently occurred only during the winter months but the yellowing and mottling persisted throughout the year (Mali and Rajegore, 1979 and Patil, 1982).

The symptoms depended on the strain of the virus pathogen and the temperature. The mild strains induced diffuse mosaic or line patterns and ring spot on the leaf lamina (Yot-Dauthy and Bove 1966; Lockhart, 1986; Niblett *et al.*, 1994). Severe strains of the virus produced more pronounced symptoms of necrosis of emerging cigar leaves. Internal tissues of the pseudostem turned necrotic with severe leaf distortion. Plants infected with severe strains of the virus might die, especially if infected soon after planting. Symptoms were known to fluctuate during the growing season depending on the temperature and rainfall. Some severe strains caused necrosis in pseudostem as heart-rot and even resulted in plant death (Bouhida and Lockhart, 1990). Occasionally the leaves were deformed and curled. These symptoms appeared sporadically and the majority of leaves were symptomless. Symptoms were generally more severe when temperature was below 24°C, which occurred during winter or in the subtropics and at high altitude in the tropics (Niblett *et al.*, 1994).

Lockhart and Jones (2000) also reported necrosis of cigar leaves due to infection of severe strains of CMV. Khan *et al.* (2011) reported the symptoms of infectious chlorosis of banana plants as typical yellow stripes on leaves, in addition to leaf distortion and stunting of the plants.

Lepcha *et al.* (2017) reported CMV infection in high altitude cultivar *Musa* × *paradisiaca* cv. Chini Champa (Family Musaceae) and observed that the plants were exhibiting yellow mosaic and interveinal chlorosis on leaves. They suspected CMV

infection based on symptoms for the first time in Rorathang village, Sikkim, North East India. The observed symptoms were yellow mosaic and interveinal chlorosis on leaves.

2.1.4 Varietal Susceptibility

Silberschmidt and Nabrege (1941) reported that the banana varieties Gross Michel, Prata, Oura S. Tome, Nanica, Nanicao, Java and Maranhao were infected by a mottling on the foliage in the form of alternate dark and light, unbroken or discontinues stripes. The leaves were with wavy appearance and in severe cases, the lamina was much reduced.

Musa, cultivars with AAA and AAB genome were the most commonly infected. Bluggoe, ABB was occasionally infected as well as the diploids (AA, AB). In Central America, cucumber mosaic incidence had been reported in the Cavendish varieties than in Gross Michel. *Musa balbisiana* was the only *Musa* Species on which symptoms of the virus infection was not observed in the field. However, *M balbisiana* seedlings from Central America were showing symptoms of whitish chlorotic mottling characteristic of virus infection even though virus symptoms were not seen on mature plants (Stover, 1972). In India *Ela vasahi Athiya kel, Kare Bale* cultivars and *M. balbisiana, M. chilicarpa, M. coccinia* and *M. accuminata* were reported to be resistant. The Basrai ad Lal Velchi banana varieties were highly susceptible (Singh *et al.,* 1995).

Capoor and Varma (1970) reported that the varieties susceptible to mosaic were Chakkarakaeli: Thella chakkarakeli, Rasthali, Raja Keli; Vamanakeli, Basarai, Harichall, Mauritius, Cavendish, Pedda Pacha Aratti, Nendran: Desi, Nendran, Singapuri; Chenkadali, Betta Bale, Lal Velchi, Lal Kel, Vella Vazhai; Rasthali, Amrithapani, Son Kel; Ney Poovan, Kadali, Safed Velchi or lady's Finger; Poovan-Yarchi bale, Metheli or Motta Poovan; Nendran Padath Thi; Vannan; Peyladdan-Peyan; Ney Mannan, Chia Monthan; Monthan-Maduraga Bale, Nir bale or Nir Bontha; Miscellanous, Alshi, Amroli, Belhe, Bhur kel, Bargi Bale, Hazari, Harnai Khasadi, Kagdi Velchi, Kangal, Lokhandi, Mumbai Bale, Mysore Mitka, Mittli, Shendurni, Surat, and Valha Sakas.

The local cultivars such as cooking bananas, Saba and Cardoba, and the fancy varieties, 'Morado' were highly susceptible to banana mosaic (San Juan, 1985).

Cucumber mosaic virus was found in all the districts of Tamil Nadu on commercial plantations of Robusta and Mysore Poovan varieties of banana. The disease was transmitted only through suckers from the diseased gardens. Among the genotypes screened, the clones with ABB genomes showed complete field resistance (Mohan and Lakshimanan, 1988).

2.1.5 Histological Changes

Allam *et al.* 1995 observed isometric particles in the cytoplasm of infected banana cells with disorganized chloroplast, degenerated mitochondria and inclusion bodies. The ultra-structural histological changes in banana plant infected with CMV were studied by El-Deeb *et al.* (1997) and reported several changes in the histological arrangements due to viral infection. The changes were reported in the mesophyll cells of young leaves where cytoplasm contained only few vacuoles and many vesicles. The plasmalemma was separated from the cell wall.

2.1.6 Impact of the Disease on Yield

Considerable yield loss was reported in banana plantations in Australia due to CMV which was transmitted by the aphid, *Pentalonia nigronervosa,* (Wardlaw, 1938).

Magee (1940) reported infectious chlorosis of banana for the first time in New South Wales in 1930, ever since several sporadic outbreaks had occurred which led to complete loss of yield and the plants.

In a severely affected plantation of Giant Cavendish banana in Honduras, about 150 acres had to be totally eradicated leading to total yield loss (Wardlaw, 1961). Cucumber mosaic virus was prevalent on bananas in Ceylon and caused considerable loss in yield even when symptoms were mild. (Ariyaranthe and Liyanage, 2002).

Joshi and Joshi (1976) reported about 5 per cent incidence of mosaic in about 15000 hectares of banana in Gujarat state and the yield loss due the disease was more than 10000 tons, which amounted to more than rupees 3 million every year and also reported that the disease associated with heart rot symptoms were widely distributed and caused reduction in yield by 10 per cent.

Lassoudière (1979) reported 3.5 per cent loss in the weight of stem when incidence was 10 per cent. Effect on yield was apparent in ratoon crop. The disease caused serious loss in a farm of about 648 acres in Honduras, where more than 2000 plants were destroyed during the first year of planting without any yield (Stover, 1972).

CMV infection caused serious yield loss in bananas and plantains in Puerto Rico and other tropical locations (Bird and Wellman, 1962; Hema and Sreenivasulu 2002).

Estelitta *et al.* (1996) reported that in CMV infected plants bunch weight reduction of 54, 45 to 62 per cent was observed in commercial banana varieties like Nendran, Palayankodan and Karpooravally respectively. Reduction was also observed in the number of hands and fingers due to infection.

Considerable yield loss due to this disease was reported in plantations of Cavendish and Mysore varieties by Selvarajan and Balasubramanian (2008) and Silva and Silva (2009) and reported that combined infection of CMV with other banana viruses led to heavy crop loss.

2.2 TRANSMISSION

2.2.1 Transmission through Planting Material

Stover (1972) reported that when infected suckers were raised, the plants showed interveinal chlorosis at an early age and the symptoms were not pronounced and tended to disappear periodically.

Shehata and El-Boroiiosy (2007) confirmed the infection of banana suckers of cultivar Maghrabi with CMV via Indirect ELISA using specific polyclonal antiserum.

Mohan and Lakshimanan (1988) reported CMV was found in all the districts of Tamil Nadu on Robusta (Dwarf Cavendish) and Poovan (Mysore) banana. The disease was transmitted only through suckers from the diseased gardens.

2.2.2 Mechanical Transmission

Benigno (1963) reported that when leaves of *Musa textilis* were crushed, the virus got in-activated by some components of the leaf sap like tannic acid. He also reported that in such cases, attempts to transmit the virus mechanically were unsuccessful. Yot-Daughty and Bove (1966) showed that the tannin rich sap from crushed banana leaves inactivated the virus and reported that the virus particles, being protein, were immediately precipitated by tannins released during leaf crushing. However, by using appropriate biochemical techniques they succeeded in extracting active virus particles from infected banana leaves. Capoor and Varma (1968) reported that the transmission of banana mosaic virus was not possible from banana to banana by the conventional leaf rubbing method with extracted sap but it could be done on seedlings of cucumber, when the sap of banana leaves was extracted in 0.1 M Na_2S0_3 solution and diluted in neutral 0.15 M phosphate buffer. Attempts to transmit the virus by sap inoculation from cucumber leaves or flower petals to banana were also proved futile. Mali and Rajegore (1980) were not able to transmit banana mosaic virus mechanically by the conventional leaf rub method from banana to banana, cucumber, tobacco (*Nicotiana glutinosa*) and other test hosts when 0.1 M sodium sulphate buffer and 0.15 M phosphate buffer (pH 7.0) were used but succeeded in doing so when 0.01 M phosphate buffer (pH 9.1) containing 3 per cent nicotine solution and 0.01 M cystaine were used.

Sulistyowatti *et al.* (2004) reported successful transmission of CMV through mechanical inoculation where tobacco leaves developed systemic symptoms. Plants like *Lycopersicon esculentum, Capsicum annuum* and *Nicotiana* spp. were dusted with carborundum powder, rub inoculated with the sap and rinsed with tap water. Symptoms were recorded 3 weeks after inoculation and also young leaves were collected from the

test plants to confirm transmission of the virus through ELISA. Differentiating symptoms such as severe mosaic and leaf distortion, stunted growth and mild mosaic were observed on *N. glutinosa, N. benthamiana* and *N. tabacum* respectively.

2.2.3 Insect Transmission

Magee (1930), Calinisan (1938) and Silberschmidt and Nobrega (1941) reported that mosaic disease virus could be transmitted from infected to healthy plants by the banana aphid, *Pentalonia negronervosa*. Magee (1940) reported transmission of the virus by the insects *Macrosiphum gei, Aphis gossypii* and with another unidentified aphid. The virus was transmitted from the Cavendish banana to *Musa ensete* and to an unnamed seeded *Musa* spp. From this unnamed species it was transmitted by *Aphis gossypii* to Cavendish and Gros Michel banana, abaca, canna, cucumber, squash and tomato.

Cucumber mosaic virus has wide host range, could be readily transmitted by insect vectors in a non-persistent manner (Bird and Wellman, 1962). Kaper *et al.* (1981) also reported that CMV could be easily transmitted by sap inoculation and by aphids in a non- persistent manner. In some virus infections, the whole leaf might become chlorotic due to decreased chlorophyll production and breakdown of chloroplasts. Banana mosaic disease was transmitted successfully by *A. gossypii* and poorly by *A. craccivora* but not at all by *P. nigronervosa* in Puerto Rico (Meiners *et al.*, 1977). Rao (1980) and Palukaitis and Garcia-Arenal, (2003) reported successful transmission of CMV by *A. gossypii*. Shukla and Govinda (2000) conducted detailed studies to establish virus-vector relationship of aphid with CMV and reported that a short pre-acquisition fasting of 2 h and acquisition threshold 2 min given to *A. pisum* increased the transmission efficiency of the aphid and that a single aphid could produce infection, and the percentage transmission increased with increase in the number of aphids. CMV is reported to be transmitted in non-persistent manner by several aphid species (Mali and Rajgore, 1980). Barbosa *et al.* (1998) reported transmission of CMV by *Myzus* spp. and developed mosaic symptoms on *N. glutinosa.*

Dheepa and Paranjothi (2010) reported successful transmission of the virus by *Aphis craccivora* and *A. gossypii* and both the species were efficient vectors transmitting the virus in non persistent manner within 20 min of acquisition and inoculation of 10-15 min. They also inoculated the virus on other host plants like tobacco, cowpea and commelina which showed symptoms after three months of inoculation.

Khaled *et al.* (2015) conducted vector transmission studies using two aphid species *A. craccivora* and *A. gossypii*, both species could efficiently transmit the virus in a nonpersistent manner within 20 min of acquisition access period and inoculation period of 10 to 15 min. The inoculated plants showed the symptoms of CMV after 13-22 days of inoculation.

2.3 PHYSICAL PROPERTIES OF VIRUS

Historically, stability of the virus as measured by the infectivity of crude extracts which was an important criterion to establish identity of viruses (Hull, 2002). The physical properties like Thermal inactivation point (TIP), Longevity *in vitro* (LIV) and Dilution end point (DEP) are important parameters to characterize a virus. Further, the techniques like virion density, sedimentation coefficient, diffusion coefficient, ultraviolet absorption spectrum, electrophoretic mobility of protein and nucleic acid are very accurate but not suitable for large scale detection of viruses.

Van-Hoof (1962) reported that CMV of banana was found to be active in the cucumber sap at dilutions of 10^{-2} and was inactivated at 55°C. The sap from the diseased plants was infectious up to 24 hours.

Mali and Rajegore (1980) reported that the virus isolated from infected banana leaves was inactivated at temperature ranging between 65-70°C and at dilution of 10^{-4}. The virus was viable up to 92 h at room temperature. Colariccio *et al.* (1996) reported DEP of 10^{-5}, TIP between 70-75°C and LIV at room temperature for 24 h for CMV. Parvin *et al.* (2007) determined the DIP, TIP and LIP of CMV as 10^{-6}, 65°C and 10 days respectively for CMV.

Chandankar *et al.* (2013) studied the physical properties of CMV and reported TIP at 60°C, DEP between 10^{-4} to 10^{-5} and LIV for two days at 28-30°C and 7 days at 6-8°C temperature.

2.4 HOST RANGE

Cucumber mosaic virus belongs to the type species of the genus Cucumovirus, family Bromoviridae, and is an economically important virus infecting about 1200 host species belonging to over 100 families including dicotyledons and monocotyledons across the world. Numerous strains of CMV have been characterized, suggesting that CMV is very successful in rapidly adapting to new hosts and new environment (Roossinck, 2002). Roossinck *et al.* (1999) and Hord *et al.* (2001) reported that CMV infects about 1287 plant species including cucurbits, solanaceous crops, cereals, fruits, vegetables, ornamentals and several weeds. These alternate hosts could serve as sources of CMV and contribute to spread of the virus.

Muharam (1987) mechanically inoculated CMV to tomato plants and after seven days of inoculation, severe mosaic, fern leaf symptoms, reduced plant growth were recorded. *Momordica charantia* developed systemic mosaic symptom within a week after mechanical inoculation of CMV.

Kiranmai *et al.* (1997) reported that *M. charantia* developed systemic mosaic symptoms within 1-3 weeks after mechanical inoculation of CMV. Subsequently, leaves showing systemic symptoms were back inoculated to *Vigna unguiculata* and *Phaseolus mungo* and tested by DAC-ELISA to confirm virus infection. They also reported that tomato plants produced systemic mosaic symptom 10 days after sap inoculation of CMV followed by leaf narrowing, fern leaf symptoms and reduction in plant size at 20 days.

Bhat *et al.* (2004) reported that some of the common weed hosts found in and around black pepper gardens such as *Ageratum conyzoides*, *Colacasia esculanta* L. Schott, *Synedrella nodiflora* L. Gaertn., *Cynodon dactylon* L. Pers and *Sonchus oleraceus* L. were tested positive for CMV infection in DAS ELISA.

Verma *et al.* (2004) mechanically transmitted CMV from naturally infected *C. annum* to different indicator plants and local lesions were produced in *Chenopodium amaranticolor* and *Vigna unguiculata* and mosaic mottle and deformity in *N. glutinosa*, *N. tabacum* cv. White Burley, *Physalis floridana*, *L. esculentum* and *Cucumis sativus* were the hosts of CMV producing different types of symptoms.

Jagadeeshwar *et al.* (2005) conducted host range studies and reported that virus could infect large number of plant species by producing systemic and localized symptoms like chlorotic ring spot on *Tridax procumbans,* chlorotic spots and mosaic on *Cucumis sativus,* mosaic on *M. charantia cv. Pusa Do Mousami,* necrotic ring spot on *V. unguiculata,* mosaic mottle on *N. tabacum* var. *Xanthi* and mosaic mottle and rat tailing on *N. glutinosa.*

Verma *et al.* (2006) reported that CMV is one of the most important widespread viruses in the world infecting the largest number of plant species.

Parvin *et al.* (2007) conducted host range studies by mechanically inoculating CMV in different plant species and could transmit CMV to *Cosmos sulphureus.* They also reported that the plant species belonging to six families were hosts of CMV producing various symptoms. The plants of Cucurbitaceae family *viz., Benincasa hispida, Trichosanthes anguina, C. sativus* and *Lagenaria siceraria* produced systemic mosaic symptoms.

Sap inoculation with CMV on *N. tabacum cv.* White burley, *N. rustica* and *N. glutinosa,* produced necrotic local lesions along with systemic mosaic and reacted positively in ELISA (Raj *et al.,* 2007).

Kelaniyangoda and Madhubashini (2008) reported that sap inoculated plants expressed symptoms three weeks after inoculation and the symptoms like leaf distortion and mottling were produced on *Capsicum annum*, *Solanum melongena* and *Datura stramonium* and local lesions were reported on *Gomphrena globosa* and *Chenopodium quinoa.*

Dheepa and Paranjothi (2010) conducted transmission studies on *Commelina* spp., *N. glutinosa*, *V. radiata*, *V. mungo* and *Chenopodium* spp. in addition to banana by mechanical inoculation with CMV and reported that *Nicotiana* spp. expressed symptoms three months after inoculation causing severe mosaic and leaf deformation on inoculated plants.

Shahid *et al.* (2012) reported that different plants act as hosts of CMV which produced different symptoms on mechanical inoculation. The specific symptoms were local lesions on *Amaranthus gracilis, A. retroflexus* and *Cucumis sativus*; vein clearing, mild mosaic, mottling, and mosaic symptoms on *S. melongena*; chlorotic lesion and mosaic on *Capsicum annum*; local lesion, mild mosaic, leaf curling, and mosaic on *N. glutinosa* and vein clearing, local lesion, and mosaic on *N. tabacum.*

Saidi and Safaeizadeh (2012) reported *Canna indica* as the host of CMV which produced mosaic symptoms after 40 days of mechanical inoculation, Rajakaruna *et al.* (2014) also reported *C. indica* as the host of CMV and produced different symptoms like yellow mosaic, striations, severe discoloration and necrosis along the veins of leaves.

Jalendar (2014) conducted host range studies and reported 29 plant and 18 weed species belonging to 13 families to be the hosts of CMV and confirmed by back inoculation on local lesion host.

Ashwini *et al.* (2016) reported that symptoms like vein clearing, puckering, deformation and reduction in leaf size were developed on *M. charantia* after 10 days of inoculation with CMV. Lepcha *et al.* (2017) performed serological assay in order to confirm the hosts of CMV and reported *Chenopodium album, N. benthamiana* and *S. lycopersicum* as host plants of CMV.

2.5 DETECTION OF VIRUS INFECTION

2.5.1 Electron Microscopy

Samad *et al.* (2008); Ali *et al.* (2012); Vishnoi *et al.* (2013); Khan *et al.* (2011) reported the morphological characters of virus particles under electron microscope

(EM). The virus particles were ~28 nm isometric under electron microscopy using leaf dip preparations. Vishnoi *et al.* (2013) also confirmed the presence of CMV particles at initial stages through electron microscopic observation. Khaled *et al.* (2015) conducted transmission electron microscopy of infected banana leaves and revealed the presence of virus particles of 28-29 nm diameter.

2.5.2 Immunodetection of the Virus

Hu *et al.* (1995) diagnosed the infected samples from Hawaii, USA with DOT-Blot assay and reported that the assay was 100 times more sensitive than ELISA and detected higher concentration of CMV in younger leaves than in older ones. Kiranmai *et al.* (1996) compared the sensitivity of different serodiagnostic tests like double antibody sandwich, simplified rapid direct antigen coating and DAC-ELISA for the detection of CMV causing infectious chlorosis of banana and reported that the sensitivity levels of three ELISA tests were comparable.

Eiras *et al.* (2001) standardized ELISA for the detection of CMV from the samples of banana in Sao Paulo State, Brazil which were further used for partial characterization of CMV CP gene.

Selvarajan *et al.* (2011) standardized DAC-ELISA for the large scale virus indexing of CMV in tissue culture plants and mother plants used for mass propagation. Kouadio *et al.* (2014) identified CMV as the causal agent of mosaic disease banana based on symptoms in most of the banana growing areas of Ivory Coast with an incidence ranging between 5 and 25 per cent and reported that 93.3 per cent of leaf samples reacted positively to polyclonal antibody of CMV during ELISA.

Khan *et al.* (2012) used the antisera produced from the coat protein soluble recombinant protein CP(r-CP) to perform ELISA instead of commercial antibody which performed better. Khan (2015) surveyed 21 banana orchards in Karnataka, Maharastra, and Uttar Pradesh for CMV infected banana and the samples were subjected using DAS-ELISA and reported that 13 samples were positive out of 300 samples tested.

Lepcha *et al.* (2017) performed serological assay in order to confirm the infection of CMV in host plants such as *Chenopodium album, N. benthamiana* and *Solanum lycopersicum.*

Different serological assays other than ELISA have been used for the identification and diagnosis of CMV worldwide. These include tube and ring precipitin tests (Scott, 1968; Mink *et al.*, 1975; Tomlinson, 1987), agar gel immunodiffusion (Scott, 1968; Rao, 1980; Tomlinson, 1987), sodium dodecyl sulphate immune diffusion (SDS-ID) (Purcifull and Batchelor, 1977). Quantitative rocket immunoelectrophoresis (Havranek, 1978), immunoelectron microscopy (Francki *et al.,* 1979), SDS immunodiffusion (Purcifull and Edwardson, 1981), ELISA (Rao, 1980; Devergne *et al.*, 1981) and western immunoblot (Hsu *et al.*, 1989). Fluorescent antibodies were also used to detect CMV (Otsuki and Takebe, 1973; Koike *et al.*, 1977).

Later more sensitive double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed. This technique, which is still widely used, makes it possible to follow epidemics not only of mosaic-inducing viruses, but also of yellows-inducing viruses such as poleroviruses, that are not detectable by SDS-ID. Madhubala *et al.* (2005) standardized DAS ELISA for the detection of CMV. Various concentrations of coating antibody (IgG) and enzyme conjugate were tried and, IgG at 1 mg/ml of coating buffer and CMV IgG alkaline phosphatase conjugate at 1:2000 dilutions successfully detected the presence of CMV in the infected samples. DAS ELISA method thus standardized was able to detect CMV infection in the vanilla plant samples. Out of 66 plants representing different locations tested, 19 showed positive reaction to CMV antiserum.

Aglave *et al.* (2007) standardized Double Antibody Sandwich ELISA (DAS-ELISA) and Direct Antigen Coating ELISA (DAC-ELISA) for the detection of CMV in Ardhapuri variety of banana.

2.5.3 Molecular Detection and Characterization

Cucumber mosaic virus (CMV) is a tripartite polyhedral virus with a diameter of 29 nm. CMV particles are isometric and composed of a coat protein shell which encapsidates the single-stranded, plus-sense RNA genome. The capsid contains 180 identical protein subunits with icosahedral symmetry. The virions contain 18 per cent RNA and 82 per cent protein. The RNA consists of three genomic RNAs and one or two subgenomic RNAs. The genomic RNAs designated as RNA1 (3.3 kb in length), RNA2 (3.0 kb) and RNA3 (2.2 kb) and are packaged in individual particles. The two subgenomic RNAs are RNA4 (1.0 kb) and possibly RNA4A (682 nucleotides) and are packaged with genomic RNA3. Many strains of CMV have been described and classified into sub-groups IA, IB and II according to the sequence similarity of the genomic RNAs (Palukaitis *et al.*, 1992).

The three genomic RNAs of CMV encode five proteins. RNA1 is monocistronic and codes for a single product of 110 kDa, the 1a protein is required for viral replication and contains methyltransferase and RNA helicase activities (Kadare and Haenni 1997; Rozanov *et al.*, 1992). RNA2 encodes the 2a protein (98 kDa) that contains the conserved amino acid sequence of many viral polymerases (Ishihama and Barbier, 1994; O'Reilly and Kao, 1998). RNA3 encodes the movement protein 3a (MP) a coat protein (CP) expressed from subgenomic RNA4. Both are required for virus movement (Canto *et al.*, 1997). RNA2 also encodes 2b, which is translated by subgenomic RNA4A that inhibits host transcriptional gene silencing (Brigneti *et al.*, 1998). CMV harbors molecular parasites known as satellites (sat RNAs) that modify the symptoms induced by the virus. The CMV sat RNAs do not encode any proteins but rely on the RNA for their biological activity (Roossinck, 2002).

CMV replication starts with the entry of the viral particles into the plant cell via aphid feeding on the host plant (Palukaitis *et al.*, 1992). After the entry into the host cell, the virus particle gets disassembled and the virion RNA gets uncoated. Then host ribosome gets translated to RNA1, RNA2 and RNA3. The translation products are

involved in viral RNA replication. The viral replicase generates (-) sense RNA strands from the (+) strand viral templates of each CMV RNAs. These (-) sense RNA strands synthesize progeny virus RNAs and RNA4 with viral replicase. Both host and viral encoded proteins have functions during this process. Translation of RNA4 produces the coat protein. The positive sense RNAs are encapsidated by the coat protein subunits. Therefore, virions are produced. These virus particles either move into a new cell with plasmodesmata or move to new host via aphid vectors (Palukaitis *et al.*, 1992).

Reverse transcription coupled with the polymerase chain reaction (RT-PCR) is a powerful and popular method for the practical diagnosis of plant RNA viruses and viroids including the members of the genus Cucumovirus (Rizos *et al.*, 1992). Nucleic acid probes and RT-PCR have been used for the detection and differentiation of CMV isolates along with biological characterization, serological relationship and electron microscopy in different crops like banana (Singh *et al.*, 1995; Kiranmai *et al.*, 1996), geranium (Verma *et al.*, 2004), carnation (Raj *et al.*, 1993), chrysanthemum (Srivastava *et al.*, 1995), gladiolus, pepper and vanilla (Madhubala *et al.*, 2005).

Hu *et al.* (1995) developed RT-PCR assay and cloned the product of coat protein for the detection of CMV subgroups from banana samples from Hawaii, USA and reported RT-PCR as a more sensitive assay when compared to dot blot or ELISA and compared nucleotide and amino acid sequence and reported that those CMV isolates belonged to CMV subgroup I. They also reported that one isolate (CMV-Hawaii), which induced mild mosaic symptom, shared 99 per cent sequence homology with CMV-C strain which induced severe mosaic and leaf distortion symptoms.

Singh *et al.* (1995) standardized the technique of hybridization with a nucleic acid probe derived by RT-PCR amplification using designed CMV specific degenerate oligonucleotide primers. The RT-PCR amplified product of CP gene of 486-488 bp was sequenced and analyzed by comparing with the sequences available in the database and revealed 76 per cent homology to sequences of CMV subgroup II isolates.

Rizos *et al.* (1992) developed a procedure based on polymerase chain reaction to classify CMV isolates into two different subgroups. Two specific primers that flank the CMV capsid protein gene were used to amplify the DNA fragment of approximately 870 bp. Restriction enzyme analysis of this fragment produced distinct restriction patterns that assigned the CMV isolate into one of the two subgroups. These two restriction groups correlated with the previously established CMV subgroupings; hence this PCR-based method might provide a simple alternative to the serological assays used for grouping CMV isolates.

Isolate of CMV infecting banana fall into two subgroups, designated DTL and T_oRS (Desvignes and Cardin, 1973; Piazzolla *et al.*, 1979). DTL serotypes were designated as WT (Piazzolla *et al.*, 1979) or subgroup I (Owen *et al.*, 1990) and T_oRS serotypes are designated S (Piazzolla *et al.*, 1979) or subgroup II (Owen *et al.*, 1990). Hu *et al.* (1995); Singh *et al.* (1995); Gafny *et al.* (1996) identified isolates of CMV from banana belonging to subgroup I which is the subgroup that includes most CMV isolates from tropic (Niblett *et al.*, 1994).

Nishiguchi (1995) reported that RT-PCR technique used for detection of specific virus strains in some plant species has greater sensitivity than ELISA. RT-PCR Restriction Fragment Length Polymorphism (RT-PCR RFLP) method was also proved to be useful in differentiating CMV strains in sub-groups IA, IB and II rather than in sub-groups I and II (Finetti-Sialer *et al.*, 1999). Thus it would provide a simple way to investigate the dynamics of CMV population in nature.

RT-PCR technique was utilized for detection of various viruses including CMV in many plant species (Fisher and Nameth, 2000). PCR amplification of the CP gene using gene specific primers resulted in the amplification of ~657 bp fragment.

Eiras *et al.* (2001) detected isolates of CMV obtained from banana in Sao Paulo State, Brazil, RT-PCR was performed with primers designed to anneal the conserved regions of the coat protein gene 3′ end, a 486-bp DNA fragment was amplified. The

sequences obtained from these CMV isolates of banana from Sao Paulo State, Brazil, were confirmed by *in silico* analysis that it showed 96-98 per cent homology with subgroup I.

RT-PCR, with specific primers for the 3′ end of RNA 3 and part of the coat protein gene resulted in 486-499 bp of DNA fragments. These results were confirmed by the sequences of the RT-PCR products, which were 92-99 per cent identical to those of subgroup I CMV isolates (Eiras *et al.*, 2004). Multiple sequence alignment of the nucleotides and translated amino acid of the CMV isolate and other strains available in the database generated three distinct cluster in phylogenetic analysis and revealed that most of the Brazilian CMV isolates were closely related among themselves and clustered with other CMV subgroups of IA isolates, one CMV isolate clustered together with CMV subgroup IB isolate. The above results indicate the prevalence of CMV subgroup I in Brazil (Eiras *et al.*, 2004).

Cherian *et al.* (2004) standardized RT-PCR protocol for the detection of CMV from infected banana leaf samples and cloned the coat protein gene in p-GEMT vector. The sequencing of the gene revealed an ORF of 657 nuclotides for 218 amino acids, starting with methionine and ending with threonine. Phylogenetic analysis revealed that it was closely related to an isolate from Israel.

Yu *et al.* (2005) performed RT-PCR, with a pair of designed generated primers to amplify coat protein-based detection of CMV infecting banana from China. Further analysis of the sequences of the genome fragments revealed that highest identities and the close relationships with Indian strains of CMV group IB.

Shehata and El-Boroiiosy (2007) standardized immunocapture RT-PCR to detect the infection of banana suckers of cultivar Maghrabi with CMV. The amplification, sequencing and phylogenetic analysis of CP gene revealed that it was related to CMV subgroup I.

Virus detection methods have improved greatly in recent years with the development of more versatile and accurate serological and molecular diagnostic techniques such as real-time PCR which could be applied directly in the field or microarrays which offered the possibility of testing concurrently a diversity of viruses or virus strains (Vincelli and Tisserat, 2008).

Kouassi (2010) developed a quantitative real-time PCR (qRT-PCR) for screening banana planting material for CMV infection in Cote d'Ivoire. A RT-LAMP assay was also established for CMV detection in banana (Peng *et al.*, 2012).

Khan *et al.* (2011) conducted RT-PCR to detect the coat protein of CMV infecting banana and reported amplication of 657 bp nucleotide fragments, coding for 219 amino acids. Further analysis of sequence of CP gene of CMV isolates showed 93-98 per cent (at nucleotide) and 94-99 per cent (at amino acid) sequence identity between the isolates. Phylogenetic analysis of these sequences with sequences available in the database revealed that the isolates belonged to subgroup IB and this isolate also infected *Physalis minima* an alternate weed host.

Selvarajan *et al.* (2011) developed and validated routine test for the detection of CMV and other banana viruses through reverse transcriptase PCR for virus-indexing and could help in early detection of virus infection in tissue culture and mother plants used for mass propagation.

Khan *et al.* (2012) demonstrated Immunocapture-RT-PCR to be more sensitive than ELISA for CMV detection.

Khaled *et al.* (2015) reported that the analysis of 120 deduced amino acid sequence of the coat protein gene shared 97.50 to 98.33 per cent similarity with those strains belonging to subgroup IA. They also reported subgroup IA as banana infecting CMV strain in Egypt. Khan (2015) mentioned that RT-PCR would be more sensitive and reliable and convenient molecular tool for detection of CMV from the infected

samples and amplified through RT-PCR and concluded its sensitivity in detecting virus was 0.02 ng.

Lepcha *et al.* (2017) isolated total RNAs using RNeasy Plant Mini Kit (Qiagen, Germany) and converted to cDNA using cDNA synthesis kit. RT-PCR was performed with One-Step RT-PCR Kit (Qiagen) to detect CMV from infected samples collected from Sikkim, Northeast India and confirmed the causal agent as CMV, amplifying the fragment of 657 bp molecular weight. The nucleotide sequences obtained were compared with the available sequences in GeneBank by BLAST and reported highest homology of 98 per cent with those available in GeneBank. A Phylogentic tree was constructed using neighbor-joining algorithm in MEGA 6 Software with 1000 bootstrap replicates to confirm isolate DP-1 as CMV belonging to subgroup IB.

2.5.4 Biosensor Based Detection

Biosensor is a bio analytic device which is associated with a physical transducer having a molecular recognition entity. Recently nanomaterials were used as diagnostic platforms called nanobiosensor, which could detect and measure the bioanalyte with high sensitivity. In such type of biosensors, nanoparticles were used as a transducer (Tothill, 2009). The main function of biosensor is to detect the bio-analyte like antibodies, proteins, enzymes, immunological molecules and called as bioreceptors. Transducer converts the interaction of bioreceptor and bio analyte into electrical signal. The amplification of signals coming from the transducer was done by detector, so that the corresponding response could be read and studied (Haun *et al.*, 2010).

Huang *et al.* (2011) developed biosensors based on graphene molecule for the detection of bacteria, where significant changes were observed in the electrical conductance of graphene based device with *Escherichia coli* at a concentration of 10 cfu/ml.

Zeng *et al.* (2013) reported biosensor based on the Surface Plasmon Resonance for the detection of *Maize chlorotic mottle virus* (MCMV). They modified gold

nanoparticles with 11-mercaptoundecanoic acid to form self-assemble monolayer and a layer of an anti-MCMV antibody and reported that the sensor was capable of detecting dynamic range of 1 to 1000 ppm antigen.

Izadi *et al.* 2016 developed electrochemical DNA-based biosensor for the detection of *Bacillus cereus*. Gold nanoparticles were modified with Polyethylene glycol (PEG) as self-assembled with single stranded DNA of *rhea* gene which could covalently bind to the gold nanoparticles by a thiol group.

2.5.4.1 Longitudinal Surface Plasmon Resonance Based Nanobiosensor (LPSR)

LSPR is an optical phenomena generated by light which interact with conductive nanoparticles (NPs) with wavelength smaller than that of incident of light. As in surface plasmon resonance, the electric field of incident light could be deposited to collectively excite electrons of a conduction band, resulted in coherent localized plasmon oscillations with a resonant frequency that strongly depended on the composition, size, geometry, dielectric environment and separation distance of NPs. Due to characteristic LSPR band, it showed different optical property according to the size of the particle (Lu *et al.*, 2009).

Sharma *et al.* (2009) and Huang *et al.* (2009) observed that metallic nanoparticles of different shape and size with different absorption property were seen in the LSPR plasmon band of a spherical nanoparticle, where one absorption band was visible in UV-Vis spectra. In the case of Gold Nanorods (GNRs) two absorption bands were present in absorbance spectrum *viz.,* longitudinal plasmon band (LPB) and transverse plasmon band (TPB). The transverse plasmon bands of GNRs were found to be not sensitive to changes in the size of the gold nanorod, whereas the longitudinal plasmon band was very sensitive. Lin *et al.* (2011) observed increase in the aspect ratio of GNRs red shift in the longitudinal plasmon band.

Hall *et al.* (2011) reported antibody-labeled gold nanoparticles increased LSPR peak shift, when it was in contact with the analyte as compared to the native antibody concentration.

Petryayeva and Krull (2011) and Le *et al.* (2012) reported that novel metals like Ag and Au were used for the production of the nanomaterial due to their LSPR phenomena in visible range. Silver displayed strongest and sharpest LSPR phenomena among all the metals. Gold particles were more preferable due to its inhabitant nature, biocompatibility and better gold thiol association for binding of biomolecules.

2.5.4.2 Gold Nanorod Based LSPR Biosensor

LSPR based biosensor is based on the interaction between light and metallic nanoparticle. The sensor configuration for both Gold nano spheres (GNS) and GNR are almost similar. Gold particles could be immobilized on the transparent substrate like glass substrate or just simply leaving the functional nanoparticle in suspended stage in a solution. In general, gold nanorod based LSPR biosensor could be categorized into three types chip-based, optical fiber-based and solution-phase-based LSPR biosensors.

Huang *et al.* (2009) demonstrated the use of various gold nanorods with different aspect ratios to fabricate the optical sensor. They choose five kinds of gold nanorods with different aspect ratios to construct five throughputs of Mean LSPR. The results showed various LSPR peaks implying different acceptor-ligand pairs which could be detected simultaneously in the wavelength ranging from 530 to 940 nm.

Guo and Chim (2012) reported sensitive approach for the detection of a protein, based on the LSPR peak shift induced by aptamer-antigen-antibody complex. The applicability was demonstrated for the detection of alfathromb protein. They observed receptor of alfathromb protein immobilizing on the gold nanorods which caused a measureable shift with the antigen.

De *et al.* (2013) used surface of the TGNPs for further modification and observed subsequent bioconjugation of TGNP films with goat anti-mouse immunoglobulins (anti-m-IgG) employed for the detection of mouse-immunoglobulin (m-IgG), based on the specific binding affinity between the antigen and antibody. The spectrophotometer sensor showed concentration-dependent binding for m-IgG.

2.5.4.3 Solution-Phase-Based LPSR Sensor

Gold nanorods were used for the development of LSPR based sensor. Two types of assays were used for the detection, one was based on the reflective index and second one was based on the aggregation of the GNRs.

In solution based LSPR biosensor, gold particles were suspended in solution rather than immobilized on the substrate (Potara *et al.*, 2011). Detection was carried out within the solution. The fabrication of such types of sensors often involved gentle mixing of the nanoparticles with functional molecule and by multiple centrifugation process which further purified the functional nanoparticles. The analyte detection was normally carried out in a small cuvate by UV-Vis spectrophotometer.

Wang *et al.* (2012) observed binding of an analyte to a nanoprobe, where local reflective index was changed when target bound to an immuno capture probe and nanobiosensor detecting wide range of analyte in Tris buffer ranging from 0.01 μ/mL to 1 μ/mL, which could not be detected in ELISA.

Wang and Tang (2013) detected human IgG through the aggregation of gold nanorods based on interaction driven by antigen-antibody. They capped surface of the CTAB with gold nanorods. Negatively charged GNRs were functionalized with the positively charged antibody through electrostatic reaction. The aggregations of functionalized GNRs were observed after the addition of human IgG, which resulted in the red-shift of the longitudinal peak and subsequently broadening the peak.

Lesniewski *et al.* (2014) reported colorometric based nanobiosensor for T7 bacteriophage based on the gold nanoparticle modified with T7 bacteriophages antibody. The T7 virion caused immunological complex which resulted in aggregation of a nanoparticles, which was visually identified by the changes in colour from red to purple with UV-Vis spectrophotometer.

Moghadam and Bijan (2015) reported visual detection of lysozome through heat induced aggregation of gold nanorods. They introduced short time exposure of heat

which greatly improved exposure of nanoprobes to a nanomolar level of lysozyme which led to the direct aggregation of nanostructure at optimum temperature.

2.5.4.4 Kinetics of GNRS along with Analyte

Soman and Giorgio (2009) studied the kinetics of quantum dot-antibody conjugation with different concentration of analyte at very low concentration for finding the aggregation pattern nano assembly. They found that aggregation percent of nanoparticles increased and after a particular interval, the kinetics curves remained flat.

Wang *et al.* (2013) demonstrated the kinetics of gold nanoparticles for the detection of copper ions and reported that the kinetic curve of Cu^{2+} ion of the sample increased over the period of time, meanwhile curve remained flat due to formation of new core assembly of nanostructure with gold nanoparticles and Cu^{2+} ion. In contrast, the kinetics curve of the blank sample remained flat over the period of time.

Song *et al.* (2013) conducted studies on the kinetic adsorption curves of rabbit anti-transferrin at different concentration of transferring which resulted in kinetics curve remained flat for some time and LSPR peak shift changed at different concentrations.

2.5.4.5 Synthesis of Gold Nanorods (GNRs)

The successful development of LSPR based biosensor depends upon the accurate synthesis GNRs. The most common method followed for gold nanorod preparation was by citrate reduction process, where aspect ratio could be maintained by adjusting the ratio between the citrate and the gold salt.

2.5.4.5a Seed-Mediated Growth Method

The seed mediated growth approach for gold nano rod preparation was first demonstrated by Jana *et al.* (2001a). They first prepared seed solution by reducing gold salt by Sodium borohydride ($NaBH_4$) in the presence of sodium citrate, which led to citrate capped Gold nano spheres. Seed solution was added into the growth solution containing Chloroauric acid ($HAuCl_4$), Cetyl trimethyl ammonium bromide (CTAB) as

the template, ascorbic acid (as reducing agent) and silver nitrate (for shape induction) for the growth of GNRs. Jana *et al.* (2001b) modified the method by introducing three steps protocol to grow the gold nanorod with larger size in the absence of silver ion.

Nikoobakht and El-Sayed (2003) created a significant modification; they initially replaced sodium citrate with CTAB and maintained the silver ion concentration in a growth solution to control the aspect ratio (AR) of the GNRs. They introduced additional surfactant benzyl dimethyl hexadecyl ammonium chloride into growth solution for synthesizing GNRs with highest aspect ratio. They could achieve GNRs of aspect ratio 10 by adjusting the concentration of silver ion in the growth solution.

Murphy *et al.* (2005) reported an improved method of GNR synthesis by using a seed mediated growth and aromatic additive and low concentration of CTAB. This approach produced GNRs with a broad longitudinal plasmon band (LPB) and lower levels of impurities. While preparing the growth solution, an appropriate amount of sodium salicylic acid gave the LPB less than 700 nm and addition of organic acid gave LPB more than 700 nm (Ye *et al.*, 2007).

Danielle and Brian (2008) conducted a study where they used CTAB from different companies for the synthesis of GNRs and concluded that the yield of nanorods depended on the purity of CTAB. GNRs were not formed in CTAB containing iodide impurities. The addition of silver nitrate in the synthesis of CTAB allowed stabilization and increased the yield significantly (Li *et al.*, 2008).

Christopher *et al.* (2014) reported that $HAuCl_4$ concentration during seed solution preparation significantly affected the morphology of the final rod morphology where higher concentration resulted in larger seed with smaller aspect ratio of nanorods. Wang *et al.* (2015) reported the facile green synthesis of gold nanorods by using epigallocatechin gallate (EGCG) extract from tea leaves as a reducing agent and stabilizing agent in aqua solution to produce gold nano structure through seed mediated approach.

Mahmoud *et al.* (2016) reported the stability of gold nanorods solution upon exposure to the human skin. They reported that the positively charged nanoparticles form aggregates when comes in contact with human skin as compared to the negatively charged gold nanorods. They concluded it was due to secretion of protein from the dermis layer of the skin.

2.5.4.5b Estimation of Aspect Ratio (AR) of Gold Nanorods

The aspect ratio (AR) is defined as the ratio of length over the diameter of the GNR. The shape of the particle caused the absorption and light scattering spectrum with two peaks: longitudinal and transverse. The interaction between the two dipoles produced the longitudinal peak observed in the visible to NIR (Near infrared region) region. The longitudinal peak was much more intense than the transverse peak and could be tuned by changing the aspect ratio of the gold nanorods (Jun *et al.*, 2006). As the AR of the gold nanorods increased, the longitudinal peak shifted to the NIR.

The optical properties of GNRs had been successfully modeled using Gans theory and Discrete Dipole Approximation (DDA) (Jain *et al.*, 2008). Two equations were derived using the two theories to output the longitudinal peak wavelength by inputting a given aspect ratio (AR). The two equations were predicted by the AR of GNRs using the absorption data collected from the UV-Vis spectrophotometer. The equations gave preliminary estimation of the AR before observing the particles on a Transmission electron microscope.

2.5.4.5c Functionalization of GNRs

Li and Rothberg (2004) reported the bi-layer of CTAB which covered the GNRs, acted as a surfactant for maintaining the stability and direct the growth of GNRs. Connor *et al.* (2005) reported that GNSs covered with citrate were stable in aqua solution and used for binding of DNA at neutral pH. GNRs capped with CTAB were stable only when it was suspended with CTAB at low pH value.

In various studies, it was found that excess CTAB on a solution were cytotoxic to human cells, but however those attached to the GNRs were non cytotoxic. Free CTAB molecules removed by centrifugation and those which were sticking to GNRs would lead to serious accumulation and sedimentation of GNRs which ultimately hamper the conjugation process. Huang *et al.* (2006) reported the complete removal of CTAB from GNR solution so as to enable the GNRs to increase the bioconjugation ability.

2.5.4.5d Surface Covering

Durr *et al.* (2004) coated the cover of the CTAB supplementary to make positively charged GNRs into negatively charged molecule with the help of anionic polyelectrolytes through electrostatic absorption such as poly (sodium-4-styrenesulfonate) (PSS), which could also enable antibody to attach to GNRs.

Gole and Murphy (2005) reported poly acrylic acid (PAA) as another polyelectrolyte agent which covered the CTAB capped GNRs and COOH group of PAA allowed protein and other amine terminate biomolecules to covalently binding to the GNRs via EDC/NHS (1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide, N-hydroxysuccinimide) linking chemistry.

Surface changes of GNRs could be achieved by the hard organic material such as silver and copper. Gorelikov (2008) reported the attachment of a silver ion on the surface of the GNRs by the reducing of silver chloride solution with hydroxylamine in presence of GNRs.

Perez-Juste *et al.* (2009) coated the citrate capped Gold Nanospheres and CTAB capped GNRs with the silica molecule. The silica coating was done by simple addition of sodium silicate to a solution consisting of gold nano spheres or GNRs with 3-mercaptopropyltrimethoxysilane (MPTMS) which ultimately changed the peak position of GNRs in UV-Vis spectra.

Xu *et al.* (2012) used 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) linking chemistry for conjugation of antibody to GNRs.

They mixed GNRs with MPA (3-Mercaptopropinic acid) solution under constant sonication at 50°C and later the use of EDC/NHS linking chemistry conjugated antibody and resulted considerable red shift from the original unmodified GNRs.

Wang and Tang (2013) reported that the CTAB capped on GNRs attracted the negatively charged protein which led to nonspecific absorption of a protein and resulted in complete replacement of CTAB caped on the GNRs

2.5.4.5e Ligand Exchange

Ligand exchange is another surface modification technique where CTAB capped on the outer surface of the GNRs were replaced by thiol-terminate molecule. This technique showed reduction of the cellular toxicity and influenced biocompatibility of GNRs when it binds to biological molecules.

Yu *et al.* (2007) observed that physiological absorption of an antibody to GNRs showing longitudinal shift of about 60 nm, after binding of an antibody to GNRs. The thiol terminate PEG is one of the common molecule used as a ligand for the modification of a GNRs (Pierrat *et al.,* 2007; Liao and Hafner, 2005).

Rostro-Kohanloo *et al.* (2009) reported that the advantage of thiolated PEG was its water solubility, so that ligand exchange occurred in aqueous solution. Such PEG modified gold particles were highly stable and used directly for biomedical application.

The thiolated PEG with bi functional group could be used for binding of protein and antibody to the GNRs (Vigderman *et al.*, 2009). Mercaptoundecanoic acid (MUA), a molecule used in exchange of the CTAB on the surface of the GNRs. MUA molecule has a COOH group desirable for bio cognition of NH_2 terminate biomolecules like protein and antibody. Here, the thiolated PEG was dissolved in an organic solution and not in water. The hydrophobic nature of the MUA challenged to the surface modification of the GNRs with MUA and caused severe aggregation.

Cao *et al.* (2012) reported the entire removal of CTAB could be done with MUA. MUA-ethanol and GNRs solution were kept under constant ultrasonication to prevent the aggregation of GNRs, along with temperature maintained at 50°C for the removal of CTAB bounded on GNRs surface.

2.5.4.5f Application of Nanobiosensors in Detection of Plant Pathogens

Boltovets *et al.* (2002) demonstrated the application of a surface plasmon resonance for detecting *Tobacco mosaic virus* (TMV) in infected leaf samples. The results obtained with the surface plasmon resonance (SPR) technique were in good correlation with ELISA and found specificity in binding between the virus and antibody.

A surface plasmon resonance (SPR) biosensor chip was developed for the rapid and sensitive detection of the *Oyster mushroom spherical virus* (OMSV) the causal agent of die-back disease of mushroom. An anti-OMSV monoclonal antibody (mAb) was generated for the bionsensor chip fabrication process. The developed biosensor chip was tested for the detection of OMSV collected from 10 different commercial farms. The chip detected less than 68 ng of viral particles in 50 µl of the sample, which corresponded to a concentration of 1.36 ng/µl (Kim *et al.*, 2008). The sensitivity and specificity of the chip was found to be superior to conventional ELISA assay.

For the detection of *Beet necrotic yellow vein virus* Hossein *et al.* (2012) developed an immunosensor, based on the principle of Fluorescence Resonance Energy Transfer (FRET). The cadmium-telluride quantum dots-rhodamine dye complex was used for the resonance to occur. The thioglicolic acid modified quantum dots were allowed to biofunctionalize with *Polymyxa betae* specific antibodies. Glutathione-S-transferase protein used as antigen and conjugated with rhodomine. The interaction between antigen-antibody brought quantum dots and rhodamine together to allow dipole-dipole coupling for the fluorescence resonance energy transfer to occur and was detected using FRET based immunosensor. The assay required only 20 µl sample and the immunosensor showed 100 per cent sensitivity. Lower limit of detection of 0.5 µg/ml whereas 2 µg/ml in ELISA assay.

A SPR based sensor was developed for the label free detection of fungal teliospores in wheat using gold disks (Singh *et al.,* 2012). The sensogram analysis showed the detection sensitivity of the developed biosensor was as low as 625 pg equivalent to 2.5 teliospores. The signal detection was done by measuring the peak shift occurred before and after binding of specific antigen to the antibody. This research work was performed in G. B. Pant University of Agriculture and Technology, Uttarakhand. This was the first nanobiosensor reported in agricultural field in India for the detection of fungal pathogens.

Cao *et al.* (2013) designed fiber optic particle Plasmon resonance based immunosensor for the detection of orchid viruses using gold nanorods (GNRs). It was the label free detection method. The GNRs were used as the sensing platform which was immobilized over the surface of optical fiber substrate. The immobilized GNRs were surface modified with 11-MUA for functionalization of orchid viruses specific antibodies and the performance of developed nanobiosensor was analyzed with antigen from different leaf samples. It was found that the limit of detection of sensor for *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ring spot virus* (ORSV) was 48 and 42 pg/mL respectively. The efficacy of nanobiosensor in term of lower limit of detection was approximately 25 times better than that of ELISA assay for both viruses. The detection time was 10 minutes with better specificity and reproducibility.

Huang *et al.* (2013) reported a surface plasmon resonance based biosensor for the detection of *Maize chlorotic mottle virus* (MCMV). A gold coated SPR chip was used as substrate over which 11- Mercaptoundecanoic acid (MUA) was applied and a layer of anti-MCMV was coated for the specific detection of MCMV, the lower detection limit of developed biosensor was 1 ppb, which was approximately two orders of magnitude higher than that of ELISA method. The biosensor showed high specificity in the recognition of both purified MCMV and crude extracts of plant samples.

