ROLE OF MIXED INFECTION OF CASSAVA MOSAIC VIRUSES IN CASSAVA MOSAIC DISEASE DEVELOPMENT

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By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled that "**Role of mixed infection of Cassava mosaic viruses in cassava mosaic disease development**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or either similar title, of any other University or society.

Place: AKHILA J.

Date: (2015-09-022)

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DEDICATED TO MY PARENTS, TEACHERS AND FRIENDS

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LIST OF ABBREVIATIONS

%	Percentage
~	Approximate
^{0}C	Degree Celsius
µ	Micro
µg	Microgram
µl	Micro litre
µM	Micro molar
A	Adenine
ACMV	*African cassava mosaic virus*
ACMBFV	*African cassava mosaic burkina faso virus*
bp	Base Pair
BRNV	*Black raspberry necrosis virus*
Ca	Calcium
CBSD	Cassava Brown Streak Disease
CGM	Cassava Green Mite
cm	Centimetre
CMD	Cassava Mosaic Disease
CMG	Cassava Mosaic Geminivirus
CMMV	*Cassava mosaic Madagascar virus*

CP	Coat Protein
CR	Common Region
CTAB	Cetyl Trimethyl Ammonium Bromide
CTCRI	Central Tuber Crops Research Institute
CGMMV	Cucumber green mottle mosaic virus
DIBA	Dot immunobinding assay
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleoside triphosphates
DsDNA	Double Stranded DNA
EACMCV	*East African cassava mosaic Camerron virus*
EACMKV	*East African cassava mosaic Kenyan virus*
EACMMV	*East African cassava Malawi virus*
EACMV-UG	*East African cassava mosaic virus- Uganda*
EACMV	*East African cassava mosaic virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immune Sorbent Assay
F	Forward Primer
FAOSTAT	Food and Agriculture Organization Statistical
Fig	Figure
g	Gram

h	Hour
ha	Hectare
ICAR	Indian Council of Agricultural Research
ICMV	*Indian cassava mosaic virus*
IR	Intergenic Region
JcMD	Jatropha Curcas Mosaic Disease
kDa	Kilodalton
Kg	Kiliogram
l	Litre
M	Molar
MEAM1	Middle East Asia Minor 1
MED	Mediterranean
min	Minute
Min	Minimum
ml	Millilitre
mM	Millimolar
MLN	Maize Lethal Necrosis
MP	Movement Protein
MW	Molecular Weight
NaOH	Sodium Hydroxide
Nacl	Sodium Chloride

No.	Number
NSP	Nuclear Shuttle Protein
nt	Nucleotide
ORF	Open Reading Frame
ORMV	Oilseed rape mosaic virus
PAMP	Pathogen Associated Molecular Pattern
PAL	Phenylalanine Amino Lyase
PALCV	*Potato apical leaf curl virus*
PCR	Polymerase Chain Reaction
PO	Peroxidase
PPO	Polyphenol oxidases
PPSMV	*Pigeonpea sterility mosaic virus*
PRSV	*Papaya ring spot virus*
qPCR	Quantitative (Real time) PCR
RLKs	Receptor- Like Kinase
Rep	Replicase Gene
RCR	Rolling Circle Replication
RF	Replication Factor
RNAi	RNA interference
RYNV	Rubus yellow net virus
SACMV	*South African cassava mosaic virus*

Sec	Second
SEGS	Sequence Enhancing Geminivirus Symptoms
SLCMV	*Sri Lankan cassava mosaic virus*
SPCSV	*Sweet potato chlorotic stunt virus*
SPFMV	*Sweet potato feathery mottle virus*
ssDNA	Single Stranded DNA
t	Tonnes
TrAP	Transcription Activator Protein
TMGMV	*Tobacco mild green mosaic virus*
ToLCBV	*Tomato leaf curl Bangalore virus*
ToLCKeV	*Tomato leaf curl Kerala virus*
TYLCV	*Tomato yellow leaf curl virus*
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
U	Unit
V	Volt
VIGS	Virus Induced Gene Silencing
YEB	Yeast Extract Broth

1. INTRODUCTION

Root and tuber crops viz., cassava, yams, sweet potato have an important role in global food and energy security. Also constitutes the important source of raw material for starch, feed and biofuel industries.

Cassava (*Manihot esculenta* Crantz), belonging to the Euphorbiaceae family is one of the most efficient vegetatively propagated perennial starchy roots that grows well in areas of acidic and low fertility soils throughout the tropical climate. It is also known as yucca, manioc and tapioca and used for curbing food security issues as well as means of income and livelihood of many nations. The cassava name is derived from the word 'Casavi or Cazabi' which means bread. The native centre of the crop is in South America, probably from Brazil (Allen, 1994; Olsen and Schaal, 2001). The crop is grown in over 90 countries and is the third most important source of calories after cereals and legumes grown in the tropics. In India, cassava is grown in an area of 2.28 lakh ha with a production of 4651000 tonnes (FAOSTAT, 2018) for food and industrial purposes.

It is a staple crop for half a billion people in Africa, Asia and Latin America. Its production faces constraints from a number of pests and diseases; among them Cassava Mosaic Disease (CMD) which was first described in Tanzania (Warburg, 1894) is a major productivity constraint throughout the world. Later the disease occurred in all cassava producing areas in Africa, India, Sri Lanka and many South East Asian countries. The disease leads to yield loss of about 88% in susceptible varieties and 50% in varieties tolerant in the field. (Edison, 2002; Obonyo *et al*., 2007). Cassava mosaic disease (CMD) is caused by several Cassava Mosaic Geminiviruses (CMGs). Initially, it was named as Cassava latent virus (Bock & Guthrie and Figueiredo, 1981) but was later characterized and renamed as *African cassava mosaic virus* (ACMV) (Stanely and Gay, 1983; Bock and Woods, 1983) belonging to the *Geminiviridae* family and *Begomovirus* genera. The virus is spread through infected planting materials or a vector, the whitefly *Bemisia tabaci*. The genome of CMGs comprises two circular bipartite single- stranded DNA molecules DNA A and DNA B. DNA A encodes the function associated with viral encapsulation, transcription enhancement and replication whereas the DNA B encoding the movement functions. The CMD is characterised as irregular yellow /yellow-green chlorotic mosaic patterns of leaves leading to deformed leaves and stunted growth have

been recognised as a threat to cassava production for a longer period of time. Eleven CMGs have been described of which 9 occur in Africa, either alone or in a combination that includes *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (ACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *African cassava mosaic Burkina Faso virus* (ACMBFV), *Cassava mosaic Madagascar virus* (CMMV) and *South African cassava mosaic virus* (SACMV) (Legg *et al.,* 2015). But only CMGs and Cassava Brown Streak Virus cause the disease of major economic significance (Legg and Raya, 1997). Among the different diseases, Cassava Mosaic Disease (CMD) is the main biotic constraint in cassava production and mostly a threat to food security. CMG in plants was detected by using serological methods such as ELISA or different formats of PCR.

The first CMG to be recorded from South Asia was the *Indian cassava mosaic virus* (ICMV) (Malathi *et al.,* 1985), followed by *Sri Lankan cassava mosaic virus* (SLCMV) several years later (Saunders *et al.,* 2002). Initially, it was reported from Sri Lanka and subsequently shown to occur in southern India also, together with ICMV (Patil *et al.,* 2005; Anitha *et al.,* 2011). Recently, the CMD has been reported and widely spread throughout Southeast Asia (Wang *et al*., 2016). Mixed infections are complex interactions between viruses and hosts. The SLCMV is capable of acquiring any viral DNA from other begomovirus results a mixed infection and generates highly virulent strains that cause more severe symptoms of CMD. To prevent the disease epidemic, need more information about the host-virus interactions in mixed infections for developing new pest management strategies. In this context, the present research work is carried out with the following objective is;

To study the effect of mixed infection of ICMV and SLCMV in cassava mosaic disease development in cassava

2. REVIEW OF LITERATURE

2.1 CASSAVA (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz) is a perennial shrub from the Euphorbiaceae family that grows well in tropical climate. Its geographical origin lies in Latin America and currently, the white and milky pulp of the tuberous root occupies the most important crop in billions of people. The cassava roots are bulky and potentially a low- cost source of starch. The leaves are also used as food and protein sources for animal feed.

The cassava crops typically reach 4m in height at maturity and roots are harvested at any time from 7-12 months after planting depending upon the cultivar's duration. Swollen storage roots which typically contain 30-40% dry matter and have 85% of starch content. It also consists of essential micronutrients such as vitamins B and C, Zinc and Calcium. The cassava is mostly made of starch as a carbohydrate content of about 70-80 % and its root consists of amylose (15-17%) and amylopectin (83%) are the two water- insoluble homoglucans (Charles *et al.* 2005; El-Sharkawy, 2003). Cassava is categorised under 'sweet and bitter' varieties due to the different levels of cyanogenic glycosides content present in the root.

The wide range of environmental adaptability of cassava and its tolerance of acid soils and sustained periods of drought were the key factors for its widespread growth throughout in the tropical regions. In Asia, the major growth of cassava has been not as a direct human food but as a low- cost source of energy in animal feed and thickeners in industries uses. In India, it is grown for direct consumption and starch grain- producing industries mainly in the southern states of Andhra Pradesh, Tamil Nadu, Kerala and some northern states. In India, cassava is grown in an area of 2.28 lakh ha with a production of 4651000 tonnes (FAOSTAT, 2018) for food and industrial purposes.

2.2 GLOBAL CASSAVA PRODUCTION

World cassava production showed a steady upgrade from 124 million tons in 1980 to 277 million tons in 2016 (FAOSTAT, 2018), which shows the enhanced demand for this crop in the food, feed and industrial sectors. Cassava production globally increases steadily and covers the area about 70% in Africa, 18% in Asia and 10% in America. About 57% of global cassava

production comes from Africa mostly from Nigeria. Asian and American countries contribute about 32% and 11% of global cassava production respectively. Among the 104 cassava producing countries the global productivity stood at 11.8t/ha (FAOSTAT, 2018).

About 26% of the world's cassava production occurred from four Asian countries (includes Thailand, Indonesia, Vietnam, Cambodia) which is 82 % of Asia's production of cassava of 89.2 million tons (FAOSTAT, 2018). However, in Asian countries, the productivity of cassava increases due to the high rate of cassava production. China is the major consumer of cassava from Southeast Asian countries. In Asia, cassava is mostly utilized for direct human consumption and feed stock for biofuel productions. Among the cassava growing countries, India ranks first in the world in terms of productivity of cassava with 20.96 t/ha as against with a world average of 11.08 t/ha (FAOSTAT, 2019). In India, cassava is the most important tuber crop and is mainly cultivated in the southern parts of India, particularly in Kerala and Tamil Nadu, which contributes over 92% of the total cassava production in the country (Government of India, 2018). In Tamil Nadu, cassava is mainly used for industries, whereas in Kerala it is used for culinary purpose. The total area under cassava cultivation in Kerala was about 61876 ha with a production of about 2325007 tons during the year 2018-19. Among the districts, Thiruvananthapuram is the highest share of area under cassava cultivation (21.07%), followed by Kollam (20.86%) and Idukki (11.25%). The production of cassava was highest in Thiruvananthapuram 475449.148 in tonnes which are less than state average of 2325007.314 in tonnes (Agricultural statistics 18-2019).

2.3 CASSAVA MOSAIC DISEASE (CMD) AND ITS DISTRIBUTION

Cassava mosaic disease is the important constraint in cassava production and a major threat to food security. Warburg, (1894) was first to report the CMD in Tanzania. *African cassava mosaic virus* (ACMV) is most significantly reducing the yield of cassava. At the end of 1940s, CMD was widely spread to most of the cassava growing countries in sub- Saharan Africa (Fauquet and Fargette, 1990). It is caused by geminivirus species which collectively called as cassava mosaic begomoviruses and are transmitted by whitefly, *Bemisia tabaci.* In Africa, cassava is mostly infected with 9 viral species and different strains. Most of these viruses are related to CMD and CBSD.

Viruses associated with CMD

Indian Cassava Mosaic Virus (ICMV), *Sri Lankan Cassava Mosaic Virus* (SLCMV), *African* Cassava Mosaic Virus (ACMV), *East African Cassava Mosaic Virus* (EACMV), *East African Cassava Mosaic Cameroon Virus* (EACMCV), *East African Cassava Mosaic Kenya Virus* (EACMKV), *East African Cassava Mosaic Malawi Virus* (EACMMV), *East African Cassava Mosaic Zanzibar Virus* (EACMZV), *African Cassava Mosaic Burkina Faso Virus* (ACMBFV), *Cassava Mosaic Madagascar Virus* (CMMV) and *South African Cassava Mosaic Virus* (SACMV) (Legg *et al.* 2015). Tiendrebeogo *et al.* (2012) reported the evolution of ACMV by recombination of bipartite and monopartite begomoviruses, *ACMV-Burkina Faso.*

Table: 2.1: Cassava mosaic geminivirus species

Viruses	Genome	Occurrence	Reference
African Cassava Mosaic Virus (ACMV)	NC_001467 NC_001468	Africa	Bocks and Woods (1983)
East African Cassava Mosaic Virus (EACMV)	NC_004674 NC_004676	East Africa	Swanson and Harrison (1994)
Indian Cassava Mosaic Virus (ICMV)	NC_001932 NC_001933	India	Hong *et al.* (1993)
Sri Lankan Cassava Mosaic Virus (SLCMV)	NC_003861 NC_003862	India, Sri Lanka, Vietnam, China, Cambodia	Saunders *et al.* (2002)
South African Cassava Mosaic Virus (SACMV)	NC_003803 NC_003804	Southern Africa	Berrie *et al.* (2001)
East African Cassava Mosaic Cameroon Virus (EACMCV)	NC_004625 NC_004630	East and West Africa	Fondong *et al.* (2000)

East African Cassava Mosaic Malawi Virus (EACMMV)	NC_022645 NC_022644	East and Southern Africa	Zhou *et al.* (1998)
East African Cassava Mosaic Zanzibar Virus (EACMZV)	NC_004655 NC_004656	East Africa	Bull *et al.* (2006)
East African Cassava Mosaic Kenya Virus (EACMKV)	NC_011583 NC_001584	East Africa	Bull *et al.* (2006)
Cassava Mosaic Madagascar Virus (CMMV)	NC_017004 NC_017005	Madagascar	Harimalala *et al.* (2012)
African Cassava Mosaic Burkina Faso Virus (ACMBFV)	HE616777 HE616778	West Africa	Tiendrebeogo *et al*. (2012)

The major constraint for cassava production in India and Sri Lanka is *Indian Cassava Mosaic Disease* (ICMD) and *Sri Lankan Cassava Mosaic Disease* (SLCMD) respectively and is caused by Geminiviruses included in the genus *Begomovirus*.

2.4 GEMINIVIRUSES

Geminiviruses are one of the most devastating viruses which infect the large number of crop species in tropical and subtropical regions. It also causes huge economic losses and is threatening the production of different crops in India.

Geminiviruses (Family: Geminiviridae) are circular, single- stranded DNA (ssDNA) viruses that are encapsidated in unique twin geminate particles (Fig. 2.1). Each having a genome size of 2.7-3.0 kb encoded with distinct genes that vary in number and function.These viruses can infect both dicot and monocot plants especially the economically important crops such as cassava, tomato, maize and beet root (Goodman. 1977, 1981; Rojas *et al.*, 2005).The yield of virus- infected crop plants is greatly affected and leads up to a huge reduction. On the basis of

genome organization, vector transmission and host range, geminiviridae family is classified into 9 genera (Zerbini *et al.*, 2017) such as: *Begomovirus, Mastrevirus, Topocuvirus, Curtovirus, Becurtovirus, Eragrovirus, Turncurtovirus* (Brown *et al*. 2012; Bernardo *et al.* 2013; Varsani *et al.* 2014). Varsani *et al.* (2017) recently reported the two genera, *Capulavirus* and *Grablovirus* related to CMGs (Fig. 2.2).

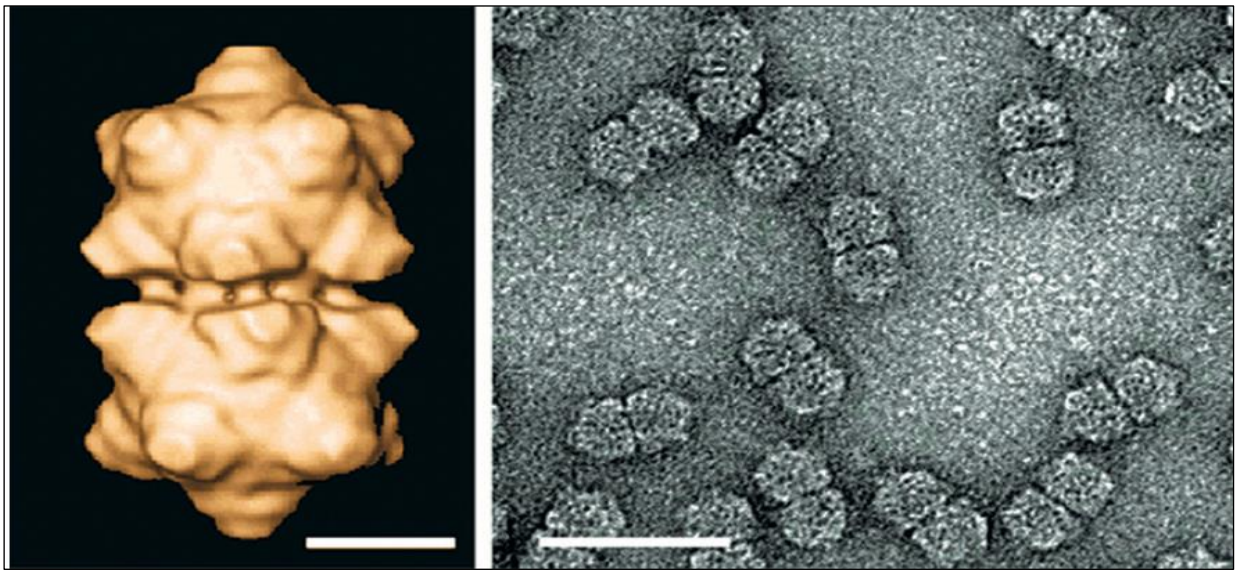


Fig. 2.1: (Left) Cryo-electron microscopic reconstruction of maize streak virus viewed along a two-fold axis of symmetry. Bar 10nm. (Right) Purified particles of maize streak virus stained with uranyl ace-tate showing typical twinned quasi-isometric subunits. Bar, 50nm (Zerbini et al., 2017).

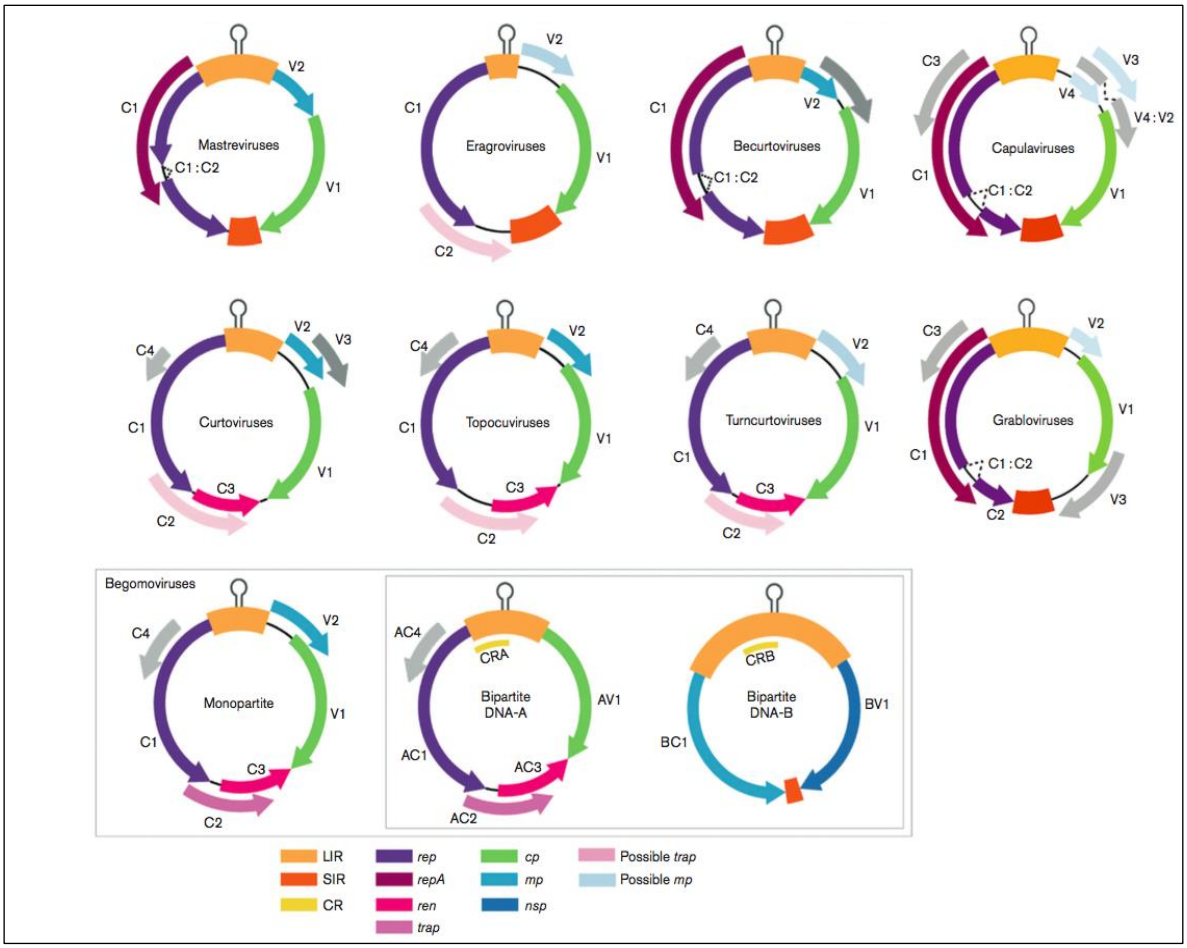


Fig. 2.2: Genome organization of isolates in various geminivirus lineages. The ORFs (V1, V2, V3, C1, etc.) are colour-coded according to the function of their protein products (rep, replication-associated protein; ren, replication enhancer protein; trap, transcriptional active- tor protein; cp, capsid protein; mp, movement protein; nsp, nuclear shuttle protein). LIR, long intergenic region; SIR, short intergenic region; CR, common region. The hairpin which includes the origin of replication is indicated in the LIR (Zerbini et al., 2017).

Begomovirus is one of the largest genus consisting of more than 410 species (Singh *et al.* 2020) infecting the host, typically persistent viruses. It devastates the sustainable development of crops in tropical and sub-tropical regions. Begomovirus complexity and satellite DNA molecules are caused by the evolution of new diseases. These viruses are transmitted by different insect species in homopterans family includes whitefly, belongs to the species *Bemisia tabaci* and others are aphids, leafhoppers, treehoppers etc causes a huge loss in the crop

production. *B. gossypiperda and B. nigeriensis*are the others species of whitefly transmitting CMD in Africa (Mayne & Ghesquiere, 1934; Golding, 1936).

2.4.1 Geminivirus Genome Organization

The genome organization of geminiviruses belonging to the genus begomovirus is having either monopartite/ bipartite DNA molecules (Fig. 2.2) are individually encapsidated in a twinned icosahedral virion containing a 110 coat protein subunit organised as 22 pentameric capsomers (Harrison *et al.*, 1977, Stanley, 1985). The majority of these viruses have bipartite genome consists of 2 circular ssDNA, DNA A and DNA B respectively.The proteins encoded by the ORFs on the complementary strand are both involved in viral DNA replication, and are translated from spliced and un-spliced versions of the same mRNA.

The monopartite genome consists of only DNA A, alone is infectious and produces typical symptoms. DNA A and DNA B encode genes necessary for viral encapsidation, replication and movement functions respectively. Both the bipartite and monopartite DNA A of begomovirus contains a viral sense strand (V strand) which codes the AV1 and AV2, coat proteins that covers the ssDNA virions as capsids and involved for the movement of virus. Other genes encoded ORFs on the complementary strand (C strand) involved in both viral replication and protein synthesis. In begomoviruses encapsidated ssDNA converted to double strand (ds) Replicated Form (RF), is template for transcription. Transcription is bidirectional and proteins are encoded in viral and complementary sense strand. Bipartite genome of begomovirus consists of 6-8 ORF whereas the monopartite genome encodes 5-6 ORF (Singh *et al.*, 2020)

DNA A carries 6 open reading frames with each encodes a specific protein the AC1, replication associated protein (Rep); AC2, the transcription activator protein (TrAP); AC3, the replication enhancer protein (RE); AC4, the RNA silencing suppressor; AV1, the coat protein (CP) and AV2, the pre-coat protein. DNA B has 2ORFs, BV1 encodes the nuclear shuttle protein and BC1 encodes the movement protein (MP).Viral ORFs are separated by a non-coding intergenic region (IR) possessing a short stretch of conserved nucleotides between the cognate DNA A and DNA B, it is called the Common Region (CR). Begomovirus has the highly specific nucleotide sequence of CR. CR possess origin of replication (Ori), invariant stem loop like non-anucleotide sequence (TAATATT-AC) conserved in all geminiviruses and two bidirectional

RNA polymerase, two promoters. Iterons (direct repeats of 5-7 nucleotides) are present upstream to the stem loop structure. Replication initiator protein (Rep) binds to iterons and produces a nick at non- anucleotide sequence to initiate the replication of viral DNA (Hanley-Bowdoin *et al.,* 2000). Replication associated protein is coded by the AC1 gene, and the coat protein (CP), coded by AV1 gene. These regions are highly conserved and act as common targets for the detection of viruses through the PCR methods.

In nature, contains both Alpha satellite and Beta satellite DNAs, whereas, the monopartite begomovirus is associated with satellite DNAs of about 1.4kbp size. Alpha satellite DNA molecule encodes replication associated protein required and beta satellite DNA encodes the multifunctional proteins, carries single ORF (β C1) for the viral replication. Both the satellite DNAs dependent on helper virus replication (Idris *et al.,* 2011). Coat protein is an important component for the survival of the begomoviruses. In dicotyledonous plants, *B. tabaci* is used as vector to carry the viruses for infection.

2.4.2 Life Cycle of Virus

The genome of geminivirus is replicated in the host cell nucleus through the Rolling Circle Replication (RCR) method (Fig. 2.3).The mode of DNA replication of geminivirus and the role of associated host factors is discussed by Pardhan *et al.* (2017).Whitefly carrying geminivirus particle, upon un-coating, to release the viral ssDNA to the cytoplasm of the infected plant cells and eventually brought into the nucleus for the viral DNA amplification. The ssDNA gets converted to double stranded (dsDNA) with the use of cellular host factors. The Rolling Circle Replication (RCR) is used several times for the duplication of dsDNA or Replicative Form (RF) and it acts as a substrate for transcription of viral factors. The dsDNA is transcribed by using the RNA polymerase II of the infected plant. The viral Rep protein nicks the DNA at specific site to initiate RCR (Stanley, 1985).Thus generated 5’ end of nick is covalently bind with Rep protein and further extension of RCR carried out through the free 3’ hydroxyl end. Progression of RCR fork is done with the help of Rep protein act as a helicase. Thus synthesised the concatemeric DNA forms are cleaved and circularized to a new viral ssDNA strand by using the activity of Rep (Yadava *et al.* 2010).

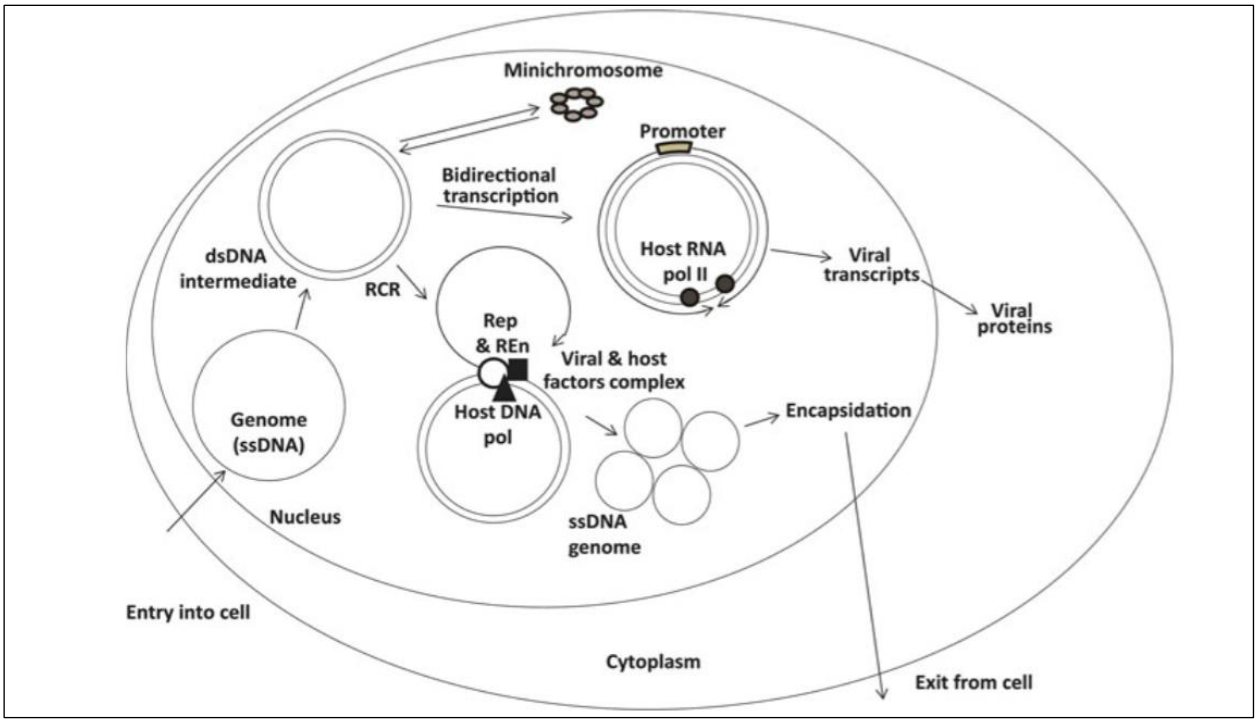


Fig. 2.3: Life cycle of geminivirus proceeds via a dsDNA intermediate which acts as a template for rolling circle replication as well as bidirectional transcription (Sharma & Ruhel, 2019).

The transcription process are repressed by its own Rep and leading to the activation of the viral coat proteins (Malik *et al.,* 2005). Circular ssDNA then be encapsulated by CP into virions, with the help of MP viral DNA transported out of the cell and crossing the nearby cells through plasmodesmatal channels, thus viral spread occurs.