Gutierrez-Aguirre *et al.* (2014) evaluated the interaction of *Potato virus Y* (PVY) in Solanaceous plants using surface plasmon resonance technique. The biosensor could

detect the presence of viral isolates of lower limit of 0.15µg/ml. A stable virus surface was generated for evaluating the interaction of corresponding monoclonal antibodies with different isolates. Stable virus surfaces were successfully interacted and it was used to explore kinetic parameters of the interaction of an array of monoclonal antibodies with two different PVY isolates *viz.,* serotype N and O. The developed biosensor proved the successful interaction and could be employed for the evaluation study of competence of two given monoclonal antibodies for the same epitope within the surface of viral particles coated.

Bio-recognition induced gold nanorods aggregation was developed as an analytical tool for detection of *Banana bract mosaic virus* (BBrMV) by Saurav (2016). In this case, due to addition of antigen to antibody labeled GNRs solution, the colour of the solution changed red to black and notable peak shift of (7-25) nm was observed both in transverse and longitudinal peak of GNRs in UV-Vis spectra. Antigen concentration up to 0.25 mg/ml and above showed stability in the peak shift and colour change in infected sample compared to healthy sample. In healthy sample no colour change were observed and with only minimum peak shift (Saurav, 2016).

The development of a nanobiosensor for the detection of banana bunchy top virus was attempted by Vinusree (2017) and the detection limit was 0.08 mg/ml. Conversion of solution phase based GNR biosensor to chip based sensor will open up more application and would help for easy commercial fabrication.

Materials & Methods

III. MATERIALS AND METHODS

The present study on "Molecular characterization of virus causing Infectious chlorosis disease of banana" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara and Banana Research Station (BRS), Kannara, Kerala Agricultural University during 2014-2017. The experimental site is located at an elevation of about 58 MSL with rainfall ranging between 2700 and 3000 mm, distributed between two rainy seasons. Relative humidity varies between 77 and 94 per cent and average temperature is about 28°C.

The details of the materials used and methodologies followed are described in this chapter.

3.1 BIOLOGICAL CHARACTERIZATION OF THE VIRUS

3.1.1 Survey, Collection and Maintenance of Virus culture

Purposive sampling surveys were conducted during 2014-2016 to collect infected samples and to record the incidence of infectious chlorosis disease of banana in selected locations of Thrissur district, Kerala. The details of places surveyed are given in Table 3 and Plate 1. The per cent disease incidence from each location was calculated as per following formula.

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Apart from documenting the disease incidence and symptoms, the infected leaf samples collected from different fields were brought to the laboratory and kept at -80°C freezer for further confirmation through ELISA as given in section 3.2.1.1.2.

The infected suckers were also collected from the surveyed fields and planted in grow bags and kept under insect proof net house of BRS, Kannara and that of Department of Plant Pathology, College of Horticulture and maintained as virus culture for further studies (Plate 4).

Table 3: Details of locations surveyed for incidence of infectious chlorosis disease in Thrissur district

Sl. No.	Locations	Latitude and Longitude
1	Cherpu	10.43236 °N, 76.20445 °W
2	Chirakkal	10.40916 °N, 76.17547 °W
3	Keezhpullikkam	10.40924 °N, 76.17536 °W
4	Thanyam	10.41588 °N, 76.12371 °W
5	Peringottukara	10.42033 °N, 76.13679 °W
6	BRS Kannara	10.53733 °N, 76.32006 °W
7	Marakkal	10.53605 °N, 76.32356 °W
8	Kannara (2 fields)	10.53733 °N, 76.32006 °W
10	Mannuthy	10.5286 °N, 76.26671 °W
11	Kandanissery (2 fields)	10.59833 °N, 76.07835 °W
13	Wadakkancherry	10.65333 °N, 76.24365 °W
14	Pudukkad	10.41781 °N, 76.2706 °W
15	Adatt	10.54507 °N, 76.14649 °W
16	Engandiyoor	10.50278 °N, 76.05949 °W
17	Wadeshari (2 fields)	10.48376 °N, 76.23001 °W
18	Thalikulam	10.44459 °N, 76.0908 °W
20	Irinjalakuda	10.32572 °N, 76.2152 °W
21	Vellangallur	10.30306 °N, 76.209 °W
22	Vellanikkara	10.54521 °N, 76.27397 °W
23	Erimapetti	10.24045 °N, 76.26325 °W
24	Mala	10.24029 °N, 76.26312 °W
25	Meloor	10.30017 °N, 76.38109 °W

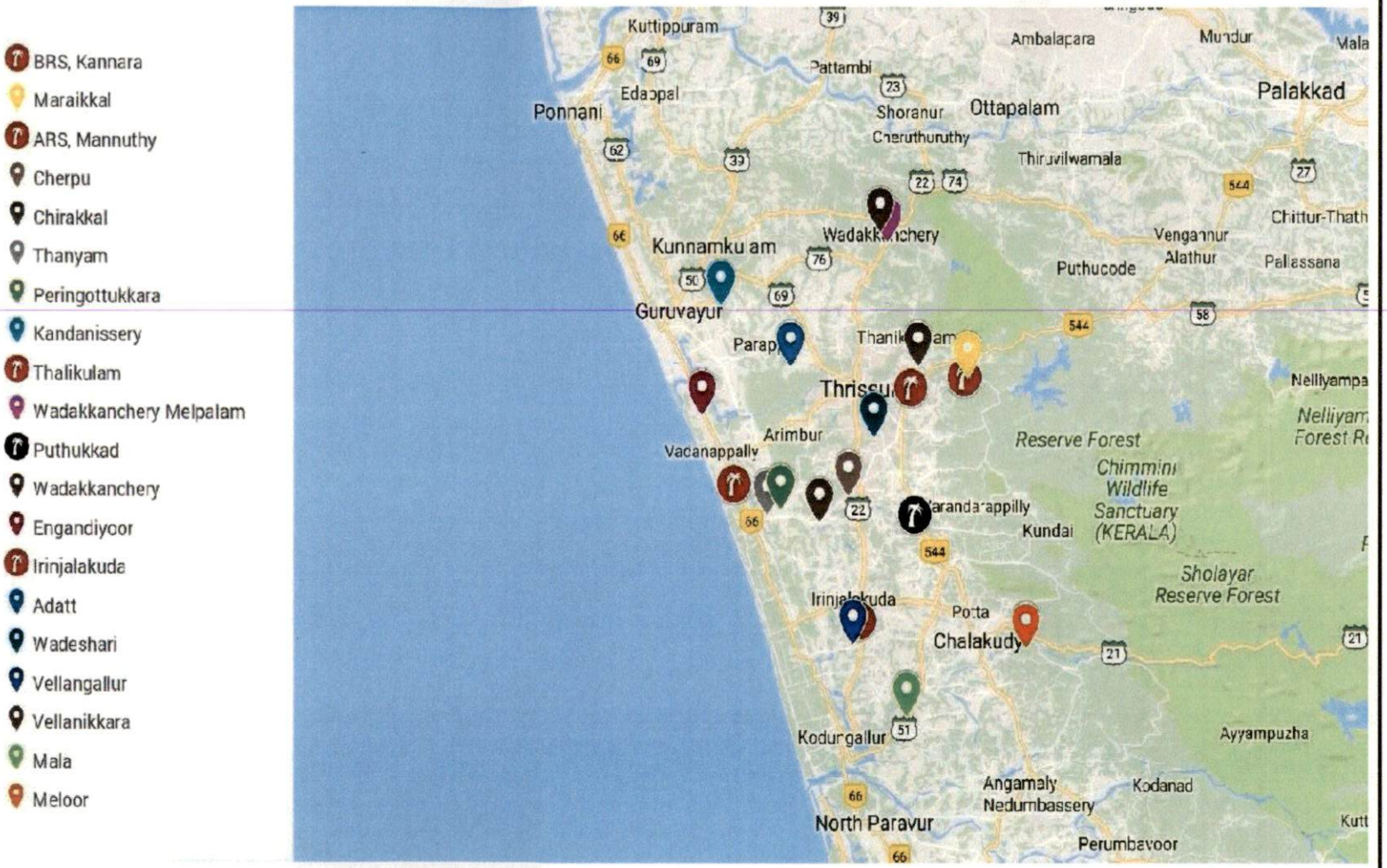


Plate 1: Map of the locations surveyed in Thrissur district

3.1.2 Symptomatology

Symptom expression of the disease was studied under natural and artificial conditions.

3.1.2.1 Symptom Expression under Natural Conditions

During the survey conducted in different places of Thrissur district the symptoms noticed on different parts of the plants were recorded along with documenting the variable symptoms if any expressed on each cultivar.

Among the fields surveyed, two fields, one each at Mannuthy and Maraikal were selected and the infected plants were tagged. The leaf samples were collected from those tagged plants and infectivity of the virus was confirmed through DAC ELISA as given in the section 3.2.1.1. These plants were observed regularly and symptoms developed on leaf lamina, midrib, petiole, pseudostem and bunch at different growth stages of plant under natural conditions were recorded.

3.1.2.2 Varietal Variation of Symptom Expression

The symptoms of the disease on different varieties grown in the germplasm and other fields of BRS, Kannara and Agricultural Research Station, Mannuthy were documented. The variation of symptoms on each variety was recorded.

3.1.2.3 Symptoms under Artificial Conditions

The symptoms produced by the virus under artificial conditions were studied by inoculating tissue culture plants of banana variety Nendran (AAB) through insect transmission as given in section 3.1.4.3. The inoculated plants were then kept under insect proof net house and observed regularly for development of symptoms.

3.1.2.4 Histological Changes due to Virus Infection

Thin sections of infected and healthy leaves of banana variety Nendran (AAB) were cut using microtome available in the College of Forestry, Vellanikkara. These sections were observed under microscope and each tissue of the leaf lamina of infected

and healthy plants was compared to study the histological changes brought about due to virus infection.

3.1.2.5 Impact of the Disease on Agronomic and Yield Characters of Banana

The infected plants of varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB) were tagged and the biometric observations were recorded at monthly intervals.

The biometric characters of the infected plants *viz.,* plant height, girth of pseudostem, number of leaves, bunch weight, number of hands and number of fingers were recorded. The chlorophyll content of the leaves was taken using SPAD chlorophyll meter. Similar observations were also taken for healthy plants of each variety.

3.1.3 Host Range Studies

The host range of the virus was studied using plants species belonging to different families and also on weeds seen in and around banana fields (Table 4). The inoculum was prepared by grinding the leaves of infected plant in pre chilled pestle and mortar with suitable buffer (5ml/g^{-1} tissue). The homogenate was filtered through double layered muslin cloth and this extract was used as inoculum for sap inoculation. Ten seedlings of each host plant were raised in polythene covers of size 10 x 15 cm filled with potting mixture. Carborandum powder (600 mesh) was uniformly dusted on the upper side of leaves of these seedlings and cotton swabs saturated with the crude extract was then rubbed gently on the surface of leaves. The inoculated leaves were washed after 2-3 min using wash bottle in order to remove any excessive inoculum and extraneous particles. The inoculated plants were kept in the insect proof net house and observed for symptom development.

The plants which developed symptoms were back inoculated to indicator host cowpea. These plants were observed for the development of symptoms and confirmed the infectivity of the virus. The infectivity was further confirmed through ELISA.

Table 4: List of plants used for host range studies

Sl. No.	Host	Common name	Family
1	*Amaranthus polygamus*	Green amaranth	Amaranthaceae
2	*Gomphrina globosa*	Globe amaranth	
3	*Momordica charantia*	Bitter gourd	Cucurbitaceae
4	*Benincasa hispida*	Ash gourd	
5	*Lagenaria siceraria*	Bottle gourd	
6	*Cucumis anguria*	Gerkins	
7	*Cucumis sativus*	Salad Cucumber	
8	*Trichosanthes cucumerina*	Snake gourd	
9	*Solanum melongena*	Brinjal	Solanaceae
10	*Capsicum annuum*	Chilli	
11	*Nicotiana tabaccum*	Tobacco	
12	*N. glutinosa*	Tobacco	
13	*N. benthamiana*	Tobacco	
14	*Vigna unguiculata*	Cowpea	Fabaceae
15	*Canavalia gladiata*	Sword bean	
16	*Centrosema pubescens*	Centro or butterfly pea	
17	*Zingiber officinale*	Ginger	Zingiberaceae
18	*Curcuma longa*	Turmeric	
19	*Heliconia platystachys*	Bird-of-paradise	Heliconiaceae
20	*Canna indica*	Canna	Cannaceae
21	*Synedrella nodiflora*	Cinderella weed	Asteraceae
22	*Sphagneticola trilobata*	Singapore daisy	
23	*Acmella radicans*	Brazil cress	
24	*Tridax procumbens*	Coat buttons	Compositae
25	*Commelina benghalensis*	Tropical spiderwort,	Commelinaceae
26	*Ichnocarpus frutescens*	Black creeper	Apocynaceae
27	*Phyllanthus amara*	Gale of the wind,	Phyllanthaceae
28	*Ludwigia hyssopifolia*	Water primrose	Onagraceae
29	*Stachytarpheta indica*	Blue porter weed,	Verbenaceae

3.1.4 Transmission Studies

3.1.4.1 Transmission through Planting Material

The suckers from infected plants of different cultivars were collected after confirming the presence of the virus in mother plants through diagnostic symptoms and serodiagnostic assay, ELISA. The suckers were planted in grow bags filled with potting mixture and kept in insect proof net house and periodically sprayed with insecticide, 0.03 per cent dimethoate to make it completely free from the access of insect vectors. The plants were observed regularly for symptom expression. Five healthy suckers of each cultivar were also planted in grow bags to serve as control. The number of suckers showing symptoms was recorded and the per cent transmission of disease through planting material was calculated.

3.1.4.2 Mechanical Transmission

3.1.4.2.1 Preparation of Inoculum

The leaf extract was prepared by grinding the leaves of infected plant in pre chilled pestle and mortar with suitable buffer ($5ml/g^{-1}$ tissue) standardized as given below. The homogenate was filtered through double layered muslin cloth and this extract was used as inoculum for sap inoculation.

3.1.4.2.2 Standardization of Buffers

The standardization of buffer for mechanical transmission of the virus causing infectious chlorosis disease of banana was carried out on the indicator plant, cowpea (*Vigna unguiculata)*. The buffers used for the experiment are given in Table 5 and the chemical composition is given in Appendix-III.

For each treatment, five plants were inoculated. The inoculation was done as detailed in section 3.1.4.2.3 and kept in insect proof net house and observed for development of local lesions. The most suitable buffer was selected based on the number

of local lesions produced and was then used for further studies in mechanical transmission.

3.1.4.2.3 Inoculation of Virus

The inoculation of the virus was done on tissue culture plants of banana. Carborandum powder (600 mesh) was uniformly dusted on the upper side of leaves of these tissue culture plants and cotton swabs saturated with the crude extract was then rubbed gently on the surface of leaves. The inoculated leaves were washed after 2-3 min using a wash bottle in order to remove any excessive inoculum. The inoculated plants were kept in the insect proof net house and observed for symptom development.

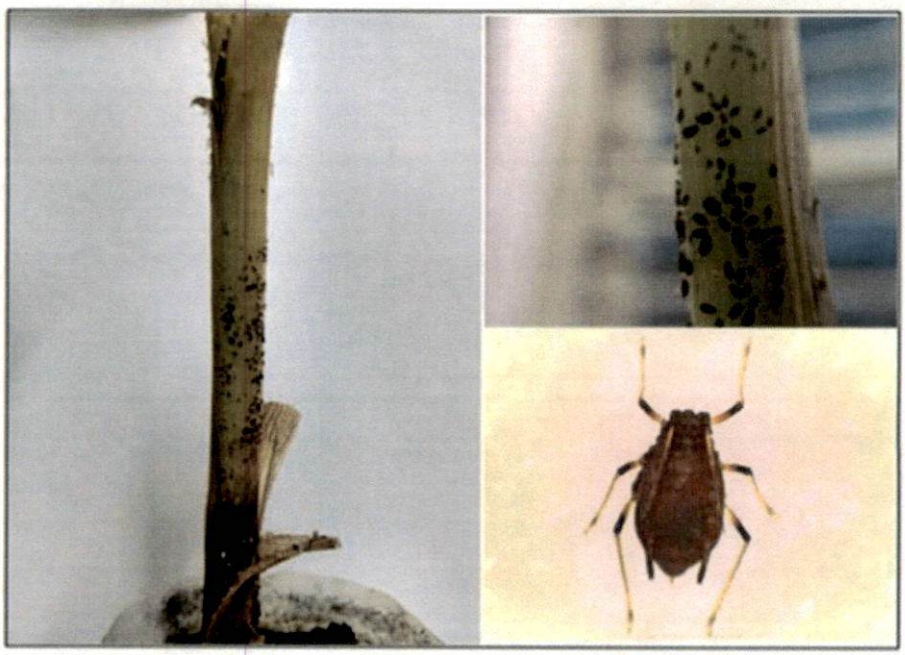
Table 5: Standardization of buffers for mechanical transmission of virus

Sl. No.	Name of the buffer	Concentration	pH
T_1	Sodium borate buffer	0.1 M	8.0
T_2	Potassium phosphate buffer	0.1 M	7.2
T_3	Potassium phosphate buffer	0.05 M	7.2
T_4	Sodium phosphate buffer	0.1 M	7.2
T_5	Citrate buffer	0.1 M	6.2
T_6	Tris buffer	0.1 M	7.2

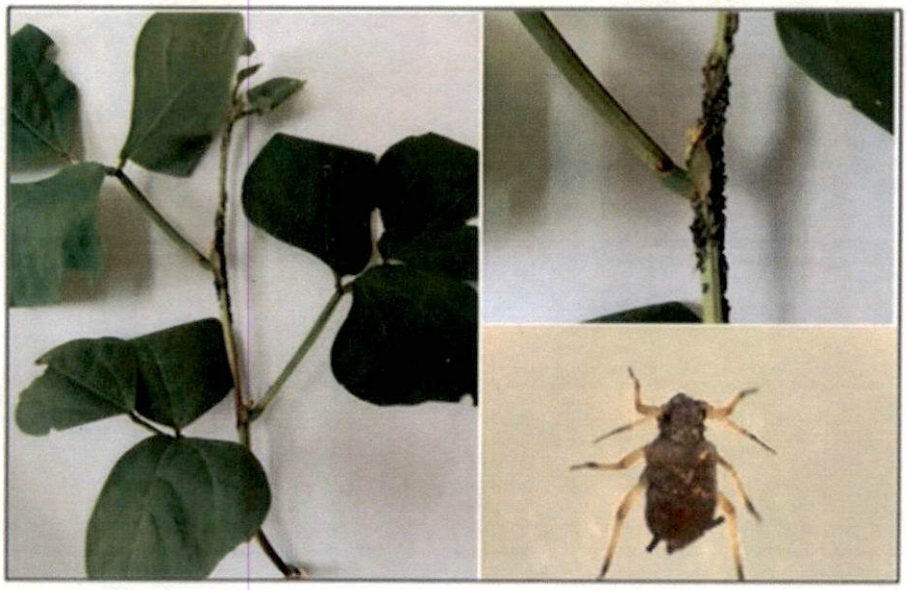
3.1.4.3 Insect Transmission

3.1.4.3.1 Collection and Rearing of Insects

Banana aphids (*Pentalonia nigronervosa*) and Cowpea aphid (*Aphis craccivora*) were collected from healthy banana plots of BRS, Kannara and cowpea fields of College of Horticulture, Vellanikkara respectively using soft camel hair brush. Banana aphids were mainly present in the heart or crown of the plants and in the outer sheath of the pseudostem, on the leaf axils of the petiole and on the lower side of the leaf midrib.



A. Rearing of banana aphid, ***Pentalonia nigronervosa***



B. Rearing of cowpea aphid, ***Aphis craccivora***

Plate 2: Collection and maintainance of colony of aphids

Cowpea aphids were found on the stem and petiole of cowpea plants. The identification of the aphids was confirmed from the Department of Entomology, College of Horticulture, Vellanikkara (Plate 2). The banana and cowpea aphids were gently released into the leaf axils of the healthy banana tissue culture plants (one month old) and cowpea seedlings respectively and kept in insect proof cages for colonization. These healthy aphid colonies were further used for vector transmission studies.

3.1.4.3.2 Effect of pre-acquisition fasting period of aphids for virus transmission

Group of 25 each of healthy adult banana and cowpea aphids were starved at different intervals of 0, 30 and 60 min. After the fasting period, these were collected and released separately on infected banana plants for acquisition of the virus. After the acquisition feeding period of 6 h, each group of adults were released on healthy tissue culture plants of banana variety Nendran and allowed to feed for 24 h. At the end of inoculation feeding period the aphids were killed by spraying 0.03 per cent dimethoate. The inoculated plants were kept in the insect proof net house for symptom expression. Time taken to express the initial symptom was recorded and incubation period was calculated by counting the number of days between the date of inoculation and the date of initial symptom expression.

3.1.4.3.3 Effect of acquisition feeding period of aphids for virus transmission

To determine the effect of acquisition feeding period on transmission, groups of 25 aphids each of *P. nigronervosa* and *A. craccivora* were starved for 1 h and allowed to feed on infected plants separately at different duration of acquisition access periods of 2, 6 and 24 h. At the end of respective acquisition access periods, the aphids were disturbed gently by tapping using moist soft camel hair brush and were transferred for inoculation on healthy tissue culture plants of banana variety Nendran and allowed to feed for 24 h. At the end of inoculation feeding period, the aphids were killed by spraying 0.03 per cent dimethoate. The inoculated plants were kept in insect proof net house for symptom expression. The time taken to express the initial symptom was recorded and the

incubation period was calculated by counting the number of days between the inoculation and the day of initial symptom expression.

3.1.4.3.4 Effect of inoculation feeding period of aphids on the transmission of virus

To determine the minimum inoculation feeding period required to render the aphids viruliferous, groups of 25 aphids were starved for 1 h and allowed to feed on infected plants for 1 h. At the end of acquisition feeding period, the aphids were transferred to healthy tissue culture plants of banana variety Nendran and allowed to feed for 10, 30 and 60 min. The insects were later killed by spraying with insecticide dimethoate 30 EC 0.03 per cent and plants were kept in insect proof net house. The observation on development of symptoms was recorded. Time taken to express the initial symptom was recorded and the incubation period was calculated by counting the number of days between inoculation and the date of initial symptom expression.

3.1.5 Physical Properties of Virus

The physical properties of the virus such as Thermal Inactivation Point (TIP), Dilution End Point (DEP), and Longevity *in vitro* (LIV) were studied (Noordam, 1973). The indicator plant used for the bioassay was cowpea (*V. unguiculata*).

3.1.5.1 Thermal Inactivation Point (TIP)

The crude sap was extracted from the infected leaf material as described in (3.1.4.2.1). Five ml of sap was taken in eleven glass test tubes and each test tube was then individually exposed to temperature of 28, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C maintained in water bath for 10 min. The treated sap from each test tube was mechanically inoculated on cowpea seedlings at two leaf stage as described in section (3.1.4.2.3). Ten seedlings were maintained for each treatment. The inoculated plants were kept in insect proof net house and observed for the development of local lesions and recorded the number of local lesions developed on each.

3.1.5.2 Dilution End Point (DEP)

The crude sap of the virus was extracted as per the method described (3.1.3.2.1). A series of dilutions *viz.,* 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} were prepared from the infected sap using distilled water. The undiluted crude sap was also used as one of the treatment.

From the crude sap prepared, one ml was taken and mixed with nine ml of distilled water in another test tube. This dilution was 10^{-1}. From this dilution, one ml of sap was pipetted out and mixed in another test tube containing nine ml of distilled water which made the dilution 10^{-2}. Likewise, different dilutions were prepared. For each dilution, separate pipette was used to avoid contamination and to get accurate dilutions. Such diluted sap was inoculated on cowpea seedlings at two leaf stage starting from the lowest dilution to highest dilution. The inoculated plants were kept in insect proof net house and observed regularly for the expression of symptoms. The observations on number of local lesions were recorded.

3.1.5.3 Longevity In vitro (LIV)

The survival of the virus in crude leaf extract was determined at room temperature (24±2°C) and at cool temperature (4°C) with different durations of storage *viz.,* 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 2 days, 3 days, 4 days, 6 days, 7 days, 8 days, and 9 days. The crude extract from infected banana leaves was prepared by grinding with 0.01 M Phosphate buffer pH 7.2 in pre chilled pestle and mortar. 10 ml of extracted crude sap was taken in each test tube, and the test tubes were plugged with rubber cork and stored at room temperature (28±2°C) and cool temperature (4°C). Immediately after extraction of sap, healthy seedlings of cowpea at two leaf stage (10 No.) were mechanically inoculated with the sap as described in section 3.1.4.2.3. The sap stored at room temperature and cool temperature (4°C) were inoculated on cowpea seedlings separately at fixed periods *i.e.* 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 2 days, 3 days, 4 days, 6 days, 7 days, 8 days and 9 days separately. Inoculation was also done soon after the preparation of crude extract *i.e.* at 0 h. The inoculated plants were labeled

and kept in insect proof net house and observations of local lesions and number of plants infected were recorded.

3.1.6 Electron Microscopy

The morphology of the virus particles associated with infectious chlorosis disease was studied using JOEL-100 EX-II Transmission Electron Microscope utilizing the facility at Indian Agricultural Research Institute (IARI), New Delhi. The infected leaves of banana, *N. glutinosa* and *N. benthamiana* were collected and sent to Advanced Centre for Plant Virology, Department of Plant Pathology, IARI, New Delhi.

The protocol followed for electron microsocopy was leaf dip method (Bhat *et al.*, 2004). One centimeter square of infected leaf tissue was taken and crushed on a clean glass slide using 0.1 phosphate buffer (pH 7.0) containing one per cent Nicotine sulphate. A drop of the extract was placed on the carbon coated grid of the electron microscope and allowed to stand for two min. Then the grid was washed with 10 drops of distilled water and stained with 2 per cent uranyl acetate. The excess stain was removed by touching the edge of the grid with a piece of filter paper and examined under Transmission electron microscope. The electron micrographs of the virus particles were taken on plate film. The morphological characters *viz.,* shape, length and breadth of the particles were recorded.

3.2 IMMUNOLOGICAL STUDIES

Immunological studies (Serological assays) were conducted at Molecular Virology Laboratory of BRS, Kannara.

3.2.1 Detection of Virus Infection by Protein Based Methods

3.2.1.1 Direct Antigen Coating ELISA (DAC-ELISA)

Protein based detection method *viz.,* DAC ELISA was validated. Infected leaf samples of banana showing typical symptoms and healthy control were tested for CMV

by Direct Antigen Coating ELISA (DAC-ELISA) using the polyclonal antiserum obtained from National Research Centre for Banana, Trichy, Tamil Nadu.

3.2.1.1.1 Determination of Titre of Antiserum and Antigen

DAC-ELISA was performed with different dilution of antiserum *viz.,* 1:100; 1:200; 1:300 and 1:500 to determine the antibody titre for polyclonal antibody specific to CMV using procedure (Clark and Adams, 1977) with slight modifications.

Antigen was prepared by grinding the leaf samples in 1X coating buffer (Annexure II) using pre-chilled pestle and mortar and centrifuged the homogenized product at 15000 rpm for 10 min at 4°C. The microtitre plates (96 well, Tarson Product Pvt. Ltd.) were coated with 100 µl of supernatant (prepared antigen) and incubated for 2h at 37°C. After incubation the plates were washed thrice with phosphate buffer saline with tween-20 (PBS-T) buffer (Annexure II) to remove the sap and unbound antigens at three min intervals followed by gently tapping the plates to remove the residual liquid. 100µl of blocking solution (Annexure II) was added to each well and incubated at 37°C for 1 h and then washed in PBS-T washing buffer. Antiserum (100 µl) with different dilutions (1:100; 1:200; 1:300 and 1:500) of antibody buffer was added to respective wells and incubated overnight at 4°C. The plates were then washed in PBS-T buffer and 100 µl of secondary antibody conjugated with alkaline phosphatase (1:10000 dilution) was added to each well and incubated at 37°C for 2 h. The unbound secondary antibody were washed with PBS-T and freshly prepared 100 µl substrate buffer (Annexure II) with *p*-Nitrophenyl phosphate (1mg ml^{-1}) was added to each well and incubated in dark at 37°C for 30 min for colour development. The absorbance value was recorded at 405 nm by ELISA reader (Biorad Imark micro titre plate reader). The optimum dilution was determined based on the absorbance value recorded.

3.2.1.1.2 Detection of Virus by DAC-ELISA

Once the primary antibody titre was standardized, DAC-ELISA was again performed using the standardized titre of primary antibody (1:200), which was

previously determined in the section 3.2.1.1.1. The absorbance values of the test sample and healthy sample along with buffer control were compared and if the absorbance value of test sample was more than twice that of healthy sample then the sample was considered as positive for virus infection.

3.2.1.2 Detection of Virus by Dot Immuno Binding Assay (DIBA)

Dot Immuno Binding Assay was performed using procedure described by Banttari and Goodwin (1985) with slight modification. A desired size of nitrocellulose membrane was cut and marked one cm^2 and the crude sap containing antigen was spotted on the marked square and air dried for 15 min.

After drying, membrane was immersed in blocking solution (Annexure II) with gently shaking for one hour. Then it was washed three times with PBS-T at intervals of 3 min. Primary antibody at 1:1000 dilution was then added on the blot and incubated for 2 h at room temperature, followed by washing with PBS-T buffer thrice at 3 min interval. Secondary antibody (Agdia USA Ltd.) conjugated with alkaline phosphatase was added on blot, incubated for one hour followed by washing three times (5 min each) with PBS-T buffer. Finally, membrane was rinsed in substrate solution (Annexure II) and incubated under dark condition for 15-20 min and then the membrane was washed with distilled water, air dried and observed for colour development.

3.3 VARIETAL SCREENING FOR DISEASE RESISTANCE

The field gene bank comprising of 175 accessions maintained at BRS, Kannara were screened at monthly interval from planting till harvest for natural occurrence of the disease and assessed their disease reaction under natural conditions. Observations on symptoms and incidence of disease were recorded.

The leaf samples from all the accessions were screened through ELISA to examine the infection of the virus in any asymptomatic plants. The accessions which showed positive for ELISA were labeled and observed regularly for symptom development and percent disease incidence was recorded.

3.4 MOLECULAR DETECTION AND CHARACTERIZATION

Studies on molecular characterization of the virus were carried out in the Molecular virology laboratory of BRS, Kannara.

3.4.1 Isolation of RNA

The isolation of RNA was done from young leaves of infected and healthy plants maintained in insect proof net house by two methods *viz.*, using TRIzol reagent (Sigma Aldrich) and RNeasy Plant mini kit® method. The RNA isolated through both the protocols were compared, analyzed and then stored at -20°C for further studies. All the materials used for RNA isolation were pretreated with 0.1 per cent (v/v) Diethyl pyrocarbonate (DEPC) over night and autoclaved.

3.4.1.1 RNA Isolation by TRIzol Reagent Method

The total plant RNA was isolated from the virus infected and healthy leaf tissues using TRIzol reagent method with slight modification (Sambrook *et al.*, 1989). 100 mg leaf sample was ground using liquid nitrogen in sterile DEPC treated pestle and mortar. The powdered tissue was transferred to RNase free micro centrifuge tube and was homogenized with 1000 µl of TRIzol reagent. It was centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was transferred to a fresh centrifuge tube and kept for 5 min at 35°C. Two hundred µl of chloroform was added to the supernatant, shaken vigorously and incubated at room temperature for 30 min. When centrifuged at 13,000 rpm for 10 min at 4°C, three separate phases were formed. Out of the three layers, colourless upper phase which contained RNA was transferred into fresh micro centrifuge tubes and 500 µl of isopropanol was added. Then incubated at 35°C for 30 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 75 per cent ethanol by gently vortexing followed by centrifugation at 7500 rpm for 5 min. Finally, air dried the pellet for 15 min and was re-suspended in RNase free water.

3.4.1.2 RNA Isolation using RNeasy Plant Mini Kit® Method

Total RNA extracted from the infected and healthy samples of banana using RNeasy Plant Mini Kit® as per the protocol supplied by the manufacturer. Thirty mg of leaf tissue from both healthy and infected plants were taken and the tissue was macerated using liquid nitrogen and homogenized the lysate in the appropriate volume of buffer RLT. The lysate was centrifuged for 3 min at 12,000 rpm. To the supernatant one volume of 70 per cent ethanol was added to the lysate and mixed well. Seven hundred µl of sample with precipitate was transferred to RNeasy Mini spin column placed in collection tube and centrifuged for 15 sec at 7,800 rpm. Then 500 µl buffer RPE was added to column and centrifuged for 15 sec at 7,800 rpm. The column was then placed in 1.5 ml collection tube and 30-50 µl RNase free water was added to elute RNA from the column. The isolated RNA was stored at -20°C for further studies.

3.4.1.3 Quality and Quantity Analysis of RNA

3.4.1.3.1 Gel Documentation

Agarose gel (0.8 %) was used to check the quality of RNA in 1X TAE buffer (Annexure IV). The electrophoresis was carried out at 50V for 30 min. The gel was then visualized and the image was documented using BioRad Gel Doc Ez Imager (Sambrook and Russel., 2001).

3.4.1.3.2 Purity of RNA

The purity of RNA samples was further assessed by determining the absorbance value using Nano drop 2000C (thermo scientific) spectrophotometer. Absorbance was recorded at 260 and 280 nm wavelength and purity was indicated by the ratio $A_{260/280}$.

3.4.2 Synthesis of First Strand Complementary DNA (cDNA)

The isolated RNA was used to synthesize cDNA by using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's instructions. The components of reaction mixture used are given in the Table 6. The reagents were added into sterile nuclease free thin walled polypropylene PCR tubes on ice. The

template RNA, Oligo $(dt)_{18}$ primer and nuclease free water were added into sterile polypropylene PCR tube and mixture was incubated at 65°C for 5 min in thermocycler. After incubation, it was immediately chilled on ice for 2 min and then remaining components were added. Finally, the mixture was incubated at 42°C for 60 min followed by incubation at 70°C for 5 min for termination.

Table 6: Reagents used for cDNA synthesis

Sl. No.	Name of the component	Volume (µl)
1	GC-rich RNA Template	1
2	Oligo $(dT)_{18}$ primer	1
3	RevertAid M-MuLV-RT (200 U/µl)	1
4	10 X reaction buffer	4
5	Ribolock RNase inhibitor 20 U/µl	1
6	10 mM dNTP Mix	2
7	Water (nuclease free)	10
	Total volume	20

3.4.3 Primers

Two sets of primers were used for Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Primer pair specific to the coat protein gene of CMV as reported by Cherian *et al.* (2004) were used for the detection of virus. Another set of primer was developed using the genomic data available in the data base.

Table 7: Sequences of reported primers used for PCR

Primer	Sequence (5'->3')	length	Tm value	Product Size (bp)
RP-CMV-F1	CATCGACCATGGACAAATCT GAATCAAC	28	62.87	750
RP-CMV-R2	CTCTCCATGGCGTTTAGTGAC TTCAGCAG	29	67.15	750

Second set of primer pair specific for the coat protein gene of CMV were designed based on standard guidelines of primer synthesis (Thompson *et al.,* 1994) as described below.

3.4.3.1 Primer Designing and Validation

Primer pairs specific for the coat protein gene of CMV were designed based on standard guidelines of primer synthesis (Thompson *et al.,* 1994). The conserved region in the coat protein was determined using complete coat protein sequences of CMV available in National Center for Biotechnology Information (NCBI) database using multiple sequence alignment of nucleotide sequences obtained using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo). Based on homology, conserved boxes of 18 to 24 bases each for both primers were selected and pair of gene specific primers that flank the coat protein gene were designed to amplify DNA fragment of viral coat protein gene. The designed primer sequences were validated using Oligo analyser 3.1 program so as to avoid nonspecific amplification. After validation the details of primer sequences were sent to Scigenome, Kochi for the synthesis of primers.

3.4.4 Standardization of PCR Conditions for Designed Primer

Various combinations of RT-PCR parameters like annealing temperature, different combinations of primer and template dilutions were used to standardize RT-PCR conditions. The different dilutions of designed primer were tested with different dilutions of cDNA template (Table 8) and gradient PCR was carried out in Matercycler gradient PCR (Eppendorf) to standardize annealing temperature.

Table 8: Dilutions of primer and cDNA template used for standardizing PCR conditions

Different combinations of primer and template dilutions	Aliquot (µl)	
	Primer	Template cDNA
1:1	0.5	0.5
$1:10^{-1}$	0.5	2.0
$10^{-1}:1$	1.0	0.5
$10^{-1}:10^{-1}$	1.0	2.0

3.4.5 Detection of the Virus by RT- PCR

RT-PCR was carried out in PCR Thermo Cycler (Eppendorf master cycler, Germany) by using coat protein specific primers reported by Cherian *et al.* (2004) and designed primers. It reaction was carried out in standard PCR conditions using reported and designed set of coat protein specific primers. The amplified product was visualized in 1.8 per cent agarose gel and documented in BIO-RAD Gel Doc® EZ Imager and compared with 1Kb DNA marker (GeNei, Bangalore).

Procedure

The composition of the reaction mixture and program used for PCR are given in table 9. The master mix was prepared by dispensing components in 0.2 ml tubes. Momentary spin was given for thorough mixing of the components. The PCR tubes were then placed in thermocycler (Eppendorf Mastercycler Gradient). The reaction program was set as presented in Table 10.

Table 9: Components of PCR master mix

PCR -Mixture	Quantity (µl)	
	Reported primer	Designed primer
Water	20	20
10X PCR buffer	2.5	2.5
20 mM dNTPs mix.	1.0	1.0
Forward Primer	1.0	1.0
Reverse Primer	1.0	1.0
Taq polymerase	0.5	0.2
cDNA	2	0.5
Total	28	26.2

Table 10: Temperature profile for PCR

PCR-reaction	Temperature and Time	
	Reported primer	**Designed primer**
Initial denaturation	94°C for 2 min	94°C for 3 min
Denaturation	94°C for 30 sec	94°C for 30 sec
Annealing	61°C for 45 sec	69°C for 45 sec
Extension	72°C for 90 sec	72°C for 90 sec
Final Extension	72°C for 10 min	72°C for 10 min
Total no. of cycle	32	32
End cycle	72°C for 10 min	72°C for 10 min
Storage	4°C	4°C

3.4.5.1 Sequencing

The amplified PCR product were purified and sent to Scigenome, Kochi for sequencing.

3.4.5.2 In silico Analysis of Sequences

Sequence information of coat protein gene obtained was further analyzed. The nucleotide sequences were used to deduce amino acid sequence using ExPASy translator tool. Homology search and phylogenetic analysis was also performed using bioinformatics tools. The sequences were compared with the sequences available in NCBI database using BLAST tool to check homology and best aligned sequence.

3.4.5.3 Phylogenetic Analysis

Phylogenetic analysis was carried out using MEGA 7.0 software by constructing phylogenetic tree by Neighbor-Joining bootstrap method (Saitou and Nei, 1987). The CP sequences of eight CMV isolates were analyzed with the top 10 hits sequences obtained

after performing BLASTn analysis and to all eight sequences to know the phylogenetic relations with the sequences available in NCBI and between the isolates.

The CP gene sequences of eight CMV isolates generated in this study was aligned with 41 CP gene sequences of CMV isolates of banana from India and elsewhere was retrieved from NCBI. Multiple Sequence Alignment of these sequences was carried out in Clustal Omega and the phylogenetic analysis was carried out combining all these sequences and the phylogenetic relationship among the isolates from different geographical region was inferred.

3.4.6 Cloning of Coat Protein Gene

The CMV coat protein gene was cloned into T-tailed pGEM-T easy vector and transformed into *Escherichia coli* cells (DH5α). The potent colonies were screened for the presence of recombinant plasmid by performing colony PCR. After colony PCR, the PCR product was run on 1.2 per cent agarose gel electrophoresis and visualized for the expected band size of 900-950 bp and documented.

3.4.6.1 Preparation of Competent Cells

The competent cells (*E. coli* DH5α) were prepared for plasmid transformation by following the protocol described by Mandel and Higa (1970). The protocol for competent cell preparation is given below.

First day:

- Single colony of 18 h old *E. coli* DH5α strain was inoculated to 100 ml Luria Bertani (LB) medium under sterile condition and incubated overnight at 37°C on a shaker at 160 rpm.

Second day:

- Three ml of the culture was transferred aseptically to 50 ml sterile LB broth and incubated for 4 h at 37°C on a shaker at 160 rpm.
- The cells were then aseptically transferred to sterile disposable ice-cold 50 ml tube.

- The culture was cooled on ice cubes for 20 min
- The cell suspension was centrifuged at 3500 rpm for 10 min at 4°C.
- The supernatant obtained was carefully discarded and the pellet was gently re-suspended in 10 ml sterile ice-cold 0.1M $CaCl_2$.
- The tubes were kept on ice for 20 min and the cell suspension was centrifuged at 5000 rpm for 10 min at 4°C.
- The supernatant was decanted and the pellet was re-suspended in 2 ml of ice cold sterilized 0.1M $CaCl_2$. The tubes were kept on ice for 18 h.

Third day:

- Four ml of chilled glycerol was added to the cell suspension and mixed well using a sterile microtip.
- The competent cells were prepared as aliquots of 100 µl in chilled 1.5 ml microfuge tubes and covered with aluminum foil. These were stored at -20°C for further use.

3.4.6.2 Screening of Competent Cells

Transformation of competent cells with plasmid having ampicillin resistance (pUC18) was carried out to check the competency and purity of competent cells. The procedure followed for screening of plasmid is as follows.

- The competent cells stored at -80°C were thawed over ice for 10 min
- Two µl of plasmid DNA was added to 100 µl competent cells. The competent cells without adding plasmid was served as negative control.
- The cells were kept on ice for 40 min followed by heat shock at 42°C for 90 sec in a water bath and then kept on ice for 5 min.
- LB medium (250 µl) was added to the cells and incubated at 37°C for 1h on a shaker set at 120 rpm.
- Hundred µl of transformed cells was placed on LBA amended with ampicillin (Annexure VII) and incubated overnight at 37°C in a shaker (100 rpm). The recombinant clones were grown on plates containing ampicillin.

3.4.6.3 Ligation

Ligation was carried out to link the DNA insert and the plasmid vector. The amplified product was ligated in pGEM-T Easy vector System (Plate 3) (Promega Corporation, USA) as per manufacturer's protocol; before use, the vector and other components were briefly centrifuged to collect the contents at bottoms of the tube. The ligation reaction was set by mixing the reaction components and incubated overnight at 4°C the reaction components are given in the Table 11.

Table 11: Components of ligation reaction

Sl. No.	Components	Volume (µl)
1	2X rapid ligation buffer	5
2	pGEM-T Easy Vector (50 ng)	1
3	DNA product	3
4	T4 DNAligase (3 U/µl)	1
	Total volume	10

3.4.6.4 Transformation

Transformation of competence cells was done with pGEM-T vector plasmid. The ligated PCR product was added to 100µl of thawed competent cells and kept on ice for 30 min followed by heat shock given at 42°C for 90 sec in a dry bath and immediately placed on ice for 5 min. 250µl of LB broth was added to the cells and incubated at 37°C for 1h on a shaker at 1600 rpm. The aliquots of the transformed cells were placed on LB agar/ampicillin/ IPTG /X-gal plates and incubated overnight at 37°C. The transformed colonies were selected based on blue/white colony selection and further confirmation of insert was done by colony PCR.

3.4.6.5 Analysis of Recombinants by Colony PCR

Single white colony was selected and resuspended in 20µl of distilled water and heated at 98°C for 3 min followed by centrifugation at 12,000 rpm for 2 min. The

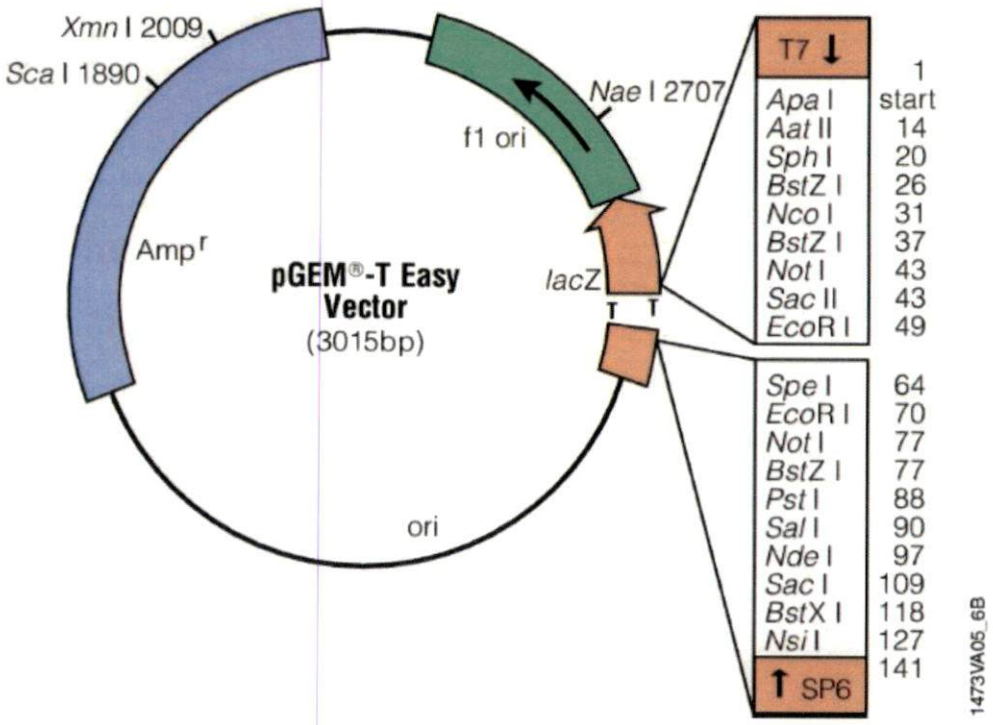


Plate 3: Restriction map showing multiple cloning site of pGEM-T vector (Promega) used for cloning

supernatant was taken in PCR tube and used as template DNA for PCR reaction. The presence and orientation of DNA insert in the recombinant clones were analyzed by colony PCR using flanking primers T7 and Sp6 (Promega, USA). The PCR reaction mixture was prepared as given in Table 12 and PCR was carried out with an initial denaturation at 94°C for two min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension step for 10 min at 72°C was also given.

The amplicons of colony PCR were resolved on 1.2 per cent agarose gel. The selected clones were confirmed through colony PCR and preserved in Department of Plant Pathology, COH, Vellanikkara for production of antiserum.

Table 12: Components of reaction mixture used in colony PCR

Sl. No.	Components	Volume (µl)
1	Template	2.0
2	10X reaction buffer	2.5
3	dNTP (10 mM)	2.0
4	Forward primer T7 (10 pM/ µl)	0.5
5	Reverse primer Sp 6 (10 pM/ µl)	0.5
6	Taq polymerase (3 U/µl)	0.2
7	Sterile distilled water	16.05
	Total volume	23.75

3.5 DEVELOPMENT OF NANOBASED BIOSENSOR FOR DETECTION OF VIRUS

3.5.1 Synthesis of Gold Nanorods (GNRs)

Synthesis of GNRs was done by seed mediated method (Nikoobakht and El-Sayed, 2003). The reagents used for this study are given in Annexure V.

Procedure

Seed solution was first prepared by adding 250 µl of $HAuCl_4$ into 7.5 ml of CTAB, mixed vigorously and 600 µl of ice-cold $NaBH_4$ was added to this mixture under vigorous stirring for 2 min. Then it was kept at 25°C for 15 min. This served as the seed solution which contained gold nano spheres. After the synthesis of such seed particles, the growth solution was prepared by mixing 50 ml of 0.2 M CTAB and 42.2 ml distilled water and 5 ml of gold solution and mixed vigorously. Then 600 µl of silver nitrate and 5.5 ml of ascorbic acid were added under gentle stirring at 25°C, followed by final addition 100 µl of prepared seed solution and kept overnight for the growth of GNRs.

3.5.2 Purification and Characterization of GNRs

The primary growth solution was centrifuged at 15000g for 35 min in 20 ml centrifuge tubes. The impurities and the supernatant were removed and the purified GNRs were re-suspended in ultrapure water (Gao *et al.*, 2004).

3.5.3 Characterization of GNRs

The characterization of GNRs was done using UV-Vis spectrophotometer and Transmission electron microscope.

3.5.3.1 Characterization of GNRs by UV-Vis Spectrophotometer

Synthesized GNRs were transferred to clean and dry cuvet for characterization using Perkinz UV-Vis spectrophotometer at wavelength of 200 to 1100 nm. The absorbance spectra readings were converted into graphical format using OriginPro 8 software. From the graph, two different bands *viz.*, longitudinal and transverse plasmon bands were analyzed for determining the peak.

3.5.3.2 Characterization of GNRs using Transmission Electron Microscope (TEM)

The centrifuged GNR solution (10 µl) was taken in carbon coated TEM grid and dried for one hour. Element analysis was carried out by JOEL – 100 EX – II transmission electron microscope at Advanced Center for Plant Virology, Indian Agricultural Research Institute, New Delhi. A total of 10 TEM images were recorded for

each sample at different resolutions. Using Image J Software, analysis of GNR particles from TEM images was done. The length and width of the particles were measured and aspect ratio was calculated.

3.5.4 Estimation of Aspect Ratio (AR) of GNRs

Aspect ratio of GNRs was estimated by two different methods as described below.

3.5.4.1 Estimation of Aspect Ratio (AR) of GNRs using TEM Images

The first method followed to calculate aspect ratio was by using TEM images. Length and breadth of twenty GNR particles from TEM images were analyzed using Image J Software and the aspect ratio was estimated by calculating the ratio of their length and breadth.

3.5.4.2 Estimation of Aspect Ratio (AR) of GNRs using GANS Theory

The aspect ratio was also determined based on GANs theory where the particle absorption was measured using Perkinz UV-Vis spectrophotometer. The LPB was assessed and put into the equation given below for the estimation of aspect ratio. (Link *et al.*, 1999).

AR=(Peak wavelength-381.49) / 97.56

3.5.5 Development of Self Assembly Monolayer (SAM) on the Surface of GNRs

Surface modification of the synthesized GNRs was done as per the procedure (Song *et al.*, 2013). The CTAB capped on the periphery of GNRs were completely replaced in this experiment, where layer of alkali-thiol group formed could act as SAM for the binding of antibody.

Reagents used

3-Mercaptopropinic acid - 20 mM (MPA)

GNR solution- 1.5 ml

Procedure

Five ml of GNR solution was taken and centrifuged for 30 min at 15000 rpm. The supernatant was discarded and the rod-pellet was dispersed in water. From the centrifuged solution 1.5 ml of gold nanorods were taken and 40 µl of MPA was added. The mixture of solution was kept for ultra-sonication for 30 min at 50°C. The mixture was centrifuged for 30 min at 13,000 rpm. The MPA modified GNRs were then dissolved in sterile distilled water.

3.5.6 Preparation of GNR-Antibody Conjugates

For functionalization of MPA modified GNRs, covalent binding of antibody to GNRs was done by using EDC/NHS (1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) linking chemistry was applied, where a carboxylic acid group attached to the gold nanorods was covalently bound to the amine group of antibody. The procedure followed was based on Song *et al.* (2013) with slight modifications. The concentration of EDC and NHS combination used was 7.5 mM and 1.5 mM respectively.

A) Reagents used

EDC- 7.5 mM

NHS- 1.5 mM

MPA modified gold nanorods- 1.5 ml

Procedure

1.5 ml of MPA modified GNRs were taken in 2 ml Eppendorf tube. Hundred µl of mixture of EDC/NHS was added to this tube. The solution was incubated for 1 h under constant sonication at 50°C.100 µl of antibody at dilutions (1:200) was added to this solution and kept for 2 h incubation for the specific binding. Then 30 min of sonication was given in order to remove non specific binding. GNRs were blocked by washing with 0.02 per cent BSA and the product was re-suspended in PBS buffer (Annexure II). GNR-Antibody conjugates were characterized by UV-Vis spectrophotometer.

3.5.7 Application of GNR Based Nanobiosensors for the Detection of Virus

3.5.7.1 Isolation of Antigen

Isolation of antigen is the initial step for the detection of CMV infected plants. Antigen was isolated by grinding the infected leaf samples with liquid nitrogen in pre-chilled mortar and pestle. The pulverized tissues were then mixed with 1X coating buffer (Annexure II). The homogenate was centrifuged at 15000 rpm for 10 min at 4°C. Finally supernatant was mixed with equal volume of acetone and kept at 4°C for precipitation of protein. The precipated protein was collected and suspended in suspension buffer.

3.5.7.2 Detection of the virus antigen through GNRs Nanobiosensors

One ml GNR solution based nanobiosensors was taken in 2 ml eppendorf tubes and 100 µl of antigen was used for the GNR probe-antigen interaction in order to detect the virus. After the addition of antigen to the GNRs nanobiosensor solution, it was incubated for 45 minutes. After incubation the interaction of GNRs nanobiosensor solution and antigen was characterized by using UV-Vis spectrophotometer. The spectrum absorbed by the interaction of GNR probe-antigen was recorded from range 200 to 1100 nm. From the generated spectral graph the peak shifts were monitored and analyzed.

3.5.8 Determination of GNRs Sensitivity to Virus Antigen

3.5.8.1 Quantification and Preparation of Different Concentrations of Antigen

Isolated antigen was quantified by nanodrop spectrophotometer based on absorbance $A_{260/280}$ ratio and was serially diluted at eight different concentrations. The eight different concentrations used to determine the sensitivity of virus detection were viz., 6, 3, 1.5, 0.75, 0.38, 0.19, 0.09 and 0.04 mg/ml.

3.5.8.2 Detection of GNRs Sensitivity to Different Antigen Concentration

To determine the sensitivity of the detection technique using GNR solution based nanobiosensors, eight different concentration viz., 6, 3, 1.5, 0.75, 0.38, 0.19, 0.09 and

0.04 mg/ml were taken and added to 1 ml of GNR solution in eight different 2 ml centrifuge tubes respectively. These were Incubated for 1h to allow probe specific target binding to reach to an equilibrium stage, which led to aggregation of gold nanorods and as well as change in the colour of the solution. Characterization was done using UV-Vis spectrophotometer and absorption spectra between 200 to 1100 nm wavelengths were analyzed for corresponding peak shifts.

Results

VI. RESULTS

The results of the investigations carried out on "Molecular characterization of virus causing infectious chlorosis disease of banana" during 2014-2017 are presented in this chapter.

4.1 BIOLOGICAL CHARACTERISATION OF THE VIRUS

4.1.1 Survey, Collection and Maintenance of Virus Culture

4.1.1.1 Survey

During 2015-2017, purposive sampling surveys were carried out in farmer's fields in different banana growing areas of Thrissur district of Kerala *viz.,* Cherpu, Chirakkal, Keezhpullikkam, Thanyam, Peringottukara, Kannara, Marakkal, Mannuthy, Kandanissery, Wadakkancherry, Pudukkad, Adatt, Engandiyoor, Wadeshari, Thalikulam, Vellangallur, Vellanikkara, Erimapetti, Mala and Meloor. A total of 28 different fields were surveyed and per cent disease incidence was recorded (Table 13). The predominant varieties cultivated in the surveyed fields were Chengalikodan Nendran (AAB), Robusta (AAA), Karpooravalli (ABB), and Grand Naine (AAA). The samples were also collected from infected plants from the surveyed locations which were and brought to the laboratory and kept in -20°C deep freezer for the confirmation of infection and for further studies.

4.1.1.2 Disease Incidence

The disease incidence in the surveyed locations ranged from 2 to 90 per cent. Among the 28 fields surveyed, maximum disease incidence (90%) was recorded in Thalikulam area on banana variety Robusta (AAA) followed by Karpooravalli (ABB) at Kannara (63.3%) and Nendran (AAB) variety at Mannuthy (28.7%). An incidence of 0.6 per cent was recorded on banana var. Grand Naine (AAA) at Kannara while disease incidence was not observed in fifteen locations. With regard to aphid colonization, there was infestation of aphids on banana plants in six locations *viz.,* Cherpu, Chirakkal, Peringottukara, Kannara, Mannuthy and Thalikulam.

Table 13: Details of survey conducted in different locations of Thrissur district, Kerala

Sl. No.	Location	Cultivar	Stage of the crop *	Mean Per cent disease incidence	Association of Aphid +/-
1	Cherpu	Robusta	H	2.0	+
2	Chirakkal	Nendran	F	0.0	+
3	Keezhpullikkam	Karpooravalli	V	0.0	-
4	Thanyam	Robusta	V	0.0	-
5	Peringottukara	Nendran	V	0.0	+
6	Kannara plot 1	Amritsagar	F	3.3	-
7	Kannara plot 2	Pelipita	V	2.5	-
8	Kannara plot 3	Grand naine	V	0.6	+
9	Kannara plot 4	Robusta	F	0.0	-
10	Kannara plot 5	Nendran	V	0.0	-
11	Kannara plot 6	Karpooravalli	V	63.3	-
12	Marakkal	Changalikodan Nendran	V	0.0	-
13	Mannuthy	Nendran	V	28.7	+
14	Kandanissery Plot 1	Nendran	V	0.0	-
15	Kandanissery Plot 2	Nendran	F	0.0	-
16	Wadakkancherry Plot 1	Changalikodan Nendran	F	0.0	-
17	Wadakkancherry Plot 2	Robusta	F	1.0	-
18	Pudukkad	Changalikodan Nendran	V	0.0	-
19	Adatt	Changalikodan Nendran	V	0.0	-
20	Engandiyoor	Changalikodan	F	0.0	-
21	Wadeshari Plot 1	Nendran	V	0.0	-
22	Wadeshari Plot 2	Changalikodan Nendran	V	0.0	-
23	Thallikullum	Robusta	V	90.0	+
24	Vellangallur	Nendran	V	4.0	-
25	Vellanikkara	Changalikodan Nendran	F	0.0	-
26	Erimapetti	Nendran	F	0.0	-
27	Mala	Robusta	V	0.0	-
28	Meloor	Robusta	F	0.0	-

*V= Vegetative stage, F= Flowering stage and H= Harvest stage

The disease was predominantly recorded on banana varieties Nendran and Robusta and the highest incidence was recorded on Robusta variety in Thalikulam which is a coastal area where there was severe infestation of aphids also.

4.1.1.3 Collection and Maintenance of Virus Culture

The infected plants maintained at BRS, Kannara under insect proof net house served as one of the sources of inoculum for further studies. The young plants which showed symptoms of the disease in different locations were uprooted and planted in growbags and these were maintained separately in insect proof net house at BRS, Kannara, and Department of Plant Pathology, College of Horticulture, Vellanikkara. Total of 20 isolates from five different banana cultivars *viz.,* Karpooravalli (ABB), Robusta (AAA), Nendran (AAB), Amritsagar (AAA) and Grand-Naine (AAA) were maintained (Plate 4).

4.1.2 Symptomatology

The symptoms of the disease expressed on different parts of banana plant *viz.,* leaf lamina, midrib, petiole, pseudostem and bunches were recorded under natural and artificial conditions.

4.1.2.1 Symptoms under Natural Conditions

4.1.2.1.1 Symptoms on Leaf Lamina

Different types of symptoms were noticed on leaf lamina under natural conditions.

Type I:

Symptoms appeared as pale yellow or chlorotic discontinuous linear mosaic streaks on the leaves running parallel to the veins from midrib towards the leaf margin. These symptoms were more pronounced on younger leaves. (Plate 5, Fig. A-D; Plate 7, Fig. B).

Plate 4: Maintenance of Virus culture- Location: BRS,Kannara

Type II:

Prominent spindle or diamond shaped chlorotic lesions were produced on leaf lamina. These spots were with yellow coloured margin and the centre of lesions remained green measuring about 1-2 cm in length and 0.3-0.5 cm width initially. These chlorotic lesions later enlarged linearly extending from midrib towards leaf margin with size varying from 1-15 cm x 1-1.5 cm length and width respectively (Plate 6a, Fig. A, B and C).

Type III:

Another type of symptom was the appearance of chlorotic large sized irregular patches which later became necrotic. Upward rolling of leaves was noticed. Young emerging leaves were with distorted leaf lamina with marginal necrosis and these necrotic spots appeared to be slender measuring about 3.4 cm length and 0.2-0.4 cm width (Plate 6a, Fig. D and E). Such leaves were thick and brittle in texture. Older leaves tend to droop and crack from the point of necrotic lesions and torn off.

Type IV:

The new emerging leaves were distorted and curled upward and in some cases the new leaves were with reduced leaf lamina leaving only midrib giving the appearance of whip (Plate 8a, Fig. A). These types of symptoms are seen on severely infected plants.

Type V:

. The leaves were distorted with yellow coloured chlorotic linear lesions running from midrib to the edge of leaf lamina which gave the appearance of green islets between the yellow chlorotic linear lesions (Plate 9, Fig. A, C, D and E). Young emerging leaves from such plants were found to curl upward and become 'saucer' shaped (Plate 9, Fig. C). On such leaves marginal necrosis and cracking of leaf lamina were also observed. The young leaves were deformed and tapered towards the leaf apex. Severe distortion of leaf narrowed down the area of leaf lamina giving an appearance of 'Strap leaf' (Plate 9, Fig. F). The plants were stunted and this type of symptoms observed on banana var. Robusta (AAA).

4.1.2.1.2 Symptoms on Midrib

The midrib of leaves of infected plants appeared purplish in colour. Such purple discolouration was more prominent towards the base of the leaf. Colour of the midrib at distal end of the leaves turned light greenish pink. Such symptoms were more pronounced on banana var. Karpooravalli (ABB). Dark longitudinal lines were seen on the lower surface of midrib (Plate 5, Fig. E).

4.1.2.1.3 Symptoms on Bunches

Infected plants produced bunches with abnormally elongated peduncle which became 'S' shaped in some cases (Plate 6b, Fig. F). Such peduncles were slender when compared to healthy ones. The bunch size was reduced. With less number of hands and fingers compared to healthy bunches. The size of the fingers was also drastically reduced with irregular arrangement (Plate 6b, Fig. F). These fingers were distorted and comma shaped (Plate 6b, Fig. H). Severe infection failed to produce bunches as in the case Nendran (AAB) and Robusta (AAA).

4.1.2.2 Varietal Variation of Symptom Expression

The visible symptoms of infectious chlorosis disease were observed on five accessions of banana which were maintained in the germplasm and also in different varietal plots at BRS, Kannara and Agricultural Research Station, Mannuthy. The various symptoms observed on each variety are described below.

4.1.2.2.1 Symptoms on Banana var. Karpooravalli

Type I symptom described above was observed on banana var. Karpooravalli. Initial symptoms observed on leaf lamina as small pale yellow or chlorotic broken streaks (Plate 5. Fig. B) giving the appearance of mild mosaic streaks. Later chlorotic mosaic symptoms developed on the leaves. These chlorotic mosaic streaks run from the mid-rib to the leaf margin parallel to the veins (Plate 5, Fig. C). Mid-rib turned purplish-black in colour (Plate 5, Fig. D and E). However, the symptoms were masked in the later stages. The yield was not affected.

4.1.2.2.2 Symptoms on Banana var. Grand Naine

In the case of banana var. Grand Naine type II and III symptoms were noticed. First symptoms observed were prominent diffused spindle or diamond shaped lesions on the leaf lamina (Plate 6a, Fig. A). These spindled shaped patterns greatly varied in size measuring about 1-15 cm length and 1-2 cm width (Plate 6a, Fig. C). As the plant growth advanced, those patterns got diffused but the newly emerging heart leaves were with distorted leaf lamina showing marginal necrosis and necrotic spots on the leaf lamina (Plate 6a, Fig. D and E). Mild mosaic and linear necrotic lesions measuring about 3.4 cm in length and 0.4 to 0.7 cm in width were also observed on younger leaves (Plate 6a, Fig. A and B).

Infected plants produced small sized bunches when compared to healthy plants (Plate 6b, Fig. E and F). The peduncle of the infected bunches were elongated abnormally, sometimes become curved and become 'S-shape'. There was also reduction in number of hands and the fingers (Plate 6b, Fig. F). The fingers were distorted, comma shaped and small (Plate 6b, Fig. G and H).

4.1.2.2.3 Symptoms on Banana variety Amritsagar

The symptoms produced on banana variety Amritsagar were type I and type III symptoms. Appearance of spindled shaped chlorotic lesions, mosaic and leaf distortion were the major symptoms. The leaves were thick and brittle in texture (Plate 7, Fig. A). Chlorotic mosaic spots or dotted line like linear lesions were also observed on top 2-3 leaves at the flowering stage. The chlorotic mosaic patterns were found to initiate at the midrib and extended towards the margin of the leaf lamina (Plate 7, Fig. B). These chlorotic patterns when observed closely appeared to be spindled shaped with tapering ends measuring about 1-1.5 cm length and 0.2-0.3 cm width and the centre of the lesion remained green (Plate 7, Fig. C). There was no significant difference in the bunch size healthy and infected plants.

4.1.2.2.4 Symptoms on Banana variety Nendran

Type III and IV symptoms were produced on leaves of banana var. Nendran. Small spindled shaped chlorotic lesions which had yellow margin pointed with tapering ends. These lesions were with yellow margin and the central portion remained green measuring about 2.5 cm and 0.3-0.5 cm length and width respectively (Plate 8a, Fig. C).

The leaves showed severe upward curling. This type of symptom was mostly observed 4 months after planting. At six months after planting, the plants produced severe leaf distortion symptom and the leaf lamina was extremely reduced retaining only midrib giving the leaf a whip like appearance. (Plate 8a, Fig. A).

The new emerging leaves were found to be severely deformed, curled upward with chlorotic spindle pattern on the leaf lamina. There was breaking of the leaf lamina from the midrib extending towards the margins of leaf blade (Plate 8a, Fig. E). These plants remained stunted (Plate 8a, Fig. B) and did not flower. Those flowered, produced small sized bunches. In case of late infection, symptoms of chlorotic spindled shaped patterns and reduced leaf area were observed.

The bunches of infected plants were with abnormally elongated 'S shaped' peduncle and produced less number of hands and fingers compared to healthy bunches. (Plate 8b, Fig. A, B and C). The fingers greatly varied in size and shape (Plate 8b, Fig. B and D). During the initial stage of finger formation, some of the fingers produced small green pin head lesion on its peel (Plate 8b, Fig. D). These small pin head lesion turned into necrotic lesions in the later stages of bunch formation. Finger associated with necrotic lesions never developed further and remained pencil sized and erect in a perpendicular fashion (Plate 8b, Fig. E). Such fingers were completely deformed and comma shaped. (Plate 8b, Fig. D and E). The impact of the disease in the agronomic yield characteristics of the variety are discussed in section 4.1.2.5.

4.1.2.2.5 Symptoms on Banana variety Robusta

Severe chlorotic linear lesions running from midrib towards the margin of leaf blade (Plate 9, Fig. A). These lesions were either generally distributed over the leaf lamina or developed in bands. The plants which were severely infected were showing dropping and buckling of younger leaves (Plate 9, Fig. B). The plants with such symptoms produced new young leaves which displayed a greenish yellow mottled appearance and severely distorted. The chlorotic linear lesions present on the leaf lamina were running from midrib towards the margin of leaves. These types of symptoms were visible on both side and the upper surface of the leaves tends to curl upward and became 'saucer' shaped. (Plate 9, Fig. C) Marginal necrosis and cracking of leaf lamina from the margin of leaves towards the midrib were also observed. In advance stage of the disease, severe distortion was observed (Plate 9, Fig. D). The leaves with green island symptoms exhibited wedge shaped appearance with sharp tapering ends (Plate 9, Fig. E). The plants with all the above symptoms produced new leaves which were deformed, thick and brittle in texture and narrowed with drastic reduction in size. Such leaves looked like strap with extreme reduction in leaf lamina sometimes leaving only midrib (Plate 9, Fig. F).

4.1.2.3 Symptoms under Artificial Conditions

Symptoms developed under artificial conditions were studied through insect transmission of virus on tissue culture plants of Nendran variety as explained in section (4.1.4.3). The initial symptoms were expressed on test plants by transmission *P. nigronervosa* and *A. craccivora* on 35-40 and 55-64 days of inoculation. The new emerging leaves produced the small linear chlorotic lesions which enlarged with the expansion of leaf lamina (Plate 12, Fig. A). Severe malformation was observed on one side of the leaf lamina which reduced the leaf size drastically (Plate 12, Fig. B and D). Linear chlorotic lesions appeared on the leaves and which were found more towards the leaf margin. Gradually the lesions broadened and developed into chlorotic band like mosaic patches on the leaves (Plate 12, Fig. C). As the disease advanced, there was

reduction in leaf lamina (Plate 12, Fig. D). There was inward rolling of leaves and the plants were stunted (Plate 12, Fig. E).

4.1.2.4 Histological Changes due to Viral Infection

An attempt was made to study the histological changes in foliar tissue due to infection of the pathogen. The microscopic observations of thin sections of infected and healthy leaves of banana variety Nendran revealed changes in the anatomy of the tissues due to virus infection. Comparative analysis was done by taking thin sections of healthy and infected leaf samples of banana using microtome. The healthy leaf samples were with intact palisade, spongy and air space layers with definite epidermis and stomata. Sections of diseased leaves exhibited spatial arrangement of tissues which were disrupted. The development of paramural abnormal tissue between the regular parenchymatous cells with black inclusion bodies was seen in the diseased plant. The comparison of healthy and infected tissue are given in (Table 14, Plate 10a and 10b).

Table 14: Histological changes due to viral infection

Tissue	Healthy	Diseased
Epidermis	Single layered, longitudinally elongated parenchyma with almost similar shape with prominent cuticle in outer wall	Single layered, irregularly shaped parenchyma with interrupted cuticle
Mesophyll parenchyma	Air chambers with prominent septa. Septum formed by circular 1-2 layered parenchyma cells. Some regions regularly arranged radially elongated rectangular parenchymatous (3-4 layers)	Air chambers present. Septum somewhat disorganized. Intracellular depositions seen in cells. Arrangement of layers irregular
Mesophyll Chlorenchyma	1-2 layers of parenchyma cells with rich chloroplast	chloroplast were less in number
Parenchyma, Vascular tissues	Well organized circular parenchyma cells. Air chambers were regular in outline. Chloroplast abundant in cells. Vascular tissues were well organized with prominent metaxylem and phloem	Disorganized parenchyma cells with less number of chloroplast. Dark depositions present in cells. Vascular tissue malformed in some regions.

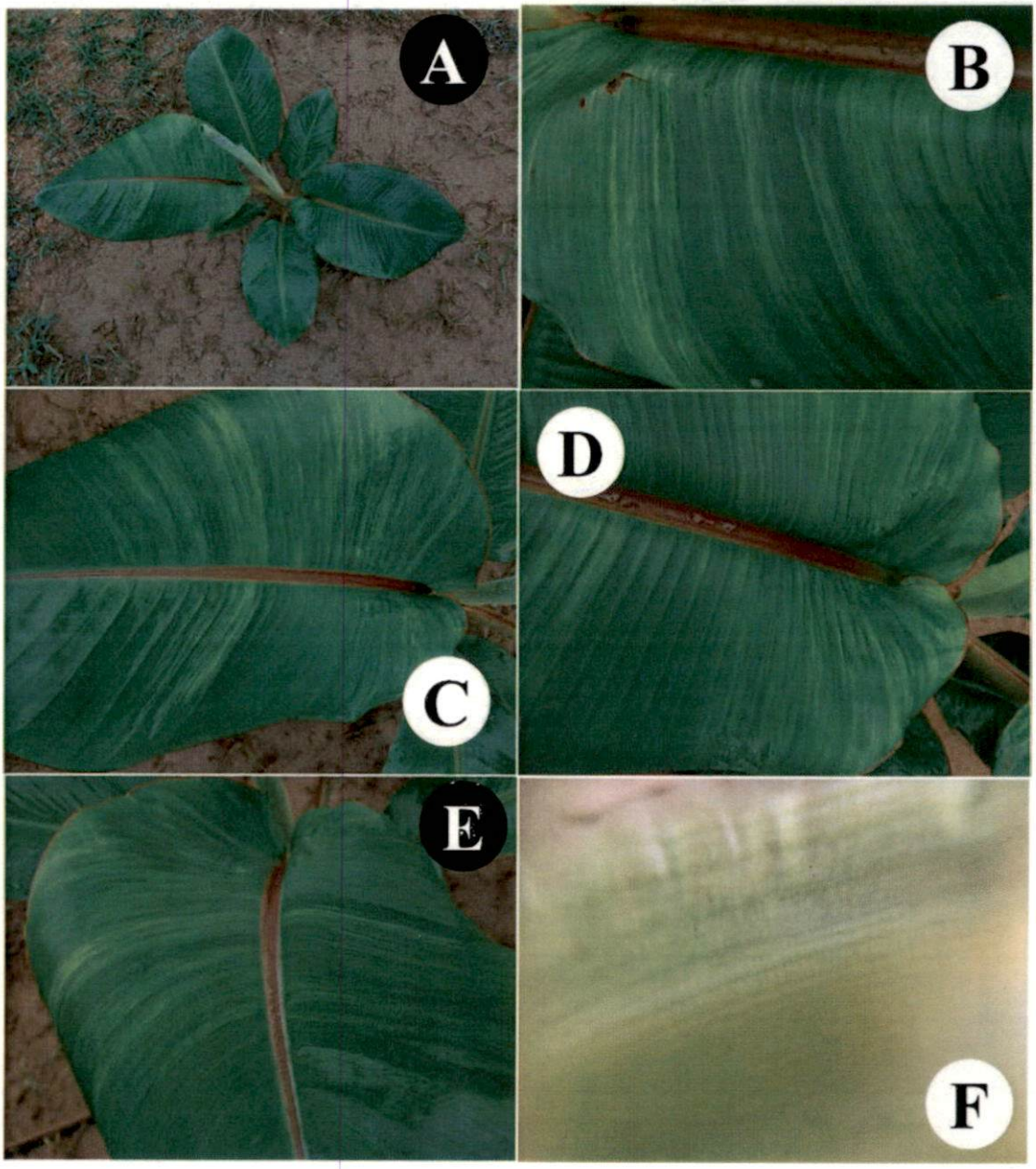


Plate 5: Symptoms of infectious chlorosis disease on banana var. Karpooravalli (A) Infected plant (B) Initial symptoms: Small pale yellow broken streaks (C) Chlorotic mosaic symptoms (D) Leaf blade near petiole region is severely distorted (E) Chlorotic mosaic streaks run continuously from the mid-rib towards leaf margin (F) Dark purplish discoloured mid-rib

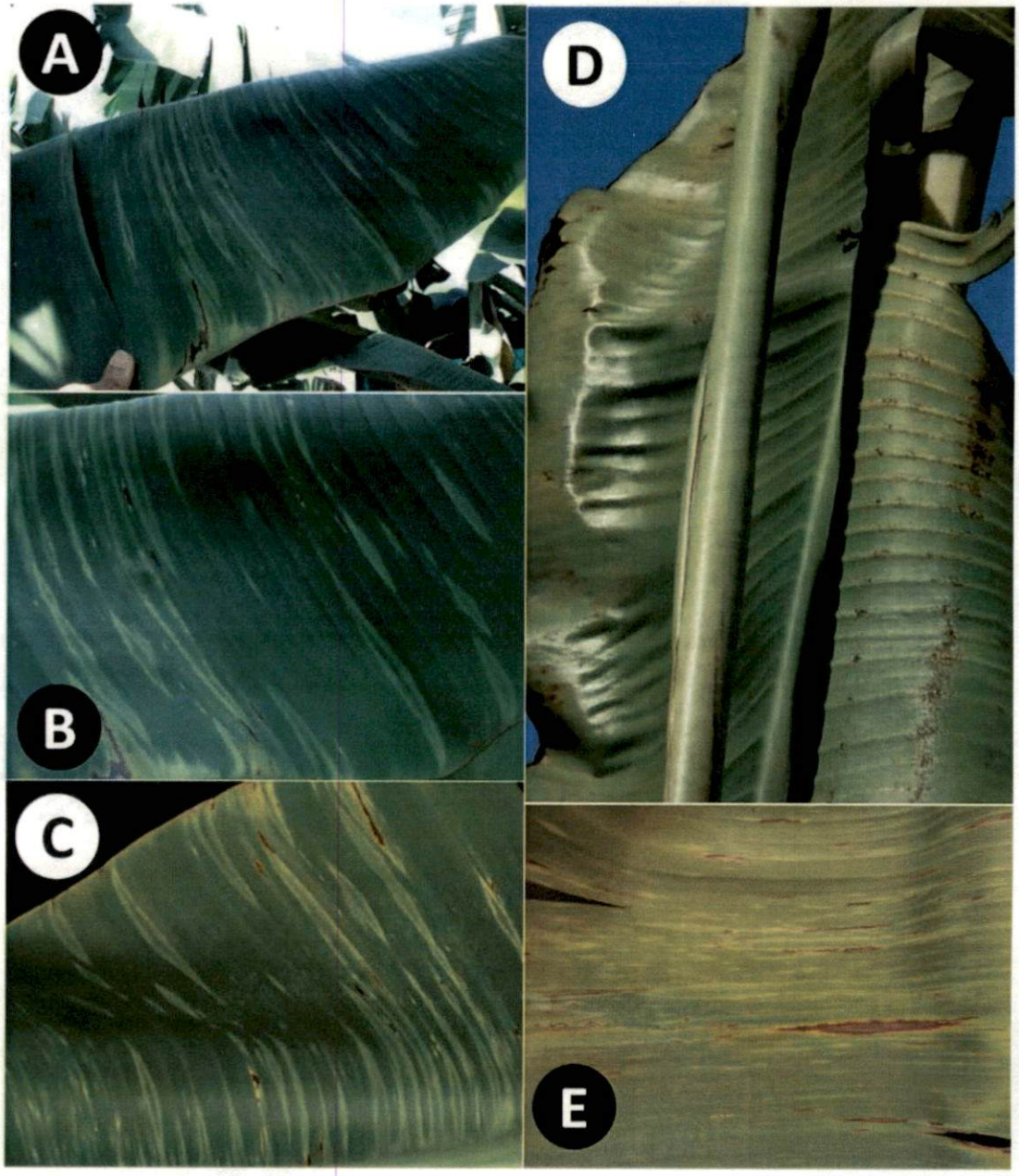


Plate 6a: Symptoms of infectious chlorosis disease on banana var. Grand Naine (A) Initial symptoms: prominent diffused spindle shaped lesions on the leaf lamina (B-C) Spindle shaped pattern on leaf lamina extending from mid-rib to the margin of leaf blade (D) Distorted leaf lamina with marginal necrosis and necrotic spots (E) slender linear necrotic lesions

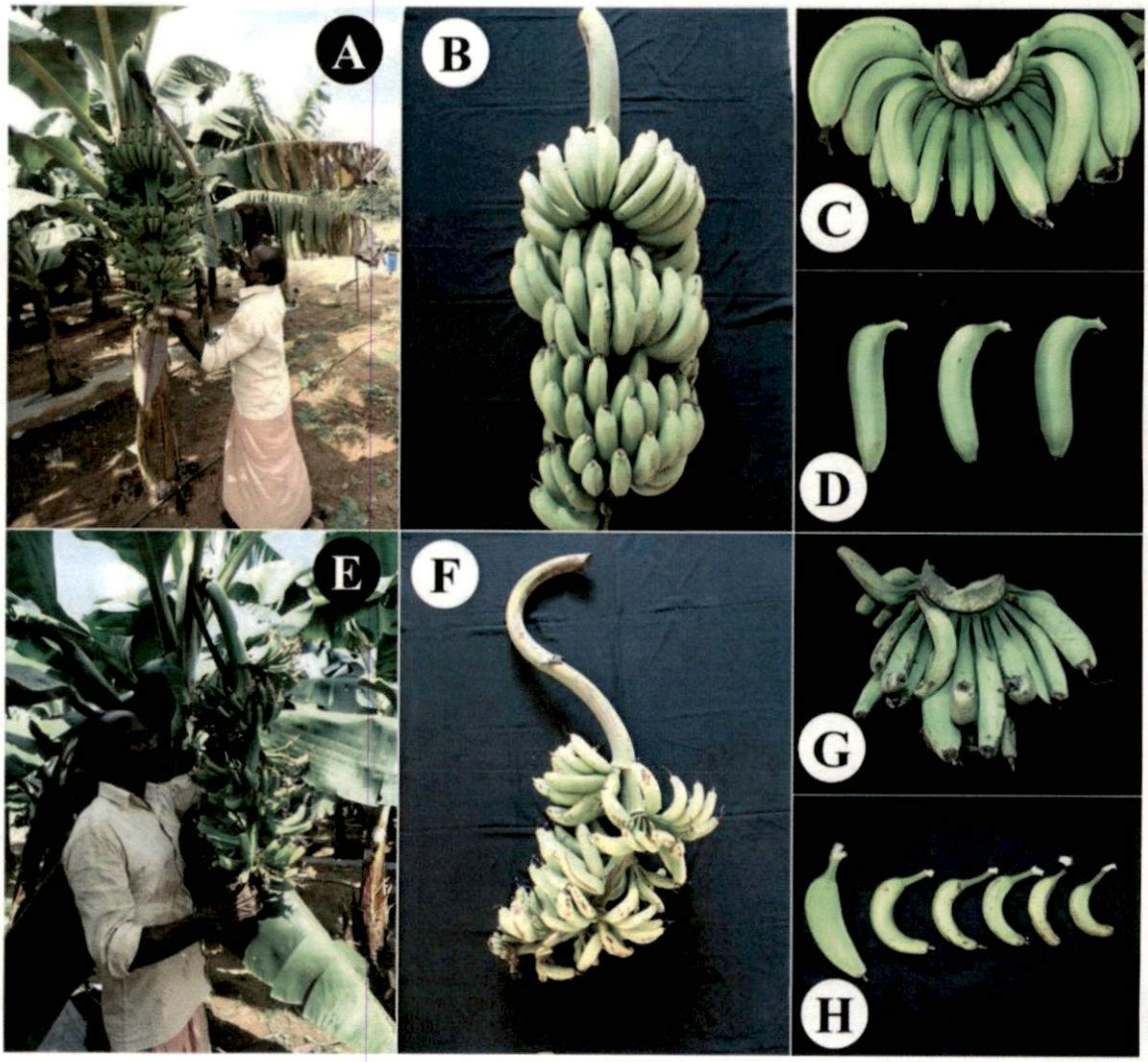


Plate 6b: Symptoms of infectious chlorosis disease on banana var. Grand Naine: (A and B) Healthy bunch, (C) Hands from healthy plant (D) Fingers from healthy plant, (E) CMV infected bunch in field conditions (F) Affected bunch with S-shaped peduncle (G) Hands from diseased plant showing distorted finger, (H) Variation in size of fingers from CMV infected plant

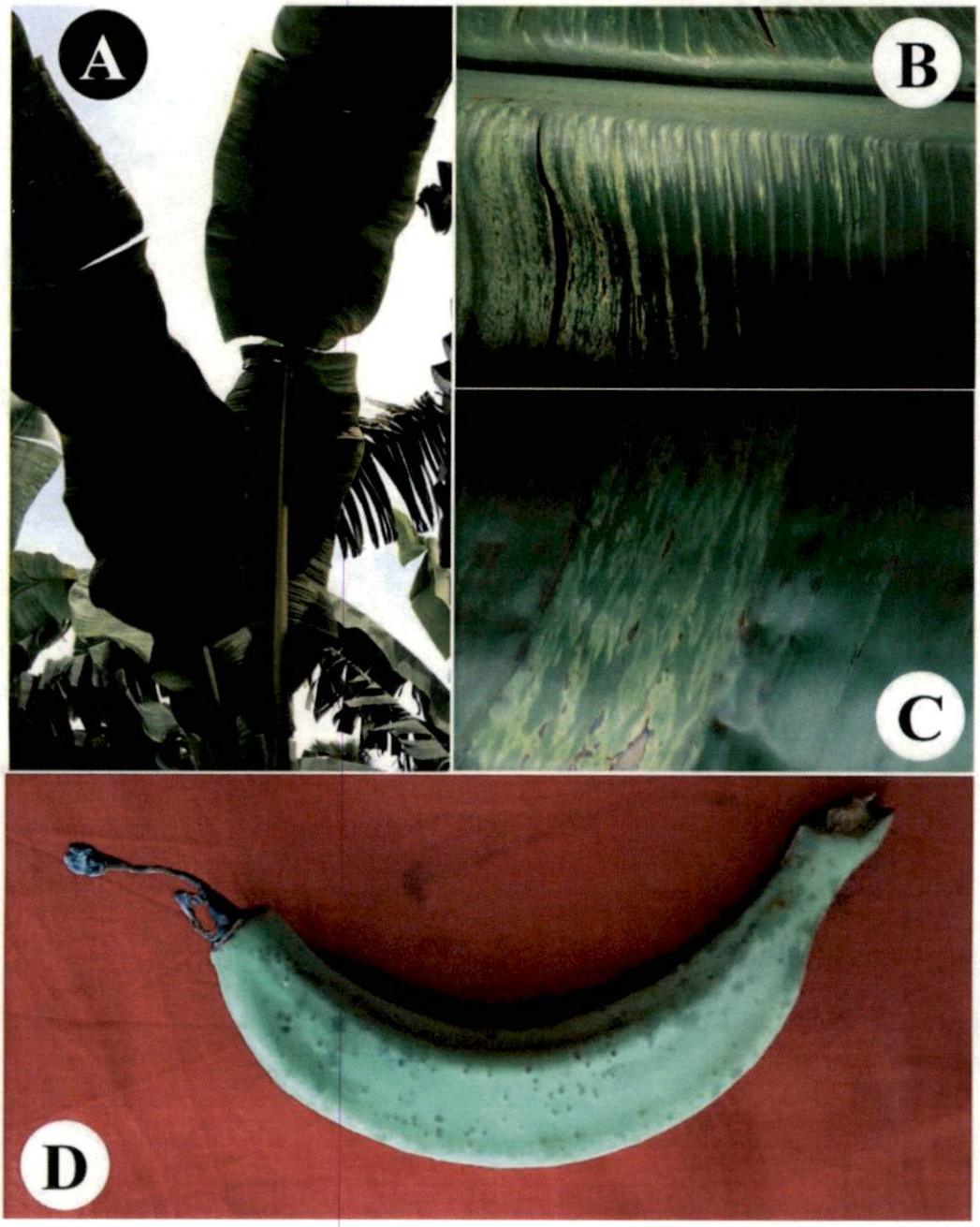


Plate 7: Symptoms of infectious chlorosis disease on banana var. Amritsagar: (A) Distant view of distorted leaves which are thick and brittle (B) Chlorotic lesions (C) spindled shaped chlorosis with tapering ends (D) pin head necrotic symptoms on fingers

Plate 8a: Symptoms of infectious chlorosis disease on banana var. Nendran: (A) Upward curling (B) Stunted plants (C) Spindle shaped chlorotic lesion (D) Chlorotic lesion associated with necrosis extending from its origin to both sides

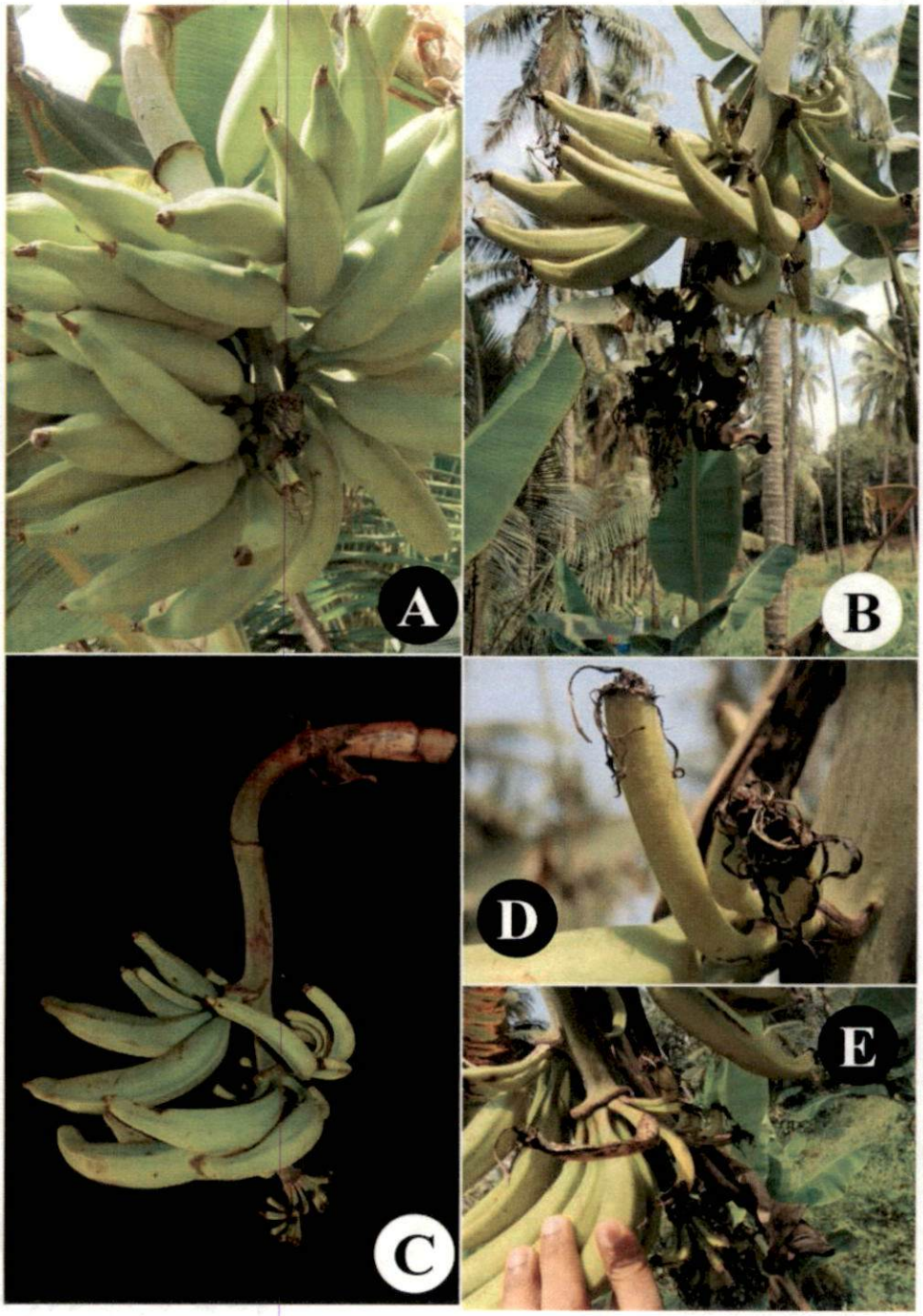


Plate 8b: Symptoms of infectious chlorosis disease on banana var. Nendran: (A) Healthy bunch (B) Deformed bunch from infected plant (C) peduncle (D) Fingers with pin head lesion on its peel (E) Fingers with necrotic lesions

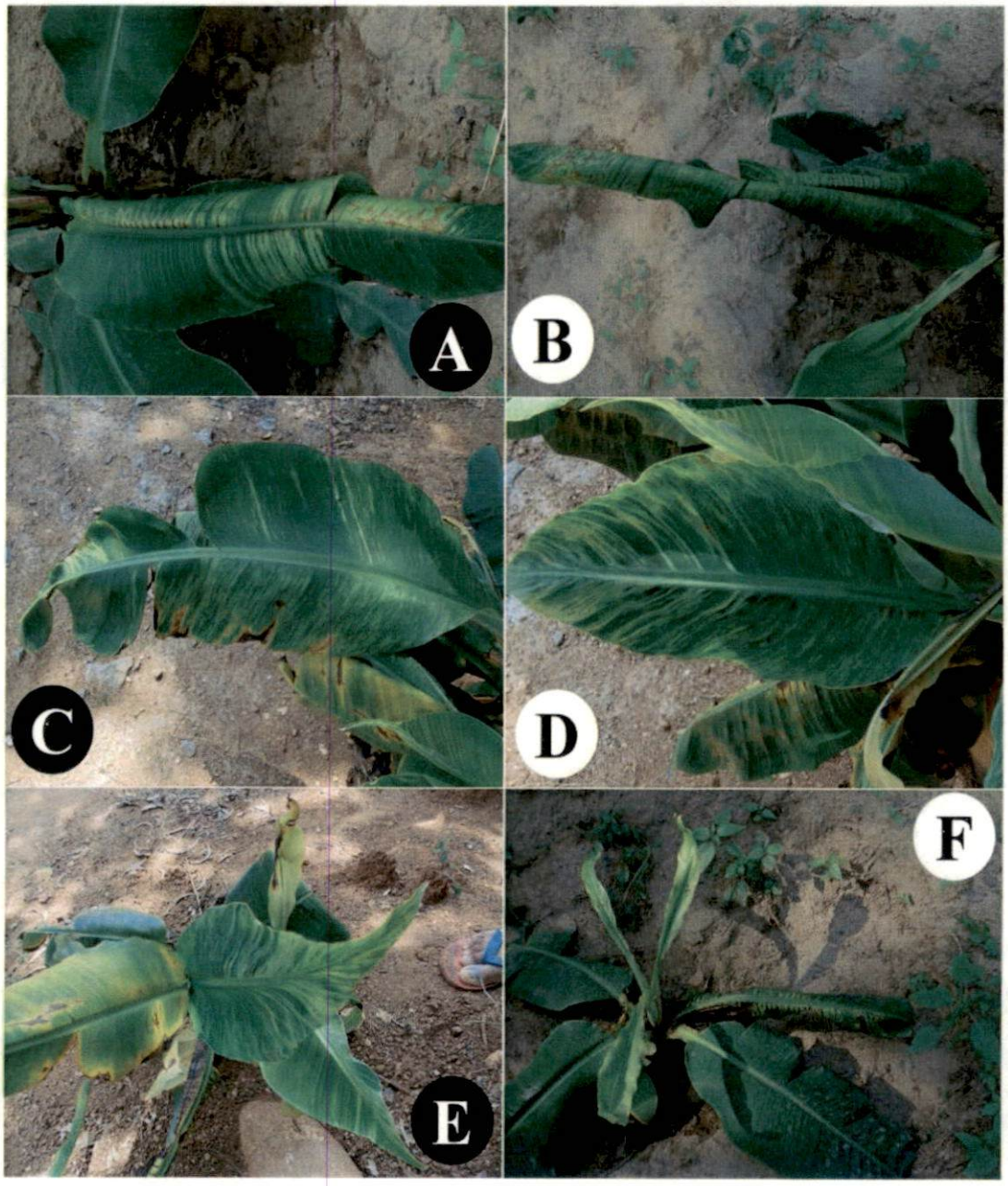
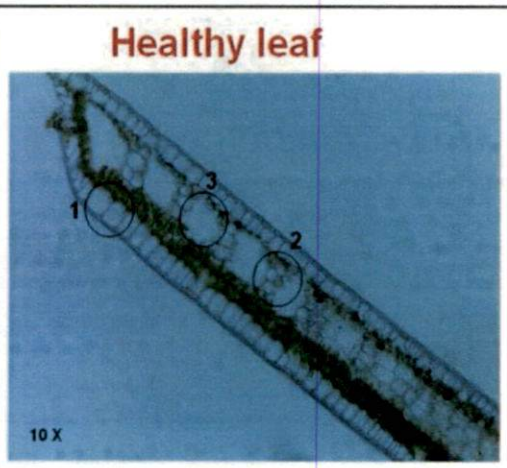
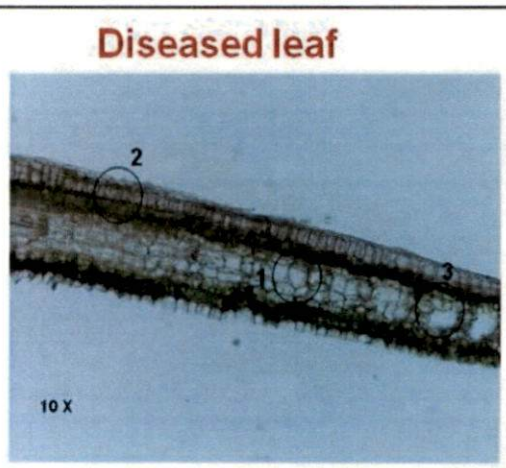


Plate 9: Symptoms of infectious chlorosis disease on banana var. Robusta: (A) Chlorotic linear lesions running from midrib towards the edge of leaf blade (B) Buckling of young leaves (C) leaves curled upward and form 'saucer' like (D) Green islets appearance (E) Malformed leaves appearance with tapering ends (F) Strap appearance.

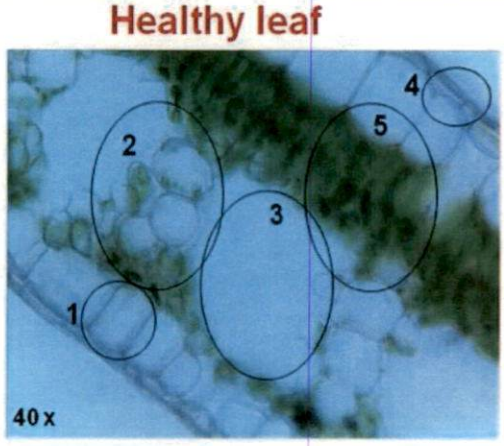


1. Longitudinal parenchyma
2. Uniform septum
3. Prominent air chambers
4. Epidermis with prominent cuticle

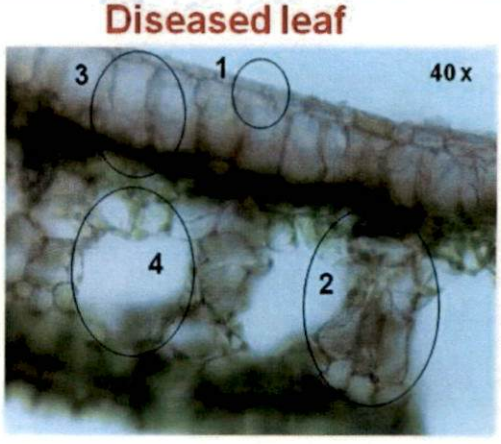


1. Disorganized septum
2. Irregular shaped parenchyma
3. Disorganized air chambers

A. 10 X magnification



1. Longitudinal parenchyma
2. Uniform spetum
3. Prominent air chambers
4. Epidermis with prominent cuticle
5. 1-2 Layers of Parenchyma cells with rich chloroplast



1. Interrupted cuticle
2. Disorganized septum
3. Irregular shaped parenchyma
4. Disorganized air chambers

B. 40 X magnification

Plate 10a. Histopathological changes in leaf anatomy due to CMV infection

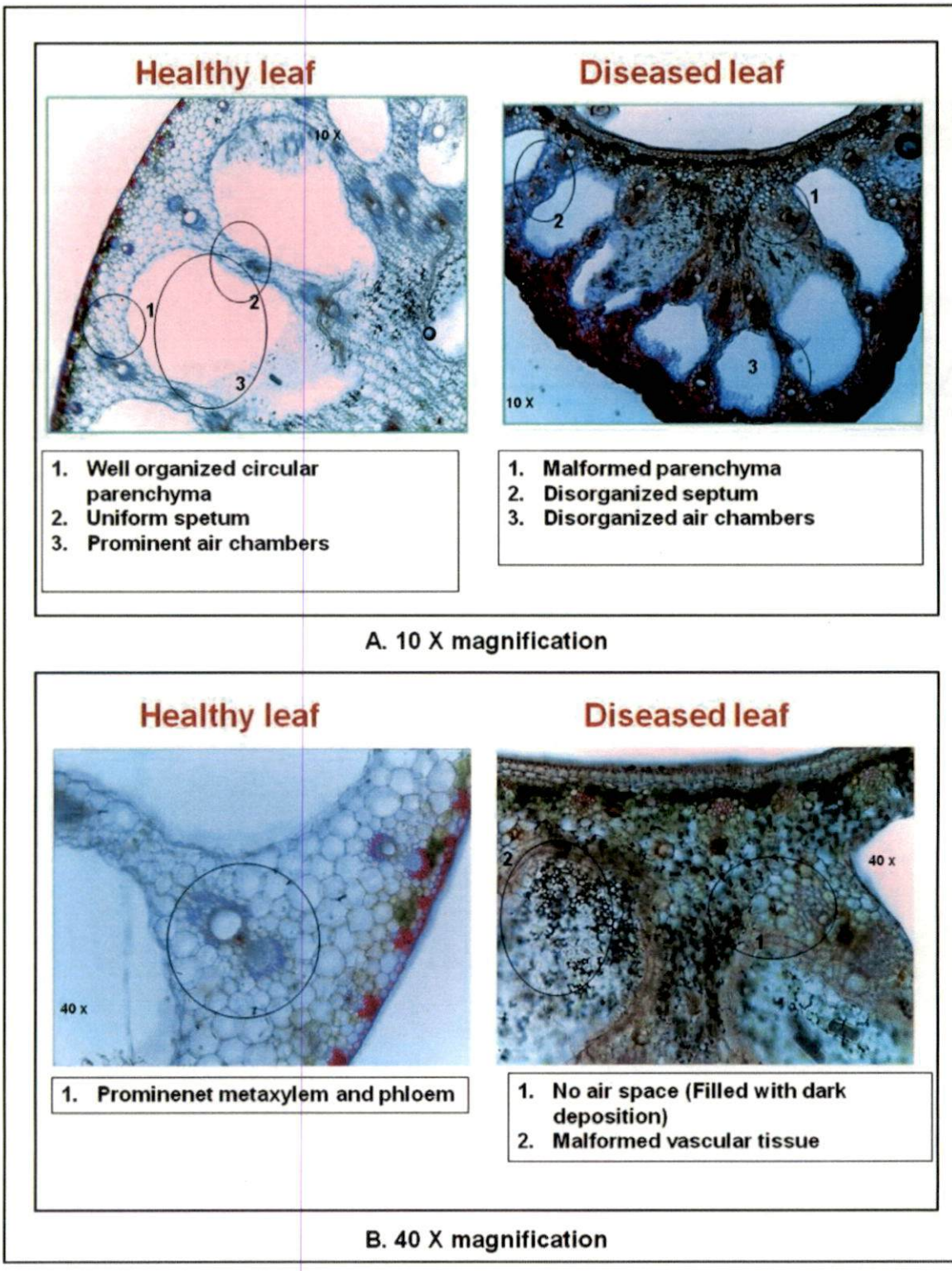


Plate 10b. Histopathological changes in the midrib anatomy due to CMV infection

4.1.2.5 Impact of the Disease on Agronomic and Yield Characters of Banana

The impacts of viral infection on the growth characters such as height of the plant, girth of pseudostem, number of leaves, leaf area, chlorophyll content, bunch weight, number of hands and fingers per bunch, finger weight, length of finger, and finger girth of different varieties of banana *viz.,* Karpooravalli (ABB), Amritsagar (AAA), Nendran (AAB), and Grand Naine (AAA) were studied and the results are presented below.

4.1.2.5.1 Height of the Plant

The mean values of the height of the plant of different varieties at shooting stage are given in (Table 15a.). The results showed that the plant height was affected due to virus infection. The per cent reduction in plant height was maximum in the variety Nendran (35.7) and lowest in Amritsagar (10.9).

4.1.2.5.2 Girth of Pseudostem

In the case of pseudostem girth, the results revealed that the virus infection adversely affected the girth of the plant (Table 15a.). Maximum per cent reduction was recorded in Nendran (AAB) (42.3) and lowest of (1.6) was recorded in the variety Grand Naine (AAA).

4.1.2.5.3 Number of Leaves

The number of functional leaves also was affected due to virus infection and maximum reduction was observed on variety Nendran (Table 15b.). The per cent reduction in number of leaves were 8.3, 11.5, 17.7 and 22.7 respectively on varieties like Karpooravalli (ABB), Amritsagar (AAA), Grand Naine (AAA) and Nendran (AAB) respectively.