2.4.3 Viral Factors Associated With Replication of Geminivirus

Assay of the genes is important for understanding the virus-host interaction and develop an antiviral strategies. For the replication of geminiviruses required the presence of several essential proteins coded with viral genes such as;

2.4.3.1 Rep Protein (AC1)

Open reading frame encodes ~41 kDa Replication (Rep) protein which is multifunctional protein involved in viral genome replication, interaction of host factors, ATP/ GTPase activity (Hanley- Bowdoin *et al.,* 2000; Nagar *et al.,* 1995; Desbiez *et al.,*1995). Rep protein initiates the replication by binding to the iterons located in the conserved non- anucleotide sequence,

which produce a nick at the 7th and 8th nucleotides and ligated after replication process. Pant *et al.,* 2001 reported the Rep protein also act as a site specific type- I topoisomerase. A study conducted by Choudhury *et al.* (2006) with the Rep protein of *Mungbean yellow mosaic Indian Virus* (MYMIV), showed the DNA helicase activity. DNA methyltransferase activity of host down regulated with the Rep of *Tomato yellow leaf curl virus* (TYLCV) (Rodriguez-Negrete *et al.,* 2013).

2.4.3.2 Replication Enhancer Protein (AC3)

REP is a ~16kDa oligomeric protein interact with Rep and enhances ATPase activity for efficient viral genome replication. Pasumarthy *et al.* (2010) was studied in *Tomato leaf curl Kerala virus* (ToLCkeV) have been demonstrated the high order oligomer and ATPase activity of AC3 protein.

2.4.3.3 Coat Protein (AV1)

It encodes for ~29 kDa protein. The viral infection will affected with the absence of CP, because it facilitates the nuclear transport of viral genome. In monopartite begomovirus, Priyadarshini *et al.* (2011) found the CP act as a nuclear shuttle protein. Importance of coat protein for viral transmission evaluated by Kanakala and Ghanim, (2016) with the CP of TYLCV and cyclophilin B protein of *B.tabaci.*

2.5 CHARACTERIZATION AND DETECTION OF BEGOMOVIRUS

Efficient detection methods are required for developing controlled management strategies of viral infection for farmers. Begomoviruses was early detected by Enzyme Linked Immune Sorbent Assay (ELISA) using polyclonal antibodies. Phaneendra *et al.* (2014) studied on begomoviruses for the first time with Maltose Binding Protein (MBP) - tag fusion CP to produce an antibody against the virus. *Tomato Leaf Curl Bangalore Virus* (ToLCBV) in tomato and other species of crops detected by using Monoclonal antibodies (Devaraja *et al.,* 2003). Double and trible antibody sandwich ELISA (DAS-ELISA and TAS-ELISA), were used for the detection of begomoviruses includes the *Potato Apical Leaf Curl Virus* (PALCV) and ToLCBV

respectively (Venkatasalam *et al.*, 2005; Devaraja *et al.*, 2003, 2005). Due to some drawbacks in ELISA, other techniques performed for the diagnosis of begomovirus.

Dot- immunobinding assay (DIBA) highly sensitive and reproducible method for detection of virus and required less antibodies than ELISA. Nagendran *et al.* (2017) studied on different begomovirus and various types of cucurbits such as *Cucumber Mosaic Virus* (CMV), Zucchini yellow mosaic virus (ZYMV) *Papaya Ring Spot Virus* (PRSV), *Cucumber Green Mottle Mosaic Virus* (CGMMV) using DIBA and showed (about 98.6%) high incidence. Hybridization based methods such as southern blotting, Microarray, an effective detection methods using with hybridization probes for specific DNA sequence in the various samples of viruses. Then PCR became a powerful tool for the detection and characterization of viruses by using the conserved region. But with the known sequences, the analysis of viruses became failed due to the difficulty in extraction of DNA required for the PCR and the low concentration of virus. Geminiviruses are classified using complete genome sequences comparison, analysing highly conserved region for clones of PCR primer and to identify new viruses (Padidam *et al.*, 1995).

Snehi *et al.* (2018) first reported the Yellow Vein Net Diseases on *Malva parviflora* caused by *Tomato leaf curl kerala virus* (ToLCKeV), a new begomovirus host from India is detected by PCR using specific begomovirus primers. After that Rolling Circle Amplification (RCA) technique was developed. The replicative viral ssDNA amplified by RCA with high fidelity polymerase (Dean *et al.*, 2001, Haible *et al*., 2006).The begomovirus was isolated on chilli first reported by Saeed *et al*. (2014), *Chilli leaf curl india virus* infecting *Menthaspicata* in India. The full length sequence of both DNA A and DNA B of begomovirus was amplified by using RCA (Saeed *et al.*, 2014). Pakkianathan *et al.* (2015) used Fluorescence *Insitu* Hybridization technique for determine the localization and replication of TYLCV in the insect vector.

2.6 SYMPTOMS OF CMD

The symptoms of CMD were firstly described by Storey and Nicholas in 1938. It occurs as characteristic leaf mosaic patterns in various parts of the leaf and they are determined at an initial stage of leaf development. The chlorotic parts of the leaf is fail to expand fully results a

stresses setup by unequal expansion of the lamina cause malformation and distortion. In case of several infection, the leaves gets reduced in size, misshapen, twisted with bright yellow specks surrounded by normal green area. The growths of plants are stunted and immature leaves absciss. CMD affected plant leaves undergo reduction and distortions of the chloroplast, decrease the rate of photosynthesis and total carbohydrate content.

The chlorotic zones are normally differentiated and separated in size from that of a whole leaflet to small spots. Leaflets may show a uniform mosaic pattern or it localized to a few zones which are often at the bases. During the viral infection the size and number of tubers are decreased (Castillo *et al.,* 2011). The main difficulties occur in recognising CMD symptoms, when the leaves of plants were affected by arthropod pests such as Cassava Mealy Bug (*Phenacoccus manihot*, Cassava Green Mite (CMG) (*Mononychellus tanajoa* Bondar) or nutrient deficiency reported Asher *et al*. (1980) that causing severe problem in diagnosis. Usually CMD symptoms severity analysed by the standard 1-5 scale of Hahn *et al.* (1980) (Table 2.2). Most severe score symptoms represented in the 4-5 scales.

Table: 2.2: The cassava mosaic symptom scale of 1- 5 (Hahn et al., 1980)

Scale	Description
1	Unaffected shoots, no symptoms
2	Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy
3	Pronounced mosaic pattern on most leaves, monitoring and distortion of the lower one-third of the leaflets
4	Severe mosaic distortion of two- thirds of most leaves and general reduction of leaf size and stunting of shoots
5	Very severe mosaic symptoms on all leaves, distortion, twisting, misshapen and severe leaf reductions of most leaves accompanied by severe stunting of plants.

Spatial distribution of viruses in plants is first studied Mckinney, (1929) in tobacco plants and observed symptoms appeared on single or different strains of TMV infected on leaves. They found that mosaic patterns are spatially separated. Then, Takeshita *et al.* (2004) studied the

spatial distribution of different tissues after mixed infections of viral strain by using *in-situ* hybridization techniques.

2.7 CMD IN INDIAN SUBCONTINENT

In India, Abraham (1956) was first reported the causative agent of CMD is Indian Cassava Mosaic Virus followed by the report of disease by Alagianagalingam and Ramakrishnan (1966).The ICMV strain type isolated from the infected cassava in Southern India is originally regarded as a strain of ACMV. The disease can cause a huge yield loss depending on the cultivars grown (Malathi *et* al. 1985) about 45% reduction in highly susceptible varieties. Later, SLCMV (Austin, 1986) was reported from Sri Lanka had lower sequence homology to ICMV. Saunders *et al.* (2002) was first reported SLCMV DNA A has characteristics of monopartite begomovirus, and the virus probably originated by the acquisition of a DNA B component of ICMV. SLCMV DNA A sequences are more closely related to ICMV DNA B than SLCMV DNA B. The replication of ICMV DNA B and SLCMV DNA A and the appearance disease symptoms during the pseudo recombination of both the viruses in *N. benthamiana* were studied by Karthikeyan *et al*. (2016).

The distribution of ICMV transmission to the samples is limited in northern and central regions of Kerala and Tamil Nadu whereas in the SLCMV is widely spread to CMD areas (Patel *et al*., 2009; Jose *et al.*, 2011; Jayakrishnan, 2016). Dutt *et al.* (2005) were first reported the identification SLCMV (SLCMV-Adi strain) causing CMD in Sothern Indian state of Kerala. About 90% high and 80% moderate level sequence similarity showed to the genome of other begomoviruses (ICMV and SLCMV) in Indian subcontinent. DNA B component of both SLCMV and ICMV showed 94% identity in the coding region and 97% outside it. During recombination, upstream stem loop sequences of ICMV DNA B is replaced with same region of SLCMV DNA A. Due this effect, the iteron motif required for replication transferred from SLCMV clones. Presence of ICMV and SLCMV in the mosaic affected areas were founded by Patil *et al.* (2005) using PCR-RFLP. On the basis of RFLP pattern, new strains of viruses showed in the samples with high proportion. ICMV and SLCMV from the South Asian regions analysed with all other cassava geminiviruses Legg *et al.* (2015) and separated from other CMGs in African regions.

In India, the virus genetic variability among isolates of ICMV and SLCMV is high (Patil *et al.,* 2005) while, the SLCMV isolated from the infected cassava caused CMD in Tamil Nadu, India showed a low level genetic variability, mainly in the form of scattered single nucleotide changes derived by Rolling Circle Amplification analysis (Kushawaha *et al*., 2018). The infectious clones of SLCMV were infective in Arabidopsis and showing symptoms similar to the cassava including leaf distortion, stunted growth (Mittal *et al.,* 2008). Recently, another SLCMV detected from Tamil Nadu (SLCMV-Attur strain) infectious on *N.benthamiana* (Kushawaha *et al.,* 2015).

Recently, Wang *et al.* (2016) reported the CMG in Southeast Asia and founded only SLCMV species, containing a bipartite circular ssDNA genome present in the samples collected from Cambodia. Wang *et al.* (2018) reported firstly the SLCMV (SLCMV-HN7 strain) was the causal agent for the CMD in China, probably origins begin from Cambodia. CMD caused by SLCMV also reported in Vietnam (Uke *et al.,* 2018). It shows about 99.8% genome sequence (both DNA A and DNA B) similarity to the SLCMV from Cambodian that belong to strain in India. Whereas, the DNA A sequence of SLCMV had only 93.7% similarity to the SriLanka SLCMV strain. Thus the SLCMV causing devastating change in the cassava production in Vietnam of Indian strain. SLCMV is also report in Madhya Pradesh, India on Chaya (*Cnidoscolus acontifolia*) based on the analysis of coat protein gene sequences (Snehi *et al.*, 2017).

Wang *et al.* (2014) was first reported the CMGs in Southeast Asia on a *Jatropha curcas* plant, woody non edible oil seed crop belonging to the Euphorbiaceae family. *Jatropha Curcas Mosaic Disease (JcMD)* was associated with new begomovirus have been reported in Africa (Kashina *et al.,* 2013). Snehi *et al.* (2012) was characterized the stain of ICMV, (ICMV) - Dharwad is the causal agent of JcMD in southern India, about 25-47% disease incidence. Identified DNA A of highly pathogenic ICMV isolated from *Jatropha curcas* causes symptoms in *N. Benthamiana, ICMV-Singapore* was the causative agent of JcMD in Southeast Asia which highly similar to *ICMV-Dharwad* (Wang *et al.,* 2014). Ramkat *et al.* (2011) were detected the two begomoviruses ACMV and EACMV-UG in the samples collected in *Jatropha* from Kenya. EACMV-UG is more predominant than ACMV in *Jatropha.*

2.8 HOST RANGE OF ICMV AND SLCMV

Cassava to cassava ICMV and SLCMV is transmitted by *B. tabaci.* Mathew *et al.* (1993) reported the ICMV infected to 43 species of Nicotiana plants by sap inoculation such as *Nicotiana benthamiana* and *Nicotiana glutinosa* and also to *Datura stramonium, Manihotesculenta, Manihot glaziovii*, Petunia hybrid (Mathew and Muniyappa 1993), But it is failed on transmitted to *N.longiflora*. Severe symptoms were observed within 6-10 days after inoculation (Mathew and Muniyappa, 1993). The overall efficiency of transmission of ICMV from cassava to *N.benthamiana* and vice versa is about 22%. Antony *et al.* (2006) reported the halotype of *B. tabaci* adopted from the cassava plant is referred as Indian cassava biotype.When compared to ICMV, the host range of SLCMV is almost similar. Among 39 species belong to *Solanaceae* is infected with SLCMV through mechanical inoculation (Jose *et al.,* 2008). In case of ICMV, not transmitted from cassava to cassava or from other host to cassava by sap inoculation methods.

Dutt *et al.* (2005) reported SLCMV occurrence in cassava is more common than ICMV in Kerala. SLCMV having a broad host range and extended to *Arabidopsis* (Mittal *et al*. 2008). ICMV can easily infect to some species of *Nicotiana* such as *N. amplexicaulis, N. benavidesii, and N. nudicaulis* but not by SLCMV. The presence of virus in all these plants species is confirmed through SLCMV specific primers. CMD is recently reported in Southeast Asia (Wang *et al.,* 2016) regions were severely affected with the pest and diseases including Cassava Mealybug (*Phenacoccus manihoti),* Cassava Bacterial Blight (*Xanthomonas axonopodis* pv *manihotis)* and Cassava Witches' Broom Disease (Graziosi *et al.,* 2016; Alvarez *et al.,* 2013).

2.9 CMG VIRUS TRANSMISSION

Viruses are successfully transmitted from plant to plant by various methods, which required either through mechanical injury or through insects carries the viral particles that penetrated to the living cells. As compared to other CMGs, ICMV and SLCMV were introduced to new areas, through the infected planting materials and by naturally transmitting whitefly vector.

Stem cuttings were used for propagating cassava plants, that region where the viruses enter and cause infections in plant. CMD caused with several viral species as single or mixed infections. The plant viruses are capable for adapting the environmental conditions and the resistance

mechanism of the host. Begomovirus, ssDNA viruses are belonging to the Geminiviridae family mostly transmit to the host by *B. tabaci*, it carries more than 200 plant viruses in persistent manner. Some of the RNA viruses such as Ipomoviruses, Criniviruses and Carlaviruses are transmitted in a semi persistent manner. Dubern (1994) conducted a detailed study of transmission of viruses to the host through a vector, *B. tabaci* and observed that the viral particle remains in vector at least 9 days also depend on viral virulence. About 80.5% transmission efficiency were showed by *B. tabaci* while on transmitting viruses from cassava to cassava.

Virus infection is initiated in the host plant cells by manually inoculating the viral particle into the living host tissues. For successful mechanical transmission of virus to the plant cells depends on a various factors such as the degree of susceptibility of the host cell, functioning of the infectious viral particle within the cell and conditions necessary for viral replication. In this process, the extracted viral sap mechanically inoculated to the site (Kado, 1972).

Duraisamy *et al.* (2012) studied viral transmission (ICMV and SLCMV) through *B.tabaci* observed 80.5% efficiency during three months period from cassava to cassava and showed similar symptoms of CMD. Similar study were also reported by Chant, (1958) and Antony *et al.* (2005). Viral transmission confirmed with amplification of *replicase* genes carrying the viral specific primes of both the viruses through colony PCR (Duraisamy *et al.*, 2012).The viral transmission efficiency of SLCMV with three species of whitefly (Asia II1, Middle East-Asia Minor (MEAM1), Mediterranean (MED) in Asia regions compared and observed only the Asia II1 is highly capable for transmitting SLCMV to the host and induce symptoms, compare to other two species Chi *et al.* (2019). Presence of Asia II 1 biotype in cassava growing areas of Kerala was reported by Harish *et al.* (2018).

2.10 MIXED INFECTION IN CROP PLANTS

Mixed viral infections are causing an important disease in crops worldwide. The Maize Lethal Necrosis (MLN) disease mostly effect on maize by the combination of the machlomovirus *Maize chlorotic mottle virus* (MCMV) and different strains from *Potyviridae* family (Scheets, 1998; Mahuku *et al.*, 2015; Stewart *et al.*, 2017; Redinbaugh and Stewart, 2018). Renteria-Canett *et al.* (2011) studied the mixed interaction of the viruses from the family Geminiviridae

on pepper plants that have synergistic interaction between the *Pepper Huasteco Yellow Vein Virus* (PHYVV) and *Pepper Golden Mosaic Virus* (PepGMV) and were able to infect more in vascular tissues. Thus the mixed infection (synergism) is mainly due to the increasing concentration of both DNA viruses in the host plants and raises the symptoms. Viral distribution in host plants depends on the structure of plant and colonization of viruses; it is clear in single viral infection as compared to multiple infections due to the disease severity.

A study conducted in Ethiopia by Tewodros *et al.* (2011) to assess the single and mixed infection of viruses infecting to the most important crop, sweet potato. *Sweet potato feathery mottle virus* (SPFMV) followed by *Sweet potato chlorotic stunt virus* (SPCSV), were the most prevalent viruses detected from the symptomatic and non-symptomatic plant samples. SPCSV is more frequently mixed infected with SPFMV (9.3%) than single infection. In the *Oilseed rape mosaic virus* (ORMV) and *Tobacco mild green mosaic virus* (TMGMV), one virus indicated as protective function for maintaining the symptoms with no additional virus fitness results the benefits to antagonistic interaction (Aguilar *et al*., 2000).The first studied antagonistic interaction between *Papaya Ringspot Virus* and *Papaya Mosaic Virus* in *Carica papaya* (Chávez-*Calvillo et al*., 2016). The mixed infection depends on the order of infection in papaya plants. Another crop is greatly affected by multiple viral infection is grapevine. The grapevine leaf roll disease (GLD) is caused by the association of 11 *Grapevine leaf roll-associated viruses* (GLRaVs) (Naidu *et al.*, 2014, 2015).

2.11 MECHANISM OF MIXED VIRAL INFECTION IN PLANTS

Fawcett, (1931) was made the first attempt to study the role of mixed viruses in the development of diseases caused by simultaneous infection of more than one viruses and the physiological effect on their interactions. Most viruses spread with the range of type of transmission mechanism that characterized as the ability of insect vector to acquire and transmit the virus within a short time in a stylet-borne (non-persistent) manner. The viruses are transmitted in the circulative (persistent) manner throughout the life of the insect vector. In between the two extreme the semi-persistent, characterized by the retention ability of virus for a long time than the non- persistent but not as the persistent. Initially plant- virus interaction

was studied with single infection models. Later the increased report of several mixed infections is used for controlling strategies against viral diseases. Virus concentration in plants varies with mixed and single infection.

Mixed viral infections of plants are common in nature. A single plant is frequently infected with more than one virus or strain. Multiple infections allow the complex interaction among the viral species. It causes the change in the genetic makeup of the viral population due to the intra host virus-virus interaction. About half of the pathogens infecting the host are viruses (Anderson *et al.* 2004) and the mixed infection of different viruses can cause diseases in wild and cultivated plants. Some viruses are non- pathogenic, having the beneficial role in their host plants, providing mutualistic effect and also protecting the plants from abiotic stress (Lefeuvre *et al.* 2019; Roossinck, 2011). Virus interaction is categorized into a positive (facilitation) interaction and negative (interference competition) interaction more specifically synergistic and antagonistic interactions (Syller, 2012) respectively based on the plant mediated interaction and environmental factors. In mixed infection, the virus requires the adaptability to the host as well as the synergistic or antagonistic activity. Infected cassava materials transferred between the geographical regions can develop mixed infections, by evolving synergistic interactions between the dual infecting viruses and create a frequent recombination and genome re-assortment. Synergistic interaction is occurring between unrelated viral species and causing a huge yield loss economically (Syller, 2012; Mascia & Gallitelli, 2016). Whereas, the antagonistic action between the related viruses and the viral diseases managed by cross protection (Pechinger *et al.*, 2019). Multiple infections are classified as Co-infection, two viruses invade the host simultaneously or short interval of time and Super-infection, different strains of viruses re-infect the infected host plant (Miralles *et al.* 2001; Saldana *et al.* 2003; Carrillo *et al*. 2007). In case of synergistic interactions, that enhances the symptoms severity due to the complexity of diseases as compare to the individual infections. Mixed infection of same viruses in different host plants causes different results due to the host response, viral accumulation and vector transmission.

2.12 MIXED INFECTION IN CASSAVA

Cassava infecting begomovirus (CMBs) occur in either alone or mixed infection of different combination of strains. In Africa, the distribution of CMGs especially the ACMV and EACMV are spreading drastically and most probably causing the mixed infections of these viruses and results the emergence of new distinct recombinant strains such as EACMV- UG (Uganda viral variant), strain carries the genome of EACMV and coat protein gene of ACMV type (Zhou *et al.,* 1997; Pita *et al.,* 2001). During the mixed infection of EACMV-UG and ACMV showing severe disease symptoms, indicates the strong neutral synergistic interaction between the two viruses. Sometimes, the ACMV infection is suppressed by EACMV-UG. The disease severity during the single and mixed infection of both viruses (ACMV & EACMV-UG), were assessed quantitatively by Naseem & Winter, (2016). More disease symptoms occurred during the mixed infections, due to the raises of genomic DNA A and DNA B components of both viruses causing an increase in virus concentration. Severe diseased symptoms of mixed infection also reported (Fondong *et al.,* 2000) in Cameroon associated with ACMV and EACMCV. Most of the EACMV isolates along with ACMV distributed in Nigeria, was first reported by Ogbe *et al*. (2003). The symptoms occurred during the mixed infection of these viruses were similar to other cases reported (Cameroon, Tanzania) in African region. In India, mixed infection of ICMV and SLCMV occurs in cassava (Dutt *et al.,* 2005; Anitha *et al.,* 2011; Jayakrishnan, 2018), but their effect on host occurring together was not studied in detail till now.

Casinga *et al.* (2018) first reported the devastating cassava cultivation in African regions due to the mixed infection of *Cassava brown streak virus* and *Ugandan cassava brown streak virus*, causing an important disease called as *Cassava brown streak disease* (CBSD).

2.13 STRATEGIES FOR SCREENING VIRAL INFECTIONS IN PLANTS

2.13.1 Transmission through grafting

Grafting is commonly used for the propagation of plants. This technique is initially used for the transmission and detection of viruses mainly in woody plants. It can transmit all viruses to host plants systematically and can cause infection which cannot be transmitted mechanically.

Grafting can distinguish virus diseases from physiological disorders such as nutrient deficiencies.

In graft inoculation, a shoot (scion) or bud isolated from one plant is placed to the rooted part (stock) of another plant. There are different types of grafting techniques such as stem grafting, root grafting and tuber grafting that are based on the various parts of plants including root, tuber and corms used in grafting. If any of the graft inoculum either scion or stock is infected with a virus, it moves to the healthy one and visually expresses the symptoms (Matthews, 1991; Nayudu, 2008). The success of the grafting techniques is the matching of cambium layers for the formation of callus. After grafting, several days or months are required for the expression of infected viral symptoms to the healthy plants. A novel grafting technique, petiole grafting carried for screening the transmission of *Pigeon pea sterility mosaic virus* (PPSMV) (Reddy *et al.,* 2002). A new grafting technique adapted for the successful transmission of CBSV to plants by chip bud grafting (Wagaba *et al.,* 2013).

Graft transmission of ICMV was easily transmitted by top grafting (Malathi *et al.,* 1985; Madhubala, 2003) and not through true seed. ACMV was transmitted by grafting (Deighton, 1926; Storey and Nichols, 1938). Ariyo *et al.* (2003) reported that ACMV and EACMV were transmitted from cassava to cassava through grafting. Mathew *et al. (*1983) successfully transmitted a naturally occurring mosaic disease on *M. glaziovii* to cassava through grafting.

2.13.2 Transmission through vector

In- nature, plants required a biological vector for the transmission of viruses from one plant to another plant. Viruses are interact with each other in their plant tissue. Viral particles are transmitted to the host-plant cells through vectors especially insects. The virus- vector relationship is crucial in causing infection in plants. Interaction between two related or unrelated viruses in mixed infection is frequently occurred in individual plants through vectors (Choisy & de Roode, 2010). Aphids are commonly used as vectors for transmitting the virus to the host, which carry either one or more viruses at a time. Rochow, (1972) was first studied the role of mixed infection in transmitting the *Black raspberry necrosis virus* (BRNV) and *Rubus*

yellow net virus (RYNV) by Aphids. Another vector used for transmission is whitefly (*Bemisia tabaci),* complex of cryptic species. In the case of mixed infection, the whitefly viral transmission rate is also complex. Sanchez-Campos *et al.* (1999) reported the capability of two different whiteflies, one is Middle East Asia Minor 1 (MEAM1) and other is Mediterranean (MED) were transmitted to *Tomato yellow leaf curl virus*(TYLCV) and *Tomato yellow leaf curl sardinia virus* (TYLCSV). Moreover, the MED is showed better transmission the MEAM1. Later the tomato plants test results obtained by Ning *et al.* (2015) support the transmission of viruses through whiteflies.

2.14 DETECTION AND DIAGNOSIS OF CMD

In India, several species of CMG in plants were detected and characterized by using either viral nucleic acid or viral specific antigen. Serological methods such as ELISA, DIBA and TBIA are used to detect ICMV (Makeshkumar and Nair, 2001; Malathi *et al.,* 1985). Using electron microscope isolated the presence of geminate particle of ICMV from *N. benthamiana by* Mathewsand Muniyappa, (1992). Moreover, the ICMV in host is detected by developing an antibody using the coat protein. Different formats of PCR and Multiplex PCR have been applied for the diagnosis of ICMV and SLCMV in India as well as EACMV and ACMV in Africa (Makeshkumar *et al.,* 2005; Dutt *et al*., 2005, Anitha *et al*., 2011). All the begomoviruses share the high homology region in the genomes, when analysing the nucleic acid sequences. Thus the effective method for the identification of begomoviruses is done by using degenerate primer in PCR (Ieamkhang *et al*. 2005; Ruhui *et al*., 2008). Later the ICMV, SLCMV and ACMV in CMD infected plants detected by using specific primers were designed from the full length sequence of CMV (Dutt *et al.,* 2005; Patil, 2005; Makeshkumar, 2001). Alabi *et al.* (2008) developed multiplex PCR for simultaneous detection of ACMV and EACMCV in cassava plant affected with CMD. Rep specific primers and CP specific primers are more versatile in the detection of viruses. Multiplex PCR is cost effective and rapid sample preparation methods are adapted for crop improvement and epidemiological studies of mixed infection of viruses and can develop control strategies against CMD.

CMD outbreak in Southeast Asia (Wang *et al*., 2016) detected by PCR using the CP targeting primers of SLCMV (Dutt *et al.,* 2005) to amplify and clone the genomic components. Different

set of PCR primers were evaluated for specific amplification of the SLCMV and confirmed the presence of viruses and symptomless infections in the samples collected from Southeast Asia (Siriwan *et al,* 2020). Recently, complete genome sequence of SLCMV causing CMD in samples isolated from Thai regions reported by Leiva *et al.* (2020) using Nano-pore based technology.

Beyene *et al.* (2017) developed rapid Virus Induced Gene Silencing (VIGS) screening method for selecting susceptible and resistant varieties of cassava to CMD. Infectious clone of virulent EACMV are inserted in the VIGS vector in order to silencing the endogenous cassava gene. In cassava, the VIGS system was first reported (Fofana *et al*., 2004) on isolate of ACMV-CV. CMD associated geminiviruses were detected by using generic PCR primers (Alabi *et al.*, 2008) and Legg *et al.* (2015) validated this method for detecting the geminivirus in the samples of East and West Africa. PCR-RFLP can be used for detecting the variability of viruses. A study conducted by Patil *et al.* (2005) with ICMV and SLCMV infected cassava were found in India having high variability. ICMV is geographically restricted to certain areas whereas SLCMV was widespread.

Uke *et al.* (2019) establishes an efficient way to monitor the rapid spreading of SLCMV in Southeast Asia with the combination of using AGRIBUDDY in the surveillance phase and PCR in the diagnosis phase. It store high resolution images which allow discriminating CMD and herbicide effects. However, the molecular diagnoses still have some limitations. It can overcome by using on- site techniques such as Loop- mediated isothermal amplification (LAMP).

Single and mixed infection of begomoviruses (ACMV and EACMV -UG) DNA A and DNA B components in cassava plant are more common in African countries. Naseem and Winter, (2016) reported a qPCR methods for detection and quantification of virus concentration in cassava varieties. Diseased plants symptom severity showed high correlation with the concentration of virus in leaves tissues. Proteomics, a tool used for study various function of plants such as host pathogen interaction, growth and development. Expressed proteins during cassava mosaic viruses (ICMV and SLCMV) infected the leaves of cassava studied by Duraisamy *et al.* (2019) using proteomics. During viral infection, responsive proteins and differential expressed proteins are analyzed by using 2d gel electrophoresis and Matrix-

Assisted Laser Desorption/ Ionization-Time-Of- Flight (MALDI-TOF) mass spectrometry respectively. The expressed proteins involved in the defense mechanism of cassava during CMV infection can identify by proteomic analysis.

2.15 HOST -VIRAL INTERACTIONS AND ANTI- VIRAL DEFENSE MECHANISMS

Plant viruses extensively use the host biological machinery for their genome replication, viral gene expressions etc. Viruses and their interaction with plant during infection lead to severe diseases in host due to various physiological disorders. Host viral interactions are highly influenced by the multiplicity of infection (MOI) that is the number of viral genome present in the same cell (Gutierrez *et al.,* 2010). However, plants adopted several mechanisms including the signal transduction pathways, antiviral immune receptors against the viral infections. Incarbone and Dunoyer, (2013) evaluated the plant defense mechanism such as gene silencing, protein degradation, hormone mediated defense and metabolic regulation which are operated to overcome the viral infections. An antiviral defense mechanism was founded by Zorzatto *et al.* (2015) against the DNA viruses. A trans-membrane immune receptor, which is similar to co-receptor like kinase mostly involves in pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) (Korner *et al.,* 2013) to suppress the viral infection by activating the translation process of host plants.

Dominant resistance mechanisms in host plant include resistance protein of the type nucleotide-binding sequence leucine- rich repeat (NBS-LRR), accumulation of viral proteins which activate the programmed cell death through hypersensitive responses (De Ronde *et al.,* 2014; Soosaar, 2005). Whitham *et al.* (1994) reported firstly the resistance protein (R) in tobacco N gene against the *Tobacco mosaic virus* (TMV). R protein can activate the defense signaling pathway in the infected tissues. Plant have one of the defense mechanism against geminiviruses reported by Fontes *et al.* (2004), NIK1 an antiviral immune receptor, NIK1 (Nuclear Shuttle Protein (NSP)-interacting kinase) belonging the member of receptor- like kinase (RLKs) family. In addition to this recessive resistance response is carried out with host factors such as eIF4E and eIF4G (Truniger and Aranda, 2009; Schmitt-Keichinger, 2019).