4.1.2.5.4 Leaf Area

Leaf area reduction was observed in infected plants when compared to healthy plants (Table 15b.). The per cent reduction was 10.9, 20.1, 37.6 and 58.4 per cent

respectively on varieties Karpooravalli (ABB), Amritsagar (AAA), Grand Naine (AAA) and Nendran (AAB) respectively.

4.1.2.5.5 Chlorophyll Content

Reduction in chlorophyll content of leaves was observed due to infection. The per cent reduction in chlorophyll content was 45.7, 39.1, 34.1 and 1.6 per cent respectively on varieties Karpooravalli (ABB), Grand Naine (AAA), Nendran (AAB) and Amritsagar (AAA) respectively (Table 15b.).

4.1.2.5.6 Bunch Weight

Yield reduction was observed due to virus infection. The maximum per cent reduction in bunch weight due to infection was 83.4 in Nendran (AAB) followed by Grand Naine (AAA) (40%) and the least reduction was recorded in Amritsagar (AAA) and Karpooravalli (ABB) 12.1 and 8.4 per cent respectively (Table 16a.).

4.1.2.5.7 Number of Hands

Reduction in number of hands/bunch was recorded in different banana varieties due to virus infection. The per cent reduction in number of hands due to infection was 20, 19, 7.3 and 3.7 respectively on banana varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB) (Table 16a.).

4.1.2.5.8 Number of Fingers per Bunch

The number of fingers per bunch was also reduced. The per cent reduction was 47.4, 36, 16.3 and 5.6 respectively in banana varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB) (Table 16a.).

Table 15a. Effect of virus infection on biometric characters of banana

Banana Variety	*Height (cm)			*Girth (cm)		
	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction
Karpporavalli (ABB)	2.93	2.29	21.7	90.00	83.5	7.2
Amritsagar (AAA)	2.50	2.23	10.9	55.90	50.8	9.1
Nedu-Nendran (AAB)	4.98	3.20	35.7	49.14	28.3	42.3
Grand Nanine (AAA)	2.37	1.66	30.2	61.38	60.4	1.6

*Mean of 5 replications

Table 15b. Effect of virus infection on leaf parameters

Banana Variety	*Number of leaves			*Leaf area (m2)			*Chlorophyll content (nmol/cm2)		
	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction
Karpporavalli (ABB)	10.00	9.17	8.3	1.80	1.60	10.9	43.39	42.70	1.6
Amritsagar (AAA)	10.40	9.20	11.5	1.02	0.81	20.1	47.54	25.82	45.7
Nedu-Nendran (AAB)	9.80	7.57	22.7	2.08	0.87	58.4	54.72	33.33	39.1
Grand Nanine (AAA)	11.38	9.38	17.7	2.23	1.39	37.6	44.49	29.31	34.1

*Mean of 5 replications

Table 16a. Effect of virus infection on yield attributes

Banana Variety	*Bunch weight (Kg)			*Number of hands per bunch			*Number of fingers per bunch		
	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction
Karpporavalli (ABB)	26.60	24.35	8.4	9.00	8.67	3.7	130.00	122.67	5.6
Amritsagar (AAA)	9.45	8.31	12.1	8.20	7.60	7.3	98.40	82.40	16.3
Nedu-Nendran (AAB)	7.18	1.19	83.4	5.00	4.00	20.0	49.40	26.00	47.4
Grand Nanine (AAA)	18.85	11.31	40.0	10.33	8.38	19.0	156.33	100.13	36.0

***Mean of 5 replications**

Table 16b. Effect of virus infection on fruit characters

Banana Variety	Fruit weight (gm)			Lenth of Finger (cm)			Girth of finger (cm)		
	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction	(a) Healthy	(b) Infected	Per cent reduction
Karpporavalli (ABB)	275.75	245.00	11.2	14.25	12.33	13.5	16.23	16.01	1.3
Amritsagar (AAA)	165.20	154.40	6.5	17.41	15.04	13.6	17.78	15.60	12.2
Nedu-Nendran (AAB)	213.20	80.75	62.1	24.40	11.13	54.4	14.66	8.72	40.5
Grand Nanine (AAA)	196.00	139.70	28.7	17.64	12.58	28.7	19.62	12.16	38.0

***Mean of 5 replications**

4.1.2.5.9 Fruit Weight

There was reduction in fruit weight due to virus infection. Maximum per cent reduction (62.1) was observed in Nendran (AAB) (62.1) and the lowest in Amritsagar (AAA) (6.5) (Table 16b.).

4.1.2.5.10 Finger Size

Virus infection had negative influence on the length of the finger. The maximum per cent reduction was observed in Nendran (AAB) (54.4), Grand Naine (AAA) (28.7) and Amritsagar (AAA) (13.6) and the lowest was observed in Karpooravalli (ABB) (13.5) (Table 15b.). In the case of finger girth also, there was reduction due to virus infection. Results showed that the per cent reduction was 40.5, 38, 12.2 and 1.3 respectively in banana varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB). Nendran (AAB) recorded the highest per cent reduction and Karpooravalli (ABB) as lowest in the per cent reduction in length and girth of finger (Table 16b.).

4.1.3 Host Range Studies

In the present investigation, 29 plants belonging to 11 families were tested for their susceptibility to CMV through mechanical inoculation under insect proof net house conditions. Out of these, only 19 plant species expressed visible symptoms. Type of symptoms expressed, total number of days required for symptom development are presented in Table 17. The infection was further confirmed through ELISA test and also by back inoculation to indicator plant, cowpea.

The host plants like *Solanum melongena, Vigna unguiculata* and *Centrosema pubescens* produced local symptoms like necrotic local lesion and *Amaranthus polygamus* exhibited both local and systemic symptom *viz.,* pin hole necrotic lesion and mottling. Other symptoms produced by the host plants were yellowing, vein clearing, leaf distortion, stunting, mosaic, mottling, necrotic lesion, downward curling, puckering,

shoe string symptom, blisters, veinal necrosis, and cupping of leaves. The symptoms produced by each plant species are presented in Table 17 and Plate 11.

Among the different hosts, only *V. unguiculata, S. melongena* and *Centrosema pubescens* developed localized symptoms. *V. unguiculata* produced symptoms of necrotic local lesion within 48 h (Plate 11a, Fig. A). The local symptoms were also produced by *C. pubescens* and *S. melongenum* and the incubation period was 3 and 7 days respectively (Plate 11a, Fig. B and C). The per cent virus transmission on these hosts *viz. V. unguiculata, S. melongenum* and *C. pubescens* were 100, 60 and 80 per cent respectively (Table 17).

In the case of *A. polygamus* all inoculated plants initially produced local symptoms within 5 days after inoculation and symptom were appearance of necrotic lesion and the newly emerged leaves produced systemic symptoms which were expressed as mild leaf mottling (Plate 11a, Fig. D and E).

Symptoms like interveinal yellowing, vein clearing and mosaic symptoms were developed on *M. charantia*. Downward curling of leaves was expressed within 18 days followed by severe puckering and leaf deformation which developed 40 days after inoculation (Plate 11a, Fig. F-H). Yellowing and vein clearing symptoms were observed on *B. hispida* with 22 per cent transmission (Plate 11b, Fig. A and B). In case of *Lagenaria siceraria,* systemic symptoms like interveinal chlorosis was produced 20 days after inoculation with 44 per cent transmission (Plate 11b, Fig. C, D and E). Subsequently the infected leaves became reduced in size. *Cucumis sativus* (Plate 11b, Fig. F-H) and *Trichosanthes cucumerina* (Plate 11b, Fig. I) also developed symptoms like interveinal chlorosis and vein clearing which were developed within 11 and 15 days in *C. sativus* and *T. cucumerina* respectively with 44 and 22 per cent transmission.

Table 17: Host range of virus causing banana infectious chlorosis disease.

Family	Host	Symptoms	Incubation period (Days after inoculation)		Confirmation of infectivity		No. of Plants inoculated	No. of Plants infected	Per cent transmission
			Local	Systemic	Back innoculation	ELISA			
Amaranthaceae	*Amaranthus polygamus*	NL , Mot	5	20	P	+ VE	5	5	100
	Gomphrina Globosa	ns	-	-	N	- VE	10	0	0.00
Cucurbitaceae	*Momordica charantia*	Y, M, DC, P	-	18	P	+ VE	9	3	33.33
	Benincasa hispida	Y, VC	-	24	P	+ VE	9	2	22.22
	Lagenaria siceraria	M, Y	-	20	N	+ VE	9	4	44.00
	Cucumis anguria	ns	-	-	ND	- VE	5	0	0.00
	Cucumis sativus	VC, Y, M	-	11	P	+ VE	9	4	44.44
	Trichosanthes cucumerina	VC, Y, M	-	15	P	+ VE	9	2	22.22
Solanaceae	*Solanum melongena*	NL	7	-	-	+ VE	5	3	60.00
	Capsicum annuum	M, P, DC, FFL	-	8	-	+ VE	9	7	77.77
	Nicotiana tabacum	M, Y	-	10	P	+ VE	6	5	83.33
	N glutinosa	B, DC, S, LD, M	-	13	-	+ VE	10	9	90.00
	N benthamiana	B, P, LD, M, FFL, Mot	-	18	-	+ VE	10	9	90.00

Y = Yellowing, VC = Vein Clearing, LD= Leaf distortion, S= Stunting, M= Mosaic, Mot=Mottling, NL = Necrotic lesion, DC = Downward curling, P = Puckering, FFL= Filiform, B= Blister, VN= Veinal necrosis, St= striations, YM= Yellow mosaic, ns = no symptoms.

table 17 continued

Family	Host	Symptoms	Incubation period (days after inoculation)		Virus recorvery		No. of Plants inoculated	No. of Plants infected	Per cent transmission
			Local	Systemic	Back innoculation	ELISA			
Fabaceae	*Vigna unguiculata*	NLL	2	-	-	+ VE	9	9	100
	Centrosema pubescens	NLL	3	-	-	-	5	4	80
	Canavalia gladiata	B, Y, VC	-	2	-	+ VE	5	4	80
Zingiberaceae	*Zingiber officinale*	ns	-	-	-	- VE	10	0	0
	Curcuma longa	ns	-	-	-	- VE	10	0	0
Heliconiaceae	*Heliconia platystachys*	Y, M	-	20	-	+ VE	10	2	20
Cannaceae	*Canna indica*	TN, Y, M	-	68	-	+ VE	10	6	60
Asteraceae	*Synedrella nodiflora*	Y, Mot, C, S, LD	-	14	-	+ VE	10	7	70
	Sphagneticola trilobata	ns	-	-	-	- VE	5	0	0
	Acmella radicanss	M, Mot, LD	-	15	-	+ VE	9	7	77.77
Composite	*Tridax procumbens*	ns	-	-	-	+ VE	5	0	0
Coomelinaceae	*Commelina benghalensis*	ns	-	-	-	+ VE	5	0	0
Apocynaceae	*Ichnocarpus frutescens*	DC, M, P, LD	-	14	-	+ VE	5	4	80
Phyllanthaceae	*Phyllanthus amara*	M	-	6	-	+ VE	5	2	66.66
Onagraceae	*Ludwigia hyssopifolia*	ns	-	-	-	- VE	5	0	0
Verbenaceae	*Stachytarpheta indica*	ns	-	-	-	- VE	5	0	0

Y = Yellowing, VC = Vein Clearing, LD= Leaf distortion, S= Stunting, M= Mosaic, Mot=Mottling, NL = Necrotic lesion, DC = Downward curling, P = Puckering, FFL= Filiform, B= Blister, VN= Veinal necrosis, St= striations, YM= Yellow mosaic, TN= Tip necrosis, C= Cupping.

Capsicum annuum exhibited mosaic symptom within 8 days after inoculation and later developed puckering and downward curling of leaves (Plate 11b, Fig. J, K and L). As the disease advanced, the leaves become narrow with filliform tips.

The three different species of *Nicotiana, viz., N. tabacum, N. glutinosa and N. benthamiana* produced mosaic and yellowing symptoms within 10 days with 83 per cent of transmission in *N. tabacum* (Plate 11c. Fig. A and B) and 90 per cent infection was *While N. glutinosa* and *N. benthamiana*.

The symptom expression on *N. glutinosa,* is about 14 days after virus inoculation the test plants developed initial systemic symptoms like downward curling, mottling and stunting (Plate 11c. Fig. C and D). Leaves were reduced and malformed (Plate 11c. Fig. E). After 40 days the new emerging leaves were reduced in size and leaf tip got invaginated to heart shaped (Plate 11c. Fig. F-H). Necrotic lesions were also visible on new emerging leaves. These lesions later turned to large irregular patches and leaf puckering was also observed (Plate 11c. Fig. I-K). In the later stages symptoms like mosaic, leaf distortion and mottling were seen (Plate 11d. Fig. A and H).

In case of *N. benthamiana,* the initial symptoms appeared 18 days after inoculation with 90 per cent transmission. The inoculated leaves developed light green blisters (Plate 11e. Fig. B and C) which further turned dark green (Plate 11e. Fig. D).

The newly emerged leaves severe blistering, leaf distortion and puckering symptom (Plate 11e. Fig. B and D). The leaf lamina got reduced and leaves turned filiform with mosaic symptom (Plate 11e. Fig. E-H). In the case of *N. tabacum* only mosaic symptom was recorded and the initial symptom was observed 10 days after inoculation of the virus. In the case of *Canavalia gladiata,* blisters were produced on inoculated leaves within 2 days and further symptoms observed were by yellowing (Plate 11f, Fig. C and D) and vein clearing with 80 per cent transmission (Table 17).

Heliconia platystachys showed chlorosis symptom and mosaic symptom after 28 days of inoculation (Plate 11f, Fig. A and B) and per cent transmission was 20 per cent

(Table 17). In case of *Canna indica,* the symptoms were yellowing of leaves, vein clearing and necrosis of leaf tips. The necrotic patch extended to the leaf margins towards the rear end of leaf axil which was coupled with yellowing symptoms (Plate 11g, Fig. A-D). The incubation period was about 68 days (Table 17).

Among the hosts belonging to family *Asteraceae,* weeds like *S. nodiflora and Acmella radicans* developed systemic symptoms, whereas *Sphagneticola trilobata* did not produce any symptoms. The per cent transmission was 70 and 77 per cent and incubation period were 14 and 15 days respectively for *S. nodiflora* and *Acmella radicans*. Initial symptoms in *S. nodiflora* were stunting (Plate 11g, Fig. E) followed by mottling (Plate 11g, Fig. F and G) and cupping (Plate 11g, Fig. H). In case of *Acmella radicans,* yellowing (Plate 11g, Fig. I), mottling (Plate 11g, Fig. J) and severe leaf distortion (Plate 11g, Fig. K) were also observed.

Ichnocarpus frutescens commonly known as black creeper belonging to the family Apocynaceae developed systemic symptom within 14 days of inoculation and recorded 80 per cent transmission. The symptoms were leaf curling and puckering (Plate 11h, Fig. A). New leaves developed mosaic patches (Plate 11h, Fig. B-C). These leaves were distorted with mosaic symptoms (Plate 11h, Fig. D-F). *Phyllanthus amara* belonging to the family Phyllanthaceae produced mosaic symptoms within 6 days of inoculation (Plate 11h, Fig. H-I) and recorded 80 per cent transmission (Table 17).

Among the 29 host species tested, 9 plant species did not develop any visible symptoms. The leaf samples from all the plant species were subjected to ELISA and confirmed the infectivity of the virus. The 9 plant species which did not produce visible symptoms were also subjected to ELISA and confirmed the absence of infectivity of the virus and proved that these species were non-hosts of CMV.

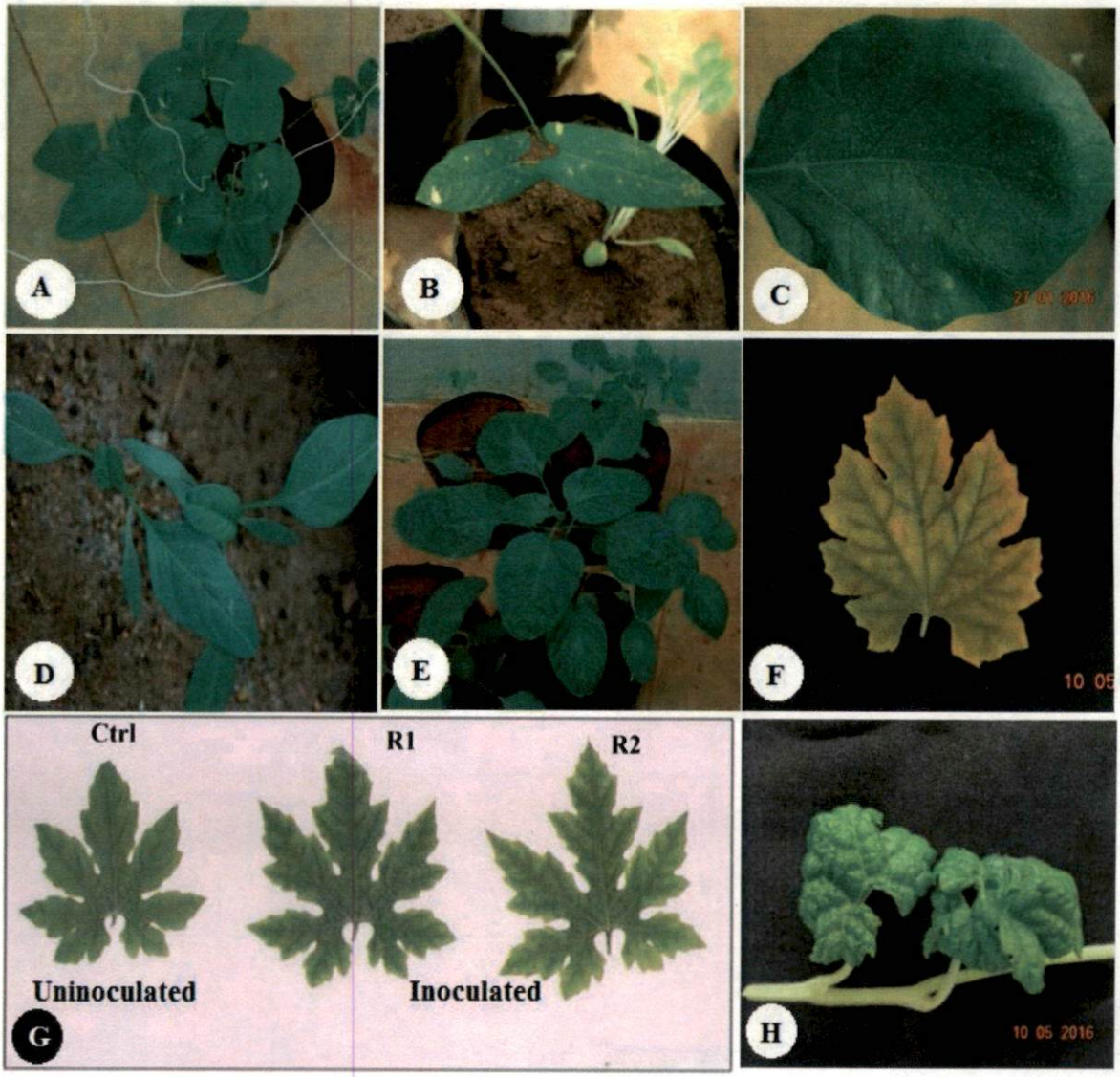


Plate 11a: Host range studies: symptoms caused by CMV (A-D) (A) *Vigna unguiculata* (B) *Centrosema pubescens* (C) *Solanum melongena* (D) *Amaranthus polygamus;* (E) Leaf Mottling on *A. polygamus;* (F) Mosaic symptom of *Momordica charantia* (H) New leaves with severe downward curling and puckering in *M. charantia*

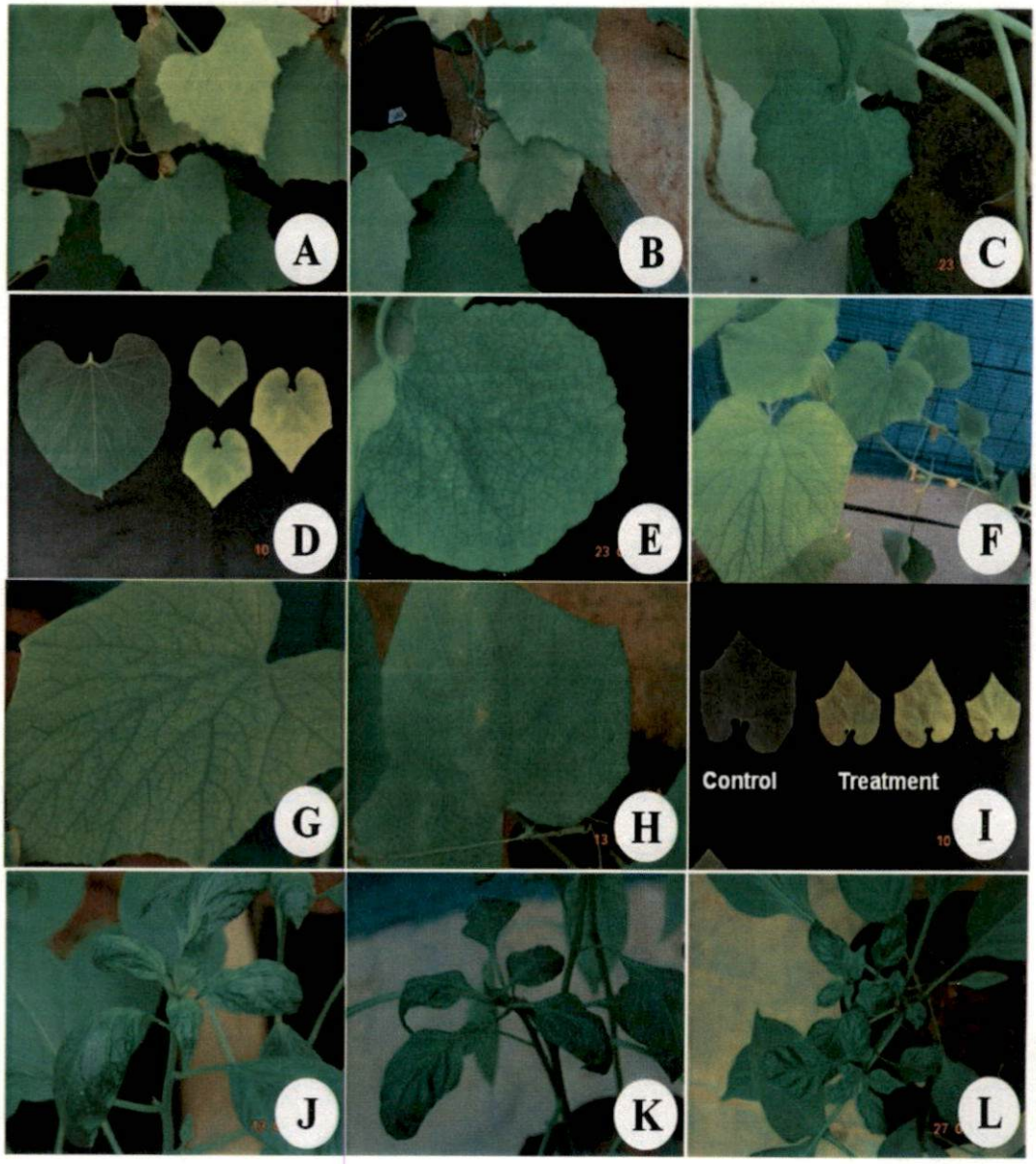


Plate 11b: Host range studies: symptoms caused by CMV (A-B) *Benincasa hispida* (A) Yellowing of *B. hispida,* (B) Vein clearing of *B. hispida, Lagenaria siceraria*, (C) Mosaic symptoms of *Lagenaria siceraria,* (D) Comparison of yellowing symptoms of *L. siceraria,* (E) Closer view of mosaic pattern on *L. siceraria,* (F-H) *Cucumis sativus,* (F) Mosaic and Yellowing symptoms on *C. sativus,* (G) Yellowing symptoms on *C. sativus,* (H) Vein clearing of *Cucumis sativus,* (I) Comparison between uninoculated and inoculated *Trichosanthes cucumerina*, where inoculated leaves show yellowing, Vein clearing and mosaic symptoms, (J-L) *Capsicum annum* L. (J) Downward curling (K) Filliform symptoms (L) Mosaic and puckering

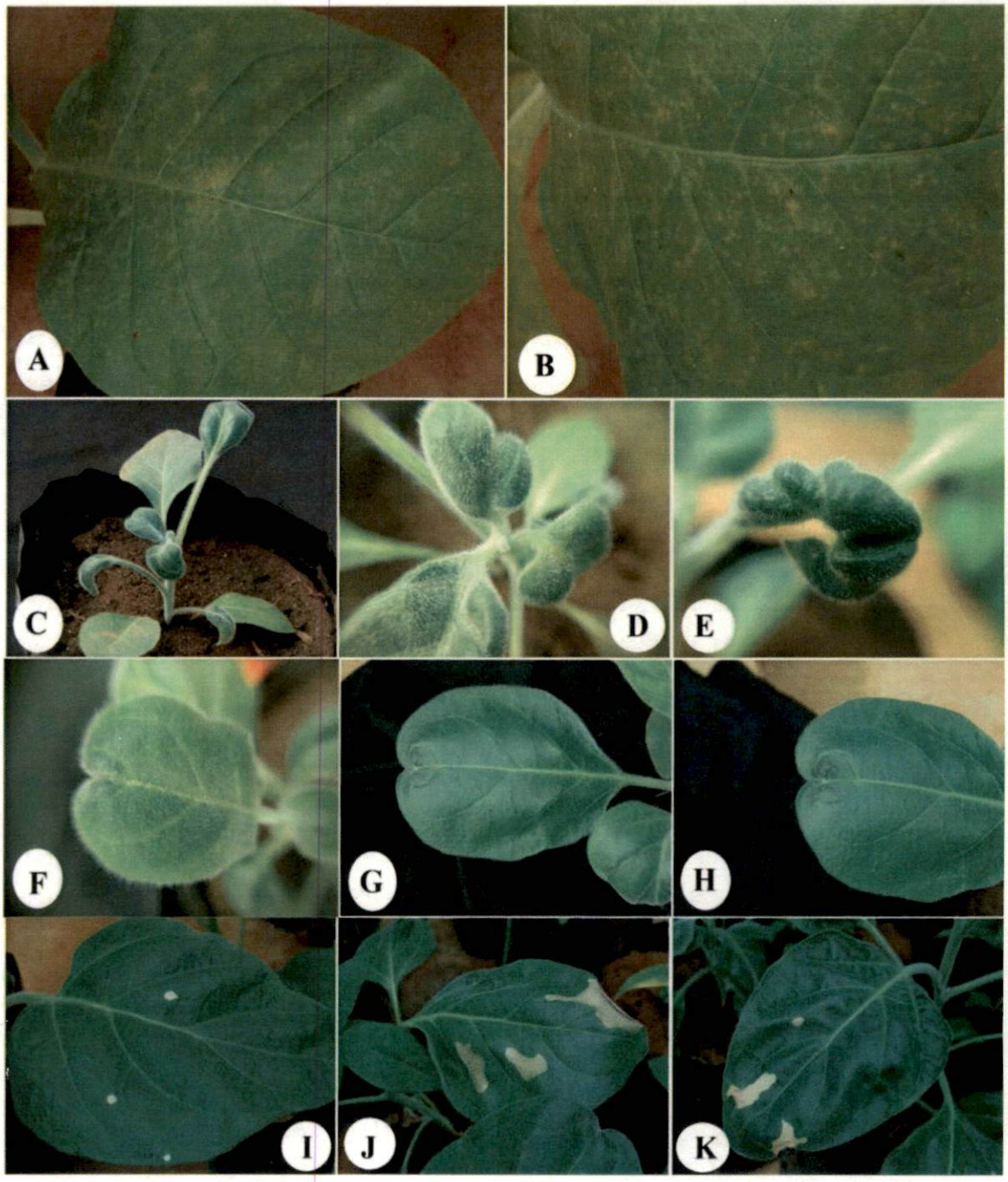


Plate 11c: Host range studies: Systemic symptom on *Nicotiana tabacum and N. glutinosa* (A-B) *N. tabacum* (mosaic and yellowing), (C-K) *N. glutinosa,* (C-E) Stunting, downward curling and mottling, (F-H) Distortion of leaf forming heart shape pattern, (I-J) Developmental stages of necrotic lesion, (K) Necrotic lesions coupled with puckering symptom

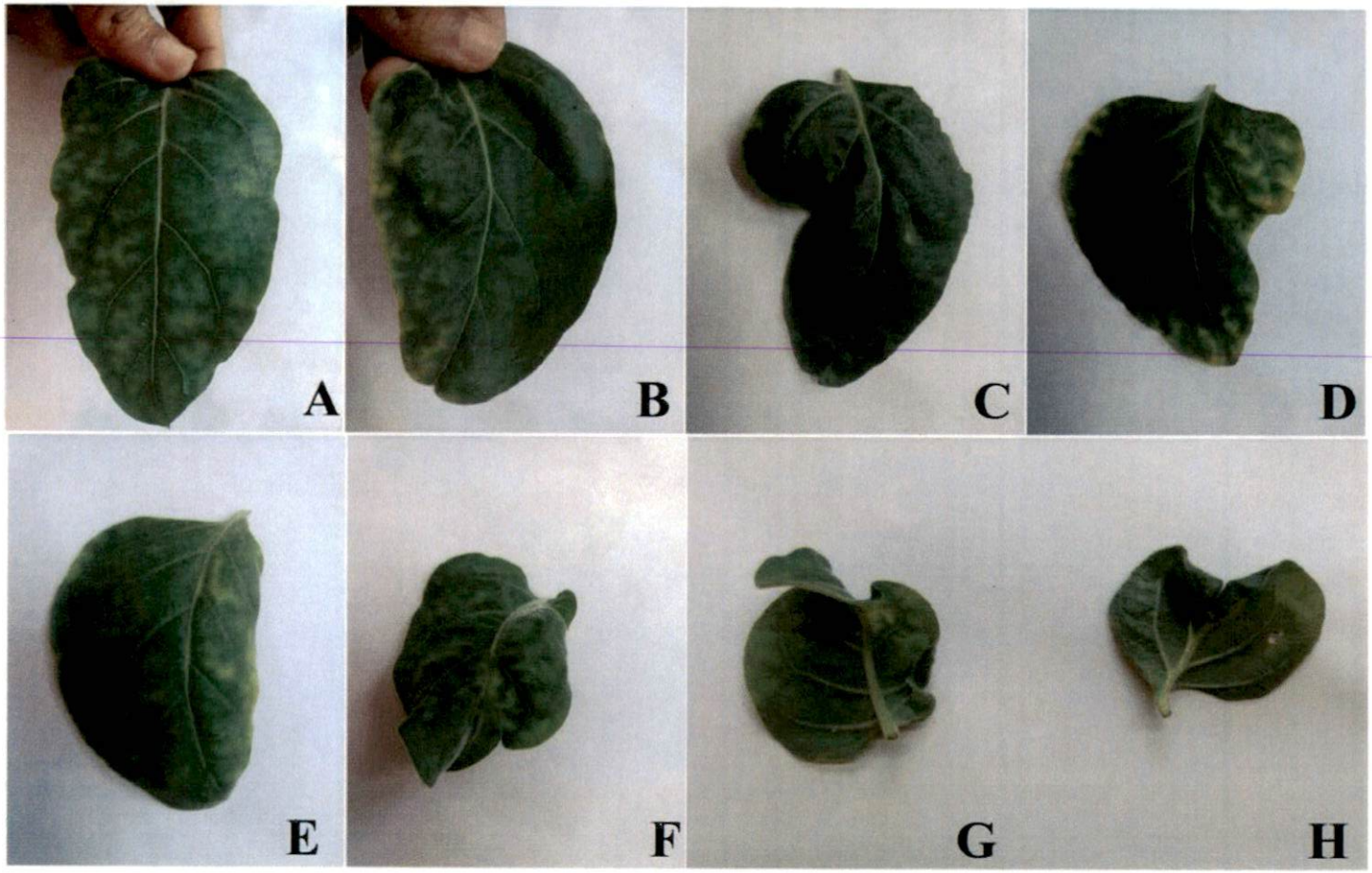


Plate 11d: Host range studies: Development of symptoms on *N. glutinosa.* (A) Mosaic symptoms, (B) leaf distortion and mosaic, (C-E) leaf distortion and mosaic, (F-H) severe mosaic, mottling and leaf distortion symptoms

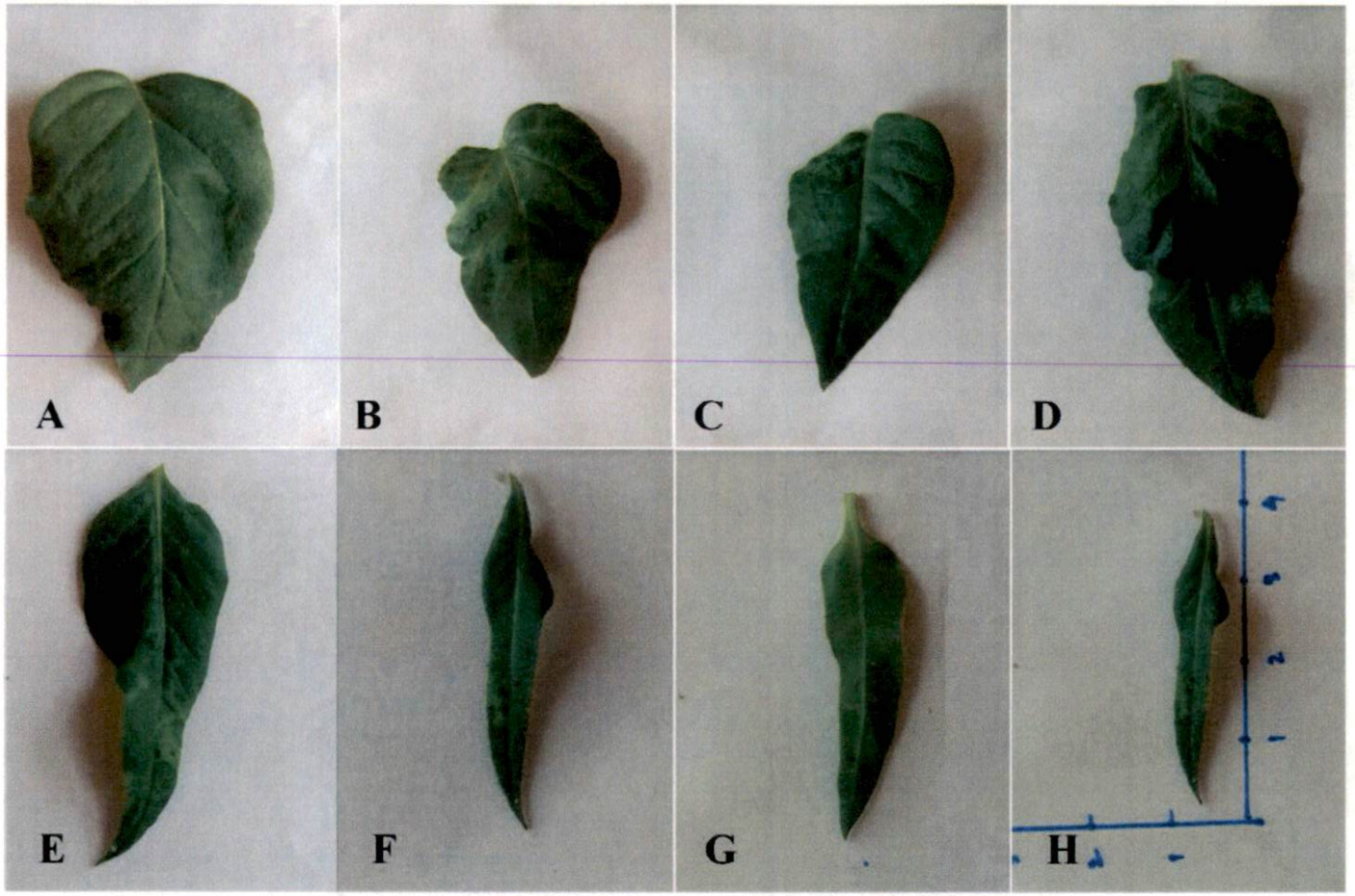


Plate 11e:Host range studies: Development of symptoms on *N. benthamiana*; (A) Distortion of leaves (B) Distortion and blister (C-D) dark green blisters, distorted leaves with puckering symptom (E-F) Initial stage of filli form leaf with mosaic symptoms, (G-H) Filliform leaves

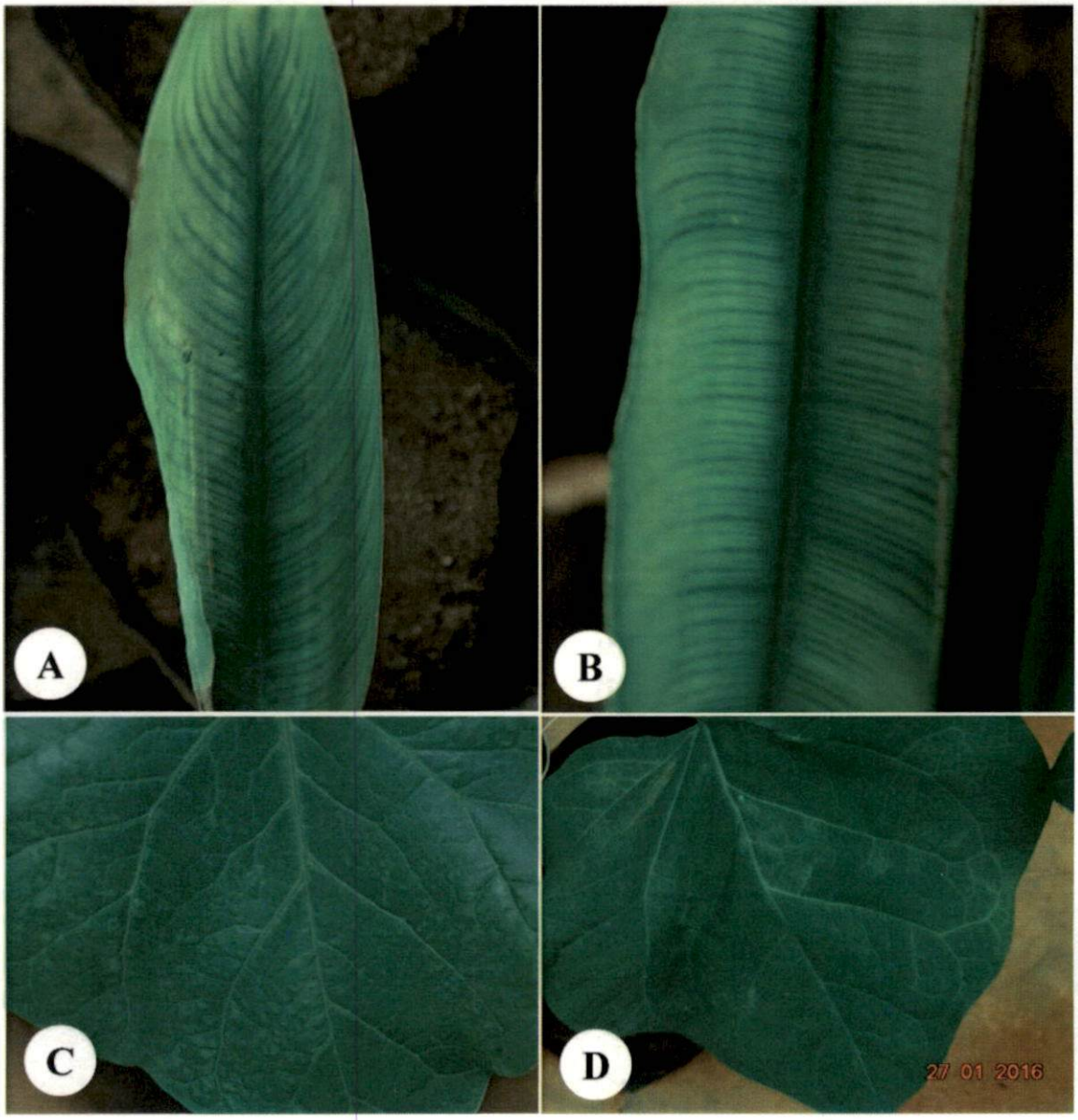


Plate 11f: Host range studies: Symptoms caused by CMV (A and B) *Heliconia platystachys* (A) Yellowing, (B) Mosaic (C and D) *Canavalia gladiata*; (C) Blisters, (D) Vein clearing

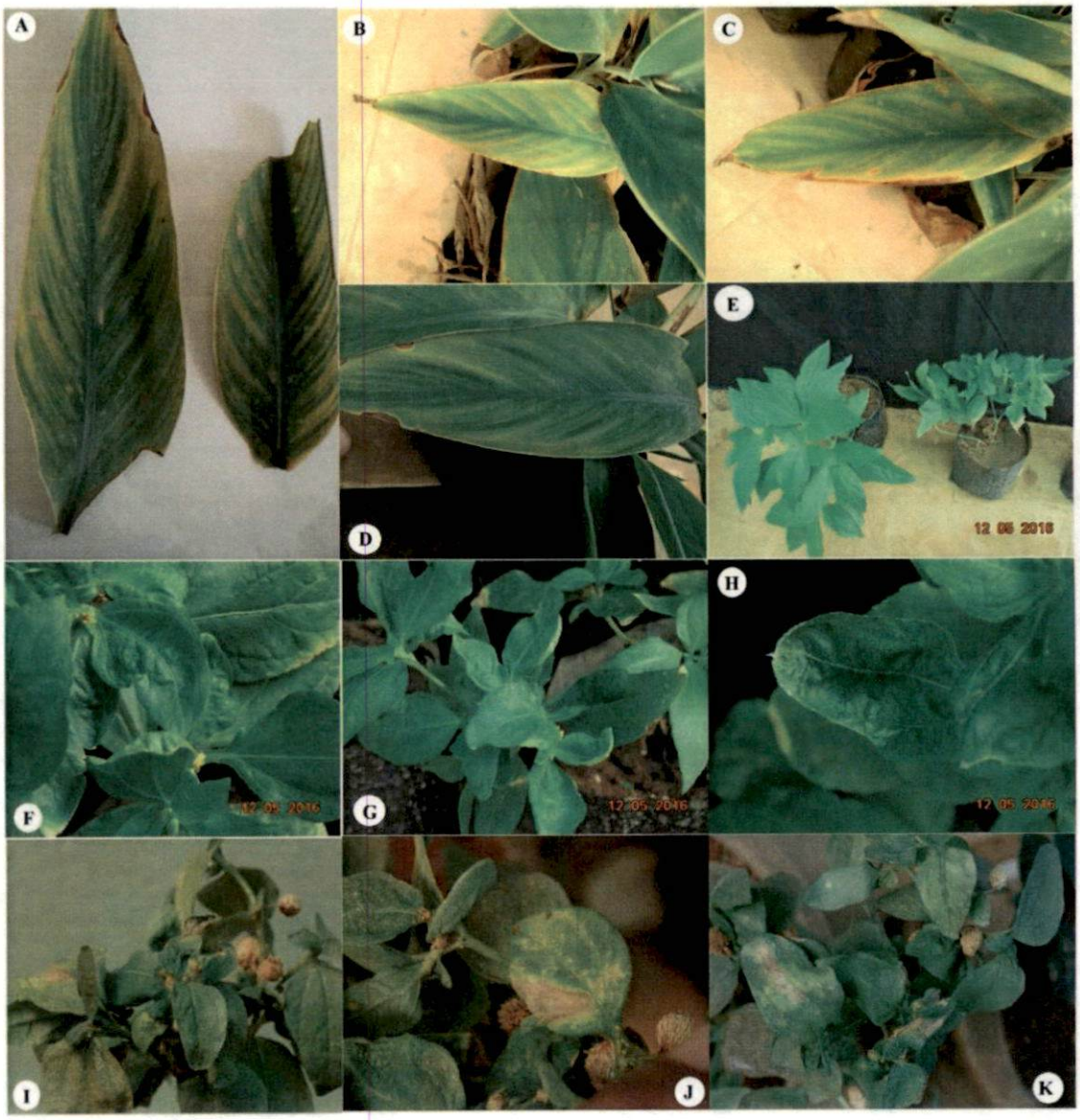


Plate 11g: Host range studies: symptoms expressed by CMV (A-D) *Canna indica;* (A) Marginal necrosis and mosaic symptoms, (B) Tip necrosis and yellowing, (C) Marginal necrosis and yellowing, (D) Mosaic symptoms. E-H *Synedrella nodiflora* (E) Healthy and stunted (F) Mottling, (G) Cupping, (H) Leaf distortion, (I-K) *Acmella radicans;* (I) Mottling, (J) Yellowing (K) leaf distortion

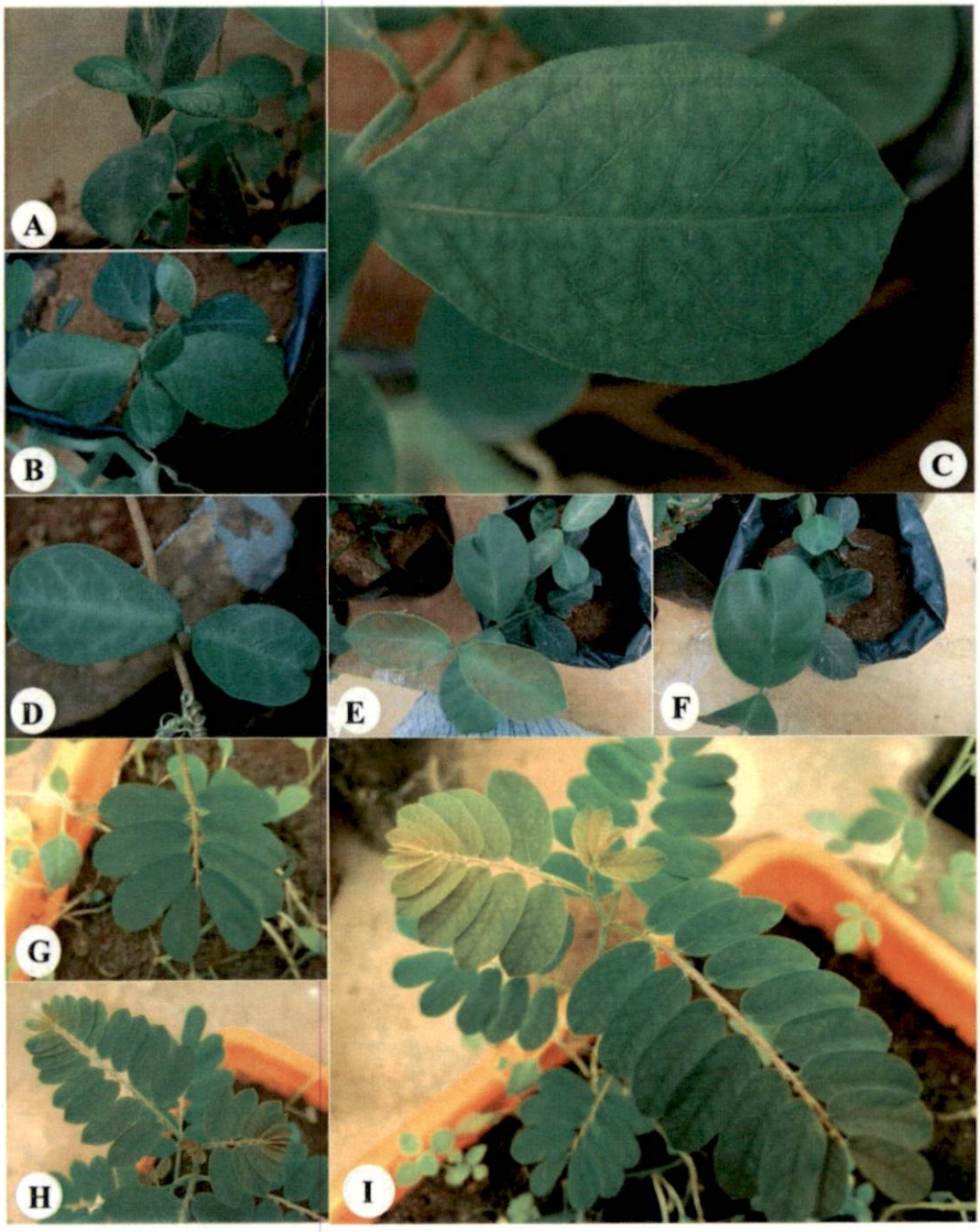


Plate 11h: Host range studies: symptoms expressed by CMV (A-F) *Ichnocarpus frutescens*; (A) Downward curling with severe puckering, (B and C) Mosaic symptoms, (D) Initial stage of leaf distortion (E-F) Distorted leaves, (G-I) *Phyllanthus amara;* (G) Uninoculated (H and I) mosaic symptoms on inoculated

4.1.4 Transmission Studies

4.1.4.1 Transmission through Planting Material

The results of the experiment on the transmission of the virus through planting material/suckers revealed that the virus is carried through the suckers of infected mother plants. The rate of transmission through sucker is presented in the Table 18. Among the four varieties, Robusta (AAA), recorded the highest per cent transmission (100) through planting material and Grand Naine (AAA) recorded the lowest (30.77). The per cent transmission recorded in other varieties such as Karpooravalli (ABB), Nendran (AAB) and Amritsagar (AAA) were 66.00, 61.90 and 37.50 per cent respectively.

Table 18: Transmission of the virus through planting material (Suckers)

Banana variety	*Mean number of suckers per plant	Mean number of infected suckers	Per centage of infected suckers
Karpooravalli (ABB)	3.33	2.20	66.00
Amritsagar (AAA)	4.00	1.50	37.50
Nendran (AAB)	3.50	2.17	61.90
Grand Naine (AAA)	3.25	1.00	30.77
Robusta (AAA)	3.50	3.50	100.00

***Mean of five replications**

4.1.4.2 Mechanical Transmission

When transmission was done from infected banana to healthy banana through sap, no symptoms were developed and hence it is concluded that this virus is not transmitted through sap from banana to banana. The inoculated plants were subjected to ELISA and found negative (Table 19).

Table 19: Mechanical transmission using different buffers

Buffer	No. of banana plants inoculated	No.of plants infected
Sodium borate (pH. 8)	9	0
Potassium phosphate (pH. 7.2)	9	0
Potassium phosphate (pH. 7.2)	9	0
Sodium phosphate (pH. 7.2)	9	0
Citrate (pH. 6.2)	9	0
Tris (pH. 7.2)	9	0

4.1.4.3 Insect Transmission

Insect vector transmission studies were conducted with banana aphid, *Pentalonia nigronervosa* Coquerel and cowpea aphid, *Aphis craccivora* on 2-3 months old tissue culture plants of banana. The results are given in Table 20a to 20c. It was observed that the per cent transmission of virus ranged from 14.29 to 85.71. With regard to incubation period, the days taken for symptom expression by banana aphid, *Pentalonia nigronervosa* and cowpea aphid *Aphis craccivora* were 35 and 55 days respectively.

The plants inoculated with the viruliferous aphids developed mild chlorosis on the leaf veins initially. The new emerging leaves produced small linear chlorotic lesions which enlarged with the expansion of leaf lamina. Severe malformation was observed on the leaf lamina which reduced the leaf size drastically (Plate 12, Fig. D). The leaves turned brittle and veins got thickened chlorotic lesions observed were running parallel to the veins from the midrib to the leaf margin (Plate 12, Fig. C).

Virus-vector relationship

Among the two different aphid species tested, banana aphids, *P. nigronervosa* was found to be efficient insect vector as it recorded maximum per cent transmission and minimum period of incubation in the host.

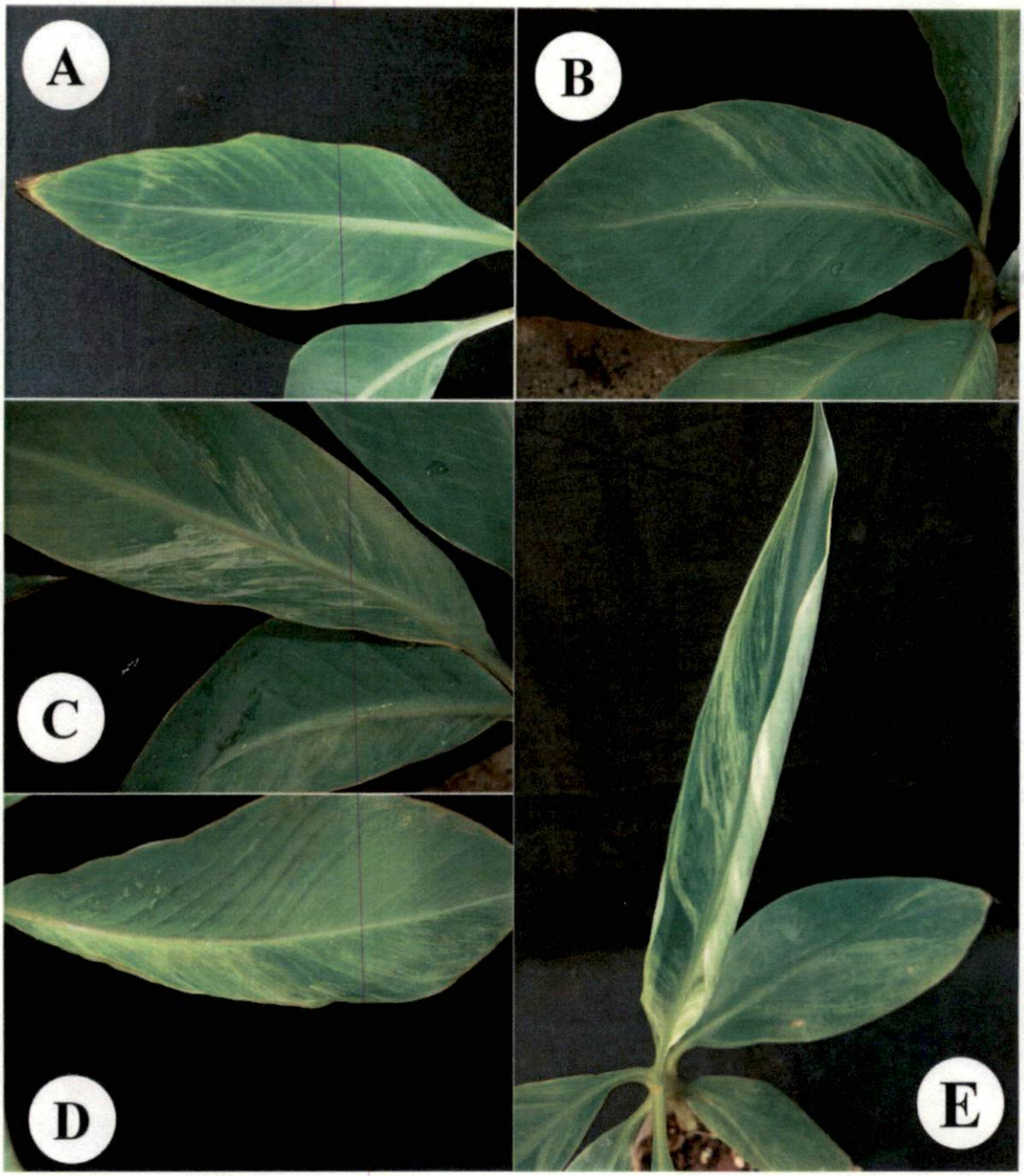


Plate 12: Symptoms developed under artificial conditions (aphid transmission) on banana Nendran var. (A) Initial symptoms (B) New leaf with chlorotic lesions (C) Expansion of leaf lamina and chlorotic lesions (D) Malformed leaf (E) Rolling of leaf

4.1.4.3.1 Effect of Pre-acquisition Fasting Period of Aphids for Virus Transmission

In the case of banana aphid *Pentalonia nigronervosa* pre-acquisition fasting period had significant effect on the per cent transmission of the virus. The aphids without pre-acquisition fasting period could not transmit the virus and recorded zero per cent transmission. Highest per cent transmission was recorded with 30 min of fasting. However, with increase in fasting period upto 60 min, the rate of transmission got reduced to 42.86 per cent.

In the case of cowpea aphids, the per cent transmission was less when compared to banana aphids to the virus was not transmitted when pre-acquisition fasting period was not given. When aphids were not subjected to pre-acquisition fasting period the per cent transmission was zero. Maximum of 28.57 per cent transmission was recorded with 60 minutes acquisition fasting and with 30 minutes of acquisition feeding period 14.29 per cent transmission was recorded (Table 20a).

4.1.4.3.2 Effect of Acquisition Feeding Period of Aphids for Virus Transmission

Results presented in Table 20b showed that in case of banana aphid a period of 2 hours is required for acquisition of virus from the source plant. It recorded 85 per cent transmission with 2 hours of acquisition feeding period which was sufficient for per cent transmission. The rate of transmission decreased as the acquisition period was increased.

The data presented in Table 20b indicates that the cowpea aphid, 6 hours of feeding was required for the acquisition of virus from the source plant and recorded 14.20 per cent transmission. However, with 24h of acquisition feeding period the per cent transmission was 14 per cent. It was observed that the rate of transmission increased as the acquisition feeding period was increased.

4.1.4.3.3 Effect of Inoculation Feeding Period of Aphids on the Transmission of Virus

A minimum of 30 minutes was required by banana aphid for successful inoculation of the virus. But the rate of transmission was only 42.86 per cent. At 60

minutes of inoculation access, it recorded 85.7 per cent transmission. In both the cases, per cent transmission increased progressively with corresponding increase of inoculation access period (Table 20c).

In case of cowpea aphid, a minimum period of 30 minutes was required for successful inoculation of the virus. But the rate of transmission was only 14.29 per cent and with 60 minutes of inoculation feeding period, it was 28.57 per cent of transmission (Table 20c).

Table 20a: Effect of pre-acquisition fasting period of aphids on the transmission of the virus

Pre-acquisition fasting period (min)	Per cent transmission	
	P. nigronervosa	*A. craccivora*
0	0	0
30	85.71	14.29
60	42.86	28.57

Table 20b: Effect of acquisition feeding period of aphids on the transmission of the virus

Acquisition feeding period (hrs)	Per cent transmission	
	P. nigronervosa	*A. craccivora*
2	85.71	0
6	42.86	14.29
24	0	28.57

Table 20c: Effect of inoculation feeding period of cowpea aphid on the transmission of virus

Inoculation feeding period (Minutes)	Per cent transmission	
	P. nigronervosa	*A. craccivora*
10	85.71	0
30	42.86	14.29
60	0	28.57

4.1.5 Physical Properties of the Virus

4.1.5.1 Thermal Inactivation Point

In the present study, it was observed that the plants inoculated with crude sap immediately after extraction at room temperature of 28°C showed 100 per cent transmission producing maximum number of local lesions. The sap exposed to 30°C and 35°C temperature showed 97 and 90 per cent transmission respectively. With the increase in temperature, there was gradual decline in the number of local lesions which showed the loss of infectivity of the virus particle at higher temperature. The crude sap subjected to 65°C was found to be least infective with only 5 per cent transmission. Beyond 65°C there was no infectivity at all. From the results it is concluded that the thermal inactivation point of the virus ranged between 65-70°C (Table 21 and Plate 13, Fig. A).

Table 21: Effect of temperature on per cent transmission of CMV (Thermal inactivation point)

Temp. (°C)	No of local lesions	Per cent Transmission
28	58	100
30	56	97
35	52	90
40	37	64
45	32	55
50	23	40
55	18	31
60	9	16
65	3	5
70	0	0
75	0	0
CD	0.97	-

4.1.5.2 Dilution End Point

The undiluted crude sap recorded 100 per cent transmission with maximum number of local lesions (54) the sap remained its infectivity up to the dilution of 10^{-4}. Hence, it was concluded that the dilution end point of the virus is 10^{-4} (Table 22 and Plate 13, Fig. B).

Table 22: Effect of dilution of crude sap on per cent transmission of CMV (Dilution end point)

Dilution	No of local lesions	% Transmission
Crude sap	54	100
10^{-1}	32	59
10^{-2}	15	28
10^{-3}	4	7
10^{-4}	1	2
10^{-5}	0	0
10^{-6}	0	0
10^{-7}	0	0
10^{-8}	0	0
CD	1.25	

4.1.5.3 Longevity in-vitro (LIV)

The Longevity *in-vitro* (LIV) was studied under room temperature and at refrigerated condition of 4°C.

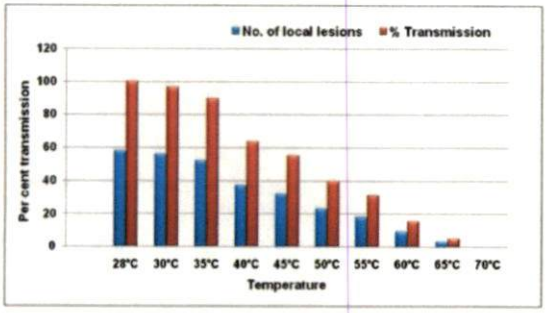
The studies on LIV done at room temperature (28°C) revealed that 100 per cent transmission was observed when inoculated with crude sap immediately after extraction from infected leaves. Gradual decrease in per cent transmission was observed when the crude sap was kept at room temperature for different periods. The results showed that the virus could retain infectivity up to 3 days and got inactivated from 3rd day onwards when kept at room temperature (Table 23 and Plate 13, Fig. C).

When stored at refrigerated conditions (4°C), the infectivity of the sap was retained up to seven days. A gradual decline in the per cent transmission was observed by storing the extract in refrigerator (4°C). However, over the period of 4 hours of storage under refrigerated conditions, the crude sap retained the infectivity with maximum transmission of 94 per cent. The infectivity of the virus was retained upto 7 days (Table 23 and Plate 13, Fig. D).

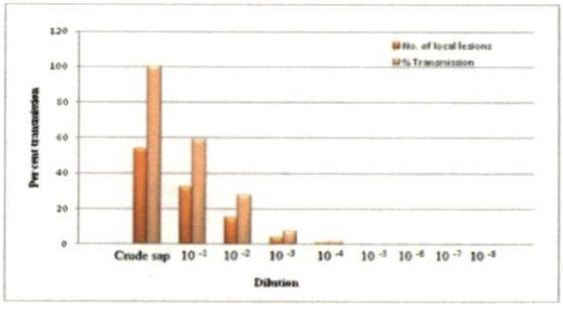
It was observed that the LIV of the crude sap was 2 and 7 days at room temperature and refrigerated conditions respectively.

Table 23: Effect of storage time on per cent transmission of CMV at room temperature and refrigerator conditions (Longevity *in-vitro*)

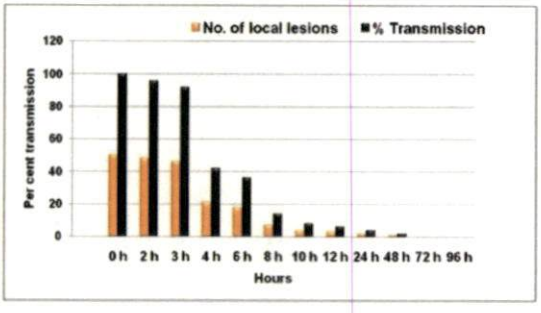
Duration	Room temperature (28^0C)		Refrigerated (4^0C)	
	No. of local lesions	Per cent Transmission	No. of local lesions	Per cent Transmission
0h	50	100	52	100
2h	48	96	50	96
3h	46	92	46	88
4h	21	42	49	94
6h	18	36	46	88
8h	7	14	44	85
10h	4	8	44	85
12h	3	6	45	87
24h	2	4	36	69
48h	1	2	35	67
3days	0	0	25	48
4days	0	0	14	27
5days	-	-	10	19
6days	-	-	6	12
7days	-	-	2	4
8days	-	-	0	0
9days	-	-	-	-
OD	0.69		0.78	



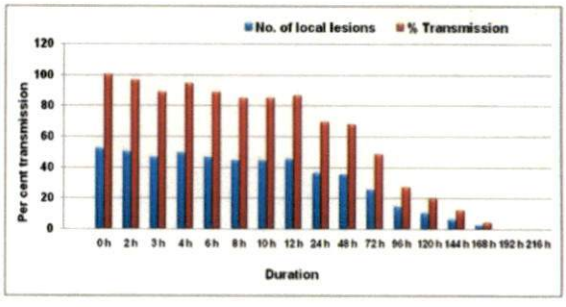
A. Effect of temperature on per cent transmission of the virus (Thermal inactivation point).



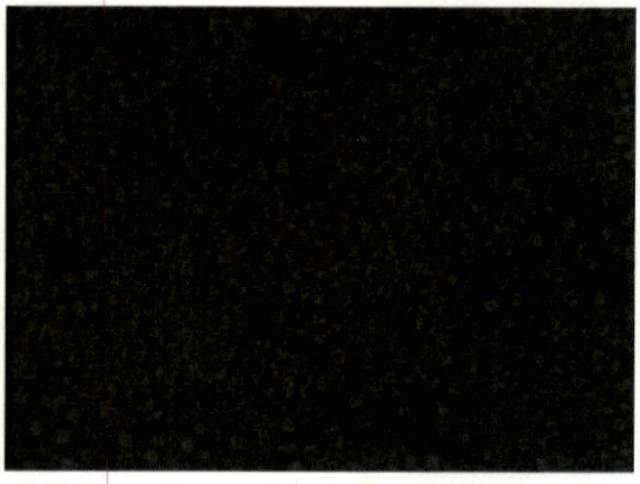
B. Effect of dilution on per cent transmission of the virus (Dilution end point).



C. Effect of storage time on per cent transmission of the virus at room temperature (Longevity *in-vitro*).



D. Effect of storage time on per cent transmission of the virus under refrigerated conditions (4°C).



E. Electron micrograph depicting spherical isometric particles of CMV (28.5 nm) (120000 X)

Plate 13: Biophysical and morphological properties of CMV

4.1.6 Electron Microscopy

Electron microscopy was done by leaf dip method (Bhat *et al.*, 2004) using JEOL- 1 OO-CF-II transmission electron microscope available at Advanced Centre of Plant Pathology, Indian Agricultural Research Institute, New Delhi. The leaf dip samples were prepared using negative stain 2 per cent uranyl acetate (pH 4.5) from infected leaves of banana, *N. benthamiana* and *N. glutinosa* The electron photomicrographs revealed the presence of numerous spherical isometric virus particles of size 28.5 nm (Plate 13, Fig. E).

Based on these characteristics of the particles, the virus causing banana infectious chlorosis disease is confirmed as *Cucumber mosaic virus*. This virus belongs to genus Cucumovirus, family Bromoviridae).

4.2 IMMUNOLOGICAL STUDIES

4.2.1 Detection of Virus Infection by Protein based methods

4.2.1.1 Direct Antigen Coating ELISA (DAC-ELISA)

4.2.1.1.1 Determination of Titre of Antiserum and Antigen

To determine the antibody titre with different dilution of 1:100; 1:200; 1:300 and 1:500 antiserum were selected to do ELISA and absorbance value was recorded. The results obtained are presented in (Table 24).

It was found that CMV could be best detected in the primary polyclonal antibody 1:200 along with 1:10000 dilution of alkaline phosphatase conjugate secondary antibody (Plate 14, Fig. A).

4.2.1.1.2 Detection of Virus by DAC-ELISA

The titre of antiserum *viz.,* 1:200 which was previously determined by DAC-ELISA was used further for virus detection. The absorbance value at 405 nm microtitre plate reader was recorded. The result of the experiment revealed that the CMV specific

antiserum gave higher reactivity and it could give clear difference between healthy and infected samples (Plate 14, Fig. B). The average absorbance value of samples was almost thrice as that of healthy samples.

4.2.1.2 Detection of Virus by Dot Immuno Binding Assay (DIBA)

Antigen extracted from both infected and healthy leaf samples was coated on nitrocellulose membrane and DIBA was conducted to detect the presence of virus in suspected and healthy sample using polyclonal antibody specific to CMV. The result of the experiment was assessed by comparing the intensity of the colour visually. The infected leaf sample showed brown coloured spots on nitrocellulose membrane indicating positive reaction which was absent in healthy samples (Plate 14, Fig. C).

Table 24: Determination of antibody titre

Absorbance at 405 nm												
Antigen dilution	Anitbody dilution											
	1:500			1:300			1:200			1:100		
	- ve ctrl	+ ve ctrl	Sample	- ve ctrl	+ ve ctrl	Sample	- ve ctrl	+ ve ctrl	Sample	- ve ctrl	+ ve ctrl	Sample
10^{-1}	0.074	0.223	0.065	0.076	0.273	0.077	0.096	0.352	0.079	0.099	0.785	0.091
10^{-2}	0.072	0.237	0.074	0.106	0.279	0.075	0.116	0.341	0.101	0.142	0.634	0.118
10^{-3}	0.074	0.215	0.080	0.104	0.265	0.071	0.123	0.498	0.090	0.134	0.651	0.105
10^{-4}	0.068	0.190	0.067	0.094	0.211	0.071	0.124	0.445	0.077	0.148	0.582	0.105
10^{-5}	0.087	0.153	0.069	0.091	0.283	0.077	0.153	0.311	0.085	0.156	0.563	0.103
10^{-6}	0.082	0.123	0.073	0.075	0.183	0.070	0.094	0.248	0.113	0.133	0.409	0.107
10^{-7}	0.068	0.118	0.064	0.089	0.176	0.084	0.085	0.197	0.084	0.094	0.308	0.106

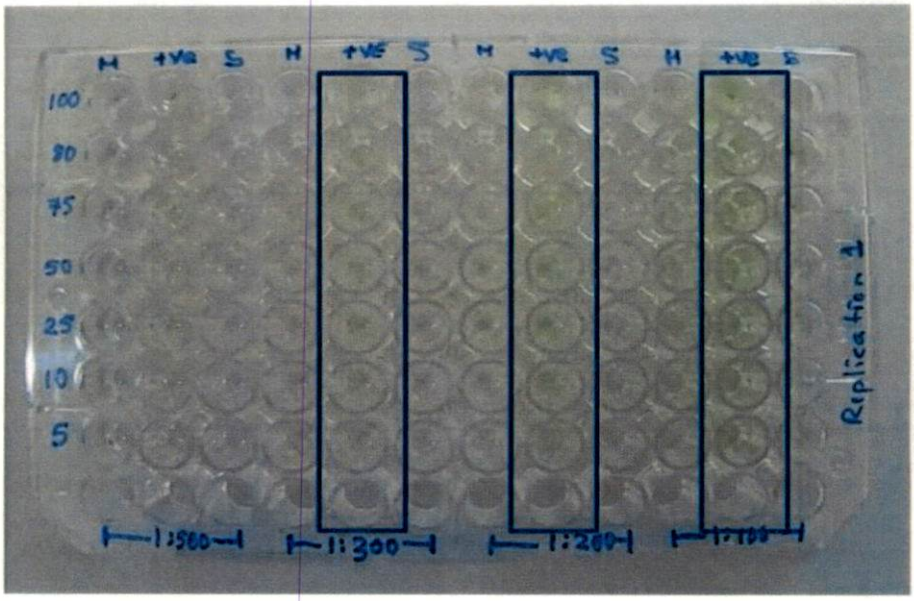


Fig. A. Determination of antibody titre: Antibody dilution 1:500: Lane1, 2 & 3; Antibody dilution 1:300: Lane 4, 5 & 6; Antibody dilution 1:200: Lane 7, 8 & 9; Antibody dilution 1:100: Lane 10, 11 & 12; Antigen dilution: Horizontal lane A to H

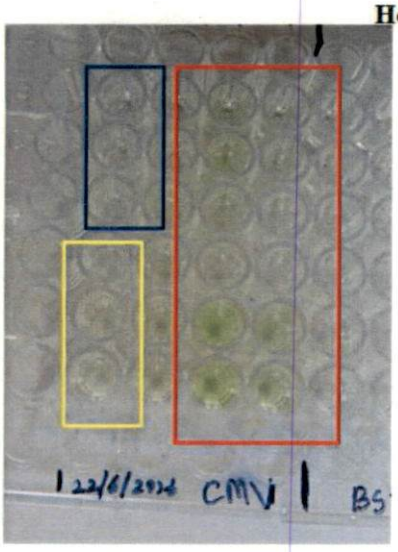


Fig. B. Detection of CMV by DAC-ELISA: Buffer: Highlighted blue; Healthy: Highlighted yellow; Infected smples: Highlighted red

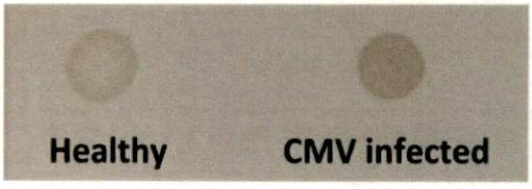


Fig. C. Detection of CMV by DIBA

Plate 14: Detection of virus by protein based methods

4.3 VARIETAL SCREENING FOR DISEASE RESISTANCE

The accessions of banana maintained in the field gene bank of Banana Research Station, Kannara were screened periodically at monthly intervals to assess their reaction to disease under natural conditions. The accessions were screened based on visible symptoms. A total of 175 accessions were screened and out of which, 12 were found to be positive (Table 25). Apart from assessing the disease reaction based on visual symptoms, the young leaf samples were also collected from all the accessions, those with and without symptoms and subjected to ELISA to confirm the presence or absence of the virus infection in asymptomatic plants also . Among the accessions banana var. Robusta recorded the highest absorbance value which showed the extent of virus particles present in the sample The accessions found positive for ELISA were varieties Amritsagar, Grand naine, Lacatan, Robusta, Karpooravalli, Nendran, Chengathalikodan, Populu, Kadali, Karivazha, Nattu Poovan and Njali Poovan. The results are given in Table 26.

Table 25: Detection of CMV in the accessions by DAC-ELISA

Sl. No.	Variety	Genome	ELISA OD Value	Inference
1	Amritsagar	(AAA)	0.333	+VE
2	Grand Naine	(AAA)	0.328	+VE
3	Lacatan	(AAA)	0.322	+VE
4	Robusta	(AAA)	0.441	+VE
5	Karpooravalli	(ABB)	0.296	+VE
6	Nendran	(AAB)	0.432	+VE
7	Changalikodan	(AAB)	0.286	+VE
8	Populu	(AAB)	0.298	+VE
9	Kadali	(AA)	0.345	+VE
10	Karivazha	(AA)	0.369	+VE
11	Nattu poovan	(AB)	0.263	+VE
12	Njali poovan	(AB)	0.275	+VE
	Negative control	-	0.098	-VE

Table 26: Occurrence of CMV in the accessions of the field gene bank

Ploidy	Number of accessions found +ve	Name of accessions	Type of Symptoms	ELISA (+/-)
AA	2	Kadali	No visible symptoms	+
		Karivazha	No visible symptoms	+
AB	2	Natu Poovan	No visible symptoms	+
		Njali Poovan	No visible symptoms	+
AAA	4	Amritsagar	Type I, II, IV and V	+
		Grand Naine	Type I, II, IV and V	+
		Robusta	Type I, II, IV and V	+
		Lacatan	Type I, II, IV and V	+
AAB	3	Changalikodan	No visible	+
		Nendran	Type I, II, IV and V	+
		Populu	No visible symptoms	+
ABB	1	Karpooravalli	Type I	+
Negative control				-

The study revealed that the accessions with AA, AB, AAA, AAB genome were found susceptible to the disease and the disease

4.4 MOLECULAR DETECTION AND CHARACTERIZATION

4.4.1 Isolation of RNA

The leaf samples from infected and healthy banana plants were used for RNA isolation using two different protocols *viz.*, TRIzol reagent method and RNaesy plant mini kit® method.

4.4.1.1 Analysis of Total RNA

The quality and quantity of RNA isolated using the two different methods *viz.*, TRIzol reagent and RNeasy Plant Mini Kit® methods were assessed by using 0.8 % agarose gel in 1X TAE buffer and Nanodrop spectrophotometer.

4.4.1.2 Gel Documentation

The RNA isolated from symptomatic leaves using two different protocols *viz.*, TRIzol reagent method and RNaesy plant mini kit® were run on 0.8 per cent agarose gel electrophoresis in 1X TAE buffer. RNA isolated using RNaesy plant mini kit® produced two dark intact bands (Plate 16, Fig. B) while RNA isolated by using TRIzol reagent method produced only faint bands (Plate 16, Fig. A).

4.4.1.3 Quantification of RNA

Nanodrop spectrophotometer was used to quantify the RNA isolated by the two different protocols *viz.*,TRIzol reagent method and RNaesy plant mini kit®. Based on the mean absorbance value $A_{260/280}$, the purity of the isolated RNA was analyzed and revealed that the RNA isolated by TRIzol method and RNaesy plant mini kit® method were 1.68 and 2.04 respectively. Among the two different methods, RNaesy plant mini kit® method yielded maximum RNA (42.96 ng/µl) compared to TRIzol method (14.86 ng/µl).

The quality of the isolated RNA assessed using Nanodrop spectrophotometer revealed that the total RNA isolated by RNaesy plant mini kit® method was better than the TRIzol reagent method (Table 27). Hence PureLink® Plant RNA Reagent method was used further to isolate RNA from the samples.

Table 27: Quality and quantity of total RNA

Particulars	Method	
	TRIzol	**PureLink® Plant RNA Reagent**
RNA yield (ng /µl)	14.86*	42.96*
Absorbance 260/280	1.68*	2.04*

*mean of three replications

4.4.2 Synthesis of First strand Complementary DNA (cDNA)

The RNA was converted into cDNA by using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) as described in section 3.4.2. The cDNA obtained was quantified and the ratio of absorbance value ($A_{260/280}$) was recorded to assess the purity of cDNA. The purity of the samples ranged between1.71 to 1.74 and yielded cDNA ranging from 33.91 to 3595.20 ng/µl (Table 28).

Table 28: Quality and quantity of cDNA

Isolate No.	Isolate	Abbrevation	Conc. (ng/µl)	Purity/ Absorbance 260/280
Isolate 1	Nendran (AAB)	3529CTRL	2025.6	1.71
Isolate 2	Nedu Nendran (AAB)	3529A1	2251.3	1.71
Isolate 3	Kadali (AA)	3529A2	3026.9	1.72
Isolate 4	Grand Naine (AAA)	3529V	33.91	1.74
Isolate 5	Robusta (AAA)	3529W	3595.2	1.75
Isolate 6	Amritsagar (AAA)	3529X	3021.5	1.74
Isolate 7	Changalikodan (AAB)	3529Y	1860.7	1.72
Isolate 8	Amritsagar (AAA)	3529Z	2076.2	1.71

4.4.3 Primers

Two set of primers were used for Reverse transcription Polymerase Chain Reaction (RT-PCR). Primers pair specific to the Coat Protein (CP) gene of CMV as reported by Cherian *et al.* (2004) was validated for the detection of virus. Also, a second set of primer pairs specific for the coat protein gene of CMV was designed as described in section 3.4.3.1 for the amplification of virus cp gene.

4.4.3.1 Primer Designing and Validation

The CP gene sequences of CMV available in NCBI were selected in FASTA format and the details of selected accessions are given in (Annexure IX). The multiple sequence alignment of nucleotide sequences was done using Clustal Omega' (Plate 15,

Fig. A). Based on the homology, conserved boxes of 18 to 24 bases each for both primers were selected and the forward and reverse primers were designed. The details such as length of primers, melting temperature and expected PCR product are presented (Table 29).

Prior to the synthesis of the primers, *in-silico* analysis was carried out to determine the specificity and other characteristics like the self-complementarity, GC content, feasible annealing temperature probability for primer dimer formation and hairpin formation were assessed using OligoAnalyzer 3.1 (Integrated DNA technologies) available at http://eu.idtdna.com/site (Plate 15, Fig. B). The details of primer validation using Oilgo analyzer 3.1 software are presented in Table 30. The primer sequences thus validated by *in silico* analysis were sent to Scigenome, Kochi for synthesis.

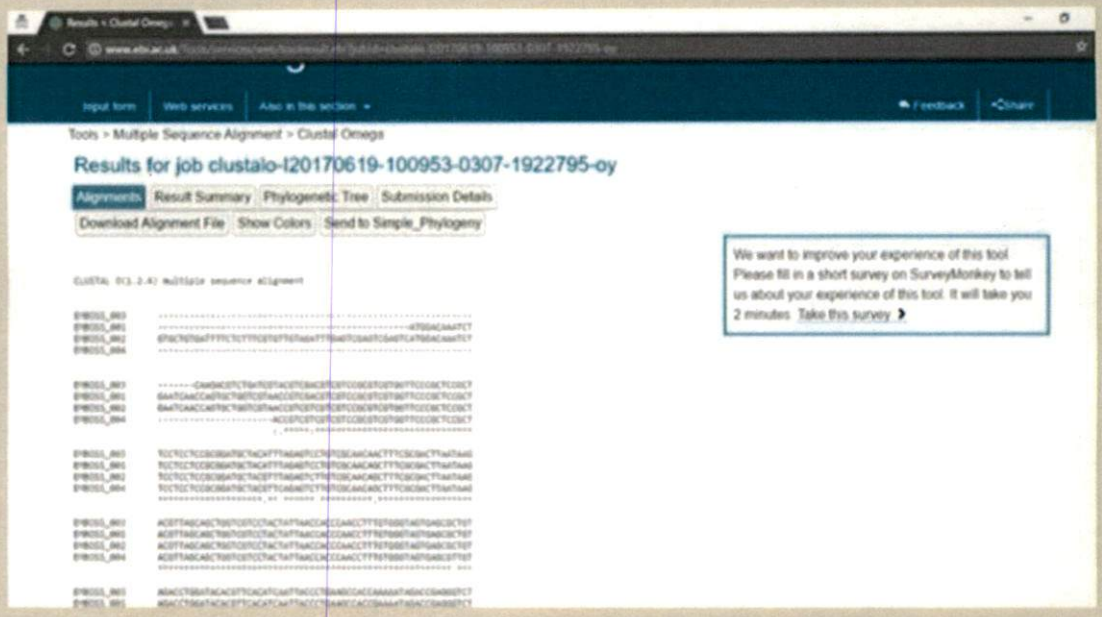
Table 29: Sequences of designed primers

Primer	Sequence (5'->3')	length	Tm	Product size
DP-CMV-F1	TCGTCCGCGTCGTGGTTCCC	20	62.21	700
DP-CMV-R2	TCAGCTCCCGCCACAGAAATC	21	62.71	700

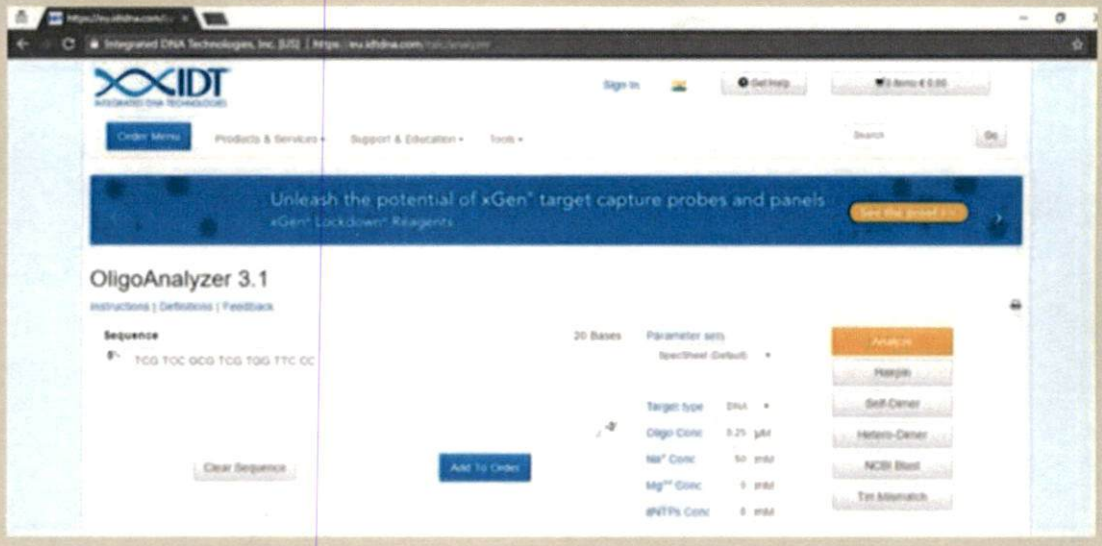
DP: reported primer F: Forward Primer R: Reverse Primer

Table 30: Validation of designed primers

Primer Sequences	Homodimer (Maximum 5bps are allowed)	Heterodimer (Maximum 5bps are allowed)	Tm mismatch	GC content	Hairpin loop (Maximum 3 loop are allowed)
Forward Primer	3	2	Moderate	70	1
Reverse Primer	2	3	Moderate	57	2



A: Clustal Omega tool used for sequence alignment to design primer



B: Oligo Analyzer 3.1 used for validation of primer

Plate 15: Primer designing and validation

4.4.4 Standardization of PCR Conditions for Designed Primer

PCR conditions such as specific primer concentration, annealing temperature and template dilution were standardized and the results are given below.

4.4.4.1 Standardization of Primer and Template Dilution

RT-PCR was carried out at different dilutions of designed set of primers and cDNA template as described in section 4.4.4. The maximum intensity of amplification was observed with 1 µl aliquot of 1:10 diluted primer and 0.5 aliquot of undiluted cDNA. There was no amplification with other dilutions (Table 31).

Table 31: Effect of different combinations of primer and template dilutions on PCR amplification

Different combinations of primer and template dilutions	Aliquot (µl)		Amplification
	Primer	template cDNA	
1:1	0.5	0.5	Not amplified
1: (1:10)	0.5	2	Not amplified
(1:10):1	**1**	**0.5**	**Amplified with good intensity band**
(1:10):(1:10)	1	2	Not amplified

4.4.4.2 Standardization of Annealing Temperature

The annealing temperature was determined by performing Gradient 4.1 RT-PCR in Matercycler gradient PCR (Eppendorf). Highest intensity of amplification was observed at 65.5°C followed by 66.3, 67.2, 68.3, 69.5, 70.6, 71.6 and 72.5°C. The lowest intensity was observed at 73.2 and 73.5°C. There was no amplification at 64.9, 65.1 and 65.5°C (Plate 16, Fig. C).

4.4.5 Detection of the Virus by RT- PCR

RT-PCR was carried out using designed primer DP-CMV-F1 and DP-CMV-R2 and master mix composition as described in section 3.4.5 for the detection of virus

which yielded an amplified product of 700 bp size which was visualized in 1.2 per cent agarose gel electrophoresis (Plate 16, Fig. E). The primer combination of reported primer RP-CMV-F1 and RP-CMV-R2 yielded an amplified product of 750 bp size. Thick and dark bands were visualized and documented in infected isolates but absent in healthy isolates (Plate 16, Fig. D).

4.4.5.1 Sequencing

The amplicons obtained in PCR were eluted, purified and sent to Scigenome, Kochi for sequencing. The nucleotide sequences obtained were analyzed and presented (Table 32, Fig. 1a to Fig. 1h).

The nucleotide sequences were used to deduce amino acid sequence using ExPASy translator tool and the deduced amino acid sequence of eight different isolates varied from each other with respect to number of amino acids present in each sequence (Table 32, Fig. 2a to Fig. 2h).

Table 32: Number of nucleotides and amino acids of the isolates

	Isolate number							
Sl. No.	1	2	3	4	5	6	7	8
Code no.	CTRL	3529A1	3529A2	3529V	3529W	3529X	3529Y	3529Z
No. of nucleotides	732	640	625	637	574	568	638	640
No. of amino acids	243	213	208	212	191	189	212	213

4.4.5.2 In silico Analysis of Sequences of the Isolates

BLASTn and BLASTx analysis was done and the sequences were compared with the sequences of CMV strain available in the GenBank database and sequence identities were obtained and presented below. Phylogenetic analysis was also performed using

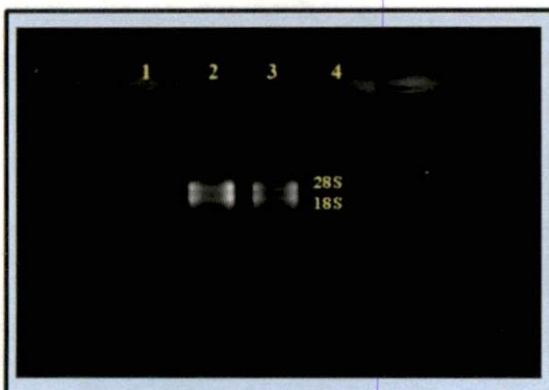


Fig A. RNA isolated using TRIzol reagent method

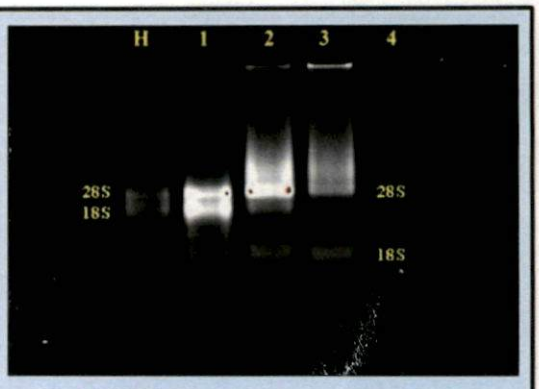


Fig B. RNA isolated using PureLink® Plant RNeasy Reagent method

Fig C. Standardization of annealing temperature using designed primer

Fig D. Detection of CMV by RT-PCR using reported primers

Fig E. Detection of CMV by RT-PCR using designed primers

Plate 16: Molecular detection of *Cucumber mosaic virus* using RT-PCR

5'TCTGAATCAACCAGTGCTGGTCGTAACCGTCGACGTCGTCCGCGTC
GTGGTTCCCGCTCCGCTTCCTCCTCCGCGGATGCTACATTTAGAGTC
CTGTCGCAACAACTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGT
CCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAGACCT
GGATACACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACCGA
GGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTCACTGAGT
TCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCC
GAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTGCCTC
CTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGACGGAG
CCTCACCGGTACTGGTTTATCAGTACGCCGCATCCGGAGTTCAAGCTA
ACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTGATATTGGCG
ACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATACGCTAG
AGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGCATTC
CCACATCTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAACCCT
CCCTCCGATTTCTGTGGCGGGAGCTGAGTTGGCAGTGTTGCTATAAA
CTGCTGAAGTCACTAA3'

Fig. 1a: Nucleotide sequence of CMV coat protein gene of isolate-1

5'CGTGGTTCCCGCTCCGCTTCCTCCTCCGCGGATGCTACATTTAGAG
TCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAGCAGCTGGTC
GTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAGAC
CTGGATACACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACC
GAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTCACTGA
GTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTG
CCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTGCC
TCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGACGG
AGCCTCACCGGTACTGGTTTATCAGTACGCCGCATCCGGAGTTCAAG
CTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTGATATTG
GCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATACG
CTAGAGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGC
ATTCCCACATCTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAAC
CCTCCCTCCGATTTCTGTG3'

Fig. 1b: Nucleotide sequence of CMV coat protein gene of isolate-2

5'CATTTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTA
GCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAG
CGCTGTAGACCTGGATACACGTTCACATCAATTACCCTGAAGCCACCA
AAAATAGACCGAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATT
CAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAG
TTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTA
AAGTTCCTGCCTCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATG
TTTGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTACGCCGCATC
CGGAGTTCAAGCTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCG
CGCTGATATTGGCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAA
AGACGATACGCTAGAGACGGATGAGCTAGTACTTCATGTCGACATTGA
GCACCAACGCATTCCCACATCTGGGGTGCTCCCAGTTTGAACTCGTG
TTTTCCAGAACCCTCCCTCCGATTTCTGTGGCGGGGAGCTGAATTGG
CAGTG3'

Fig. 1c: Nucleotide sequence of CMV coat protein gene of isolate-3

5'TGGTTCCCGCTCCGCTTCCTCCTCCGCGGATGCTACATTTAGAGTC
CTGTCGCAACAACTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGT
CCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAGACCT
GGATACACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACCGA
GGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTCACTGAGT
TCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCC
GAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTGCCTC
CTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGACGGAG
CCTCACCGGTACGGTTTATCAGTACGCCGCATCCGGAGTTCAAGCTAA
CAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTGATATTGGCGA
CATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATACGCTAGA
GACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGCATTCC
CACATCTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAACCCTC
CCTCCGATTTCTGTG3'

Fig. 1d: Nucleotide sequence of CMV coat protein gene of isolate-4

5'TTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAGCA
GCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGC
TGTAAACCTGGATACACGTTCACATCAATTACCCTGAAGCCACCAAAA
ATAGACCGAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAG
TCACTGAGTTCAATAAGAAGCTTGTTTCGCGCATTCAAATTCAAGTTAA
TCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGT
TCCTGCCTCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTG
CGGACGGAGCCTCACCGGTACTGGTTATCAGTACGCCGCATCCGGAG
TTCAAGCTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTG
ATATTGGCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACA
ATACGCTAGAGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACC
AACGCATTCCCACACTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCC3
'

Fig. 1e: Nucleotide sequence of CMV coat protein gene of isolate-5

5'ATTTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAG
CAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGC
GCTGTAGACCTGGATACACGTTCACATCAATTACCCTGAAGCCACCAA
AAATAGACCGAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTC
AGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGT
TAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAA
AGTTCCTGCCTCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTT
TGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTACGCCGCATCCG
GAGTTCAAGCTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCG
CTGATATTGGCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAG
ACGATACGCTAGAGACGGATGAGCTAGTACTTCATGTCGACATTGAGC
ACCAACGCATTCCCACATCTGGGGTGCTCCCAGTTGAACTC3'

Fig. 1f: Nucleotide sequence of CMV coat protein gene of isolate-6

5'TGGTTCCCGCTCCGCTTCCTCCTCCGCGGATGCTACATTTAGAGTC
CTGTCGCAACAACTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGT
CCTACTATTAACCACCCGACCTTTGTGGGTAGTGAGCGCTGTAGACCT
GGATACACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACCGA
GGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTCACTGAGT
TCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCC
GAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTGCCTC
CTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGACGGAG
CCTCACCGGTACTGGTTTATCAGTACGCCGCATCCGGAGTTCAAGCTA
ACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTGATATTGGCG
ACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATACGCTAG
AGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGCATTC
CCACATCTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAACCCT
CCCTCCGATTTCTGTG3'

Fig. 1g: Nucleotide sequence of CMV coat protein gene of isolate-7

5'CGTGGTTCCCGCTCCGCTTCCTCCTCCGCGGATGCTACATTTAGAG
TCCTGTCGCAACAACTTTCGCGACTTAATAAGACGTTAGCAGCTGGTC
GTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAGAC
CTGGATACACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACC
GAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTCACTGA
GTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTG
CCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTGCC
TCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGACGG
AGCCTCACCGGTACTGGTTTATCAGTACGCCGCATCCGGAGTTCAAG
CTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTGATATTG
GCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATACG
CTAGAGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGC
ATTCCCACATCTGGGGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAAC
CCTCCCTCCGATTTCTGTG3'

Fig. 1h: Nucleotide sequence of CMV coat protein gene of isolate-8

SESTSAGRNRRRRPRRGSRSASSSADATFRVLSQQLSRLNKTLAAGRP
TINHPTFVGSERCRPGYTFTSITLKPPKIDRGSYYGKRLLLPDSVTEFDKK
LVSRIQIRVNPLPKFDSTVWVTVRKVPASSDLSVAAISAMFADGASPVLVY
QYAASGVQANNKLLYDLSAMRADIGDMRKYAVLVYSKDDTLETDELVLHV
DIEHQRIPTSGVLPV*TRVFQNPPSDFCGGS*VGSVAINC*SH*

Fig. 2a: Amino acid sequence of CMV coat protein gene of isolate-1

RGSRSASSSADATFRVLSQQLSRLNKTLAAGRPTINHPTFVGSERCRPG
YTFTSITLKPPKIDRGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDS
TVWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNKLLY
DLSAMRADIGDMRKYAVLVYSKDDTLETDELVLHVDIEHQRIPTSGVLPV*
TRVFQNPPSDFC

Fig. 2b: Amino acid sequence of CMV coat protein gene of isolate-2

HLESCRNSFRDLIRR*QLVVLLLTTQPLWVVSAVDLDTRSHQLP*SHQK*T
EGLIMVKGCYFLIQSLSSIRSLFRAFKFELILCRNLILPCG*RSVKFLPPRTC
PLPPSLLCLRTEPHRYWFISTPHPEFKLTTSCCMIFRRCALILAT*ESTPCS
CIQKTIR*RRMS*YFMSTLSTNAFPHLGCSQFELVFSRTLPPISVAGS*IGS

Fig. 2c: Amino acid sequence of CMV coat protein gene of isolate-3

WFPLRFLLRGCYI*SPVATTFAT**DVSSWSSYY*PPNLCG**AL*TWIHVHI
NYPEATKNRPRVLLW*KVVTS*FSH*VR*EACFAHSNSS*SFAEI*FYRVGD
GP*SSCLLGPVRCRHLCYVCGRSLTGTVYQYAASGVQANNKLLYDLSAM
RADIGDMRKYAVLVYSKDDTLETDELVLHVDIEHQRIPTSGVLPV*TRVFQ
NPPSDFC

Fig. 2d: Amino acid sequence of CMV coat protein gene of isolate-4

LESCRNSFRDLIRR*QLVVLLLTTQPLWVVSAVNLDTRSHQLP*SHQK*TE
GLIMVKGCYFLIQSLSSIRSLFRAFKFKLILCRNLILPCG*RSVKFLPPRTCP
LPPSLLCLRTEPHRYWLSVRRIRSSS*QQVVV*SFGDAR*YWRHEKVRR
ARVFKRQYARDG*ASTSCRH*APTHSHTGVLPV*TRVF

Fig. 2e: Amino acid sequence of CMV coat protein gene of isolate-5

I*SPVATAFAT**DVSSWSSYY*PPNLCG**AL*TWIHVHINYPEATKNRPRVL
LW*KVVTS*FSH*VR*EACFAHSNSS*SFAEI*FYRVGDGP*SSCLLGPVRC
RHLCYVCGRSLTGTGLSVRRIRSSS*QQVVV*SFGDAR*YWRHEKVRRA
RVFKRRYARDG*ASTSCRH*APTHSHIWGAPS*T

Fig. 2f: Amino acid sequence of CMV coat protein gene of isolate-6

WFPLRFLLRGCYI*SPVATTFAT**DVSSWSSYY*PPDLCG**AL*TWIHVHI
NYPEATKNRPRVLLW*KVVTS*FSH*VR*EACFAHSNSS*SFAEI*FYRVGD
GP*SSCLLGPVRCRHLCYVCGRSLTGTGLSVRRIRSSS*QQVVV*SFGDA
R*YWRHEKVRRARVFKRRYARDG*ASTSCRH*APTHSHIWGAPSLNSCF
PEPSLRFL

Fig. 2g: Amino acid sequence of CMV coat protein gene of isolate-7

RGSRSASSSADATFRVLSQQLSRLNKTLAAGRPTINHPTFVGSERCRPG
YTFTSITLKPPKIDRGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDS
TVWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNKLLY
DLSAMRADIGDMRKYAVLVYSKDDTLETDELVLHVDIEHQRIPTSGVLPV*
TRVFQNPPSDFC

Fig. 2h: Amino acid sequence of CMV coat protein gene of isolate-8

bioinformatics tools for the characterization of virus. The results of analysis are presented below.

4.4.5.2a In silico Analysis of CMV Coat Protein gene of Isolate-1

The nucleotide sequence of coat protein gene of isolate-1 (3529CTRL) collected from Kannara, Thrissur district, Kerala infecting Nendran cultivar amplified and subjected to NCBI-BLASTn and BLASTx. The BLASTn analysis showed the highest sequence similarity of 99 per cent was observed with Coat protein gene of *Cucumber mosaic virus* (accession AY125575) isolated from banana. It also showed 97 per cent similarity with other sequences *viz.,* LC066476, KJ874250 and LC066464 accessions of CMV coat protein infecting tumble weed mustard, bottle gourd and cucumber respectively (Plate 17a). Whereas, BLASTx results showed that the sequence had 92 per cent homology with accession CEF39516 (Plate 17b) belonging to *Cucumber mosaic virus.*

The phylogenetic analysis, based on the alignment of CMV coat protein nucleotide sequence of isolate-1 (3529CTRL) with top 10 selected accessions obtained from BLASTn analysis, clearly revealed that thus particular isolate infecting banana and this was well supported by 100 bootstrapping value. From the phylogenetic tree it was inferred that the isolate no 2 (3529CTRL) out grouped and clustered with DQ006805 and AYI25575. The sequence was most similar to CMV coat protein accessions AY125575 and DQ006805 infecting banana in India and yellow oleander (*Cascabela thevetia*) in Italy respectively (Fig. 3a).

4.4.5.2b In silico Analysis of CMV Coat Protein gene of Isolate -2

The nucleotide sequence of coat protein gene of isolate (3529A1) collected from Mannuthy, Thrissur district, Kerala infecting Nendran banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with accession AY125575 of CMV coat protein infecting banana. The query sequence also showed 99 and 97 per cent

similarity with CMV accession JX995139 and KJ874250 respectively from Pune and Tamil Nadu infecting banana and bottle gourd (Plate 17c). From the BLASTx analysis, 99 per cent similarity was observed for the amino acid sequences *viz.,* CAA07411, AAY19285, BAV25270, BAV25270, ALT66628, CBG22867 and APB09218 (Plate 17d).

During phylogenetic analysis of nucleotide sequence-2 (3529A1) of CMV coat protein gene along with top 10 accessions from GeneBank database obtained from BLASTn analysis, clearly revealed that it belonged to CMV coat protein gene infecting banana and this is well supported by 87 bootstrapping value. From the phylogenetic tree it was inferred that the isolate 3529A1 clustered with AY125575, JX995139, JF279609, KJ874250, KJ874249 and KJ874248 in to two internal nodes with low imbalance and remained closely clustered with AY125575 and JX995139 accession of CMV coat protein gene infecting banana from India and *N. glutinosa* from Pune, India. The other internal nodes consisted leaf node with KJ874250 accession which infects bottle gourd and with KJ874249 and KJ874248 sequences infecting snake gourd from Tamil Nadu, India (Fig. 3b).

4.4.5.2c In silico Analysis of CMV Coat Protein gene of Isolate -3

The nucleotide sequence of coat protein gene of isolate (3529A2) positive sample Palakkad, Kerala infecting Kadali banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with CMV coat protein gene belonged to accession AY125575 infecting banana (Plate 17e). BLASTx analysis of sequence 3 revealed 100 per cent similarity with amino acid sequence of CMV coat protein gene of the accession CAA07411 (Plate 17f).

The phylogenetic analysis of nucleotide sequence-3 (3529A2) with other 10 sequences from BLASTn analysis clustered forming in to two nodes, where the sequence out grouped with AY125575. This virus strain was more closely related to the

CMV coat protein gene infecting banana (Fig. 3c). This analysis revealed that the internal node of phylogenetic tree was bootstrapping value of 95.

4.4.5.2d In silico Analysis of CMV Coat Protein gene of Isolate -4

The nucleotide sequence of coat protein gene of isolate (3529V) collected from Mannuthy, Thrissur district, Kerala infecting Grand Naine banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with CMV coat protein gene belongs to accession AY125575 and JX995139 infecting banana and *Nicotiana glutinosa* (Plate 17g). BLASTx analysis of sequence 4 revealed 100 per cent similarity with amino acid sequence of CMV coat protein gene of the accession CBG22867, AAY192856 and AAW71977 (Plate 17h).