Plant hormones play an essential role in development of plant and pathogen defense. Plants introduce several antagonistic hormones suddenly as a defense to viral infections against of the occurrence of hormonal change (Alazem and Lin, 2015). During viral infection, plants adopted three types of resistance mechanisms of which an important one is hormone mediated resistance. Plant hormones such as salicylic acid and ethylene are mainly involved in this type of resistance response which gets activated only after the binding of viral protein particles to the receptor regions present in host. Two strains of SLCMV (SLCMV-Col and SLCMV-HN7) were identified by Wang et al. (2018) in China and studied the comparative genome analysis of both strains. SLCMV-Col strain is more virulent than SLCMV-HN7 strain and also shows high rate of symptom severity due to DNA A component. Rep protein is required for the virulence of virus in host cells. It contains a motif comprising 7-amino acids at the carboxyl terminal region which triggers the Salicylic acid (SA) synthesis in plants as defense mechanism.

An induced systemic resistance triggers the plant defense mechanism against the pathogen invasion. It is an important mechanism of bio-control. It mediates the synthesis of phenolic compounds, production of phytoalexins and deposition of structural barriers (Chen *et al.,* 2000; Benhamou *et al.,* 1996). Moreover the synthesis of phenolics and phytoalexins from the lignin and phenyalanine aminolyase (PAL) is catalyzed by the activity of defense enzymes such as peroxidase (PO) and polyphenol oxidases (PPO). Induction of this kind of resistance response in host plants has been observed against the infection of leaf blight pathogen (Karthikeyan *et al.,* 2005).

During viral infection, the genes encoding components of innate and adaptive immunity is differentially expressed during synergism and antagonism. Post- transcriptional gene silencing (PTGS) is one of the defense mechanism in plants against viruses and is considered as a part of innate immune system in plants. Gene silencing suppress the expression of viral genes by producing small interfering RNA through a range of enzymes and its complexes. Recently, the activation accumulation of adaptive and innate immunity of the plant during mixed infection was studied in *Papaya ring spot virus* (PRSV) and *Papaya mosaic virus* (PapMV), non related viruses (Vargas-Mejia *et al.,* 2020). During antagonism RNAi mediated transcripts activate the adaptive immunity, in addition to innate immunity whereas in synergistic condition, only innate

immunity is activated. Duraisamy *et al.* (2017) studied host-pathogen interaction of cassava and cassava mosaic viruses (ICMV and SLCMV) in response to the induction of systemic resistance in cassava. Significant reduction in the chlorophyll content and accumulation of phenolic compounds was observed in host plants due to viral infection. Moreover the defense gene products PO and PPO activity increases rapidly in infected plants compared to normal plant.

Other natural defense action in plants against the virus is RNA interference. The viruses encode silencing suppressor proteins which act against the plant RNA silencing mechanism (Wieczorek and Obrepalska- steplowska, 2015). Both ICMV and SLCMV genomes have the AC4 gene, located within the AC1 gene with similar function. In ACMV, AC4 gene seen upstream of AC1 gene start site. The replication associated protein in the Geminiviruses down regulate the enzymes Methyltransferase1 and Chromomethylase3 (MET1 and CMT3) necessary for the plant DNA methylation during the viral infection. Rodriguez-Negrete *et al.* (2013) employed an alternative mechanism against geminivirus induced variation in the plant DNA methylation cycle. Ubiquitin proteasome pathway (UPS) has a central role in elimination of viral components (Alcaide-Loridan and Jupin, 2012) and act as another layer of resistance in plants.

Sequences Enhance CMD Symptoms

Ndunguru *et al.* (2016) discovered the two novel DNAs, SEGS1 and SEGS2 (sequence enhancing geminivirus symptoms) have no homology to geminivirus that enhance the CMD symptoms and overcome CMD2 resistance to CMD when it is co-inocultaed with EACMV-Uganda. SEGS contains GC rich regions and lacks long open reading frames. SEGS1 is 99% identical to cassava genome sequences whereas SEGS2, three sequences are 84-89%, also found in virions and whiteflies. SEGS associated genes involved in the host regulatory response of geminivirus. Long duration period of CMD2 tolerance can maintained with the ability of SEGS1 to overcome the CMD2 resistance and transmission of SEGS2 by whitefly (Legg *et al*., 2011). The ability of the SEG1 and SEG2 to distribute their episomes for enhances the CMD

and overcome the CMD2 resistance. Moreover, these can develop an effective management measures for geminiviruses causing disease in cassava.

2.16 MANAGEMENT STRATEGIES OF CMD

Due to the huge spreading of CMD in different regions associated with the sustainable reduction of yield of crops in agriculture. Intrinsic defense responses in host plant as described in previous section may not be sufficient to combat the devastating effects of disease. Thus efficient management strategies are required for controlling wide spread damage caused by CMD in field. Cassava plants basically using symptomless mother plant of stem cutting for propagation for managing the spread of viruses. Only causes problem when difficulty to distinguish the plant having nutrient deficiency symptoms. Most feasible method adapted for controlling CMD is phytosanitation, is crop- hygiene. It involves the removal of diseased planting materials from the field and carrying diseases free plants in new area (Fargette *et al.,* 990). It is difficult to manage the CMD in planting stage. CMD can also be reduced by rouging of the infected and susceptible plant varieties. However, developing a disease- free planting material became a new strategy for managing the CMD and it is successfully carried out in Tanzania have been achieved with increased cassava yield (IITA, 2017).

Breeding techniques has a great role in developing high yield varieties of cassava that are resistant or tolerant to CMGs. Currently there are three genetically distinct CMD resistance/tolerance mechanism have been reported in cassava (Okogbenin *et al.,* 2012; Rabbi *et al.,* 2014). Fregene and Puonti-Kaerlas, (2002) reported the polygenic and recessive variety in nature through CMD1 type resistance mechanism, accessed from *Manihot glaziovii* Muell. Arg. (ceara rubber). Whereas, the CMD2 type resistance obtained from a single locus of the tropical *Manihot esculenta* series (TME) that are founded in different accessions of West Africa landraces (Akano *et al.,* 2002; Rabbi *et al.,* 2014). Through breeding techniques highly stable CMD resistant varieties against of CMGs are developed by using the CMD2 locus in Africa and Latin America (Okogbenin *et al.,* 2012; Rabbi *et al.,* 2014). Okogbenin *et al.* (2012) was described the CMD3, a new CMD resistant cultivar, TMS 97/2205 which is derived from the crosses of TMS 30572 (CMD1 resistant type) and TME 6 (CMD2 resistant type) (Dixon *et*

al., 2010). Also reported the presence of CMD2 locus and additional locus in the same linkage group of TMS 97/2205. A Field report indicates, the genotype of all the three CMD (CMD1, CMD2, and CMD3) loci completely recovered the disease by developing moderate or severe CMD symptoms.

CMD can control by molecular breeding techniques with natural resistance genes. CMD resistant cassava varieties first developed by crossing the resistant progeny from *Manihot esculenta* × *Manihot glaziovii* and were backcrosses with cultivated cassava varieties. However this was initiated for the production of CMD resistant within the breeding work. Genetic mapping of the single dominant resistance genes CMD2 used for managing CMD in cassava plant (Akano *et al.,* 2002). This development made an effective role in breeding through marker- assisted selection (MAS) Fregene *et al*. (2006) identified molecular marker (SSR and RFLP) based breeding techniques for selecting useful traits of cassava against the diseases. Recently, whole genome sequencing of wild and cultivated cassava and related species has revealed genetic diversity for resistance of virus (Bredeson *et al.,* 2016).

Rabbi *et al.* (2014) was used high resolution SNP mapping of resistance to CMGs in cassava using genotyping-by-sequencing revealed a single dominant resistance gene. Novel resistance genes discovery from plants against of the CMGs is essential for the development of cassava varieties by conventional breeding or genetic engineering techniques. Novel plant breeding techniques such as Cisgenesis, genetically modified cassava plant is produced by transferring the genes or alleles of interest from closely related *Manihot* species (Hou *et al*., 2014). Resistant varieties can be developed by editing the plant genome with several editing tools such as clustered regularly interspaced short palindromic sequence (CRISPR/Cas9) (Belhaj *et al.,* 2013), Zinc Finger Nucleases. CRISPR/Cas9 mediated a molecular immunity system limits the ability of viral variants to replicate and infect, a long time viral resistant. The machinery provides a viral interference for generating viral variants by targeting the coding and noncoding sequences of geminiviruses (Ali *et al.,* 2016). High yielding resistant varieties of cassava can evokes by changing the gene expression and epigenetic marks through meristem sub culturing and transformation of friable embryo genic callus (Ma *et al.,* 2015; Kitimu *et al.,* 2015).

Identification of natural resistance genes and immunity related genes of plants can act on viral infection. Leal *et al.* (2013) identified an immunity related genes in cassava which could develop resistant to CMGs. Viral disease resistant plants can be engineered through RNA silencing mechanism (Duan *et al.,* 2012). Chloroplast is the main target area of the plant viruses during infection. The genetic diversity of the genes encoded in the chloroplast region during the infection of CMGs studied Marchi *et al. (*2020).

CMGs are mostly transmitted through a vector, in order to reduce the spread of CMD in cassava required suitable methods for controlling the white fly, *B. tabaci.* Recently RNAi are becoming a powerful tool for controlling CMD caused through whitefly (Raza *et al.,* 2016). Genetically engineered cassava variety (Cassava cultivar KU50) developed by Ntui *et al.,* (2015) resistant of SLCMV through RNAi. The resistant variety widely used for the production of biomass. Developed transgenic lines express a dsRNA homologous between the AV2 and AV1 region of DNA A of SLCMV. Cassava *cv* KU50 virus load is detected by RT-PCR with un-inoculated leaves of cassava.

In-vitro clonal propagation of cassava (Neama *et al.,* 2012) is used as an alternative method for the production of cassava. Due to some limitations of production of cassava plantlet derived from tissue culture. An efficient method for rapid propagation and multiplication of disease free cassava planting materials adopted an aeroponic culture techniques were developed by Tokunga *et al*. (2020). Maruthi *et al.* (2019) was reported first attempt in southern and Eastern Africa to control the two major viral infections in cassava plants such as cassava mosaic and brown streak diseases. By using the combination of tissue culture, chemotherapy and thermotherapy methods are adapted for generating virus free plants. This methods can applied for other vegetatively propagated crops to cleaning and germplasm exchange at the regional levels.

3. MATERIALS AND METHODS

The study entitled "Role of mixed infection of cassava mosaic viruses in cassava mosaic disease development" conducted at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2019-2020. Details regarding the experimental materials and procedures used in the study are elaborated in this chapter.

3.1 COLLECTION OF INFECTED LEAF SAMPLES

Cassava mosaic virus-infected leaf samples were collected from the fields of ICAR-CTCRI and Thiruvananthapuram district. The leaf samples showing symptoms were scored as described by Hahn *et al.,* 1980 (Table. 2.2) and kept in polythene bags and stored at 80ºC for further analysis and uses.

3.2 PLANT DNA ISOLATION

Cassava plant DNA was isolated from the leaf samples using CTAB method (Lodhi *et al.,* 1994). CTAB extraction buffer was prepared prior to DNA isolation. β- mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix I) to give a final concentration of 2% (v/v). Then pre-warmed the CTAB buffer at 60ºC in a water bath (ROTEK, India) for 5 min. 0.1g infected leaf samples were weighed and grinded to fine powder in liquid nitrogen using pre-chilled sterile motor and pestle. The powder was then transferred into sterile 2ml centrifuge tubes and then added 1ml of CTAB buffer (pre-warmed) to the ground samples. The tubes were incubated at 60ºC in a water bath for 30 minutes after mixing the contents well by gentle inversion. Then centrifuged the tubes at 13000 rpm for 10 min at 4 ºC. Transferred the supernatant to new Eppendorf tubes (2ml) using sterile pipette tips. After that activated RNAase (10mg/ml) and protease (20mg/ml) were added to the supernatant and incubated at 37 ºC for 1 hour. Then, added an equal volume of phenol: chloroform: Isoamyl alcohol to the mixture and mixed well by gentle inversion until the colour of the solution turns milky white. Again, the tubes were centrifuged at 13000 rpm for 10 minute at 4 ºC. Chloroform: Isoamyl alcohol (Appendix II) mixture of the equal volume was added to the supernatant taken in the tube and mixed well by inversion. Centrifuged the tubes again at 13000 rpm for 10 minute at 4

ºC until the aqueous layers were separated clearly. To the upper aqueous phase, added and mixed of 0.8 volume of ice- cold isopropanol and $1/10^{th}$ sodium acetate (pH 5.2). To precipitate the nucleic acid present in the samples, tubes were kept at -20ºC for overnight incubation. On the next day, the tubes were centrifuged at 13000 rpm for 10 minutes at 4 ºC. In order to collect the pellet, the supernatant was discarded from the tube. Then added 500µl (70%) ethanol to the tubes and mixed well by tapping. Again centrifuged the tubes at 13000 rpm for 10 minutes at 4ºC. Discarded the supernatant and air- dried the pellet at 37ºC. The pellet was then dissolved in 35µl nuclease-free water or TE buffer (Appendix II) and stored at 20 ºC.

3.3 ANALYSIS OF THE EXTRACTED DNA

3.3.1 Agarose Gel Electrophoresis

The quality of the plant DNA extracted with CTAB method was analysed by Agarose gel electrophoresis (AGE). Agarose gel of 0.8% was prepared by using 1X TAE buffer (Appendix III) and added ethidium bromide (EtBr) ($0.5gml^{-1}$) to it. DNA sample of about 2µl was mixed with gel loading dye and loaded into the wells. The agarose gel is run at 70V for about 40-45 minutes. Then the gel was visualized under UV light and imaged by using Azure gel documentation system. By using UV spectrophotometer, the quantity of DNA was measured.

3.4 VIRUS INDEXING OF THE COLLECTED SAMPLES

3.4.1 Molecular detection of viruses with polymerase chain reaction

Polymerase Chain Reaction was used to identify the type of viruses present in the collected samples (ten samples from each five varieties) with the presence of a different set of primers synthesised from Eurofins (India). The synthesized primers (100pM) were diluted to a final concentration of 10pM with sterile distilled water to obtain the working solution.

Table 3.1: Details of primers used for the diagnosis of cassava mosaic viruses (SLCMV/ICMV) from collected samples:

Virus target	**Primer name**	**Sequence (5'-3')**	**Amplicon size**	**Reference**
Multiplex	ICMV A-F	GCTGATTCTGGCATTTGTAN	900bp(ICMV)	(Patil *et al.*, 2005; Makeshkumar *et al*., 2005, 2007; Hedge *et al.*, 2010)
	SLCMV A-F	TGTAATTCTCAAAAGTTACAGTCN	600bp(SLCMV)	
	ICMV/SLCMV	ATATGGACCACATCGTGTCN		
Coat protein gene	CP-F	GGATCCATGTCGAAGCGACCA	770bp	
	CP-R	AAGCTTTTAATTGCTGACCGA		
Movement protein gene	MP-F	ATGGAGAATAATAGTAGCAA	900bp	
	MP-R	TTATACATTTTTGGATACAT		

3.4.2 Detection and differentiation of ICMV and SLCMV

Multiplex PCR was carried out with the total DNA isolated from the five varieties leaf samples for the detection and differentiation of ICMV and SLCMV. For this, three primers were used (Table. 3.1): SLCMV-A-F, ICMV-A-F, ICMV/SLCMV-A-R (Patil *et al*., 2005; Makeshkumar *et al*., 2007).