The phylogenetic analysis of nucleotide sequence-4 of 3529V with top 10 BLASTn hit sequences revealed that two accessions AY125575 and JX995139 were shown to be closely related and these were well supported with bootstrap value (89). From the phylogenetic tree it was inferred that the isolate 3529V is closely related to the CMV coat protein gene infecting banana and *N. glutinosa* from India (Fig. 3d).

4.4.5.2e In silico Analysis of CMV Coat Protein gene of Isolate -5

The nucleotide sequence of coat protein gene of isolate (3529W) collected from Thalikulum, Thrissur district, Kerala infecting Robusta banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 98 per cent similarity with CMV coat protein gene belongs to accession AY125575, JX995139 and JF279609 infecting banana and *Nicotiana glutinosa* (Plate 17i). BLASTx analysis of sequence 5 revealed 98 per cent similarity with amino acid sequence of CMV coat protein gene of the accession AII01134 (Plate 17j).

The phylogenetic analysis based on the alignment of the sequence-5 of isolate 3529W with other CMV coat protein sequences of top 10 BLASTn hits revealed that

3529W clustered with well supported bootstrap value (91). The sequence clustered clustering into two internal nodes in isolate 3529W out grouped alone with other leaf nodes belonging to different cluster. This indicated the similarities of the sequences similar to accessions AY125575, JX995139 and JF279609 belonging to CMV coat protein from India infecting banana, tobacco and musk melon respectively (Fig. 3e).

4.4.5.2f In silico Analysis of CMV Coat Protein gene of Isolate -6

The nucleotide sequence of coat protein gene of isolate (3529X) collected from Thrissur district, Kerala infecting Amritsagar banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTX. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with CMV coat protein gene belongs to accession AY125575, JX995139 and JF279609 infecting banana and *Nicotiana glutinosa* (Plate 17k). BLASTx analysis of sequence 6 revealed 100 per cent similarity with amino acid sequence of CMV coat protein gene of the accession CAA07411 (Plate 17l).

The phylogenetic analysis revealed that the sequence aligned with top 10 sequences obtained from BLASTn clustered with well supported bootstrap value 88 which branched internally with two internal nodes. This analysis shows that 3529X to be most similar to accession JF27609 of CMV coat protein gene infecting musk melon in India. The clustering was well supported with two internal nodes branching with bootstrap value (88) (Fig. 3f).

4.4.5.2g In silico Analysis of CMV Coat Protein gene of Isolate -7

The nucleotide sequence of coat protein gene of isolate (3529Y) collected from Kannara, Thrissur district, Kerala infecting Changalikodan banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with CMV coat protein gene belongs to accession AY125575 and JX995139 infecting banana and *Nicotiana glutinosa* (Plate 17m). BLASTx analysis of sequence 7 revealed 99 per cent

similarity with amino acid sequence of CMV coat protein gene of the accession AAY19285 (Plate 17n).

The phylogenetic analysis based upon nucleotide alignment of sequence belonging to isolate-7 (3529Y) clearly revealed that it belonged to CMV coat protein gene and this was well support by 87 bootstrapping value. From the phylogenetic tree it was inferred that the isolate 3529Y was found to be most closely related to AY125575 and JX995139 infecting banana and tobacco from India (Fig. 3g).

4.4.5.2h In silico Analysis of CMV Coat Protein gene of Isolate -8

The nucleotide sequence of coat protein gene of isolate (3529Z) collected from Marakkal, Thrissur district, Kerala infecting Amritsagar banana cultivar amplified using designed primer was subjected to NCBI BLASTn and BLASTx. The comparison of coat protein nucleotide sequence showed 99 per cent similarity with CMV coat protein gene belongs to accession AY125575, JX995139 and JF279609 infecting banana and *Nicotiana glutinosa* (Plate 17o). BLASTx analysis of sequence 8 revealed 99 per cent similarity with amino acid sequence of CMV coat protein gene of the accession CAA07411 (Plate 17p).

During phylogenetic analysis of nucleotide sequence of sequence-8 of isolate 3529Z, similar trend was observed like in case of isolate 3529Y. It was clear that the sequence belonged to CMV coat protein gene and this clustered with the bootstrapping value 85. From the phylogenetic tree it was inferred that the isolate 3529Z was found to be most similar to accessions AY125575 and JX995139 infecting banana and tobacco from India (Fig. 3h).

Summing up the findings obtained during *in silico* analysis, it was observed that, all sequences were having highest identity with CMV coat protein gene belonging to Cucumovirus.

A neighboring-joining phylogenetic tree upon an alignment of nucleotide sequence of eight isolates from the study and other sequences (42) reported from India and elsewhere available in the GeneBank database. From the phylogenetic tree, it was revealed that the tree was rooted on CMV coat protein gene strains, belonging to cucumovirus. Phylogenetic analysis clearly divided CMV strains into four clusters of subgroup IA, IB, II and other CMV group. During analysis it was inferred that all eight isolates clustered showing close relationship with the subgroup IB strains and clustered with the sequences which showed highest homology with CMV strain AY125575 infecting banana followed by AY690620 and AY690621 infecting Beetle vine (*Piper beetle*) and pepper (*Piper longum*) from Calicut, Kerala, India. Phylogenetic branching of CMV-CP clad of group IB showed closest relationships along with the other member of genus CMV Cucumovirus: D49496, D42079 and AF013291 forming an out group cluster which belong to unknown group of CMV. Sequences of CMV strains belonging to subgroup IB and II clustered in different clade (Fig. 3i).

4.4.6 Cloning of Coat Protein Gene

The amplified product of CMV coat protein gene belonging to isolate-1 (3529CTRL) was cloned in to the T-tailed pGEM-T easy vector and transformed in to *Escherichia coli* cells (DH5α) strain. The transformed colonies were selected based on blue/white colony selection and were sub-cultured individually (Plate 18, Fig. A).

4.4.6.1 Analysis of Recombinants by Colony PCR

The potent colonies were screened for the presence of recombinant plasmid by performing colony PCR by using flanking primers T7 and Sp6 (Promega, USA) following the protocol as in section 3.5.6.5. The transformed colonies were subjected to colony PCR by using flanking primers T7 and Sp6 (Promega, USA) following the protocol as in section 3.5.6.5. The amplicons obtained after performing colony PCR were visualized and documented with 1.2 per cent agarose gel electrophoresis with an expected band size of 900-950 bp for cloned colonies and 750 bp for cDNA product alone (Plate 18, Fig. B). The non recombinant cells (blue colony) were documented with

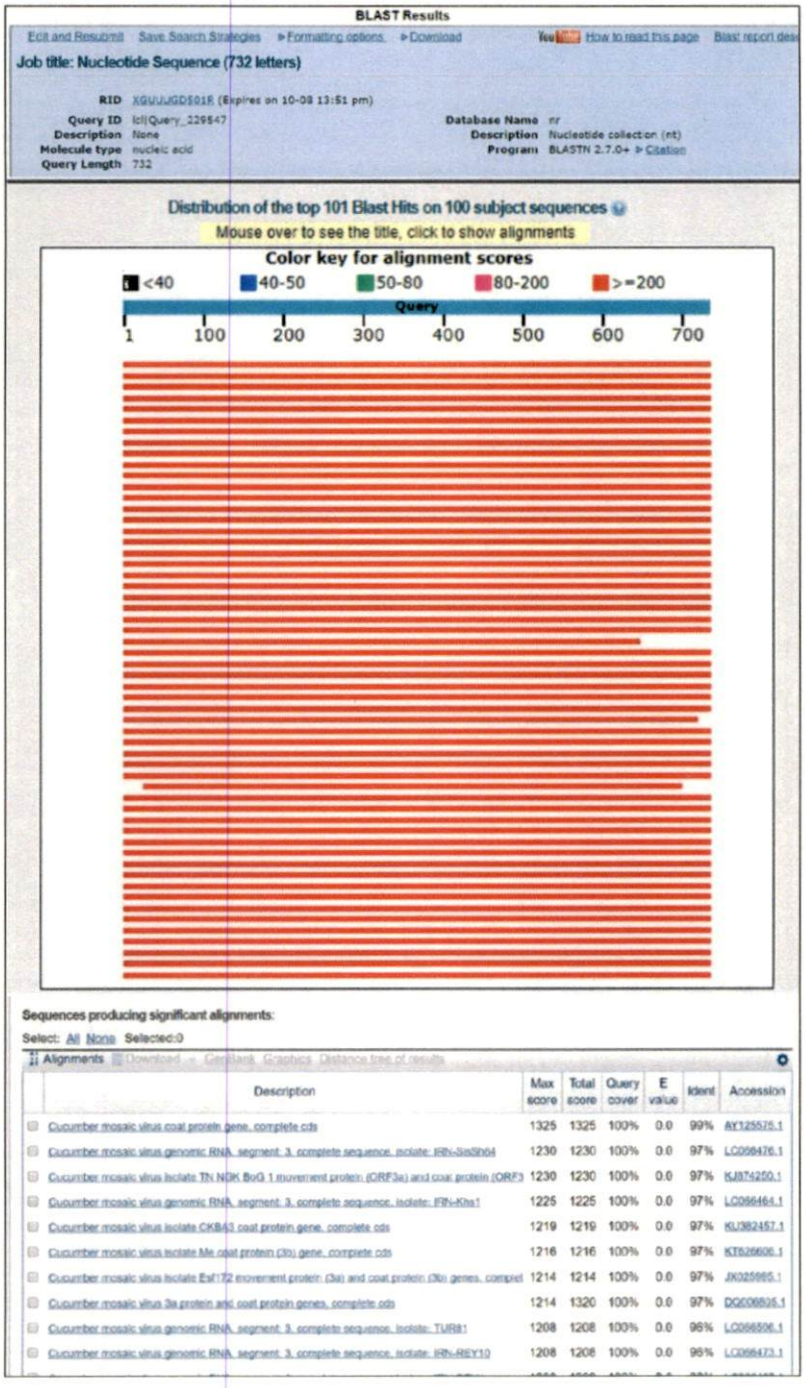


Plate 17a: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 1

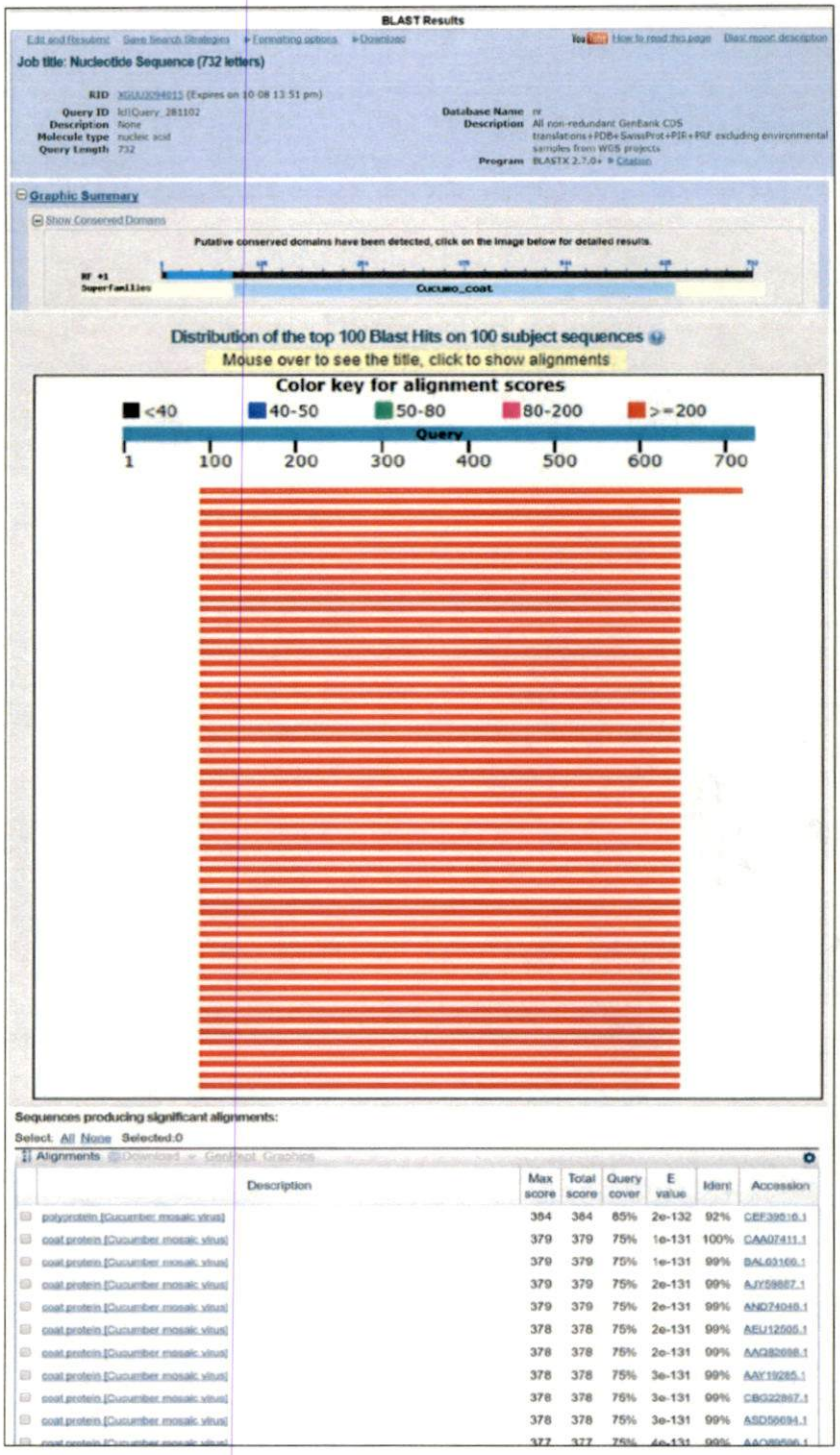


Plate 17b: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 1

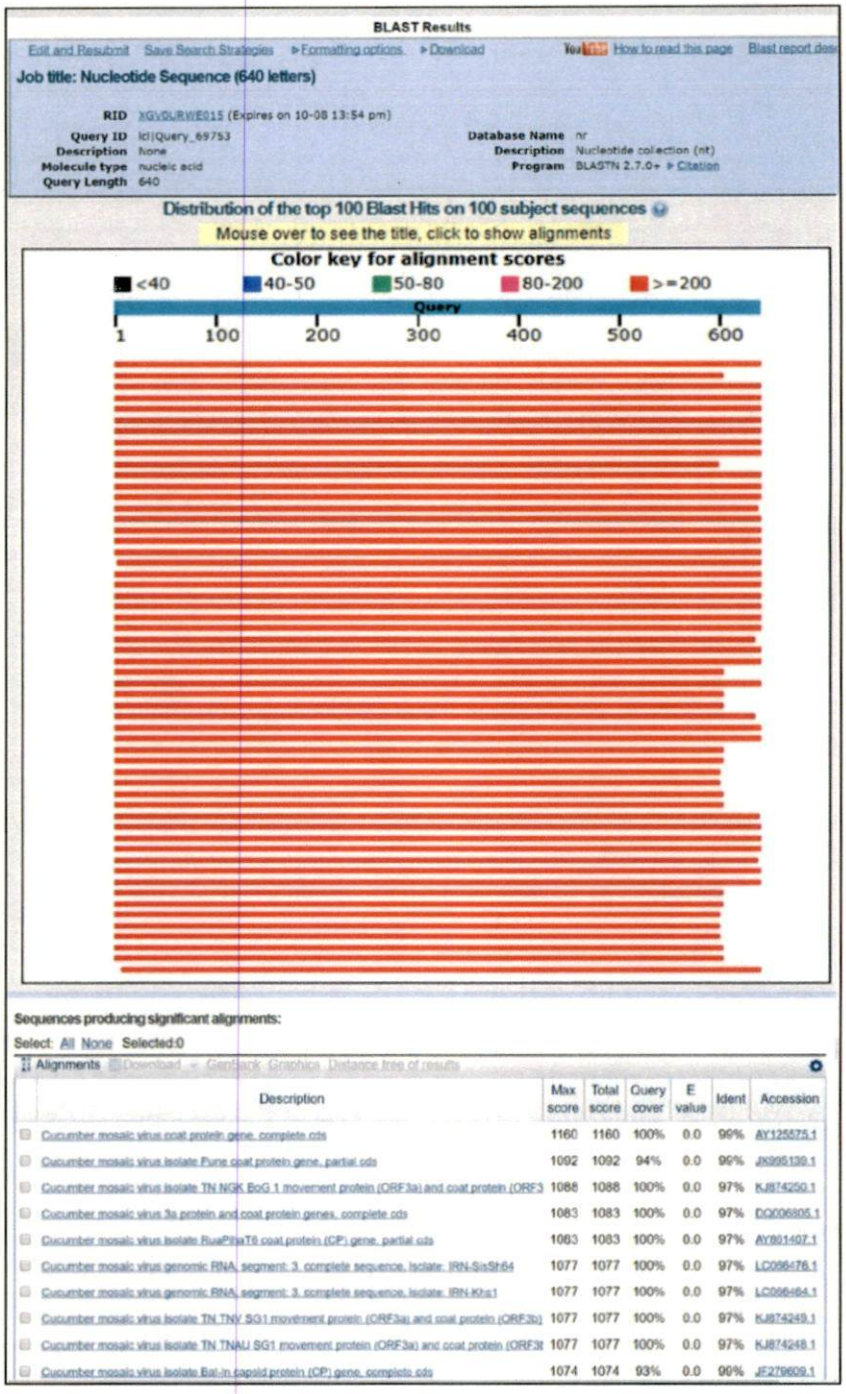


Plate 17c: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 2

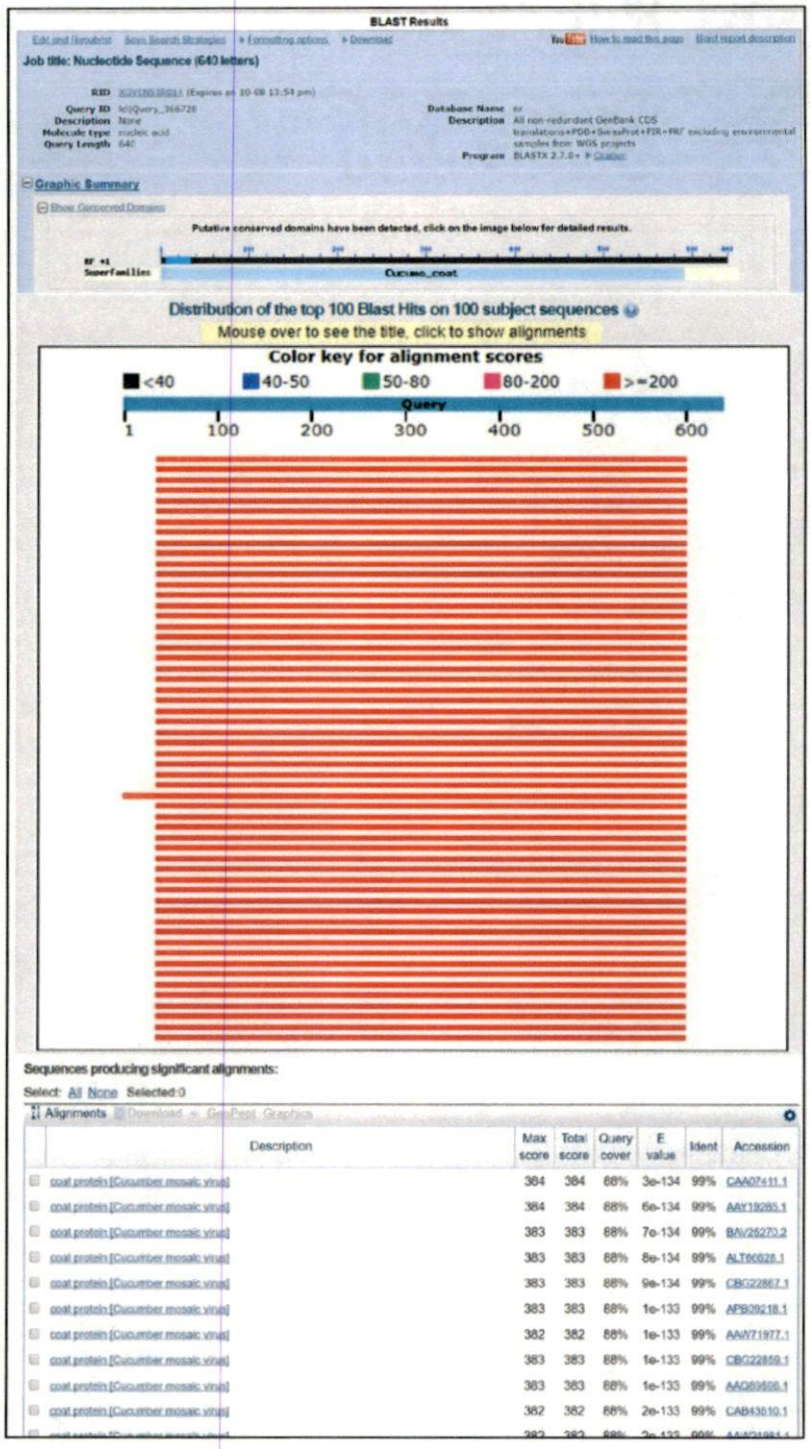


Plate 17d: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 2

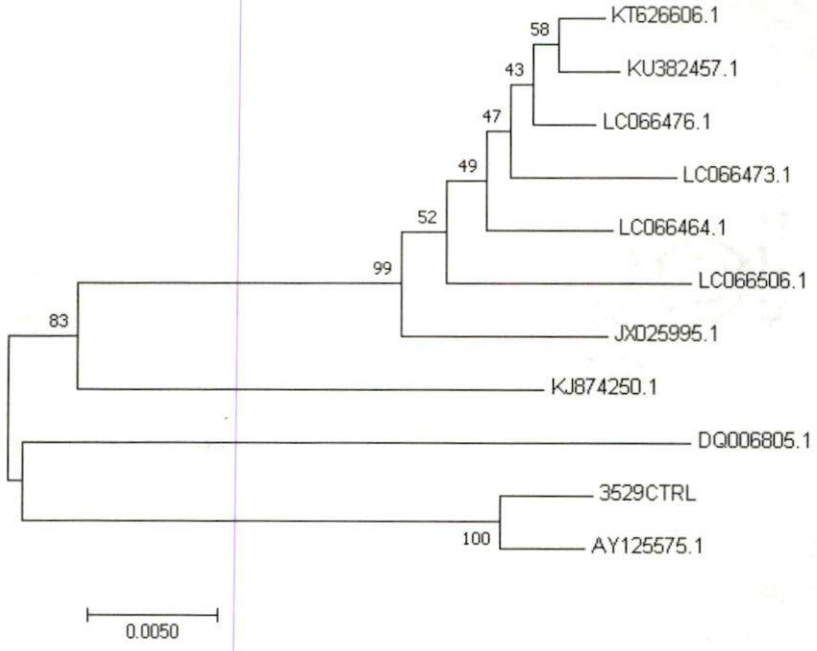


Fig. 3a: Phylogenetic analysis of CMV-coat protein sequence of isolate-1 (3529CTRL) with top 10 hits of BLASTn

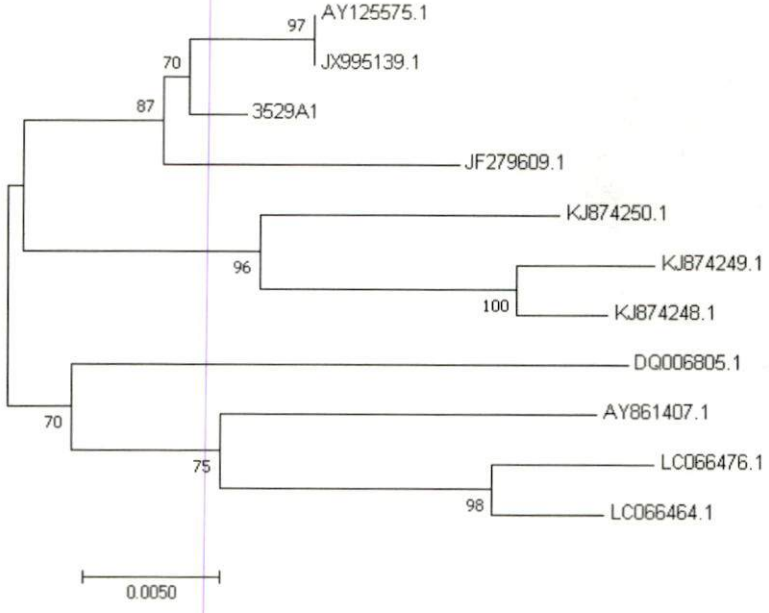


Fig. 3b: Phylogenetic analysis of CMV-coat protein sequence of isolate-2 (3529A1) with top 10 hits of BLASTn

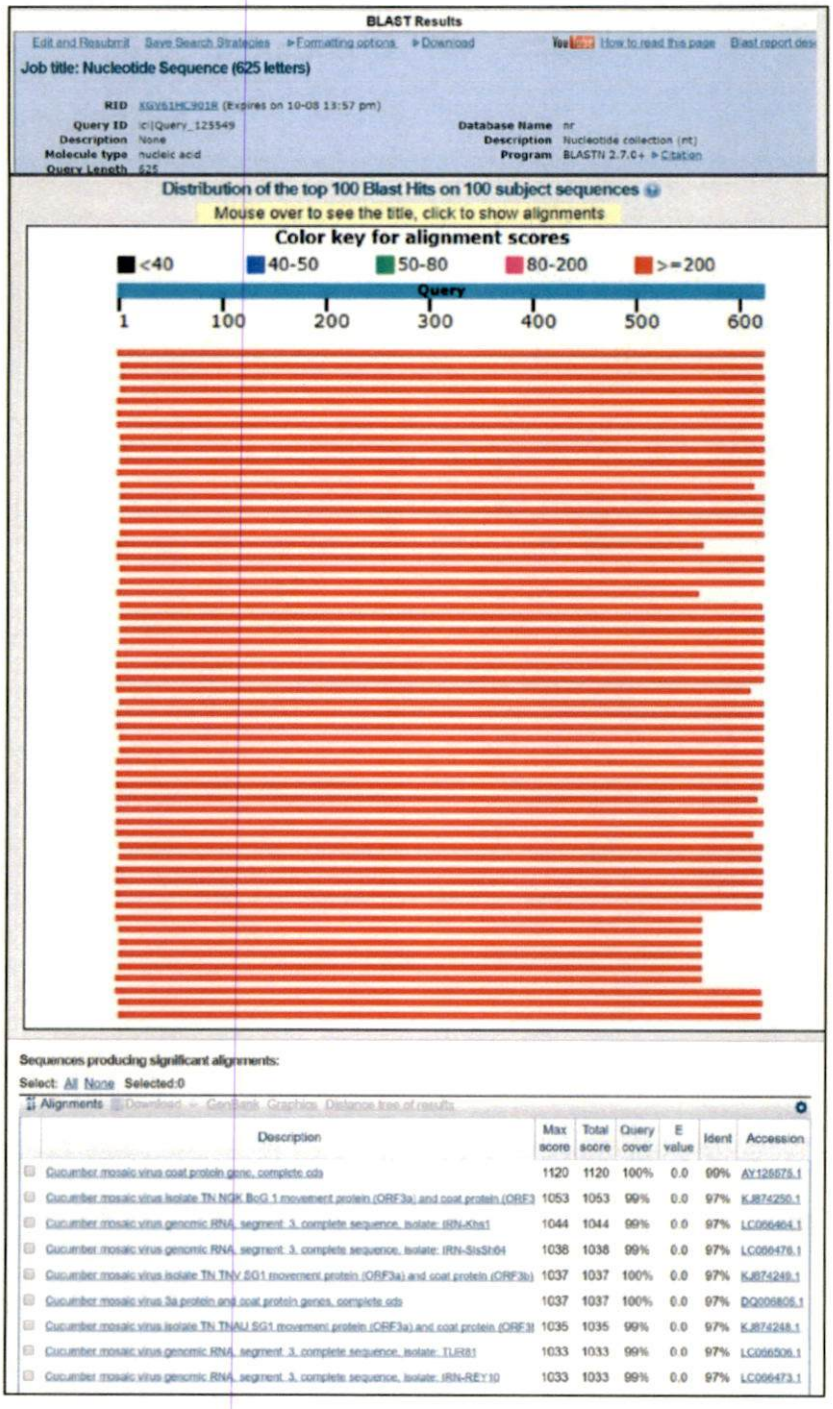


Plate 17e: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 3

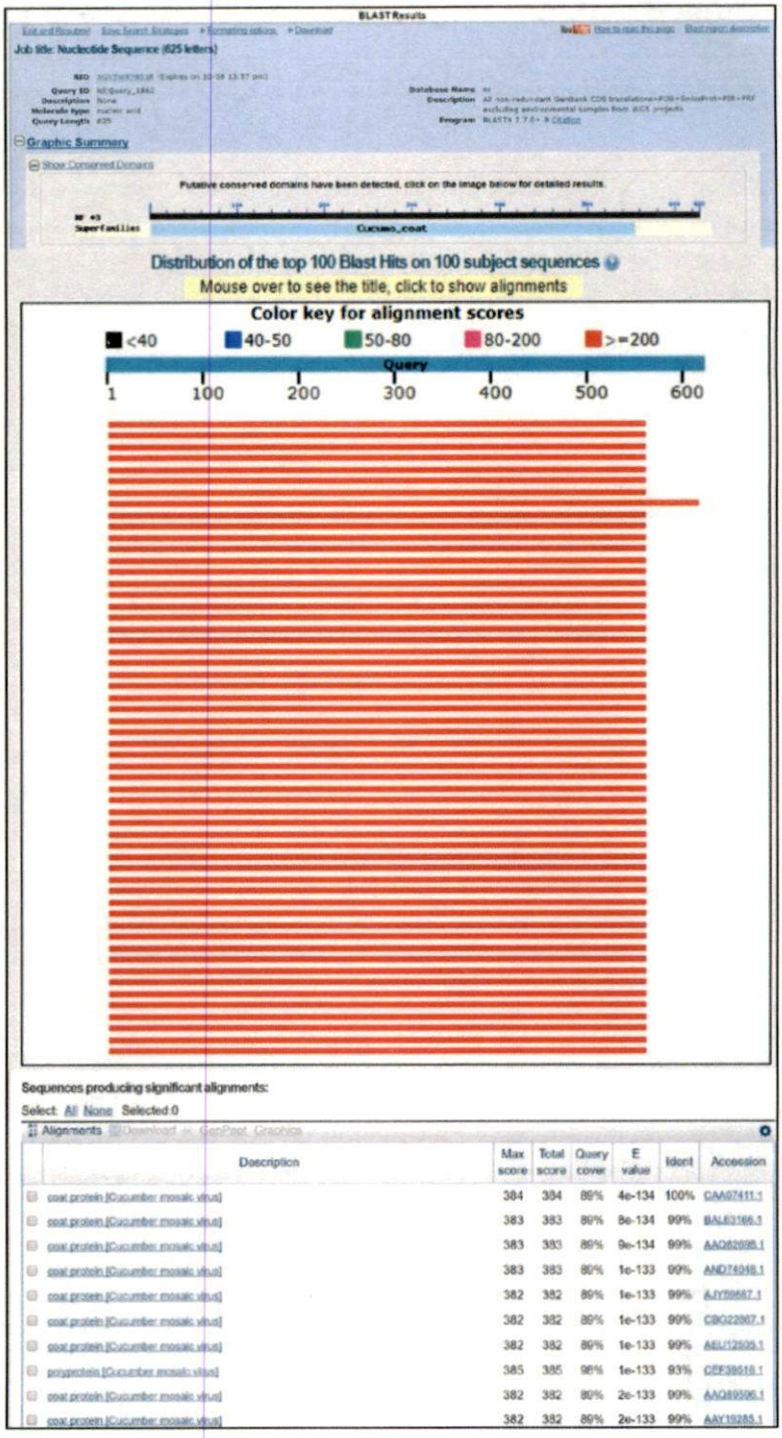


Plate 17f: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 3

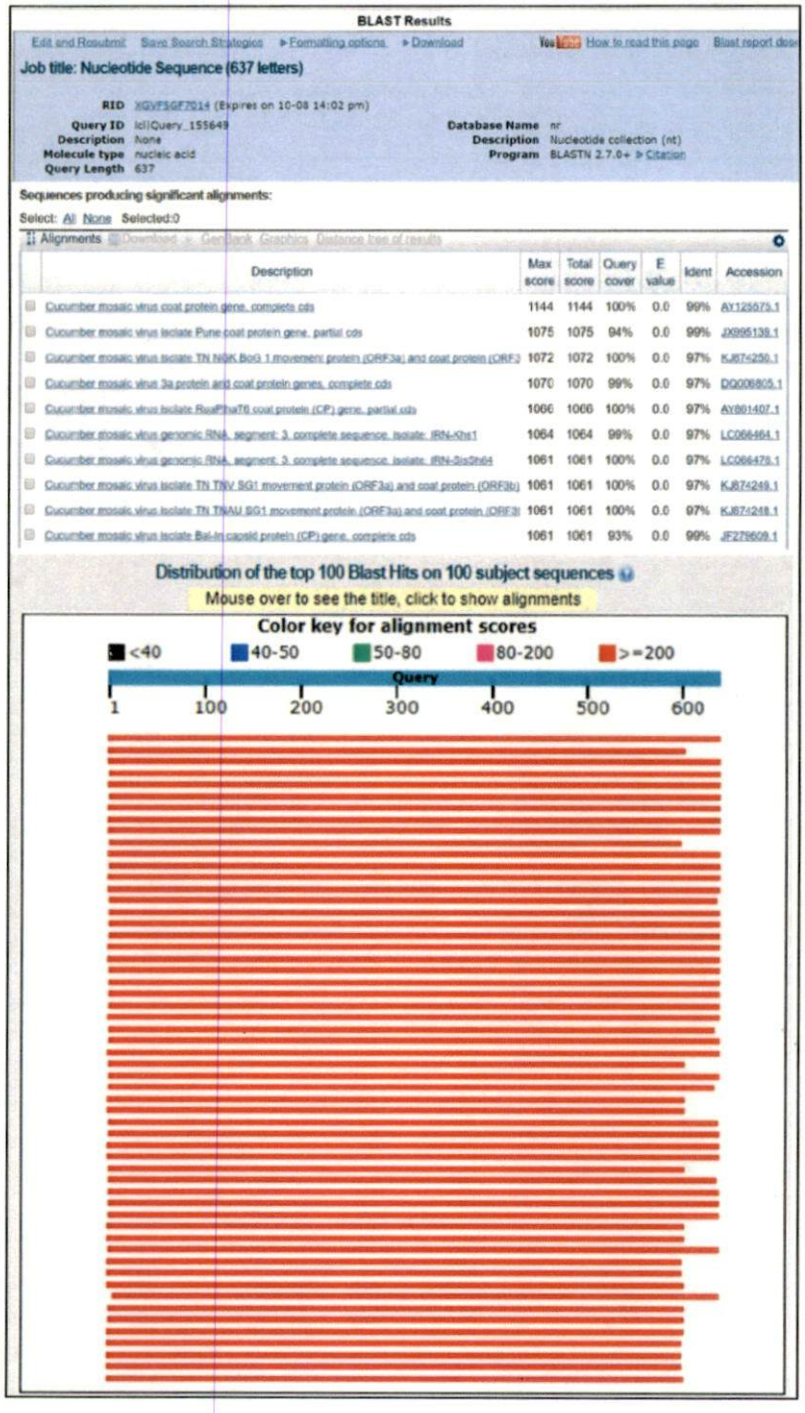


Plate 17g: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 4

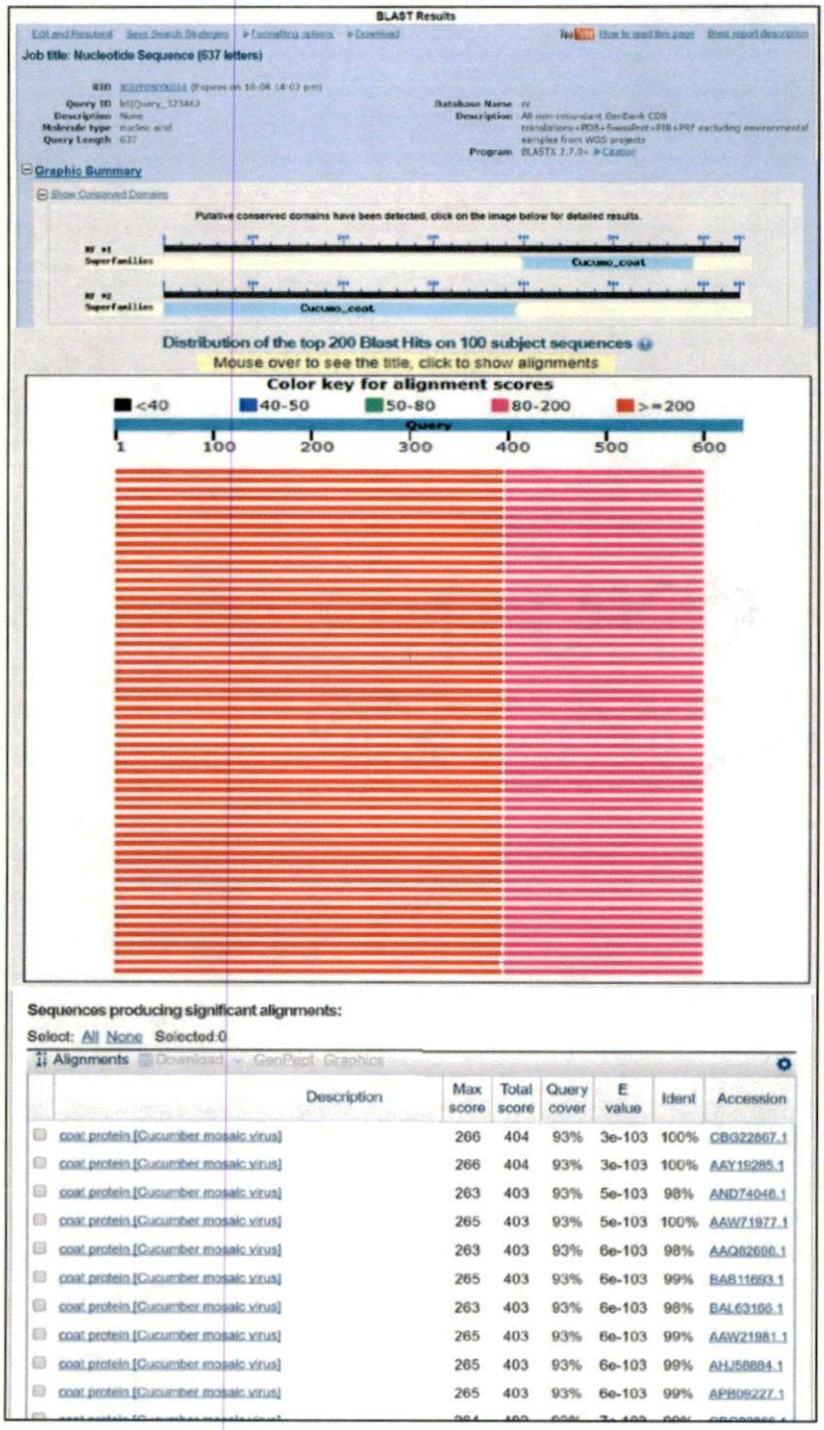


Plate 17h: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 4

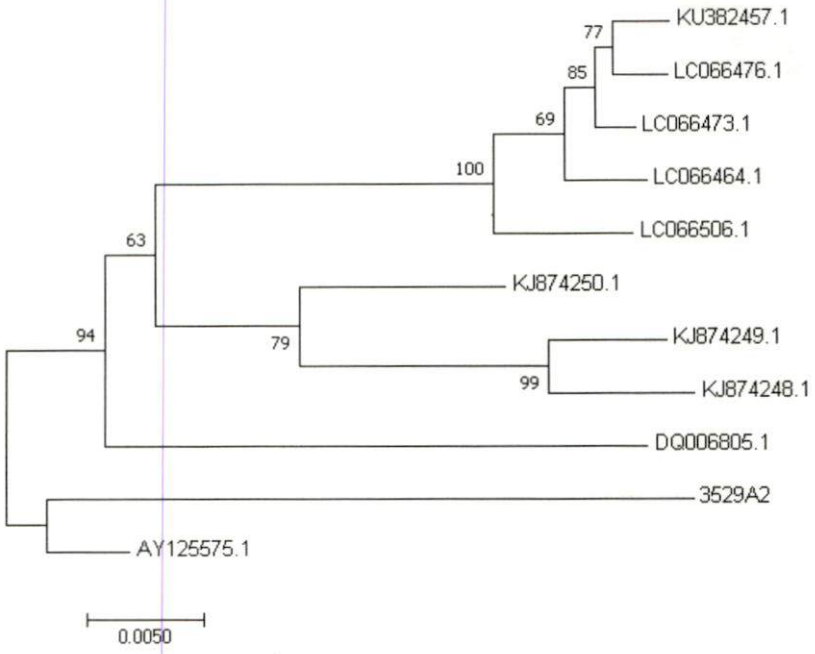


Fig. 3c: Phylogenetic analysis of CMV-coat protein sequence of isolate-3 (3529A2) with top 10 hits of BLASTn

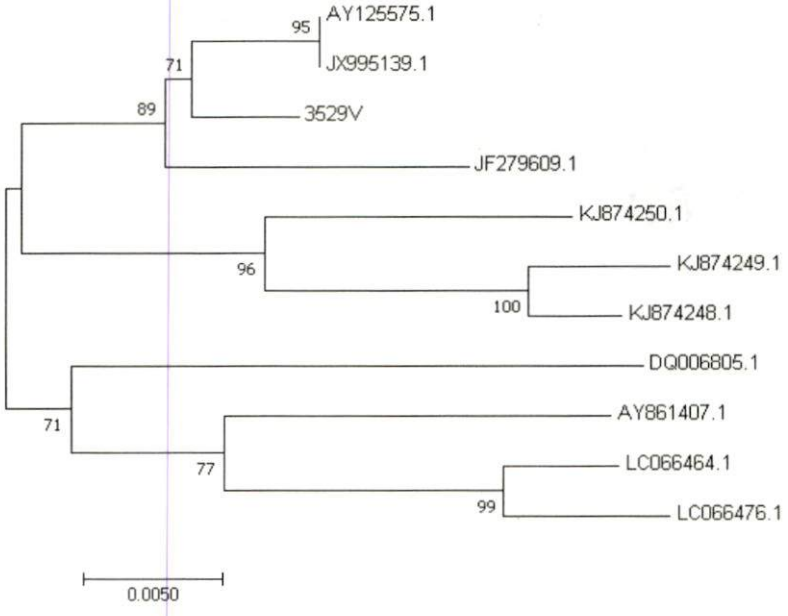


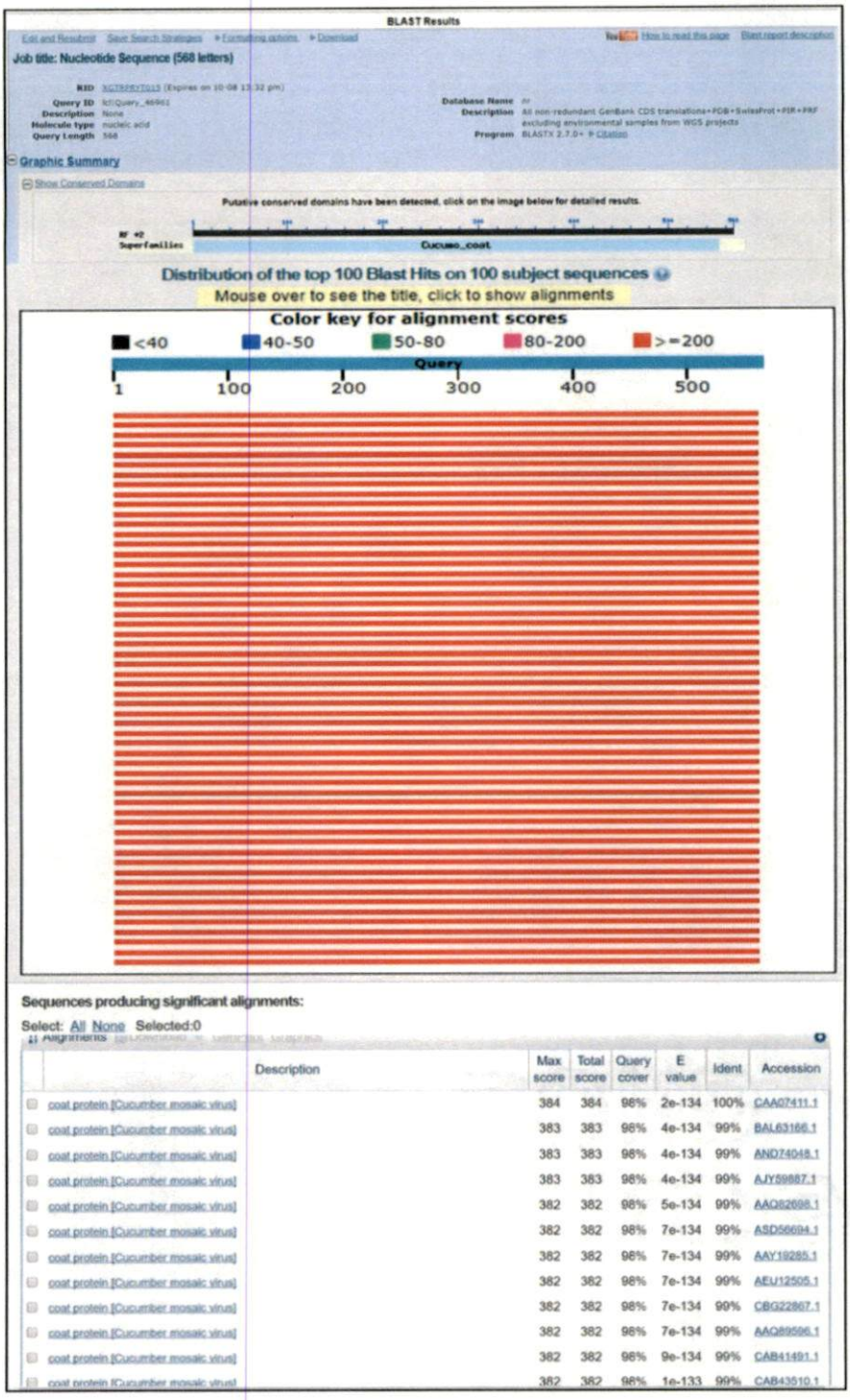
Fig. 3d: Phylogenetic analysis of CMV-coat protein sequence of isolate-4 (3529V) with top 10 hits of BLASTn

Plate 17i: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 5

Plate 17j: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 5

Description	Max score	Total score	Query cover	E value	Ident	Accession
Cucumber mosaic virus coat protein gene, complete cds	1022	1022	100%	0.0	99%	AY125575.1
Cucumber mosaic virus isolate Pune coat protein gene, partial cds	1016	1016	98%	0.0	99%	JX995139.1
Cucumber mosaic virus isolate Bal-In capsid protein (CP) gene, complete cds	1014	1014	98%	0.0	99%	JF279609.1
Cucumber mosaic virus isolate TN NGK BoG 1 movement protein (ORF3a) and coat protein (ORF3	959	959	99%	0.0	97%	KJ874250.1
Cucumber mosaic virus from India coat protein gene, complete cds	955	955	98%	0.0	97%	DQ285569.1
Cucumber mosaic virus isolate TN NGK BoG1 coat protein (ORF3b) gene, complete cds	953	953	98%	0.0	97%	KF891361.1
Cucumber mosaic virus isolate TN TNAU SG1 movement protein (ORF3a) and coat protein (ORF3	950	950	100%	0.0	97%	KJ874248.1
Cucumber mosaic virus RNA3 complete segment, genomic RNA, strain Vir	950	950	100%	0.0	97%	HE962480.1
Cucumber mosaic virus 3a protein and coat protein genes, complete cds	950	950	100%	0.0	97%	DQ306805.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: IRN-Khs1	948	948	99%	0.0	97%	LC066464.1
Cucumber mosaic virus isolate LOR_C coat protein (CP) gene, complete cds	948	948	98%	0.0	97%	KT279567.1

Plate 17k: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 6



Description	Max score	Total score	Query cover	E value	Ident	Accession
coat protein [Cucumber mosaic virus]	384	384	98%	2e-134	100%	CAA07411.1
coat protein [Cucumber mosaic virus]	383	383	98%	4e-134	99%	BAL63166.1
coat protein [Cucumber mosaic virus]	383	383	98%	4e-134	99%	AND74048.1
coat protein [Cucumber mosaic virus]	383	383	98%	4e-134	99%	AJY59887.1
coat protein [Cucumber mosaic virus]	382	382	98%	5e-134	99%	AAQ82698.1
coat protein [Cucumber mosaic virus]	382	382	98%	7e-134	99%	ASD56694.1
coat protein [Cucumber mosaic virus]	382	382	98%	7e-134	99%	AAY19285.1
coat protein [Cucumber mosaic virus]	382	382	98%	7e-134	99%	AEU12505.1
coat protein [Cucumber mosaic virus]	382	382	98%	7e-134	99%	CBG22867.1
coat protein [Cucumber mosaic virus]	382	382	98%	7e-134	99%	AAQ89596.1
coat protein [Cucumber mosaic virus]	382	382	98%	9e-134	99%	CAB41491.1
coat protein [Cucumber mosaic virus]	382	382	98%	1e-133	99%	CAB43510.1

Plate 17l: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 6

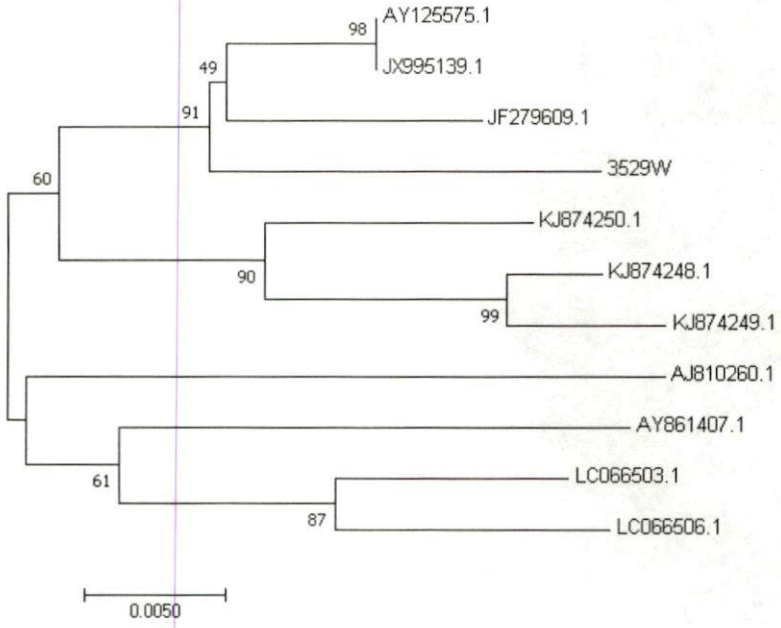


Fig. 3e: Phylogenetic analysis of CMV-coat protein sequence of isolate-5 (3529W) with top 10 hits of BLASTn