3.4.3 Multiplex PCR Analysis with ICMV/SLCMV specific primers

The components of the PCR reaction mixture were optimized as listed below:

Components	**Volume**
10X buffer	2.5µl
dNTP (10mM)	1µl
SLCMV-A-F (10pmol/µl)	0.5µl
ICMV-A-F (10pmol/µl)	0.5µl
ICMV/SLCMV-A-R (10pmol/µl)	0.5µl
Taq polymerase (0.05U/µl)	0.3µl
Water	12.7µl
Template DNA	2µl
Total volume	**20µl**

PCR was carried out in BioRad C1000 Touch Thermo cycles (Germany). PCR was set with conditions such as initial denaturation at 94 ºC for 5 minutes followed by 35 cycles of denaturation at 94 ºC for 45 seconds, annealing at 55ºC for 30 seconds and extension at 72 ºC

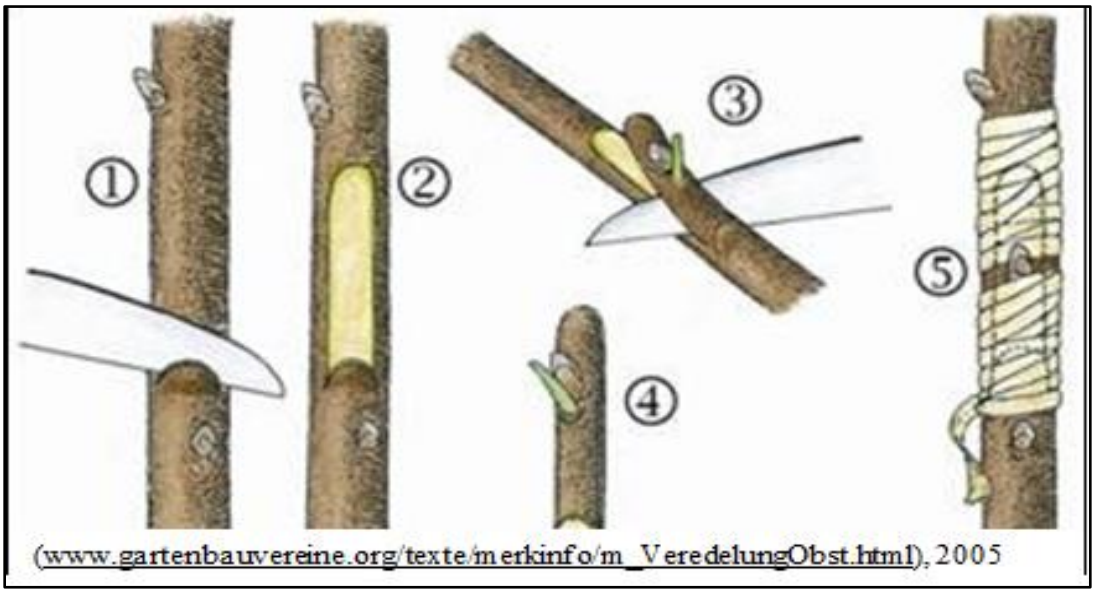
for 1 minute. Then the final extension was done at 72ºC for 5 minutes and infinitely holds at 4ºC. Using the controls can separate the target products from non- target products and primer dimers. Agaorse gel 1% was used to separate the amplified products along with marker 1kb plus from 'Thermo Scientific'. Then the gel was visualized under the gel documentation system.

3.5 VIRUS CULTURE

The Plants showing cassava mosaic diseases infected with ICMV/SLCMV were confirmed through multiplex PCR and were maintained in an insect-proof glass-house at ICAR-CTCRI and utilized for the mixed viral infection study through grafting.

3.6 MIXED VIRAL INFECTION THROUGH GRAFTING

Chip bud grafting (Wagaba *et al.*, 2013), a modified method of top and side grafting was used for transmission of the virus from one cassava plant (ICMV/SLCMV) to another infected cassava plant (ICMV/SLCMV). The axillary buds excised from the non-lignified stem of one virus- infected plant (ICMV/SLCMV) (scion) with petiole attached to the removal stem portion of the other virus-infected plant (SLCMV/ICMV) (stock) to expose the cambium tissue. The bud graft secured with a Para-film and grafted plants were kept in an insect-proof green-house for successful grafting with the formation of callus tissues.



(www.gartenbauvereine.org/texte/merkinfo/m_VeredelungObst.html), 2005

3.7AGRO INOCULATION WITH INFECTIOUS CLONES ON *Nicotiana tabacum* FOR SYMPTOM ASSESSMENT

3.7.1 Raising healthy Nicotiana tabacum plants

The seeds were collected from healthy *Nicotiana tabacum* plants and sowed in fertile soil. After about 2-3 weeks, three or four leaves staged seedlings were planted in separate pots and kept in plant growth chamber. These plantlets were used for Agro- inoculation studies.

3.8 MAINTENANCE OF INFECTIOUS CLONES OF ICMV AND SLCMV

Infectious clones of ICMV and SLCMV in *Agrobacterium* strains (Ach5 and AGL0) available in the transgenic lab of ICAR-CTCRI were used for the study (Table: 3.2). The strains were in glycerol stock, maintained at -80°C for long term storage. The stab cultures were revived to produce fresh cultures.

Table: 3.2. Agrobacterium strains with infectious viral clones used for the study

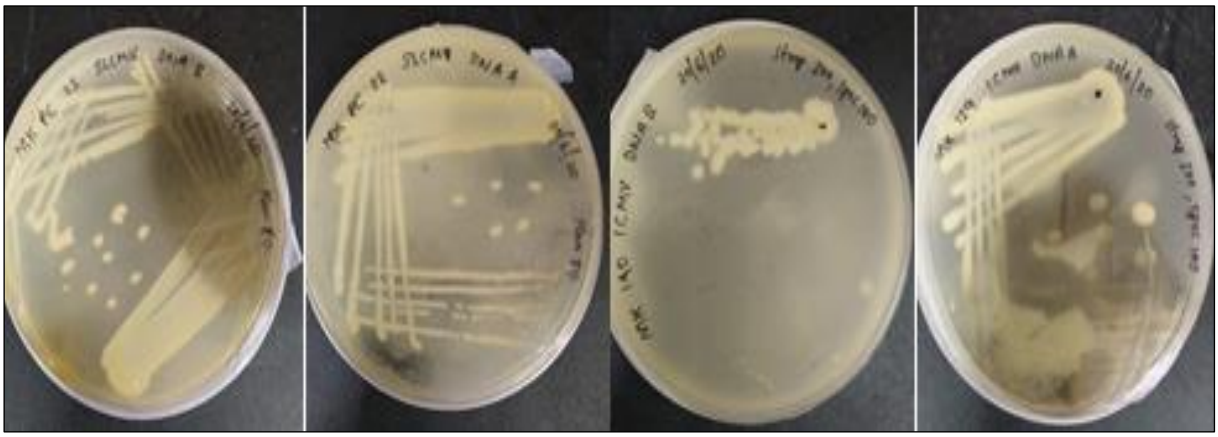
Strain no.	**Details**	**Host**	**Antibiotics**
MK 139	ICMV DNA A	Ach5	Spec100,Strep300
MK 140	ICMV DNA B	Ach5	Spec100,Strep300
MK PC 22	SLCMV DNA A	AGL0	Kan 80
MK PC 23	SLCMV DNA B	AGL0	Kan 80

3.9 AGRO INOCULATION IN *Nicotiana tabacum* LEAVES

The Agro inoculation protocol (Bull *et al.,* 2006) was performed for three consecutive days.

3.9.1 Day 1: Pre-culture

A fresh single colony of *Agrobacterium* strains containing infectious clones (SLCMV DNA A and SLCMV DNA B or ICMV DNA A and ICMV DNA B) from a YEB agar plate or an aliquot from glycerol stock of the same strains was selected and added into 3ml YEB (Appendix III) containing the required antibiotics (3µl each) (Appendix IV) and incubated for 48 hours at 28°C with shaking at 200 rpm.



3.9.2 Day 2: Main culture

From the well-grown pre-culture, 2µl was used to inoculate 100ml YEB medium containing required antibiotics (100µl each) and incubated overnight in an incubator shaker at 28°C with 200 rpm.

3.9.3 Day 3: Agro inoculation

The cultures were then transferred to oak ridge tube and cloudy thick culture suspensions were centrifuged at 3000rpm for 10 min at room temperature. The cultures were cloudy prior to centrifugation. The pellet was gently-re-suspended in 3ml of cold 100mM $MgCl_{2.}$ The Optical density (OD) at 600nm was measured. The re-suspended culture was diluted using an infiltration solution to obtain an OD of approximately 1. To the induction culture 150µg/ml acetosyringone was added and left for 2h-3h at room temperature. In the induction culture DNA A and DNA B of both SLCMV and ICMV were present separately. These were mixed in different combinations, prior to agro inoculation. For agro-infiltration with infectious virus clones, 2-3 weeks old *Nicotiana tabacum* plants were selected. The plants should have 4-5 developed leaves that are approximately 15 mm in diameter. Two lower oldest leaves in each plant were inoculated and ten plants per treatment were used. The plants were agro inoculated by infiltrating the lower surface of the oldest leaves with mixtures of respective components using a needle-less 1ml syringe and was gently pressed against the supported leaves. Control plants were inoculated with an infiltration solution devoid of *Agrobacterium*. The plants were maintained in a glass house at ambient temperature and humidity for symptom development.

Combinations of Agrobacterium strains and individual strains with infection clones used for the study were as follows:

MK PC 22: SLCMV DNA A

MK PC 23: SLCMV DNA B

MK 139: ICMV DNA A

MK 140: ICMV DNA B

MK PC 22+MK PC 23: SLCMV DNA A & DNA B

MK 139+MK 140: ICMV DNA A & DNA B

Mix of both SLCMV & ICMV (all the four MK PC 22+ MK PC 23+ MK 139+ MK 140)

3.10 AGRO INOCULATION IN CASSAVA

3.10.1 Preparation of Agro bacterium solution for Agro inoculation

To make the starter cultures, inoculate sterile test tubes containing 3ml of YEB with 80 mgl^{-1} kanamycin, 300 mgl^{-1} strepmycin and 100 mgl^{-1} spectinomycin with *Agrobacterium* colonies were transferred with the agro clones separately, and grow them for 24- 36h at 28°C and 150 rpm until the OD_{600} nm reaches >1. Then, added 2ml of the starter cultures to 1L Erlenmeyer flasks containing 100ml of YEB with required antibiotics. Then, grow the cultures for 24-36 h at 28°C and 150 rpm until the OD_{600} nm of 1-1.5. After, transfer the cultures into sterile 50ml tubes, and centrifuge the agrobacterium suspensions at 4000rpm for 20 min at room temperature. Again centrifuge and carefully re-suspend the bacterial pellets in 25ml of sterile deionised water (each in tube) at room temperature, then again centrifuge at 4000rpm for 20 min. Then, re-suspend the washed pellets in 10-20ml of LB (Appendix V), and adjust the final value of OD_{600} nm at 4 with LB. Mix an equal volume of agrobacterium carrying the ICMV (DNA A & DNA B) and SLCMV (DNA A & DNA B). Finally, add acetosyringone in the bacterial suspensions to a final concentration of 200µM and incubated at room temperature for 3h on a shaker at 100rpm.

3.10.2 Agroinocualtion in Cassava Plants

One ml of Agrobacterium suspension was carefully injected three times near the axillary buds without damaging the meristems. Using the same syringe gently punctured the stem 5-7 times. The inoculated plantlets were covered and incubated at 20-24°C in the dark for 12h. Ten plantlets per treatment were used.

3.11 PCR BASED DETECTION OF ICMV AND SLCMV

The healthy and infected *Nicotiana tabacum* and cassava leaf samples were labelled and the symptom severity of each leaf sample was recorded by visual observation as per the standard 1-5 cassava mosaic scale of Hahn *et al*. (1980) and the scale is represented in Table: 2.2

3.11.1 Nucleic Acid Extraction and Confirmation of Virus Infection through PCR

Multiplex PCR was employed to differentially detect the presence of ICMV, SLCMV and mixed infections of both viruses from the infected samples. For carrying out polymerase chain reaction (PCR) based detection of the viruses infecting *Nicotiana,* DNA isolation is a prerequisite. CTAB method of extraction (Lodhi *et al.,* 1994) was carried out for genomic DNA isolation. PCR was performed on DNA isolated from infected leaf samples using different virus-specific primers.

4. RESULTS

The results of the study entitled "Role of mixed infection of Cassava mosaic viruses in cassava mosaic disease development" conducted at ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2019-2020 are presented in this chapter.

4.1 COLLECTION OF INFECTED LEAF SAMPLES

A total of 50 Cassava mosaic infected leaf samples were collected from the field of ICAR-CTCRI and Thiruvananthapuram. A total of five varieties of cassava were selected and from each variety, ten leaf samples were collected based on the visual observation as per the cassava mosaic symptom scale of Hahn *et al.* (1980). The variety, place of collection, sample score are given in table 4.1. Out of the samples randomly collected, most of them are shown high score symptoms scale of very severe mosaic having distorted and misshapen leaves with stunted growth of plants. The leaves samples showing severe symptoms are shown in Fig: 4.1

Table: 4.1. Showing the varieties of cassava collected from different fields.

Sl No.	Variety name	Place of sampling	Symptom features	Score
1	Kochangamuttan	Vizhijam, Thiruvanathapuram	Chlorosis, leaf curling, stunting	4
2	Kariyilappothiyan	Vizhijam, Thiruvanathapuram	Chlorosis, leaf curling, distortion	3
3	H226	ICAR-CTCRI	Chlorosis, leaf curling	3
4	Manja noorumuttan	Neyyattinkara, Thiruvanathapuram	Chlorosis, stunting, leaf curling	3
5	Karutha gandharipadappan	Nedumangad, Thiruvanathapuram	Chlorosis, leaf curling	3