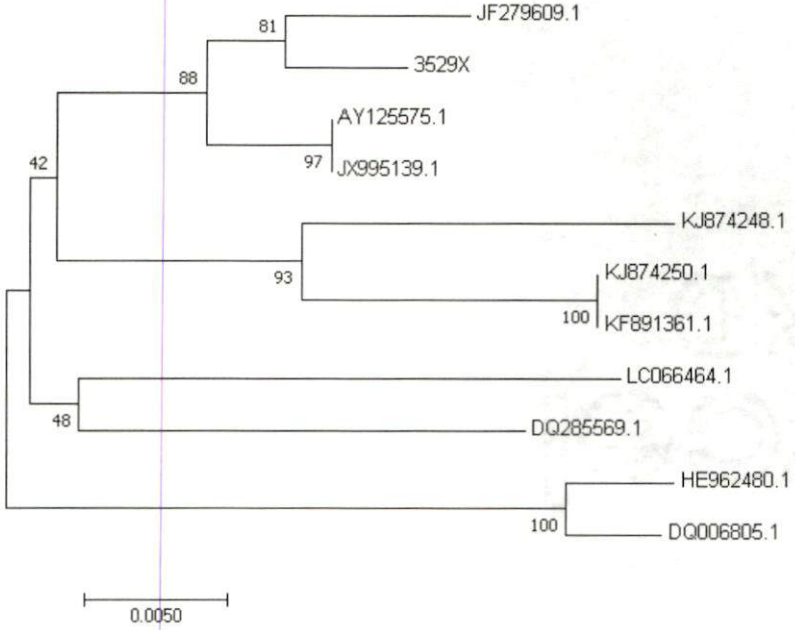


Fig. 3f: Phylogenetic analysis of CMV-coat protein sequence of isolate-6 (3529X) with top 10 hits of BLASTn

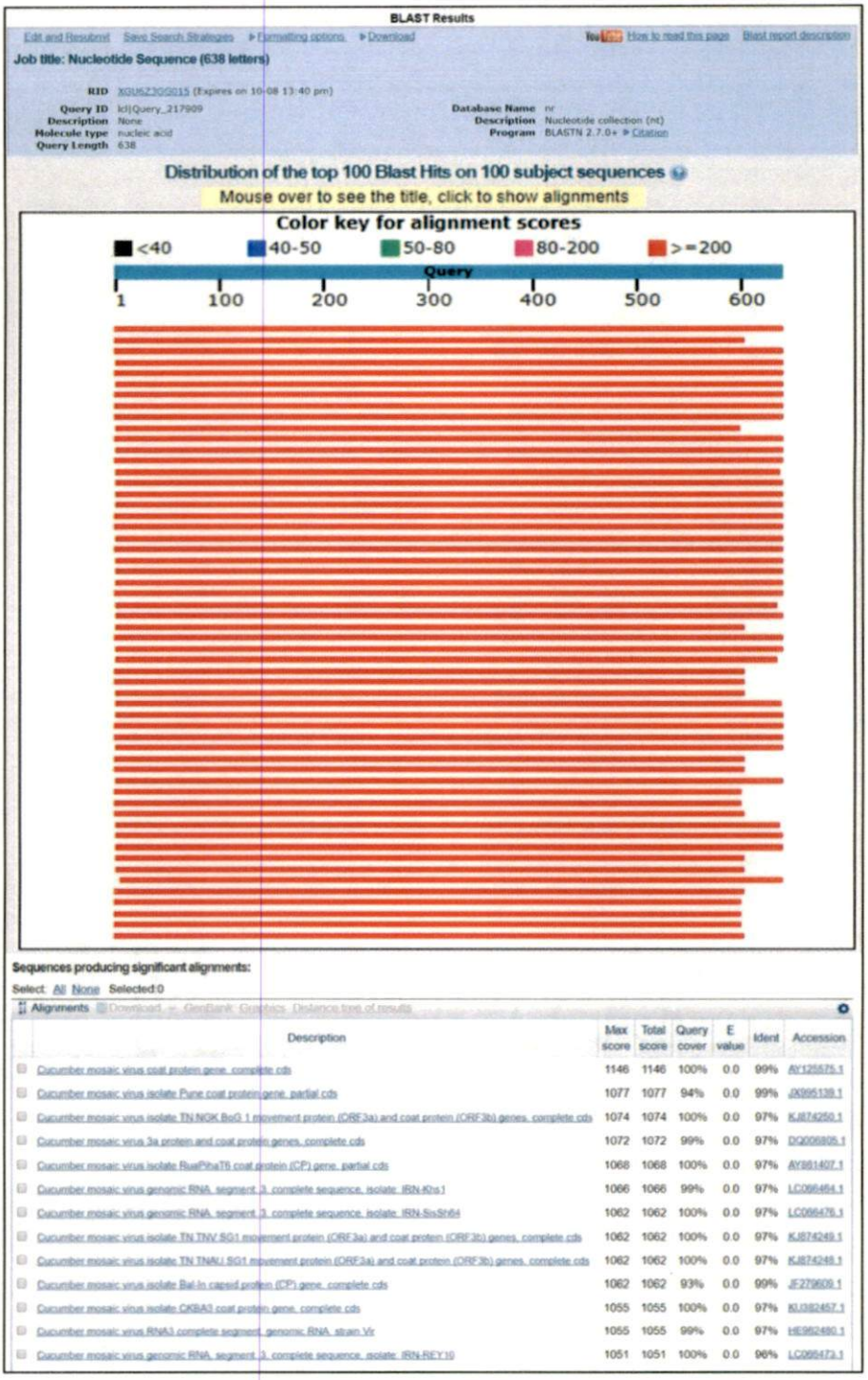
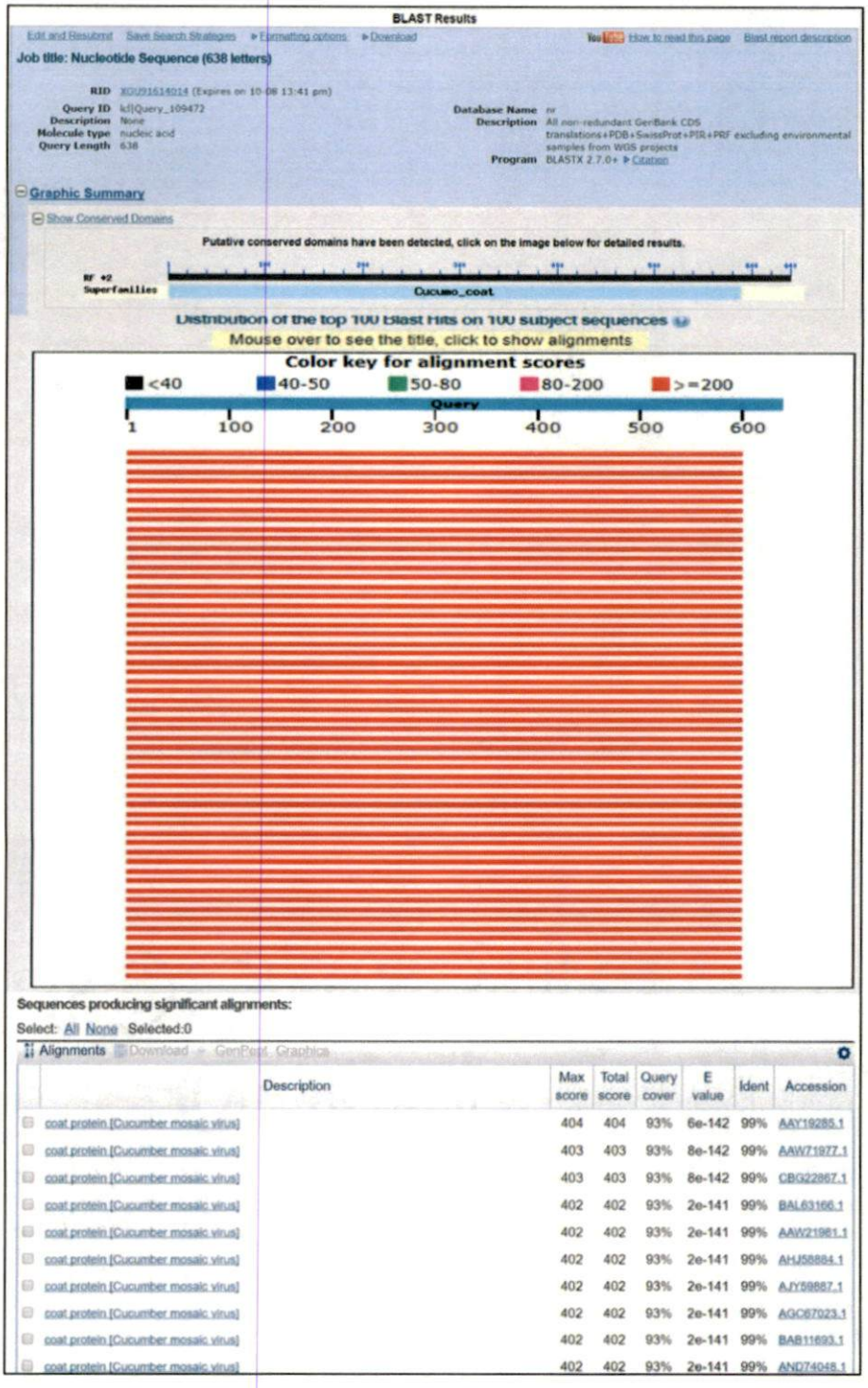


Plate 17m: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 7



Description	Max score	Total score	Query cover	E value	Ident	Accession
coat protein [Cucumber mosaic virus]	404	404	93%	6e-142	99%	AAY19285.1
coat protein [Cucumber mosaic virus]	403	403	93%	8e-142	99%	AAW71977.1
coat protein [Cucumber mosaic virus]	403	403	93%	8e-142	99%	CBG22867.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	BAL63166.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	AAW21981.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	AHJ58884.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	AJY59887.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	AGC67023.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	BAB11693.1
coat protein [Cucumber mosaic virus]	402	402	93%	2e-141	99%	AND74048.1

Plate 17n: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 7

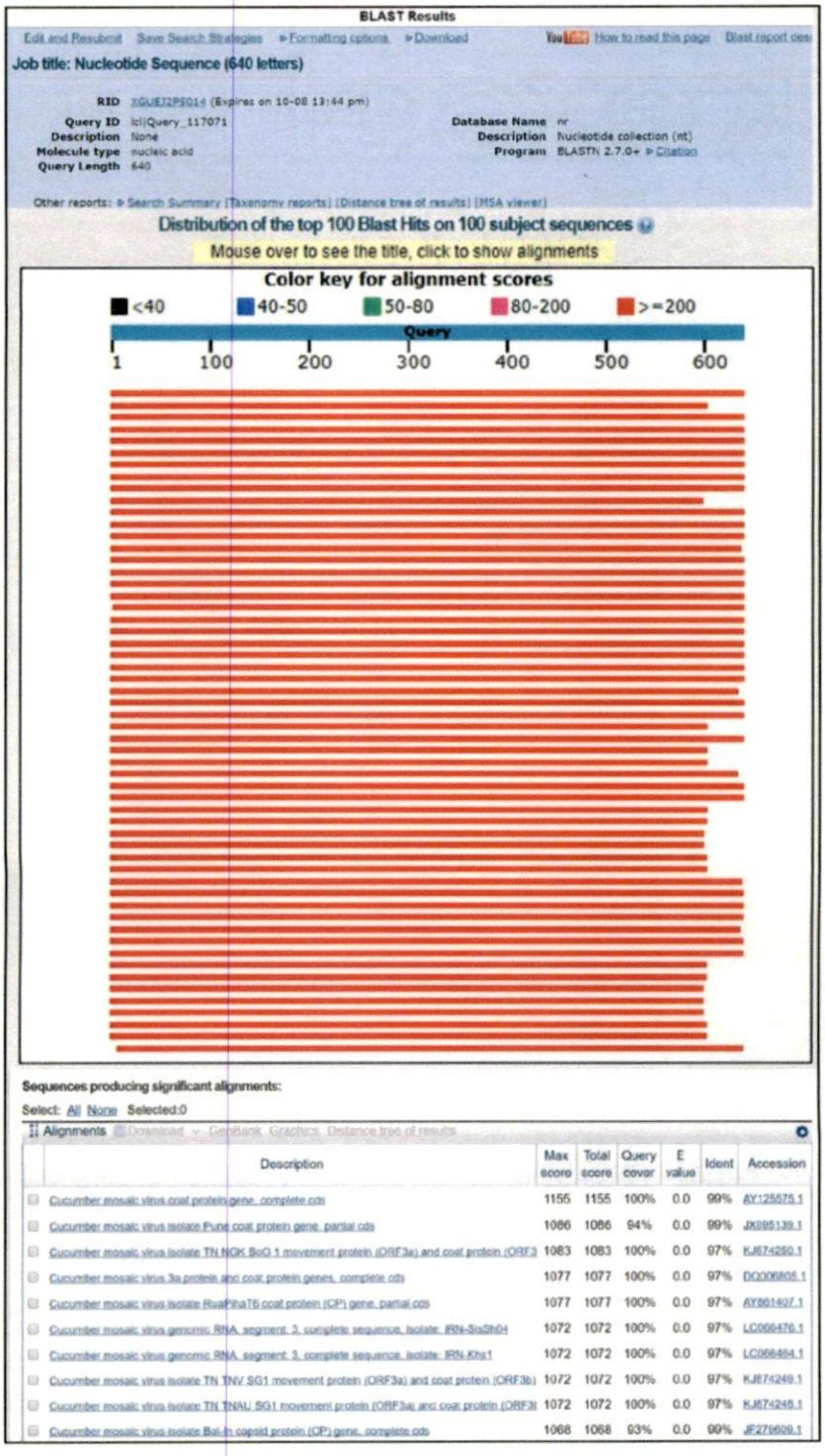


Plate 17o: BLASTn graphical and text output of nucleotide sequence of partial coat protein gene of isolate 8

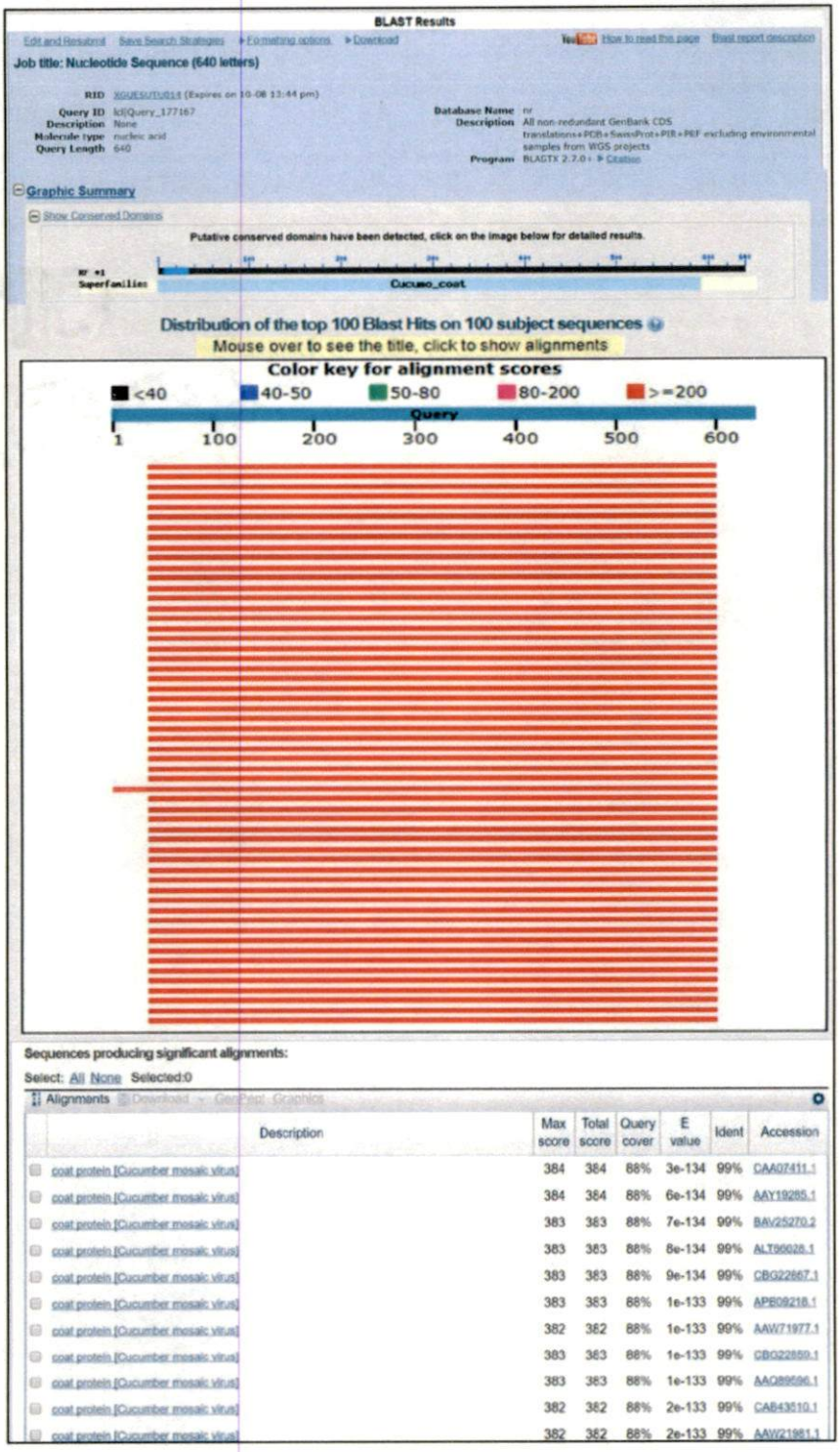


Plate 17p: BLASTx graphical and text output of amino acid sequence of partial coat protein gene of isolate 8

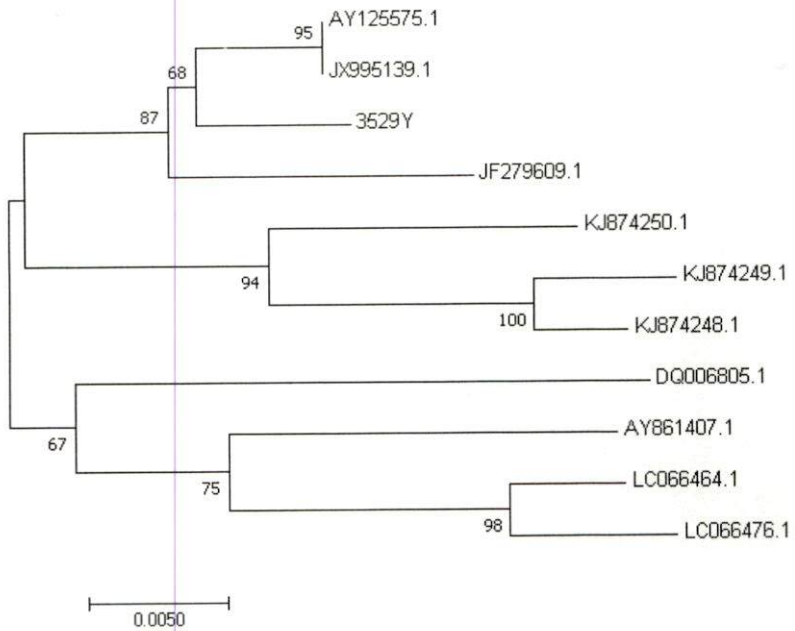


Fig. 3g: Phylogenetic analysis of CMV-coat protein sequence of isolate-7 (3529Y) with top 10 hits of BLASTn

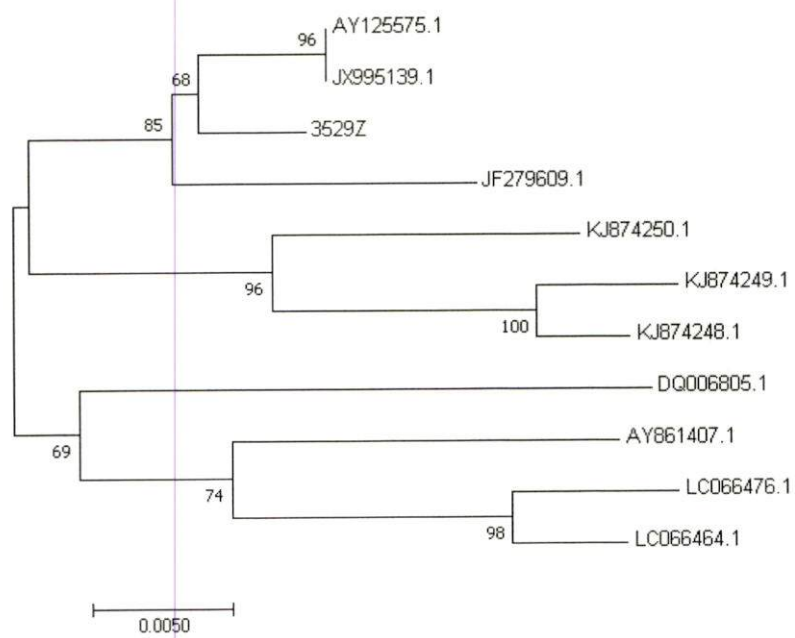


Fig. 3h: Phylogenetic analysis of CMV-coat protein sequence of isolate-8 (3529Z) with top 10 hits of BLASTn

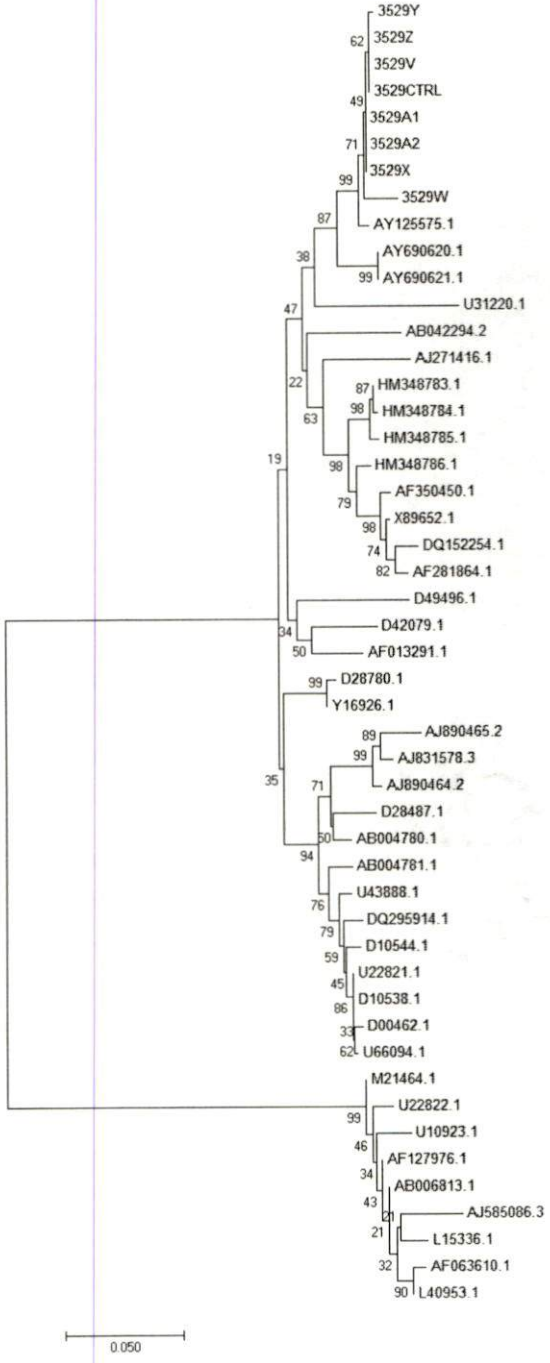


Fig. 3i: Phylogenetic tree showing relationship of CMV-coat protein sequence of all 8 isolates of CMV infecting banana

Fig. A: *E. coli* DH5α cells transformed (white colonies) with CMV CP gene and non recombinants (blue colonies) on LB agar media

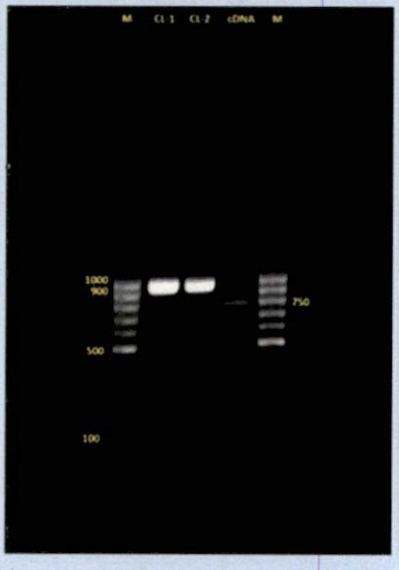


Fig. B: Analysis of recombinant clones by colony PCR:
M- Marker (100bp Plus); Lane 2and 3- recombinant plasmid; lane 4- PCR product

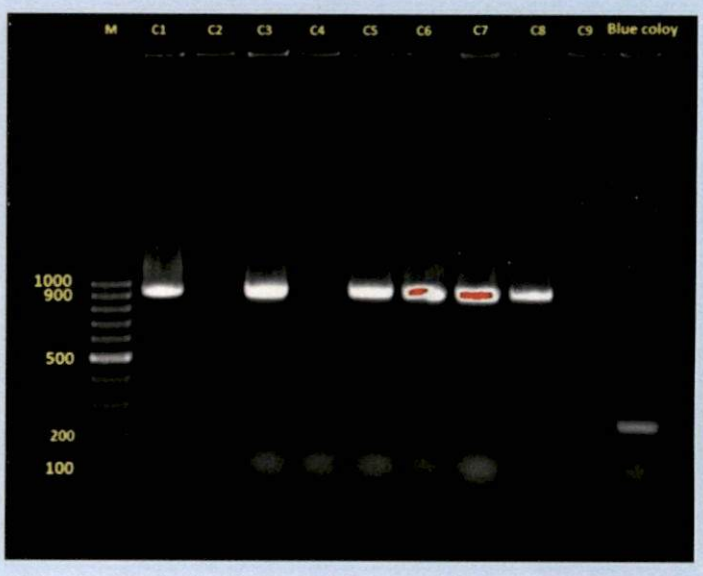


Fig. C: Analysis of recombinant and non recombinant clones by colony PCR:
M- Marker (100bp Plus); C1-C9 (recombinant Clones); Blue Colony (non recombinant cell)

Plate 18: Confirmation of cDNA insert using colony PCR

200 bp (Plate 18, Fig. C). Pure cultures of recombinant clones were maintained in Luria Bertani Agar (LBA) stab and glycerol stock and deposited in Department of Plant Pathology, College of Horticulture, Vellanikkara.

4.5 DEVELOPMENT OF NANOBASED BIOSENSOR FOR DETECTION OF VIRUS

4.5.1 Synthesis of Gold Nanorod (GNRs)

The synthesis of gold nanorods was carried out by following the procedure as described by Nokoobakht and El-Sayed (2003). It involved the preparation of two solution *viz.,* seed solution and growth solution.

4.5.1.1 Preparation of Seed Solution

Seed solution was prepared using Hydrogen Aurium Chloro Tetra Hydrate ($HAuCl_4$), Cetyl Trimethyl Ammonium Bromide (CTAB) and Sodium borohydrate ($NaBH_4$) (Plate 19, Fig. A).

4.5.1.2 Preparation of Growth Solution

Growth solution prepared by using different chemical components such as Aurium Chlorohydrate ($HAuCl_4$), CTAB, Silver Nitrate ($AgNo_3$) and Ascorbic acid (Plate 19, Fig. B).

4.5.1.3 Synthesis of GNR Solution

To prepare GNRs, synthesized (100 µl) seed solution was added to the growth solution (50 ml) which led to the formation of pinkish red colour within 20 min. The change of colour from transparent growth solution to pinkish red colour was due to formation and growth of GNRs within 20 min (Plate 20, Fig. A).

4.5.2 Purification and Characterization of GNRs

The primary growth solution was centrifuged at 15000g for 35 min in 20 ml centrifuge tubes. The gravitational force on the particles caused the particles to sediment

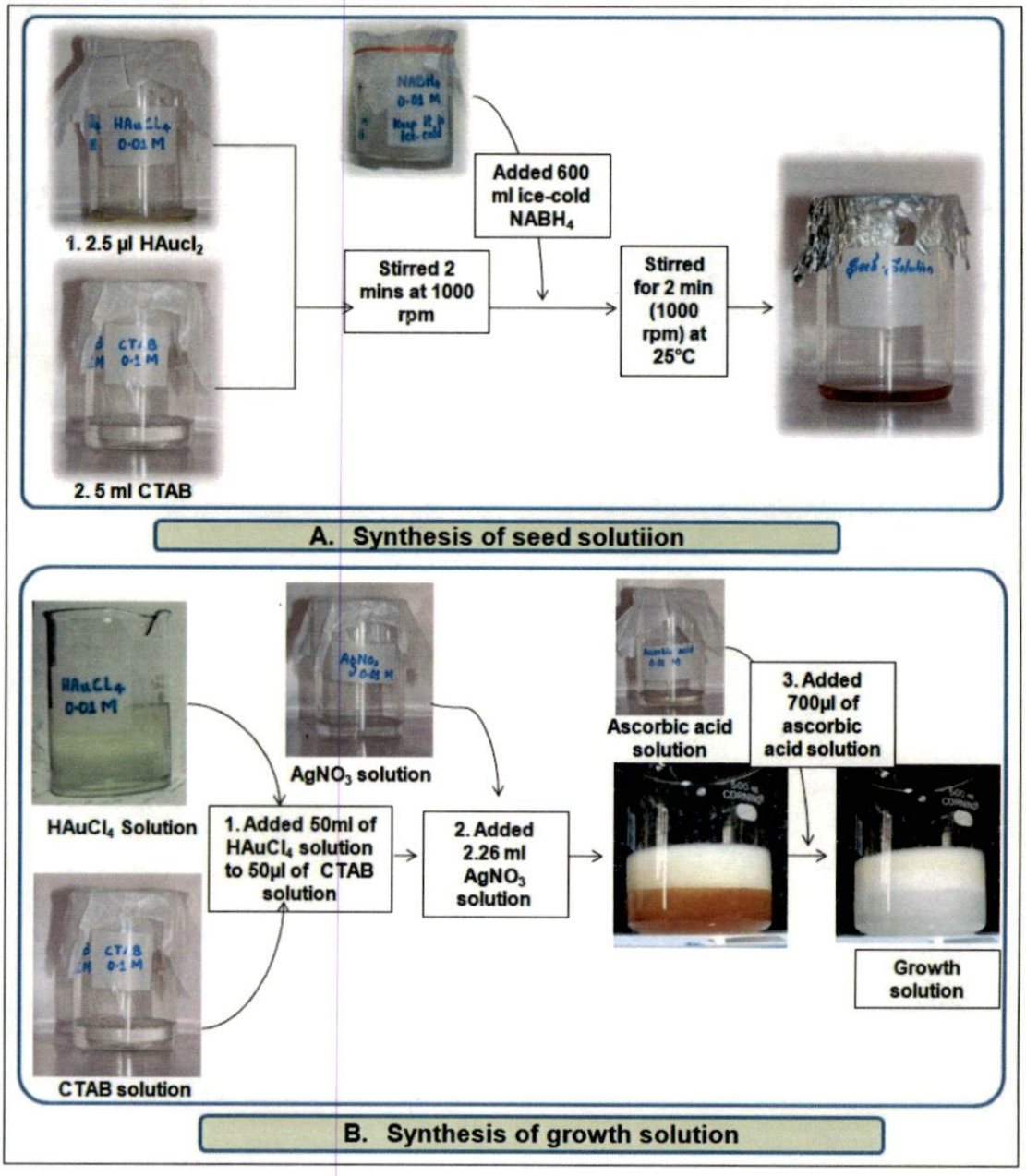


Plate 19: Flow chart showing the synthesis of seed and growth solutions

to the bottom of the tube. The difference in mass between the GNRs and the impurities resulted in the separation of the particles. The GNRs move towards the side of the centrifuge tube and the gold nanospheres remained at the bottom of the tube. The impurities and the supernatant were removed and the purified GNRs were re-suspended in ultrapure water.

4.5.3 Characterization of GNRs

The GNRs particles were characterized using UV-Vis spectrophotometer and Transmission electron microscope.

4.5.3.1 Characterization of GNRs by UV-Vis Spectrophotometer

The characterization of GNRs particles was done using UV-Vis spectrophotometer. The absorption spectra of the synthesized GNRs were taken at the wavelength ranging between 200 nm to 1100 nm. The absorption spectrum was observed in two bands. One being longitudinal plasmon band (LPB) and the other transverse plasmon band (TPB). Synthesized GNRs recorded LPB at 710.70 nm and TPB at 523.58 nm (Plate 20, Fig, B) confirming the typical characteristic absorptive peak of GNRs.

4.5.3.2 Characterization of GNRs using Transmission Electron Microscope (TEM)

From the TEM images of GNRs (Plate 20, Fig, B), twenty different GNR particles were randomly selected to measure their length and width using Image J software. The length and width of GNR particles ranged between 49 to 61 nm and 11 to 21 nm respectively (Table 33) and mean length and width were 54.96 nm and 14.75 nm respectively.

4.5.4 Estimation of Aspect Ratio (AR) of GNRs

4.5.4.1 Estimation of Aspect Ratio (AR) of GNRs using TEM Images

The aspect ratio of GNR particles was estimated by calculating the ratio of length to width of 20 GNRs. The mean aspect ratio was 3.85 (Table 33).

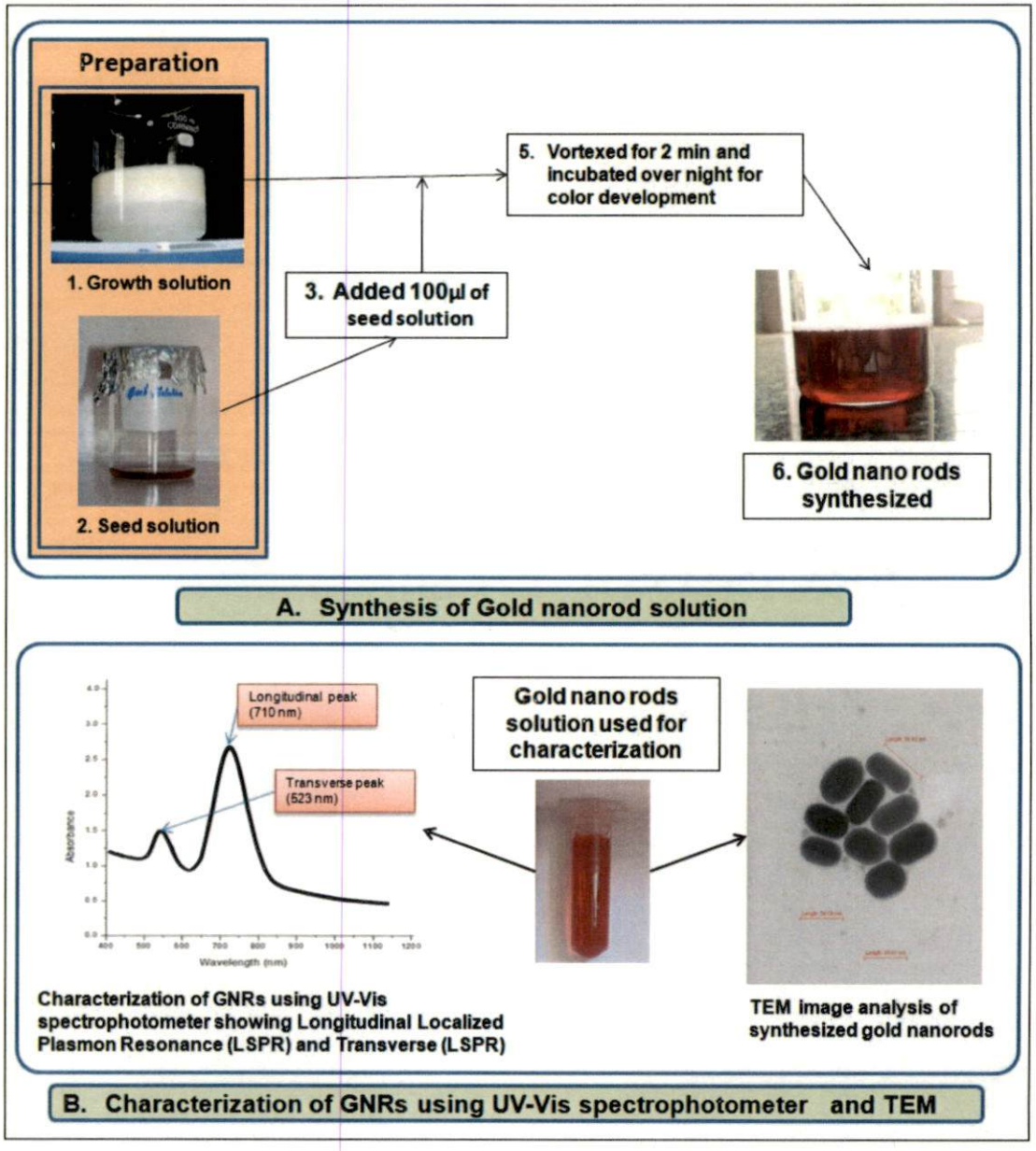


Plate 20: Flow chart showing synthesis and characterization of GNRs

Table 33: Characteristics of GNRs using TEM imges

Particle	Length (nm)	Breadth (nm)	TEM aspect ratio
1	53.0	21	2.52
2	56.9	18	3.16
3	52.8	15	3.53
4	50.8	14	3.63
5	58.6	12	4.88
6	54.0	16	3.38
7	59.0	15	3.93
8	60.0	18	3.33
9	59.0	17	3.47
10	50.0	11	4.55
11	55.0	11	5.00
12	56.0	12	4.67
13	58.0	14	4.14
14	54.0	15	3.60
15	61.0	13	4.69
16	49.0	12	4.08
17	52.0	11	4.73
18	51.0	18	2.83
19	53.0	17	3.12
20	56.0	15	3.73
Average	**54.96**	**14.75**	**3.85**

4.5.4.2 Estimation of Aspect Ratio (AR) of GNRs using GANs Theory

The aspect ratio estimated by GANs theory was 3.42 (Table 34). The aspect ratio calculated through TEM images and using Gans theory was found to be correlated.

4.5.5 Development of Self Assembly Monolayer (SAM) on the Surface of GNRs

The excess CTAB in the GNR solution was removed by repeated centrifugation. Surface modification of the synthesized GNRs was done and CTAB capped on the

periphery of GNRs were completely replaced in this experiment, where layer of alkali-thiol group formed could act as SAM for the binding of antibody (Plate 21, Fig. A).

Table 34: Aspect ratio of GNRS obtained from GANs theory

Sl. No	Longitudinal peak band	Aspect ratio (nm)
1	710.26	3.37
2	713.19	3.40
3	709.29	3.36
4	712.21	3.39
5	713.19	3.40
6	702.46	3.29
7	760.99	3.89
8	712.21	3.39
9	707.34	3.34
10	708.31	3.35
Average	714.43	3.42

4.5.6 Preparation of GNR-Antibody Conjugates

The MPA modified GNRs had thiol terminated groups with free end of carboxyl group. Combination of coupling agents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) could to activate the carboxyl (-COOH) group of MPA modified GNRs and made it to bind with amine ($-NH_2$) group of antibodies (Plate 21, Fig. A). This process enhanced the binding affinity between GNRs and antibodies. The phenomenon of biomolecular interaction changed the local reflective index on the surface of the GNRs which was monitored by LSPR shift of peak position. The shift in the peak position of GNRs from 710.39 nm to 719.17 nm which was due to binding of antibody to SAM of GNRs (Plate 21, Fig. B).

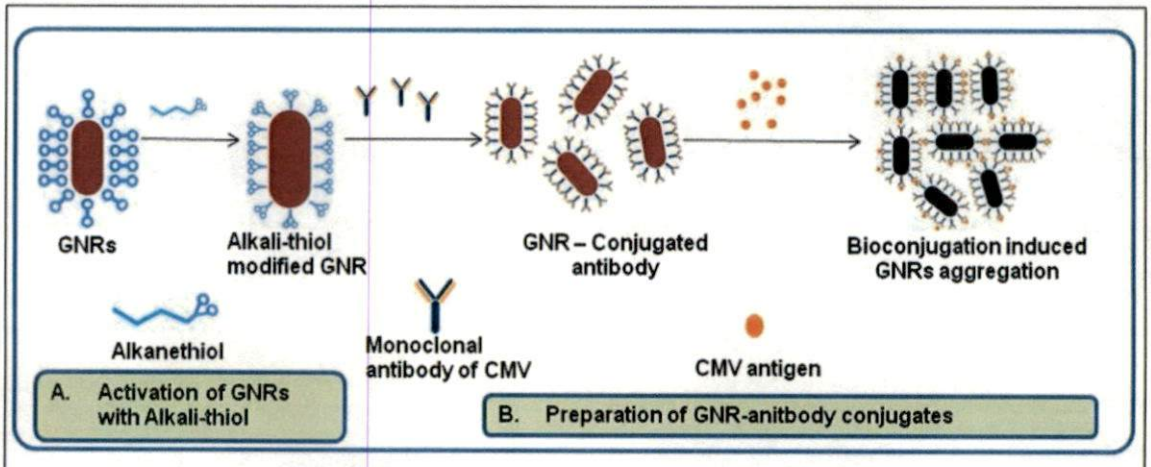


Fig. A. Schematic representation of bioconjugation-recognition based immunoassay for detection of CMV infecting banana

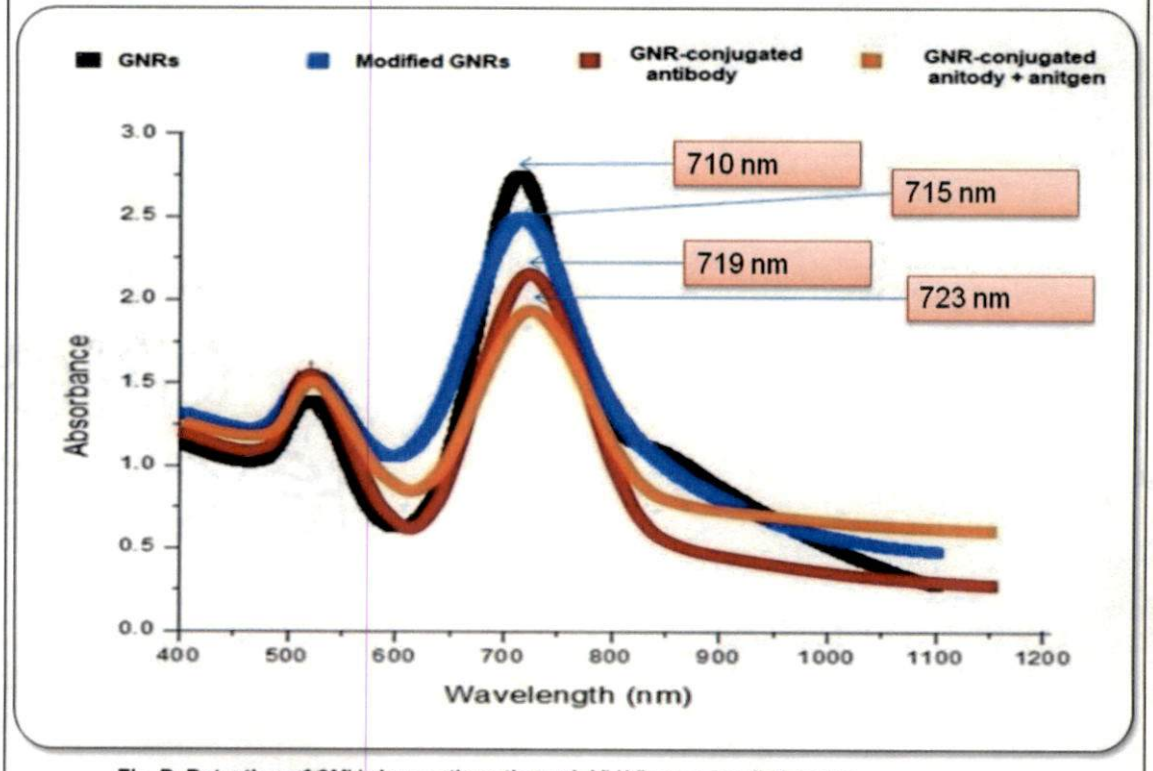


Fig. B. Detection of CMV virus antigen through UV-Vis spectrophotometer

Plate 21: Functionalization and Detection of virus antigen

4.5.7 Application of GNR Solution Based Nanobiosensors for the Detection of Virus

4.5.7.1 Isolation of Antigen

The CMV specific antigens were extracted from CMV infected banana leaf samples maintained in insect proof net house at BRS, Kannara.

4.5.7.2 Detection of the virus antigen through GNRs Nanobiosensors

In this experiment, biomolecular interaction of antigen and GNRs antibody conjugates induced aggregation of GNRs in the solution and also led to change in colour of the GNR solution (Plate 22, Fig A). During this process position of longitudinal plasmon band (LPB) peak shift was observed in the absorbance spectrum generated from absorbance value obtained during characterization using UV-Vis spectrophotometer. This was the analytical tool for the detection of CMV based on specific binding reaction between GNRs-antibody conjugates and antigen.

When the antigen was added to GNR solution, colour of the solution changed and varied from pinkish red to pale grey and finally sediment as black aggregates. The solution turned more or less colourless after their aggregation and sedimented. UV-Vis spectra of this solution revealed the shift of longitudinal plasmon band peak from 719 nm to 723 nm (Plate 22, Fig. B). But in case of healthy samples no colour changes was observed and without change in peak shift compared to infected plant sample (Plate 22, Fig. B).

4.5.8 Determination of GNRs Sensitivity to Virus Antigen

4.5.8.1 Quantification and Standardization of Different Concentrations of Antigen

The antigen concentration of 7.71 mg/ml was quantified using nanodrop spectrophotometer and different antigen concentrations were prepared and presented in Table 35.

Table 35: Preparation of different antigen concentration

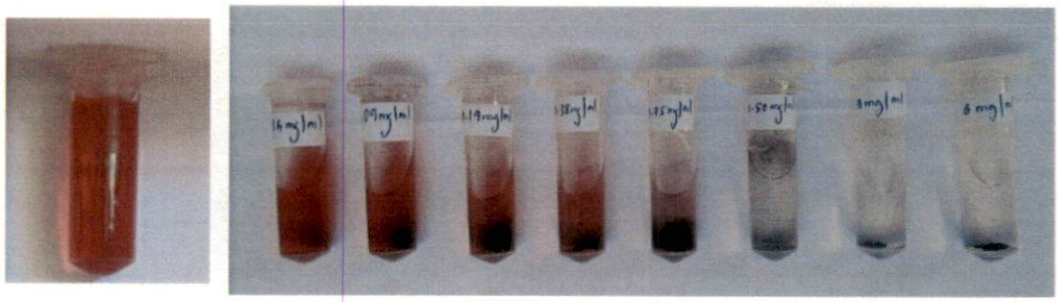
Required Protein conc. (mg/ml)	Volume of Crude sap taken (ml) *A	Volume of Distilled Water taken (ml) *B
6.0	0.837	0.163
3.0	0.418	0.582
1.50	0.209	0.791
0.75	0.105	0.895
0.38	0.052	0.948
0.19	0.026	0.974
0.09	0.013	0.987
0.04	0.006	0.994

*Conc, of protein in working (7.171 mg/ml)

4.5.8.2 Detection of GNRs Sensitivity to Different Antigen Concentration

The experiment was conducted with eight different concentrations of antigen (Table 35) and their interaction with GNR probes was analyzed using UV-Vis spectrophotometer. The absorbance values were used to convert into a graphical format using OriginPro 8 software and recorded longitudinal plasmon band for eight different antigen concentration. Gradual LPB peak shift was observed in UV-Vis spectra when the antigen concentration was increased from 0.04 mg/ml to 6 mg/ml (Plate 22, Fig. B). GNR probe could detect antigen with lowest antigen concentration of 0.04 mg/ml. Flat LPB was observed in case of antigen with 6 mg/ml.

The absorbance values, peak values and peak shifts for LPB with respect to different antigen concentration were recorded and presented in Table 36, Plate 22, Fig. B. It was observed that in both LPB and TPB, the peak shift had decreased with increase in the concentration of antigen. The shifted peaks were found to be widened for the higher concentration of antigen whereas the peaks remained sharpened for the lower concentration of antigen. The colour change due to interaction of GNR probe with



GNRs+Healthy sample

GNRs + infected sample

Fig. A. Colorimetric changes of GNR-antibody conjugates upon addition of CMV antigen with range of concentration

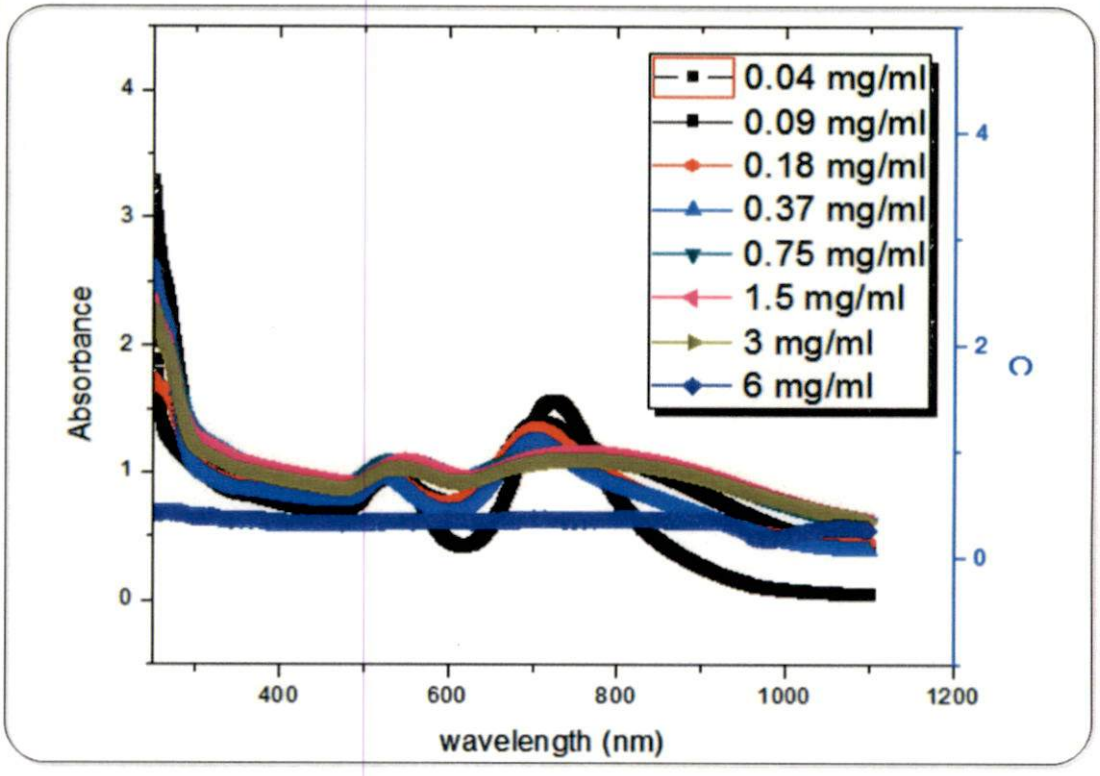


Fig. B. Spectral plot of longitudinal LSPR Peak shift over a different range of antigen concentration

Plate 22. Detection of CMV antigen with range of concentration

different antigen concentration was also recorded. There was not much change in colour with lower antigen concentration but with the increase in the antigen concentration, the colour of the GNR probe solution turned from reddish pink to purple to blackish colour. At higher antigen concentration, the sedimentation of aggregates was at faster rate which could be observed when the GNR probe solution turned colourless along with formation of sediments (Plate 22, Fig. A). On analyzing the cost/benefit of the GNR nanobiosensor based diagnostic method, it proved to be a rapid, economic and more sentitive technique for the detection of CMV in infected samples. Provided the infrasturucture is available, the cost of virus indexing comes approximately 15 rupees per sample and the detection procedure could be completed within very short period of about 2-3 h unlike other immune and molecular diagnostic techniques.