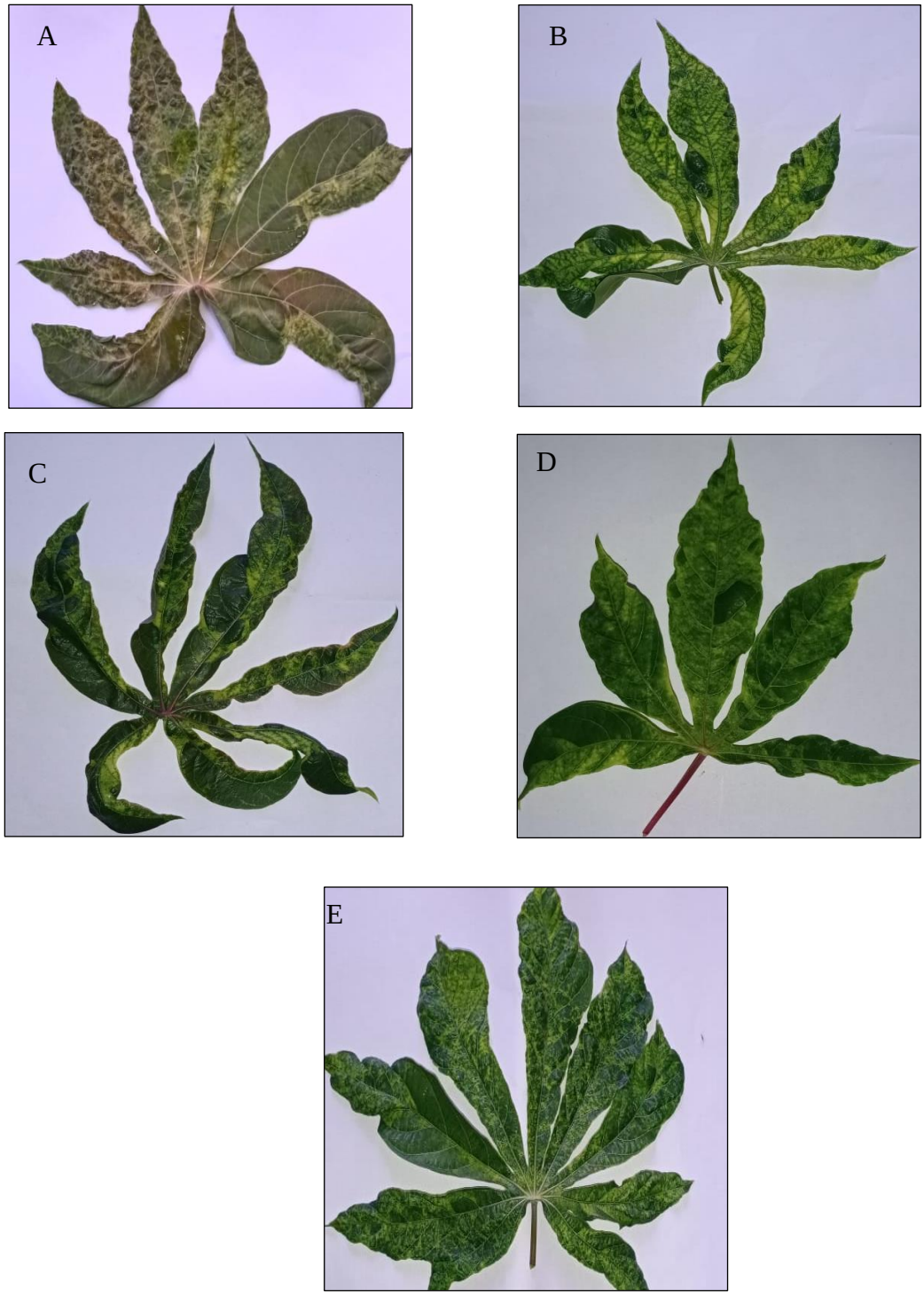


Fig: 4.1. Infected leaves samples of five varieties collected from the field showing chlorosis, leaf curling, and misshapen leaves: A-Kochangamuttan, B- Kariyilappothiyan, C-Karutha gandharipadappan, D- H226, E - Manja noorumuttan,

4.2 PLANT DNA ISOLATION

Total DNA was isolated separately from the eleven leaf samples (S1 to S11) of five different cassava varieties using CTAB method. The quality and quantity of the samples were analyzed using agarose gel electrophoresis and spectrophotometer readings respectively. Concentration as well as purity of DNA isolated was represented in Table: 4.2. From the gel image that, DNA obtained as bright bands were evident for good quality (Fig. 4.2).

Table: 4.2. Yield and purity of isolated DNA samples

SL No.	Variety of leaf samples collected	Concentration (ng/µl)	A $_{260/280}$
1	S1-Karutha gandharipadappan	5670.257	1.83
2	S2-Karutha gandharipadappan	4734.500	1.84
3	S3-Manja noorumuttan	1538.886	1.79
4	S4-Manja noorumuttan	1013.978	1.81
5	S5-Kariyilappothiyan	1202.864	1.821
6	S6-Kochangamuttan	1977.171	1.82
7	S7-Kochangamuttan	4048.221	1.82
8	S8-Kochangamuttan	4270.767	1.81
9	S9-H226	3517.088	1.811
10	S10-H226	1571.050	1.84
11	S11-H226	1976.45	1.8

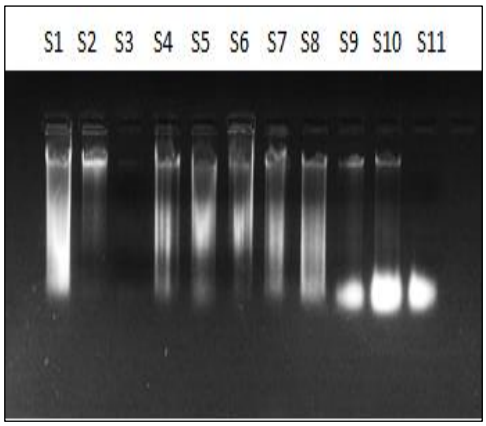


Fig: 4.2. Gel image of DNA samples isolated from the cassava mosaic infected leaf samples: Lanes S1-S11 represents the cassava mosaic infected leaf samples collected from 5 varieties

4.3 MOLECULAR DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

4.3.1 Multiplex PCR for detection and differentiation of ICMV and SLCMV

Multiplex PCR was performed for the detection and differentiation of ICMV and SLCMV in the fifty infected samples from the five varieties, only 11 samples represented in the gel image (Fig. 4.3). Of the samples collected, only one variety (Kariyilappothiyan) showed as ICMV infected (band size of 900bp) with severe symptoms such as chlorosis, leaf curling, distortion and the rest of them were infected with SLCMV (band size of 600bp). The sample, H226 collected from CTCRI also resulted as SLCMV infected.

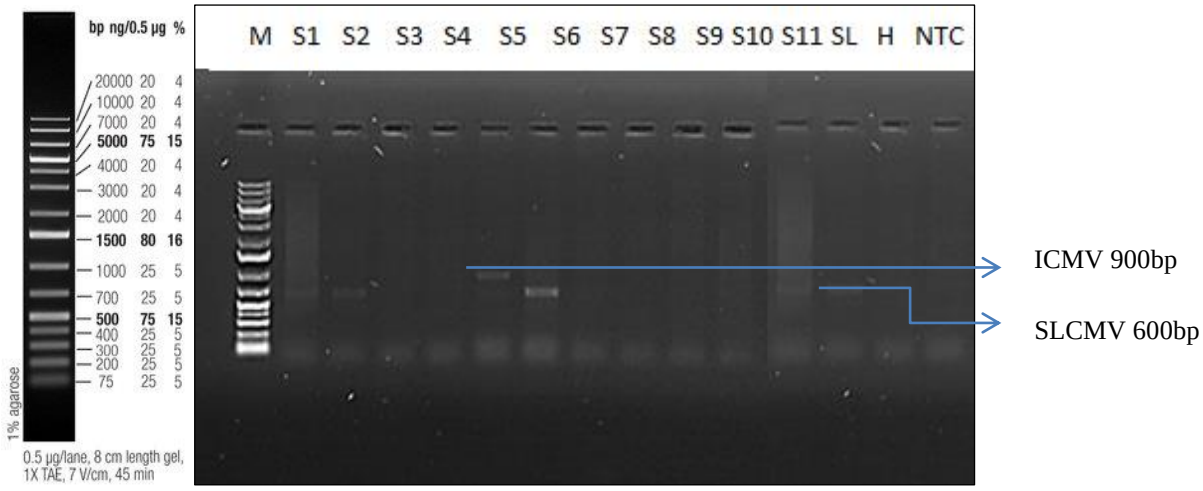


Fig: 4.3. Gel electrophoresis analysis: multiplex PCR amplified products of samples collected from the infected plants in 1% gel. M- 1kb plus DNA ladder (Thermo Scientific), lanes S1,S2- Karutha gandharipadappan, lanes S3-S4- Manja noorumuttan, lane S5- Kariyilappothiyan, lanes S6-S8- Kochangamuttan, S9-S11- H226, SL- positive control , H- negative control (samples from tissue culture-derived in vitro cassava leaf).

The leaf samples collected from the varieties, samples S3 and S4 from Manja noorumuttan did not show any band in multiplex PCR results while the rest of the varieties showed bands at 900bp and 600bp indicating the ICMV and SLCMV infected leaf samples respectively. The mosaic symptoms appeared in the leaves of the variety may be due to physiological disorders such as nutritional deficiency

Out of the 11 DNA samples subjected to multiplex PCR, all the samples showed a single band in agarose gel electrophoresis indicating the presence of only one virus in the samples tested. Out of 11 samples from 5 cassava varieties, only one sample (S5-Kariyilappothiyan) showed amplification of 900bp size DNA indicating the presence of ICMV whereas the rest of the samples except S4-Manja noorumuttan, other 3 varieties showed amplification of 600bp indicating the presence of SLCMV.

4.4 PLANTING THE CASSAVA INFECTED SAMPLES FOR GRAFTING

Stem cuttings of the confirmed virus (SLCMV and ICMV) infected plants (Fig: 4.4) were collected and planted in glass house to maintain it as an inoculum source for study the mixed infection through grafting.

Fig: 4.4. Cassava mosaic virus-infected plants (A- SLCMV and B-ICMV) in the field.

4.5 CONFIRMATION OF VIRUS INFECTION IN GLASS HOUSE PLANTS

4.5.1 DNA isolation

Established cassava plants in glass-house after one month the symptomatic leaves were tested for confirming the presence of viruses. Total DNA isolated from the infected leaf samples were analyzed by using CTAB method. The quality and quantity of DNA were analyzed using 0.8% agarose gel electrophoresis and spectrophotometer reading respectively. Concentration as well as the purity values of DNA isolated was represented in Table: 4.3. Analysis of the isolated DNA in agarose gel electrophoresis showed good quality (Fig: 4.5).

Table: 4.3. Yield and purity of isolated DNA samples from the established plants in glasshouse.

SL no.	Infected leaf Samples	Concentration (ng/l)	$A_{260/280}$
1	C1	2468.480	1.84
2	C2	2226.068	1.815
3	C3	1186.416	1.819
4	C4	1587.628	1.8
5	C5	2265.895	1.79
6	C6	1577.630	1.82
7	C7	2459.320	1.8

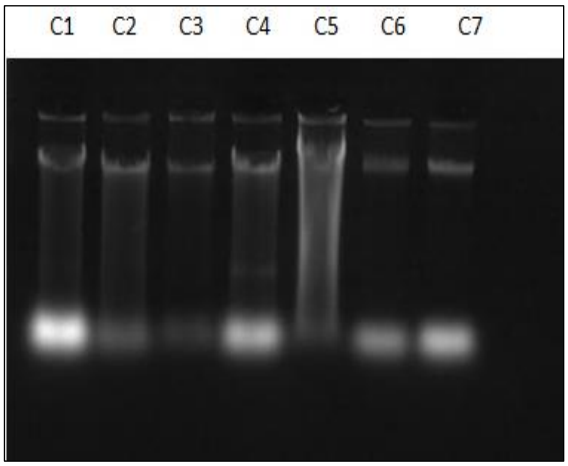


Fig: 4.5. Gel image showing the DNA samples obtained from the infected leaf samples planted in glasshouse. Lanes C1, C2- Karutha gandharipadappan, lanes C3, C4- Kariyilappothiyan, lanes C5, C6- Kochangamuttan, lane C7-H226 represents the infected leaf samples.

Leaf samples from the infected cassava plants in the glass house showed bright bands of DNA.

4.5.2 Multiplex PCR

Multiplex PCR was carried out to detect the presence of viruses (SLCMV and ICMV) in the planted samples. Of the samples collected from five varieties, one variety (Kariyilappothiyan) had only ICMV infection and samples from the other three varieties viz. Kochangamuttan, H226 and Karutha gandharipadappan had only showed the presence of SLCMV infection. Gel image showing the Multiplex PCR results of the samples collected from the glasshouse which showed the symptoms of viral infection (Fig: 4.6).

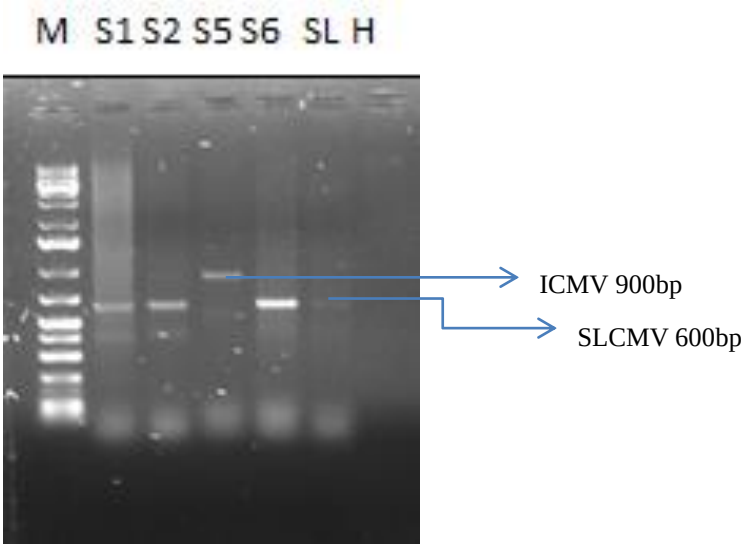


Fig: 4.6. Gel image showing multiplex PCR products of infected leaf samples planted in the glasshouse. Lane S5 represents the band at 900bp it indicates the ICMV and lanes S1,S2 and S6 represent the SLCMV with band size of 600bp. SL is positive control and H- negative control.

4.6 GRAFTING OF SLCMV AND ICMV INFECTED PLANTS FOR MIXED INFECTION

Both SLCMV and ICMV infected plants (Fig: 4.7) are used as stock and scion in grafting for mixed infection. Fig. 4.8, shows the steps involved in the chip bud grafting. The inoculation of one virus (SLCMV) infected bud to another virus (ICMV) infected plant and vice versa was done through grafting. After completion of chip bud grafting (Fig 4.9), the plants were maintained in the glasshouse for analyzing the bud emergence.

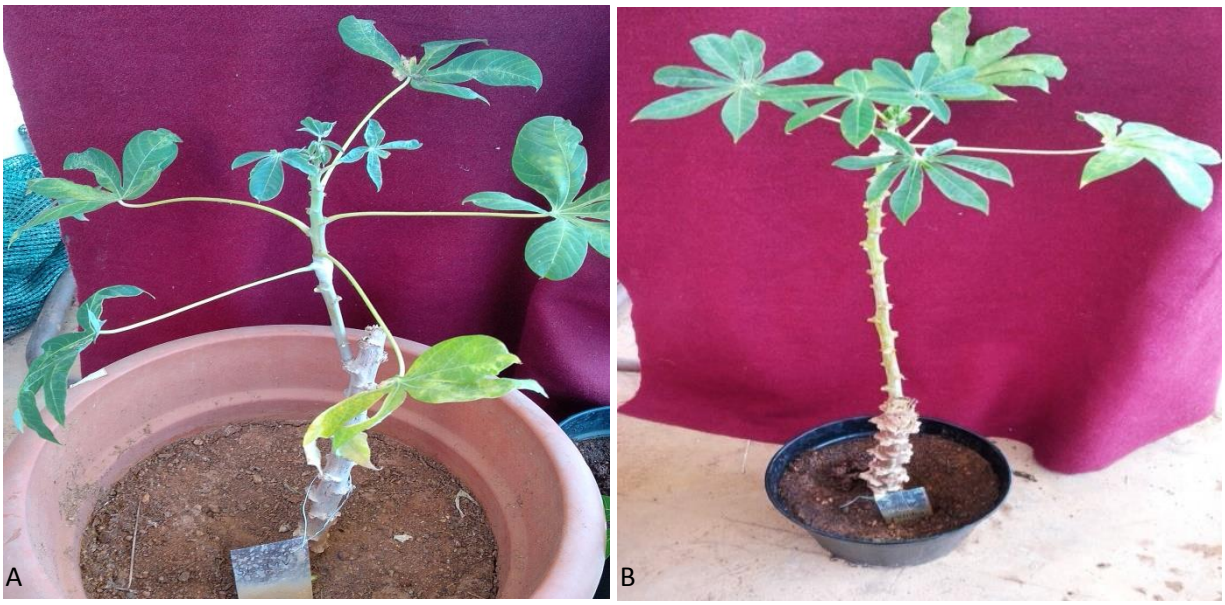


Fig: 4.7. Cassava mosaic infected plant: A- SLCMV infected plant, B- ICMV infected plant

Fig: 4.8. Steps in chip bud grafting in cassava: SLCMV, ICMV infected plant (A, 1), axillary bud is removed from the infected plants (B, 2), bud graft secured with para film (C, 3)

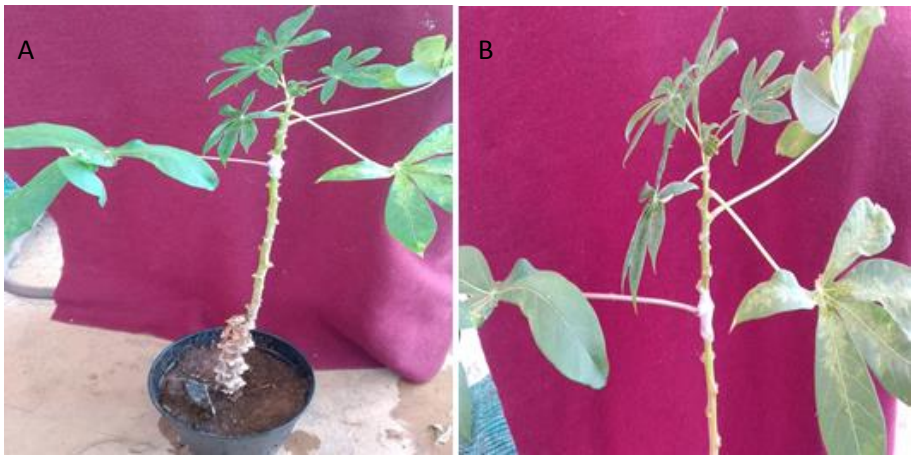


Fig: 4.9. Whole plant after completion of chip bud grafting: A-ICMV plant infected with SLCMV bud and B- SLCMV plant infected with ICMV.