Table 36: Absorbance and LPB peak values due to interaction of GNR probe with different concentration of antigen

Anitgen conc.	Absorbance (OD Value)			
	Transverse Plasmon Band		Longitudinal Plasmon Band	
	X Axis	Y Axis	X Axis	Y Axis
0.04	531.88	0.9847	727.55	1.6083
0.09	525.78	0.8632	708.50	1.3395
0.18	522.11	0.7969	707.52	1.2423
0.37	524.56	0.8123	705.08	1.1427
0.75	545.56	0.9733	747.34	1.0521
1.5	545.56	0.9436	756.87	1.0122
3	548.01	0.8750	754.43	0.9538
6	475.21	0.2880	1066.62	0.28376

Discussion

V. DISCUSSION

Banana and plantains are the important tropical fruit crops which are grown in many developing countries and contribute to the food and livelihood security of millions of people across the globe. Though banana is widely cultivated, the high susceptibility of crop to the diseases is a major constraint to its profitable and sustainable cultivation. Diseases especially those caused by virus are the major threat to banana cultivation because of abundance of insect vectors and prevalence of varied alternate hosts (Magnaya and Valmayor, 1995). The mode of propagation through vegetative means also contributed to the widespread distribution of viral diseases on this crop. Banana is infected by four major viral diseases *viz.,* banana bract mosaic, banana bunchy top, banana streak and banana mosaic. Among these, banana mosaic or infectious chlorosis caused by *Cucumber mosaic virus* (CMV) is becoming a serious and emerging threat affecting yield and is widespread in Kerala (Estelitta *et al.,* 1996). The potential effect of climate change also aggravated the spread of viruses through insect vector.

Infectious chlorosis disease is also known as heart rot, virus sheath rot, cucumber mosaic and banana mosaic (Stover, 1972). The occurrence of this disease was first reported in New South Wales, Australia (Magee, 1940) and subsequently spread to different banana cultivating regions worldwide (Lockhart and Jones, 2000).

In India, the disease was first reported in Maharashtra by Kamat and Patel (1951) and subsequently reported in different states of the country *viz.,* Gujarat (Joshi and Joshi, 1976), Karnataka (Thammaiah *et al.,* 2004), Andhra Pradesh (Ramesh, 2009), Uttar Pradesh (Khan *et al.,* 2011), and North East (Lepcha *et al.*, 2017).

In Kerala, it was first reported by Estelitta (1996) and subsequently by Cherian *et al.* (2004). Considering the unique cropping system prevailing in Kerala, infectious chlorosis disease caused by CMV is becoming a serious threat to banana cultivation in the state. Intercropping of banana with cucurbitaceous vegetables is a common practice among the farmers. Also the symptoms of this disease are similar to that of nutritional disorders especially that of micronutrient deficiency. Considering the importance of this

disease, the present study was undertaken with various aspects like biological characterization of the virus, molecular characterization and immunological diagnostics, disease resistance and development of Nanobiosensors for early detection of virus infection.

The research was initiated with a purposive sampling survey to collect infected samples and to record the incidence of infectious chlorosis disease of banana in selected locations of Thrissur district of Kerala. The disease incidence varied from location to location but maximum disease incidence of 90 per cent was recorded on banana variety Robusta in Thallikulam panchayat coming under coastal belt of Thrissur district with sandy loam soil. This might be due to high prevalence of the aphid vectors because of the climate conditions prevailing in the coastal area. This was followed by an incidence of 63.3 per cent on banana variety Karpooravalli at Kannara, Thrissur and 28.7 per cent on Nendran at Mannuthy. Out of theses 28 fields surveyed, in 19 fields there was no disease incidence. This might be due to absence of infected hosts or weeds which harbor the virus inoculum. The results also showed that the presence of inoculum could lead to the maximum disease incidence upto 90 per cent which highlights that the disease could be emerged as a serious threat to banana cultivation if there is source of virus inoculum. During the survey, it was noticed that the plants were severely infested with aphids which are the insect vectors.

Mali and Deshpande (1976) reported an incidence of upto 23 per cent in Parbhani district of Maharashtra. Kadhirvel *et al.* (1986) reported 20-50 per cent incidence of banana mosaic on banana variety Poovan from Tamil Nadu. They also reported that the incidence was more on ratoon crop. Desai (1963) reported that out of 9047 gardens surveyed in Maharashtra, 7661 banana fields were free from the disease. Singh (2003) reported that when fresh planting was done soon after banana crop, the incidence was less in contrast to ratoon plants or those surrounded by bush and weeds or fallowed lands which highlight the importance of virus inoculum for the high incidence of the disease in any field. The development of symptoms of the disease was investigated in detail under natural and artificial conditions. Under natural conditions,

different types of symptoms were expressed on leaf lamina, petiole, midrib and bunch. The salient symptoms recorded were different patterns of mosaic, linear discoloration on midrib and petiole and various degrees of leaf deformation. Similar type of symptoms was also reported by Magee (1940). This was one of the early reports of the disease. Subsequently other researchers also reported similar symptoms on leaf lamina such as yellow streak like mosaic patterns, reduction in size and inward curling of leaf lamina and necrosis of leaves (Yot-Dauthy and Bove, 1966; Capoor and Varma, 1968; Capoor and Varma, 1970; Stover, 1972; Mali and Deshpande, 1975; Mali and Rajegore, 1979; Bouhida and Lockhart, 1990; Jones, 1991; Jones and Lockhart, 1993; Lepcha *et al.,* 2017; Vishnoi *et al.,* 2013).

The symptoms developed on plants under artificial conditions when inoculated through aphids were documented and the plants developed initial symptoms of linear chlorotic lesions 35-40 days after inoculation of the virus through banana aphid *Pentalonia nigronervosa.* Later the plants produced symptoms like reduction in leaf lamina, inward rolling of leaves and stunted growth. These symptoms were similar to those expressed under natural conditions. Singh (2003) reported that when suckers of virus infected plants were planted and kept under glass house conditions, though there was predictable pattern of symptom expression, the plant growth was slower and stunted. In the present study, when the suckers from infected symptomatic mother plants were collected and planted in grow bags kept under insect proof net house exhibited distinct symptoms like reduction in leaf lamina, interveinal chlorosis, mosaic and leaf narrowing with strap like appearance of leaf lamina followed by extreme stunting of plants. The bunches of the infected plants were smaller in size and produced small, comma shaped, under developed fruits with reduced bunch weight.

In general, symptom expression on leaves and bunches leads to yield reduction. This was confirmed by comparing the biometric and yield characters of healthy and infected plants. The results revealed that there was reduction in plant height, girth of pseudostem, number of leaves, bunch weight, and number of hands and finger of infected plants when compared to healthy plants. The leaf area was also drastically

reduced and the maximum per cent reduction (58.4%) was recorded in banana var. Nendran. This reduction in functional leaf area leads to reduction in photosynthetic efficiency of the plant. This is reflected on the reduction of bunch weight and yield of the crop. The maximum yield reduction of 83.4 per cent was recorded on banana var. Nendran which showed 58.4 per cent reduction in leaf area and the 39.1 per cent reduction in chlorophyll content. However, on banana var. Karpooravalli, the leaves expressed only mild mosaic symptoms and there was less reduction in chlorophyll content and leaf area and hence yield reduction was only 8.4 per cent. Estelitta *et al.* (1996) reported a yield reduction of 54 per cent on banana variety Nendran which confirms the results of the present study. However, they reported about 62 per cent reduction of bunch weight on banana var. Karpooravalli while in the present study; the per cent reduction of yield of banana var. Karpooravalli was only 8.4 per cent. This might be due to variation in the morphotypes of Karpooravalli used for the studies. The yield reduction on banana var. Cavendish and Mysore was also reported by Selvarajan and Balasubramanian (2008). Multiple infections of other viruses along with CMV could also lead to heavy crop loss as reported by Silva and Silva (2009).

The histological and cytological changes on foliage tissue due to CMV infection were studied and documented. The comparative observations revealed that the healthy leaf tissues were with intact palisade, spongy and air space layer with definite lower and upper epidermis and stomata. While thin sections of infected leaf samples revealed that the spatial arrangement of tissue was disrupted. There was formation of paramural tissue layer between spongy tissue layers, shrunken spongy tissue, lack of air space between spongy tissue and black deposition on the midrib were also observed in infected leaves. Similar ultra structural anatomical changes have been documented by El-Deeb *et al.* (1997). They also reported that the cytoplasm contained few vacuoles and the plasmolemma was separated from the cell wall.

Study on the transmission of virus is an important criterion to establish the identity of the virus and several experiments were carried out elsewhere in this line and transmission of this virus was reported with aphids like *Aphis gossypii, Rhopalosiphum*

maydis, R. prunifolia and *Myzus persicae.* Apart from insect vectors transmission of the virus through planting materials and mechanical inoculation were reported by many researchers (Magee, 1940; Capoor and Varma, 1968; Mali and Rajegore, 1980). In the present study, the transmission of CMV through suckers (planting material) from infected mother plants were confirmed in different varieties viz., Grand Naine (AAA), Karpooravalli (ABB), Robusta (AAA), Nendran (AAB) and Amritsagar (AAA). The transmission of CMV through planting material was reported earlier by Estelitta (1996) and Preeti (2015). The symptoms produced by the plants raised through suckers collected from infected mother plants were interveinal chlorosis, reduction in leaf lamina, distortion of leaves, severe mosaic and broom like appearance and narrowing of leaves giving out strap like appearance. These symptoms were similar to those observed under natural field conditions. Similarly, Niblett *et al.* (1994); Preeti (2015) also reported interveinal chlorosis, reduction in leaf lamina, distortion of leaves along with stunting of plants infected with CMV.

The transmission of CMV from banana to banana was tried by mechanical transmission using different buffers at different pH levels. However, the virus could not be transmitted from banana to banana through sap. Mechanical transmission tried by earlier workers also showed that the virus could not be transmitted through sap from banana to banana (Capoor and Varma, 1968; Mali and Rajagore, 1980). The failure of mechanical transmission by the leaf rub method might be due to production of tannins and phenols in the inoculated test plants due to injury caused during the process. It may also activate the host response at molecular level to synthesize defense activating chemical substances against any foreign substance (Benigno, 1963; Yot- Dhauthy and Bove, 1966).

The present study on aphid transmission indicated that the CMV was transmitted by *Pentalonia nigronervosa* and *Aphis craccivora.* The inoculated test plants developed symptoms of the disease which were similar to those under the natural field conditions. The inoculated plants developed mosaic, interveinal chlorosis and distorted leaves. Similar symptoms were documented by Magee, (1930); Calinisan, (1938); Silberachmidt

and Nobrega, (1941) and Dheepa and Paranjothi (2010). They reported that mosaic disease could be transmitted from infected to healthy plants by banana aphids, *P. nigronervosa*. There are contradictory reports with negative results (Wardlaw, 1934; Magee, 1957). Magee (1940) reported transmission of CMV by *Macrosiphum gei*, *Aphis gossypi* and with another unidentified aphid. Similar results were obtained from the transmission experiment conducted with cowpea aphid, *Aphis craccivora. P. nigronervosa* was the only capricious vector and seemed to be important under natural conditions. Under artificial conditions, *A. craccivora* also proved to be the vector. The incubation period got prolonged. CMV was reported to infect wide range of hosts (Roossinck *et al.,* 1999 and Hord *et al.,* 2001). Efforts were taken to assess the role of host plants and the weeds seen in and around banana fields to be the reservoir of the virus inoculum. The hosts which were proved to be infected by CMV were reported as hosts of different CMV strains. These include host plants from Amaranthaceae, Cucurbitaceae, Solanaceae, Fabaceae, Zingerberaceae and others as depicted in the host range studies. Host plants were *Amaranthus polygamus* and *Gomphrina globosa* (Kelaniyangoda and Madhubashini, 2008; Shahid *et al.,* 2012), *Momordica charantia* (Muharam, 1987; Kiranmai *et al.,* 1997; Jagadeeshwar *et al*, 2005; Ashwini *et al.,* 2016), *Benincasa hispida* and *Lagenaria siceraria* (Parvin *et al.,* 2007), *Cucumis anguria and C. sativus* (Shahid *et al.,* 2012; Raj Verma *et al.,* 2004), *Trichosanthus cucumerina* (Parvin *et al.,* 2007), *Solanum melongena* (Kelaniyangoda and Madhubashini, 2008), *Capsicum annum* (Shahid *et al.,* 2012), *Nicotiana* spp. (Lepcha *et al.,* 2017; Sulistyowati *et al.,* 2004; Jagadeeshwar *et al.,* 2005; Yardmc and Eryigit, 2006; Dheepa and Paranjothi, 2010), *Vigna unguiculata* (Eiras *et al.,* 2001; Raj Verma *et al.,* 2004), *Canna indica* (Saidi and Safaeizadeh, 2012; Magee, 1940), *Synedrella nodiflora* L. (Bhat *et al.,* 2004), *Tridax procumbans* (Jagadeeshwar *et al.,* 2005), *Commelina* spp. (Dheepa and paranjothi, 2010) were reported to be the hosts of CMV by various researchers. However, the perusal of literature showed that the hosts like *Canavalia gladiata, Heliconia platystachys*, *Acemella radicans, Ichnocarpus fructescens, Phyllanthus amara and Centrosema pubense* were new hosts for *Cucumber mosaic virus*.

The physical properties of the virus *viz.,* Thermal inactivation point (TIP), Dilution end point (DEP) and Longevity *in vitro* (LIV) fall in the range as reported earlier for CMV strains elsewhere (Hull, 2002, Van-Hoof, 1962; Mali and Rajagore, 1980; Parvin *et al.,* 2007; Chandankar *et al.,* 2013).

The morphology of the virus associated with infectious chlorosis disease of banana was studied by taking electron photomicrographs and revealed the presence of spherical isometric virus particles of size 28.5 nm. This confirms that the pathogen is *Cucumber mosaic virus* which belonged to genus Cucumovirus, Family Bromoviridae. Similar particle size of 28-30 nm, and morphology for CMV (Banana) was reported earlier by many workers (Lockhart and Fischer, 1976; Holcomb and Valverde, 1991; Kiranmai *et al*., 1997; Doomar *et al*., 1990; Vishnoi *et al*., 2013; Madhubala *et al.,* 2005; Rostami *et al.*, 2014; Samad *et al.*, 2008 and Ali *et al.*, 2012).

Diagnostics is important in the development and implementation of disease management strategies. For the detection of virus infection, techniques such as biological indexing using symptomatology, serological detection using specific antigen-antibody recognition and molecular detection based on virus nucleic acids can be used for early detection. These techniques are necessary and mandatory in tissue culture laboratories which undertake mass multiplication of banana plants.

In the present study, serological methods viz., DAC-ELISA, DIBA and Nucleic acid based method (RT-PCR) were validated to detect CMV in the plant samples. Out of the three techniques employed, DAC-ELISA and DIBA were protein-based and relied entirely on the use of antiserum. But, RT-PCR is entirely based on the nucleic acid to amplify the viral RNA followed by subsequent separation and visualization.

ELISA is one of the sensitive tests for detection of virus. In the present study, DAC-ELISA was performed using different dilutions of primary antibody. Absorbance value read in ELISA reader were compared with that of healthy samples. The dilution of 1:200 gave the highest mean absorbance value (nearly three times more than that of healthy sample) compared to other dilutions *viz*., 1:1200, 1:1300 and 1:500. In ELISA,

generally the replicated samples having mean absorbance value which is double that of healthy sample was considered as infected. Accordingly, the CMV infection could be easily detected up to maximum antiserum dilutions of 1:500 and this was previously reported by Kiranmai *et al.* (1996) and Selvarajan *et al.* (2011). Also, standardized DAC-ELISA was used for the large scale virus indexing of CMV in tissue culture plants and mother plants used for mass propagation, which showed clear difference between healthy and infected samples with the absorbance value of infected samples almost thrice as that of healthy samples. Many workers have standardized the detection of CMV in infected samples by ELISA (Rao, 1980; Devergne *et al.,* 1981; Hu *et al.,* 1995; Eiras *et al.,* 2001; Rao and Singh., 2008; Dheepa and Paranjothi, 2010; Khan *et al.,* 2012; Kouadio *et al.,* 2014; Lepcha *et al.,* 2017).

DIBA was standardized to detect the presence of virus in infected samples using polyclonal antibody specific to CMV. The virus infection in the crude sap were detected when a drop of crude sap spotted on nitrocellulose membrane was probed with polyclonal antibody diluted in phosphate buffered saline (PBS) (10x) pH 7.4 and here anti-rabbit alkaline phosphatase conjugate diluted (1:10.000) was used as secondary antibody. On nitrocellulose membrane, the colour was developed using the substrate *p*-Nitrophenyl phosphate (1mg:1ml). The infected leaf samples gave positive reaction for DIBA and this could be detected by the purple coloured spots on nitrocellulose membrane which was absent in healthy samples. This technique was very convenient because sample preparation and the subsequent procedures for virus detection were simple and sophisticated instruments such as ELISA-reader or Thermo cycler were not required.

DIBA and ELISA are solid phase serological detection methods and depend on the concentration of the virus particles. The sensitivity of DIBA in relation to ELISA depends on the ELISA format, type of plant tissue sampled, plant sap components as well as the virus itself. Both ELISA and DIBA could be considered as efficient methods for detection of CMV, although probability of non-specific reaction of antibody and components of plant sap was higher in DIBA than in ELISA. These serological assays

proved that the causal agent of virus causing infectious chlorosis disease in banana is *Cucumber mosaic virus* belonging to genus Cucumovirus and family Bromoviridae.

The observation in the symptomatology and the appearance of different types of symptoms in different banana cultivars strengthened the probable thought of existence of different strains or variants of CMV. Traditionally electron microscopy, host plant assay and serological technique are generally time-consuming. The detection, identification and confirming low titre virus in the plant samples is a problem in traditional diagnostic technique. Hence, to improve the sensitivity molecular based PCR detection was standardized to find out the existence of different variants of CMV. Newer and advanced molecular approaches have gained more importance in the detection and characterization of plant virus. The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) provided a simple and ingenious method to exponentially amplify specific cDNA sequences *in vitro* by using reported and designed primer. Molecular based techniques overcome the problems associated in serological detection which are tedious and time consuming protocols.

Nucleic acid based detection techniques generally depends on good quality RNA which is a pre-requisite for the reliable RT-PCR and PCR reaction. Young leaves from infected plants were found to be more suitable than older leaves for RNA isolation as they contain reduced amount of tannins and phenolics when compared to older leaves. In the present study, total RNA from the infected and healthy samples was isolated by using two methods *viz.,* TRIzol reagent and RNeasy plant mini kit®. The protocol using RNeasy plant mini kit® provided highest quality and quantity of RNA when assessed by spectrophotometer analysis and gel electrophoresis compared to TRIzol reagent protocol. Although RNA yield obtained from leaf samples using two different protocols *viz.,* TRIzol reagent method and RNaesy plant mini kit® method was almost similar, the Purelink RNA reagent provided higher quality RNA recorded absorbance value of 2.04 when compared to TRIzol reagent method which showed the absorbance value 1.68 as indicated by $A_{260/280}$ ratio. The RNA analyzed in the agarose gel having two thick bands confirmed the purity of RNA without any DNA and protein contamination. The TRIzol

method which is the most common RNA isolation method was easy to conduct and time saving. However, the low $A_{260/280}$ ratio indicated that RNA isolated by this method might contain contaminants, such as phenols, polysaccharides and salts which could yield poor quality RNA (Zhang *et al.,* 2013). Among the two methods evaluated for RNA isolation, RNaesy plant mini kit® method provided better quality and quantity of RNA. Hence, this method could be used for the isolation of high quality RNA from host plant tissues, especially those rich in polyphenolics or starch like in banana, potato tuber and other tuber crops.

The total RNA isolated from the infected and healthy samples were converted into cDNA (first strand) using thermo scientific RevertAid H Minus first strand cDNA synthesis kit. The cDNA were further utilized in PCR for the characterization of CMV. The primer targeting the coat protein region of CMV were designed and synthesis at Sciegenome, Kochi. The primers designed by primer software also validated by using the software IDT oligoAnalyzer 3.1 software in order to find the physical properties such as GC content, melting temperature, feasible annealing temperature and probability for primer dimer formation, hairpin formation. The primer sequence thus validated by *in silico* analysis was sent to SciGenome, Kochi for synthesis. RT-PCR technique was employed for the detection of CMV using synthesized cDNA from diseased and healthy leaf samples. DNA fragment of 750 bp and 700 bp was amplified and visualized by agarose gel electrophoresis for reported set of primers and designed primers, respectively. No amplification was obtained from the healthy leaf sample. Similarly, Hu *et al.* (1995); Singh *et al,* (1995); Eiras *et al.* (2001); Khan *et al.* (2011) successfully amplified, visualized and documented the bands of various length. The second strand of cDNA of infected and healthy samples was amplified with the coat protein specific reported and designed primer in the present study. Both the primer pairs used for detection of virus by performing RT-PCR yielded an amplified product of 750 and 700 bp size bands. Similar results were also reported by Cherian *et al.,* (2004). These primers could be successfully used for the detection and characterization of CMV from infected plant samples.

The virus amplicons of band size 750 and 700 bp products were sequenced and subjected to similarity search using bioinformatics tools such as BLASTn and BLASTx to compare with various existing sequence of CMV strain in the GeneBank database and sequences identities were obtained. The finding obtained during *in silico* analysis it was observed that all eight sequences were having highest similarity with CMV coat protein gene belonging to cucumovirus. Similarly, the BLASTx was performed with the same sequence and showed 98 per cent sequence similarity with CMV sequences CAA07411, AAY19285, BAV25270, BAV25270, ALT66628, CBG22867 and APB09218. The blast hits showed that all sequences showed 98 per cent homology with partial and complete nucleotide sequences of coat protein of CMV. This is in accordance with the findings obtained by Hu *et al.* (1995); Singh *et al.* (1995); Boari *et al.* (2000); Eiras *et al.* (2001); Eiras *et al.* (2004); Cherian *et al.* (2004); Shehata and El-Boroiiosy, (2007); Khan *et al.* (2011); Selvarajan *et al.* (2011); Khan *et al.* (2012); Lepcha *et al.* (2017) who observed CMV virus belonging to subgroup IB among south Indian isolates. The nucleotide sequences obtained with primers specific to coat protein gene used in this experiment were used for phylogenetic analysis in order to study the genetic diversity.

In the field of genetic engineering, coat protein sequences are used to develop the pathogen derived resistance against the viruses. CMV coat protein diversity could help in predicting the risk of resistance breakdown in the field of development of transgenic banana lines. This would help to devise long term management strategies preventing the loss of resistance due to evolution of new strains of virus. Coat protein gene sequences in the present study were compared with 42 previously reported isolates from India and from other geographical area available in the GenBank database. Later, phylogenetic tree was developed by aligning the nucleotide sequences. From the phylogenetic tree it was revealed that the tree was rooted on CMV coat protein gene strains belonging to cucumovirus subgroup IB. Vishnoi *et al.* (2013) reported similar diversity analysis of the sequence data in order to know the phylogenetic and evolutionary relationships of the banana virus isolates causing infectious chlorosis and they established CMV strains belonging to subgroup IA, IB, II and other groups.

In this study, the sequence data analysis revealed 97-99 per cent identities at both nucleotide and amino levels with CMV strains of subgroup IB reported worldwide. Based on the high sequence identities and CMV sequences of different subgroups infecting different crops, phylogenetic relationships was analyzed and phylogenetic tree was constructed, the virus under study has clustered within subgroup IB and showed close relationships with Indian isolates of CMV AY125575, AY690620, U31220, AB042294, etc. However, subgroup IA and II strains showed distant relationships with banana isolate. The results of the present investigation showed most similarly with CMV isolate infecting banana reported from Maratwada region where this disease was reported for the first time in India. Though, CMV was noticed first in Maratwada region, Maharastra (Kamat and Patil 1951), was later spread to other states of the country *viz.,* Karnataka, Gujarat, Tamil Nadu, Uttar Pradesh, Kerala and North East during past five decade either through infected suckers or planting material from the diseased symptomless mother plants (Mohan and Lakshimanan, 1988) as there is no domestic quarantine enforced to restrict the movement of banana suckers between the states. Another reason could be due to prolonged presence of infected plants which acts as source of inoculum which could be readily be transmitted by aphid vectors. Also there is the prevalence of wide host range (Bird and Wellman, 1962). This virus had recently been reported to infect *Piper longum* which was grown with banana and coconut as intercultivated crop (Bhat, 2004).

Molecular cloning was done with the amplified PCR product of CMV. Amplified CMV coat protein gene was cloned in pGEM-T® easy vector *Escherichia coli,* DH5α. The transformed colonies were identified and selected based on blue and white selection. Then, these colonies were then confirmed using colony PCR and based on band shift assay clones which yielded distinct bands between 850-950 bp was sent for sequencing and sequences obtained were used for *in silico* analysis. Molecular cloning of CP gene of CMV was attempted by many workers. (Eiras *et al.,* 2004; Khan *et al.,* 2011; Vishnoi *et al.,* 2013; Ali *et al.,* 2014; Khan *et al.*, 2015; Buitron-Bustamante and Velastegui,

2017). These recombinant clones could be further utilized for the production of antibodies through expression vectors.

SYNTHESIS AND CHARACTERIZATION OF GOLD NANORODS (GNRs)

In the recent years, novel metallic nanoparticles used as biosensor for the detection of the analyte (antigen) gains more importance due to their novel plasmonic property (Saurav, 2016 and Vinushree, 2017). Though several nanoparticles exist, gold nanorods are mostly preferred due to ease in synthesizing in varying size and shapes and also due to its unique LSPR (Localized Surface Plasmon Resonance) which exhibited different colour due to its optical property in infrared region (Kissinger, 2005).

For the successful utilization of LSPR based sensors, an accurate method of synthesis has to be standardized. In the case of GNRs, seed mediated method is the most commonly preferred. In this experiment, the synthesis of GNRs was done by following the protocol as described by Saurav, (2016) with slight modifications and GNRs of average (54±5) nm length and diameter (14±3) nm were developed and these dimensions were similar to those reports earlier (Li *et al.,* 2008 and Saurav, 2016). The results were confirmed by TEM image analysis of GNRs and were in tune with earlier report by Li *et al.* (2008) and Saurav, (2016).

The quality of GNRs could also be assessed by using UV-Vis spectrophotometer. Gold nanorods present in the solution were with positive charges on their surface. If light falls on these nano particles, dipole movement is created due to electron oscillation which results in the formation of two visible peaks. This interaction is visualized only in Near Infrared Region (NIR) using UV-Vis spectrophotometer. Among the two peaks formed in UV-Vis spectra, the longitudinal peak of the GNRs is important than the transverse peak. The longitudinal peak could be shifted by changing aspect ratio of GNRs, which in turn depends upon the size of the GNRs and GNR-bioconjuagtes used for the detection.

The GNRs synthesis procedure described by Li *et al.* (2008) and Saurav, (2016) reported plasmonic longitudinal peak of GNRs at 780 and 710 nm respectively. In the current study, the longitudinal peak was found at 710 nm. The seed solution containing concentrated gold nanospheres of 5 – 10 nm act as templates for further attachment of gold ions to produce GNRs of optimum size which decides the synthesis of good quality GNRs. The optimum volume of 100 µl seed solution was required for 50 ml growth solution for obtaining maximum yield of GNRs which was indicated by UV-Vis spectrophotometer analysis.

The aspect ratio of GNRs was calculated for assessing the quality of GNRs. In this study, comparison was made between the aspect ratio calculated by GANs theory and TEM image analysis of GNRs as described by Jain *et al.* (2008) and Saurav, (2016). It was found that the results obtained by experimental data i.e. TEM image was on par with the theoretical data developed by GANs theory which indicated that the GNRs developed by this method were of optimum quality. The GNRs with distinct optical property has several advantages compared to other metallic nano-particles. Advantage such as biocompatibility and optical property of GNRs makes it a versatile and reliable technique for industrial application and as diagnostic tools.

The excess of CTAB in the GNR solution will act as the barrier and avoid establishing the linking between linker molecule and antibody. Hence, activation of the GNRs for effective binding of antibody, excess of CTAB present in the solution should be completely removed from the GNR solution by repeated centrifugation as described by Nikoobakht and El-Sayeed, (2003) and Saurav, (2016). Later, Mercapto propanoic acid (MPA) was used to create a self assembly monolayer (SAM) which could act as a linker molecule between antibody and GNRs in the conjugation process (Song *et al.*, 2013). However, due to change in ionic environment and pH of the solution, electrostatic and hydrophobic interaction occurred which resulted in aggregation of GNRs. Similar observations were also reported by Wang *et al.* (2012).

In order to achieve effective detection of analyte i.e. antigen, the antibodies are to be bound to GNRs prior to detection. These GNRs were conjugated with antibodies by covalent binding. The chemistry involved during conjugation process is the covalent binding of antibodies to GNRs. During this process, the chemical EDC/NHS (1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) activated the CooH group of Self assembly monolayer (SAM) which covalently links to the NH_2 - group of antibodies. This results were similar to the reports described by Song *et al.* (2013).

Huang *et al.* (2009) reported that when the GNRs are functionalized with antibody *i.e.* the biomolecules, there was a change in the absorption pattern of the GNRs which could be visualized in UV-Vis spectrophotometer. Accordingly, in the current study characterization of antibody conjugated GNRs were carried out using UV-Vis spectrophotometer which resulted in LSPR peak shift in UV-Vis spectra. This confirmed that there was effective binding of antibodies to the activated GNRs. Due to noncovalent binding of antibodies to GNRs a longitudinal shift of 16 nm was observed. Similarly, peak shift of about 60 nm and 15 nm was also reported by Yu *et al.* (2007) and Saurav (2016).

In this experiment, GNR antibody conjugates were used as nanoprobe which aggregated, when specific antigen binds with the GNRs-antibody conjugates. This leads to formation of complex structure due to aggregation of GNRs induced by antibody molecule and antigen interaction resulting in change in the plasmonic property which was visualized by changes in the colour of GNRs-antibody conjugation solution. This was utilized for colorimetric detection of CMV which was further confirmed by using UV-Vis spectrophotometer analysis. These results obtained were similar with the results reported by Yu *et al.* (2007) and Saurav (2016).

The binding of analyte (Antigen) to antibody resulted cross linking of GNRs followed by its aggregation. The increase of antigen concentration in the sample resulted in reduced absorption with shift in LSPR peak due to aggregation of nanoparticles. In

the samples with high concentration of antigen, the GNRs nanoprobe solution turned bluish black to complete transparent due to binding of antigen to antibody resulting in cross linking of GNRs followed by its aggregation at the bottom of the tube. Stewart *et al.* (2008) reported the changes in the absorption of GNRs nanoprobe solution after the addition of target molecule which completely depended upon the concentration of the antigen.

LSPR peak shift of GNRs using UV-Vis spectrophotometer is another method for detection of a target or antigen. When antigen is added to GNRs-nanoprobe solution, changes occured in plasmonic coupling between particles and the property of aggregation which leads to change in the peak position of GNRs both in longitudinal and transverse peak along with reduction of absorption. Wang *et al.* (2012) reported only longitudinal peak shift during antibody antigen interaction. In the present study, similar observations were recorded. Addition of antigen in GNRs nanoprobe solution resulted in reduction of absorption and shift in longitudinal LSPR peak. When the concentration of antigen was increased in the sample, the longitudinal peak shift was observed which remained flat in the UV-Vis spectra due to increase in aggregation of GNRs.

To conclude, the studies undertaken have led to the development or standardization of immunological and molecular techniques for the identification of CMV. As banana is a vegetatively propagated crop, there is an increased chance of virus introduction into new areas through the planting material. Naturally infected asymptomatic banana plants might also become the source of inoculum when used as planting materials. Among the assays, detection through nano-biosensor could be the most reliable in terms of detecting virus with specificity, sensitivity, speed and cost. Hence, Nano-biosensor based detection could be one of the most efficient techniques for large scale plant virus indexing which guarantees sensitivity and sustainability for the detection of CMV infection in the planting materials. This could be further upgraded to solid based nanobased immunostrips which could be more user friendly and could be used for insitu virus detection and diagnosis. Further cloning of CP gene was done successfully and these molecular clones of coat protein gene could help in

characterization and also help in the production of good quality antiserum through recombinant DNA technology. These clones can also used further for developing transgenic line resistant to CMV.

Summary

VI. SUMMARY

The study entitled "Molecular characterization of virus causing Infectious chlorosis disease of banana" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara and Banana Research Station (BRS), Kannara, Kerala Agricultural University during 2014-2017. The present research was envisaged to study the diagnostic symptoms of the disease, biophysical and molecular characterization and immuno-molecular detection of the virus. As the part of research work, nanobiosensor was developed for the quick and more sensitive detection of virus.

Banana is infected by four known relatively well characterized virus *viz.,* BBTV, CMV, BSV and BBrMV. Among these viral diseases, banana infectious chlorosis disease caused by CMV is having devastating effect on the production of banana. Survey on incidence of infectious chlorosis disease revealed the presence of CMV infection from 28 fields surveyed and the maximum disease incidence of 90 per cent was recorded in Thalikulam on banana variety Robusta (AAA) followed by Karpooravalli (ABB) at Kannara (63.3) and Nendran (AAB) variety at Mannuthy (28.7). An incidence of 0.6 per cent was recorded at Kannara on banana var. Grand Naine (AAA) while disease incidence was not observed in fifteen other locations. The disease was predominantly recorded on banana varieties Nendran (AAB) and Robusta (AAA) and the highest incidence was recorded on Robusta variety in Thalikulum area which is a coastal belt with severe infestation of aphids.

The symptoms of CMV observed under natural conditions were linear discontinuous chlorotic lesions, interveinal chlorosis, spindle shaped chlorotic lesions on leaves, severe mosaic with extreme distortion and reduction of leaf lamina. This leads to upward rolling of leaves along with marginal necrosis and cracking of leaf lamina. The infected bunches were small sized with slender elongated peduncle. Similar foliar symptoms were also developed when inoculated with viruliferous aphids on healthy banana.

The midrib of leaves of infected plants appeared to be purplish in colour and more prominent towards the base of the leaf. Black longitudinal lines were seen on lower surface of midrib. Chlorotic streaks were seen on the petiole of var. Nendran (AAB) and Robusta (AAA). Infected plant produced elongated 'S' shaped peduncle and such peduncles were slender when compared to healthy. Bunch size was reduced, had less number of hands and fingers compared to healthy. Fingers were drastically reduced with severe distortion and appeared comma shaped. Severely infected plants failed to produce bunches as in the case of Nendran (AAB) and Robusta (AAA).

Changes in leaf anatomy were also observed due to viral infection. Parenchymatous cells were turned to irregular shape with interrupted cuticle, disorganized arrangement of cells near septum and air chambers with intercellular depositions. Sections of diseased leaves exhibited spatial arrangement of tissues which were disrupted compared to healthy sample. The development of paramural abnormal tissue between the regular parenchymatous cells with black inclusion bodies was seen in the infected plants. The chloroplasts were lesser in number in infected leaves when compared to healthy plants.

Impact of viral infection on plant growth characters such as height, girth of pseudostem, number of leaves, leaf area, chlorophyll content, bunch weight, number of hands and fingers per bunch, finger characteristics of different varieties of banana *viz.* Karpooravalli, Amritsagar, Nendran, and Grand Naine were recorded. The per cent reduction in plant height was the highest in the variety Nendran (35.7) and the lowest in Amritsagar (10.9). The per cent reduction in number of leaves were 8.3, 11.5, 17.7 and 22.7 respectively on varieties like Karpooravalli, Amritsagar, Grand Naine and Nendran respectively. The leaf area was reduced due to infection 10.9, 20.1, 37.6 and 58.4 per cent on varieties Karpooravalli, Amritsagar, Grand Naine and Nendran respectively.

The per cent reduction in chlorophyll content due to infection was 45.7, 39.1, 34.1 and 1.6 nmol/cm^2 on banana varieties Karpooravalli (ABB), Grand Naine (AAA), Nendran (AAB) and Amritsagar (AAA) respectively. The maximum per cent reduction

in bunch weight due to infection was 83.4 in Nendran (AAB) followed by Grand Naine (AAA) (40 %) and the least reduction was recorded in Amritsagar (AAA) and Karpooravalli (ABB) *i.e.* 12.1 and 8.4 respectively. The per cent reduction in number of hands due to infection was 20, 19, 7.3 and 3.7 respectively on banana varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB). The per cent reduction due to infection was 47.4, 36, 16.3 and 5.6 respectively in banana varieties *viz.,* Nendran (AAB), Grand Naine (AAA), Amritsagar (AAA) and Karpooravalli (ABB).

Host range studies conducted on 29 host plants belonging to 11 families against CMV indicated that infection was recorded in 19 different plants and weeds which was later confirmed by ELISA. The perusal of literature showed that the hosts like *Canavalia gladiata, Heliconia platystachys*, *Acemella radicans, Ichnocarpus fructescens, Phyllanthus amara and Centrosema pubescens* were new hosts for *Cucumber mosaic virus*.

The role of virus transmission through planting material was evaluated which was 100 per cent transmission of the virus through infected planting material. CMV was mainly transmitted through infected suckers and insect vectors which produced small linear chlorotic lesions on new emerging leaves which enlarged with the expansion of leaf lamina and exhibited the symptoms similar to the symptoms developed under natural conditions. In the case of mechanical transmission it was confirmed that the virus was not transmitted through sap inoculation from banana to banana.

Insect transmission studies conducted with aphid *i.e. Pentalonia nigronervosa* and *Aphis craccivora* confirmed that the virus was transmitted through aphid species and *P. nigronervosa* was the most efficient vector of CMV (85.71%). Pre-acquisition fasting period of 30 min was found to be optimum for the efficient transmission of CMV by *P. nigronervosa.*

Biophysical properties of the virus were determined and it was found that thermal inactivation point ranged between 65-70°C, dilution end point of 10^{-4} and the

longevity *in vitro* (LIV) was up to 3 days and got inactivated from 3rd and 7th day onwards when kept at room temperature and refrigerated conditions respectively.

Electron microscopy of the CMV infected samples by leaf dip method revealed the presence of virus particles with isometric shape and 28 nm size. Hence, the etiology of infectious chlorosis disease of banana was confirmed as (*cucumber mosaic virus* (CMV) belonging to the genus *Cucumovirus* and family Bromoviridae.

The immunodiagnostic assays like DAC-ELISA and DIBA protocol were validated by using the antiserum specific to CMV. The virus was detected in plants which expressed the typical symptom and also in asymptomatic plants.

Screening of germplasm accessions maintained at Banana Research Station, Kannara revealed that out of 175 screened, only 6 accessions showed characteristic visual symptoms of CMV and 12 accessions showed positive under ELISA. It was observed that varieties with AAB genome could be more susceptible to the disease. Some of the varieties *viz.,* Kadali, Karivazha, Natu Poovan, Njali Poovan and Changalikodan did not produce any visual symptoms but were found to be positive under ELISA. This might be due to environmental conditions prevailing in that location which masked the symptoms though virus inoculation was present in the plants.

Molecular diagnosis was standardized for the detection of CMV. RNA extraction was optimized using RNeasy Plant mini kit® method and cDNA from viral RNA was also synthesized. Reverse transcription polymerase chain reaction (RT-PCR) for detection of CMV isolate was standardized. PCR product of approximately 700 bp and 700 bp were amplified using reported and designed primers specific to coat protein gene of CMV which confirmed the etiology of the disease as CMV. *In silico* analysis of eight CMV isolates revealed 96-98 per cent nucleotide homology with banana CMV strains of subgroup IB which clearly showed that all the isolates belonged to CMV group IB.

The amplified PCR product was cloned in pGEM-T easy cloning vector. The recombinant plasmid was transferred into *E. coli* DH5α cells. The transferred cells were

plated on LB plates containing ampicillin, X-gal and IPTG. Recombinant white colonies were selected to carry out colony PCR to confirm the transformation. These were further confirmed by band shift assay and colony PCR.

Development of gold nanorods based biosensors was also attempted. Gold nanorods were synthesized by adding synthesized seed solution to the growth solution which led to the formation of pinkish red colour. The change from transparent growth solution to pinkish red colour was due to formation and growth of GNRs. Synthesized GNRs was characterized using two different methods *viz.,* Transmission electron microscopy and UV-Vis spectrophotometer. UV-Vis spectrophotometer showed characteristic 710.25 nm longitudinal and 520 nm transverse bands in the spectrum. The nanorods were further confirmed with transmission electron microscopic images. From the TEM images, the mean length and width of GNR particles were assessed and found to be 54.96 nm and 14.75 nm respectively. The aspect ratio was calculated using the GANs theory and by TEM image dimensions and found to be 3.42 and 3.85 respectively.

The synthesized GNRs were further purified and conjugated with antibodies after modifying with alkanitol which showed the drift in longitudinal band from 710.25 to 719 nm. Diagnosis of CMV infection in healthy and infected samples was carried out by adding the crude sap from both samples to the conjugated GNRs. Then the colour of GNRs solution turned from reddish pink to black in the case of infected samples while in healthy samples, the colour remained the same. The results obtained were further confirmed by UV-Vis spectral analysis and observed the shift in longitudinal band in case of infected samples while in healthy samples there was no colour change or shift in longitudinal bands. Hence, it can be used in virus indexing of plants selected for tissue culture allowing us for the diagnosis which contributes significantly towards a rapid and clear identification of the virus in banana plants and suckers selected for tissue culture.

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ANNEXURE I

List of laboratory equipments used for the studies

Distillation Unit:	:	Purelab option-Q (ELGA)
Autoclave	:	Labline
Water Bath	:	Rotek
NanoDrop spectrophotometer 2000c	:	Thermo scientific
Laminar air flow	:	Labline industries,Kochi
Shaker incubator	:	JEIO Tceh, Korea
ELISA reader	:	Bio-Rad
Yarco Bacteriological Incubator	:	Yorco
High speed refrigerator centrifuge	:	Heraeus Biofuge Stratos
UV-Vis spectrophotometer	:	Perkinz USA
Geldoc EZ Imager	:	Bio-rad
Centrifuge	:	KUBATOO
Magnetic shaker	:	IKA laboratory technology
Electrophoresis unit	:	Genei

ANNEXURE II

Chemical composition of buffers used for ELISA and DIBA

Coating Buffer (10x) pH 9.2

Sodium carbonate	:	15.9 g
Sodium bicarbonate	:	29.3 g
Sodium nitrate	:	2.8 g
Distilled water	:	1000 ml

Phosphate buffered saline (PBS) (10x) pH 7.4

Sodium chloride	:	80 g
Potassium di-hydrogen phosphate	:	2 g
Disodium hydrogen phosphate	:	11.63 g
Potassium Chloride	:	2 g
Distilled water	:	1000 ml

Washing buffer(PBS-T)

PBS buffer (pH- 7.4)	:	100 ml
Distilled water	:	900 ml
Tween 20 (0.05%)	:	0.5 ml

Substrate buffer pH- 9.8 (Store in amber colored bottle)

Diethanolamine (9.8 %)	:	98 ml
Sodium Azide	:	0.2 g
Water	:	800 ml

Blocking Buffer (1x)

PBST(1x)	:	100 ml
Spray dried milk	:	5g

Antibody dilution buffer

PBS-T (1X0	:	100ml
BSA (0.2%)	:	0.2gm
PVP (2%)	:	2gm

ANNEXURE III

Composition of different buffers used in mechanical transmission

0.1 Sodium borate buffer (pH 8.0)

A. Boric acid (0.2 M) : 1.237g/100ml

B. Borax Na2B4O7 : 1.907g/100ml

3 ml of A mixed with 7 ml of B, diluted to a total of 20 ml.

Potassium phosphate buffer

A. 0.1 M Potassium dihydrogen phospahte : 6.084g/500ml

B. 0.1 M Dipotassium hydrogen phosphate : 8.079g/500ml

0.1 M (pH 7.2) - 28ml of A mixed with 72 ml of B.

0.05 M (pH 7.2) - 28ml of A mixed with 72ml of B, diluted to a total of 200 ml.

0.1M Sodium phosphate buffer (pH 7.2)

A. 0.1 M Sodium phosphate monobasic anhydrous : 5.999g/500ml

B. 0.1 M Sodium phosphate dibasic dehydrate : 8.899g/500ml

28 ml of A mixed with 72 ml of B

0.1 Citrate buffer (pH 6.2)

A. 0.1 M Citric acid : 2.1010g/100ml

B. 0.1 M Sodium Citrate : 2.940g/100ml

1.6 ml of A mixed with 18.4 ml of B

0.1M Tris buffer (pH 7.2)

Tris : 24.23g/1000ml

22.5 ml of 0.2 N HCL mixed with 25 ml of Tris, diluted to a total of 50 ml.

ANNEXURE IV

Composition of buffers and dyes used in gel electrophoresis

1. 6X Loading/Tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

The dye was prepared and stored at 4°C

2. Ethidium Bromide (intercalating dye)

The dye was prepared as stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

3. 50X TAE buffer (pH 8.0)

2 M Tris buffer pH-8	24.2 g
1 M Glacial acetic acid	5.7 ml
0.5 M ETDA pH-8	18.6 g in 100 ml DEPC water to make 0.5 M (pH-8)
DEPC water	100 ml

372.24 g of EDTA in a liter of water makes 1 M.

So 18.6 g is dissolved in 100 ml DEPC water.

ANNEXURE V

Chemical composition used for Goldnanorods (GNRs) synthesis

Chemical	Concentration (M)	Quantity(gm)	Volume(ml)
CTAB	0.1	0.2733	7.5
CTAB	0.2	3.640	50
$NABH_4$	0.01	0.0037	10
$HAuCl_4$	0.01	0.027	7
$AgNO_3$	0.01	0.008	10
Ascorbic acid	0.01	0.01	6

ANNEXURE VI

Chemical composition used for conjugation of antibody to Goldnanorods (GNRs)

1) **MPA (20 mM)** - 200 µl

   Make up volume dissolved in a 9.8 ml of ethanol for making 10 ml of solution

2) **EDC(7.5 mM)** – 0.007 gm mix with 5ml of water

3) **NHS (1.5 mM)-** 0.008 gm mix with 5ml 0f water

Annexure VII

LB (Luria-Bertini) broth

Tryptone	:	1.0 % (w/v)
Yeast extract	:	0.5 % (w/v)
NaCl	:	0.5 % (w/v)

Adjust pH 6.2-7.2 with NaOH and autoclave.

LB agar (Luria-Bertini) medium

Prepared as above with the addition of 1.5% agar prior to autoclaving.

Annexure VIII

IPTG (isopropyle-thio-β-D-galactoside) Stock solution (0.1M):

Make a stock solution of 50 mg/ml in distilled water. Use 100µl/100ml.

X-Gal stock solution:

X-Gal (5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside) is available as 40 mg/ml stock in N, N dimethyle formamide. Use 0.5 ml/100 ml LB.

Annexure IX

Details of sequences used for primer designing

NCBI Code	bp #	Country	Location	Host
AY125575	770	India	Thrissur (Kerala)	Banana
KJ874250	2053	India	Tamil Nadu (coimbatore)	Banana
KU382457	880	Iran	-	Cucumber
AM055602	657	India	Karnataka	Banana
DQ152254	657	India	Lucknow	Banana
CMU43888	957	Isreal	Bet-Dagan	Banana
AM158321	657	India	Lucknow	Banana
AF541918	406	India	New delhi	Banana
EU128723	657	India	Trichy	Banana
DQ640743	657	India	Maharastra	Banana
KT447515	657	India	Gangtok	Banana

MOLECULAR CHARACTERIZATION OF VIRUS CAUSING INFECTIOUS CHLOROSIS DISEASE OF BANANA

by

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ABSTRACT OF THE THESIS

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ABSTRACT

Banana (*Musa* spp.), a fruit crop of global importance in terms of income and food security to millions of marginal farmers throughout the developing countries and is the world's fourth most important commodity after rice, wheat and corn. It is infected by several diseases caused by fungi, bacteria and viruses. Among the viral diseases, infectious chlorosis disease is now emerging as a major threat affecting banana production worldwide. The disease also assumes significance because it affects plant growth, yield and the symptoms are often confusing with that of nutritional disorders.

The present project entitled "Molecular characterization of virus causing infectious chlorosis disease of banana" was envisaged to study the diagnostic symptoms, biophysical and molecular characterization and immuno-molecular diagnosis of the virus. Development of nanobiosensors for the quick and more sensitive detection of virus was also attempted.

The project research was initiated with purposive sampling surveys conducted in different banana growing areas of Thrissur district for documenting the symptoms under natural conditions, collection of infected samples of various virus isolates and to assess the extent of disease incidence. The highest disease incidence of 90 per cent was recorded on banana variety Robusta. The salient symptoms of the disease were mosaic, leaf distortion, stunting of plants and yield reduction. Histological changes in the anatomy of the leaf were also observed due to virus infection. Parenchyma was irregular shaped with interrupted cuticle, disorganized arrangement of cells near septum and air chambers with intercellular depositions. The chloroplasts were lesser in number. The transmission studies confirmed that the virus was transmitted through aphid species *viz*. *Pentalonia nigronervosa* and *Aphis craccivora* and also through the suckers of infected mother plants of banana. But the virus was not mechanically transmitted from banana to banana. Twenty one plant species including weeds seen in banana ecosystem were proved to be the hosts of this pathogen. Out of which *Acmella radicans, Cucumis anguria, Centrosema pubense, Canavalia gladiate, Ichnocarpus frutescens* and *Phyllanthus amara* were found to be the new host of this pathogen.

The physical properties of the virus such as Thermal Inactivation Point (TIP), Dilution End Point (DEP) and Longevity *in vitro* (LIV) were determined and it was found that thermal inactivation point ranged between 65-70°C, dilution end point of 10^{-4} and the longevity *in vitro* (LIV) was up to 3 days and got inactivated from 3rd and 7th day onwards when kept at room temperature and refrigerated conditions respectively. The morphological characteristics of the virus particles were studied by imaging the infected leaf samples through Electron microscope by leaf dip technique. It revealed the presence of spherical, isometric virus particles of size 28 nm, thus confirming the etiology of infectious chlorosis disease of banana as *Cucumber mosaic virus* (CMV) belonging to the genus Cucumovirus and family Bromoviridae.

The molecular characterization of the coat protein gene of the virus was carried out through Reverse Transcription PCR. The coat protein (CP) gene was amplified using designed and reported primer pairs which yielded amplicons of approximate size of 700 bp and 750 bp respectively. The CP gene of eight isolates were sequenced and subjected to *in silico* analysis which revealed that the sequences of CMV exhibited significant nucleotide identity (99 to 96 per cent) with the sequences of CMV available in the data base of genebank. The phylogenetic analysis revealed that all the isolates belong to the subgroup IB of Genus Cucumovirus. The molecular clones of CP gene of CMV were also developed which could be used as probes for the detection of virus.

The immunodetection techniques like Direct Antigen Coating-Enzyme Linked Immuno Sorbent Assay (DAC-ELISA) was validated and it was found that CMV could be best detected at 1;200 dilution along with 1:1000 dilution of secondary antibody. Dot Immuno Binding Assay (DIBA) was standardized to detect CMV infection of leaf samples and showed positive reaction on nitrocellulose membrane.

The field gene bank comprising about 175 accessions maintained at Banana Research Station, Kannara was screened to assess their disease reaction under natural conditions and the disease was recorded on 12 accessions with AA, AB, AAA, AAB and

AAAB genome. The accessions with ABB genome were comparatively free of the disease.

Nanobiosensor were also developed for the quick and more reliable detection of virus infection. Gold nanorods (GNRs) were fabricated through seed mediated procedure and UV-Vis spectra of GNRs solution indicated characteristic longitudinal and transverse bands at 710 and 523 nm respectively. The image under Transmission Electron microscope revealed that the solution contained rod shaped gold nanoparticles of length 54.96 nm and diameter 14.75 nm. The principle of Bio-recognition induced aggregation of antibody conjugated GNRs was used for the detection of CMV. This study describes a useful method that allows us for same day diagnosis and contributes significantly towards a rapid and clear identification of the virus in banana plant and suckers selected for tissue culture.

The outcome of this study would facilitate early detection and elimination of CMV infected plants and ensure distribution of healthy planting materials to the farmers of Kerala, thereby increasing the production as well as the productivity of banana in the state.