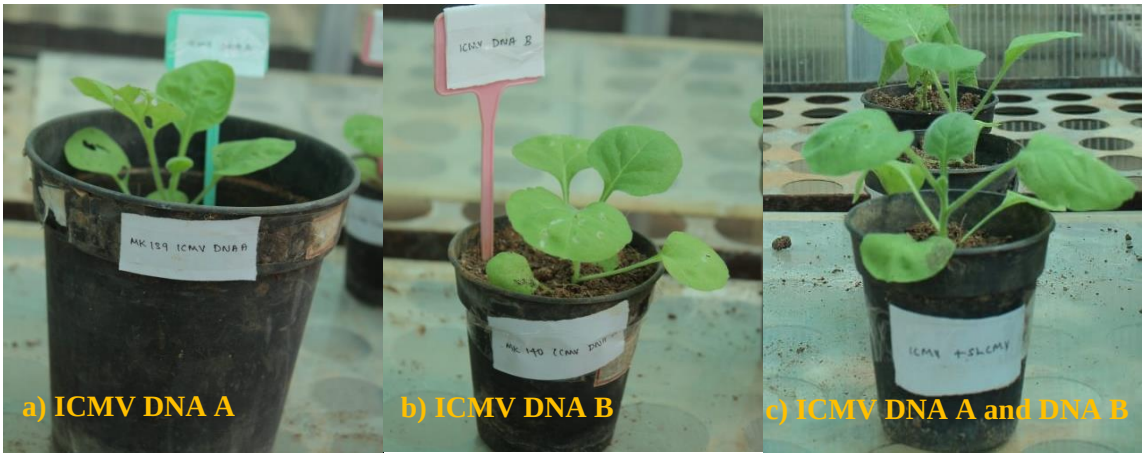
Successful bud graft emergence occurred at 2nd week after chip bud grafting (Fig: 4.10). Yellow mosaic pattern appeared on the newly emerged leaves. When ICMV infected plants grafted with buds from SLCMV infected plants showed severe symptoms with score of 3 in ICMV and score 4 in SLCMV but no substantial change observed in the SLCMV infected plant grafted with ICVM bud. This reveals that SLCMV is more severe than ICMV.

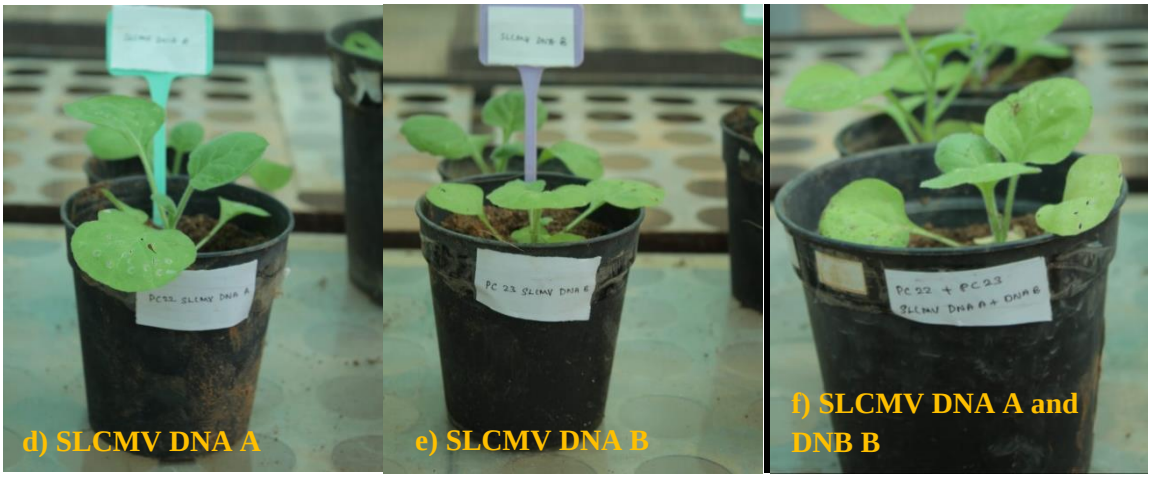
Fig: 4.10. The emergence of new leaves after successful chip bud grafting: SLCMV plant infected with ICMV bud

4.7 AGRO INOCULATION WITH INFECTIOUS CLONES ON *Nicotiana tabacum FOR SYMPTOM ANALYSIS*

Agro-infectious clones (SLCMV DNA A and SLCMV DNA B or ICMV DNA A and ICMV DNA B) were cultured from a freshly YEB agar plate.

Nicotiana tabacum plants with 4 to 5 leaves were infiltrated on the lower surface of the oldest leaves with mixtures of respective components using a needle-less 1ml syringe. Control plants were inoculated with the infiltration solution devoid of agrobacterium. *N.tabacum* plants inoculated with a partial dimer of DNA A and DNA B alone and also with the combination of both DNA A and DNA B partial dimers of ICMV and SLCMV. These plants were maintained in the glasshouse and monitored for symptom development (Fig: 4.11).





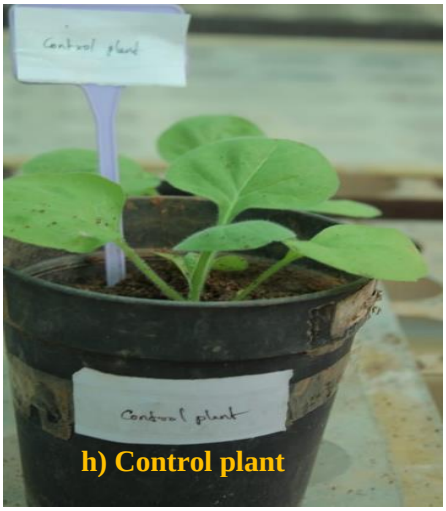


Fig: 4.11. Agro-inoculation of cloned genomic components (DNA A and DNA B) agro-infiltrated into Nicotiana tabacum: ICMV DNA A, DNA B and both (a, b, c), SLCMV DNA A, DNA B and both (d, e, f), g- mixed infection of both ICMV and SLCMV were inoculated and h- control plant without Agrobacterium

After two weeks, mild symptoms were observed in the leaves where cloned genomic components (DNA A and DNA B) were introduced into the *N. tabacum* by Agro-inoculation. Initially, all the infected leaves of agro inoculated plant showed mild chlorosis. The plants inoculated with both the components of SLCMV showed a severe spot of chlorosis to the newly emerging leaves. However both components of ICMV infiltrated plants showed mild chlorosis. Young leaves of tobacco plants inoculated with DNA A component of SLCMV and ICMV showed the spot of chlorosis and mid vein chlorosis. Whereas, the DNA B component of SLCMV and ICMV has only mild chlorosis to the newly emerged leaves. No symptoms developed on the control plants. (Fig: 4.12).

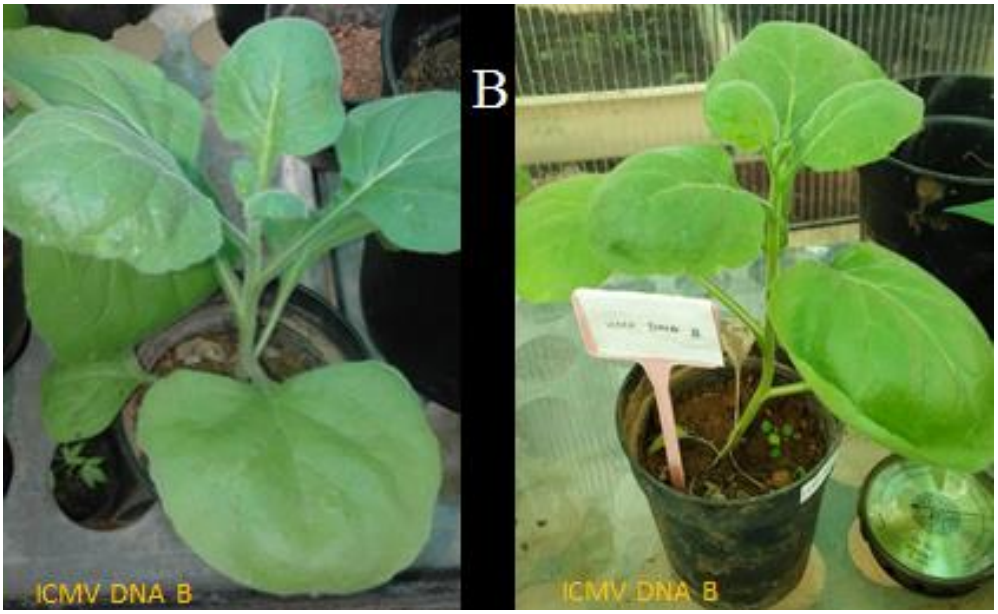
Fig: 4.12. After two weeks chlorosis developed to agroinoculated leaves of Nicotiana: SLCMV DNA A, DNA B and both (a, b, c), ICMV DNA A, DNA B and both (d, e, f), g- mixed

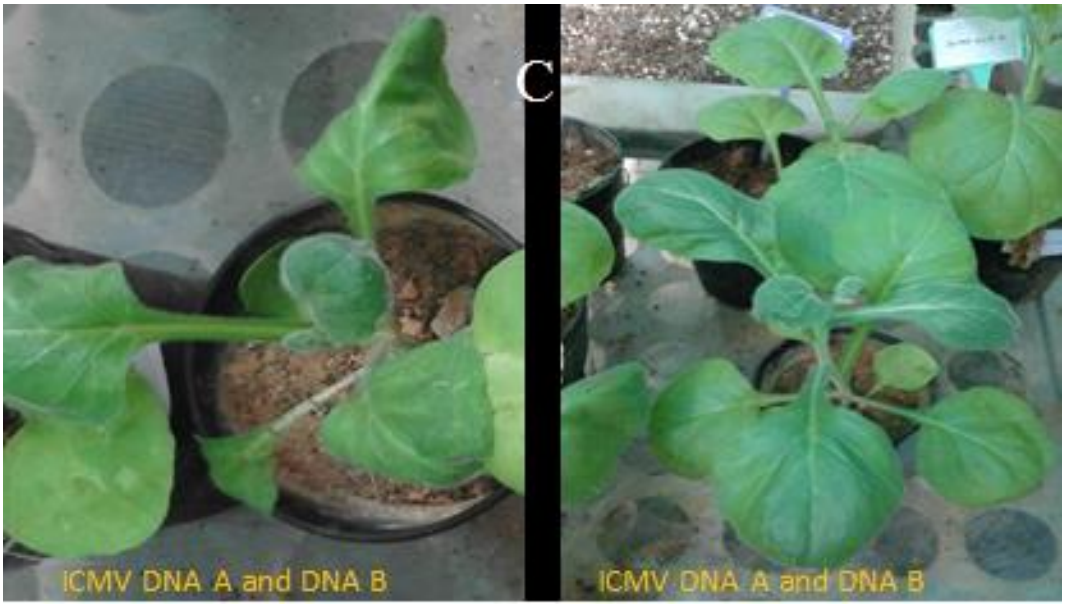
infection of both ICMV and SLCMV were showing chlorosis symptoms and h- control plant without symptoms

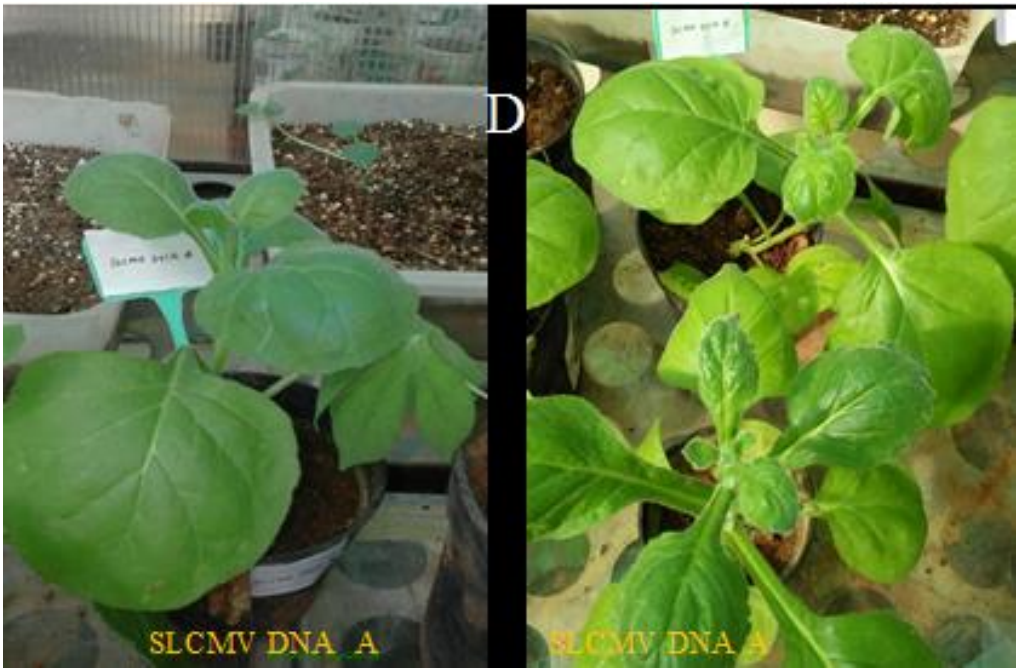
After 3 weeks of agro-inoculation, young leaves of *N. tabacum* started showing mild to severe symptoms. The plants inoculated with both the components of SLCMV, showed severe stunting, curling of leaves, thickening of mid-veins. However, the plants infiltrated with both components of ICMV showed mild mosaic and mild curling of leaves and reduction in size. In the case of plants infiltrated with DNA-A component only, the curling of young leaves occurred in the case of SLCMV and mild mosaic symptom in case of ICMV infection. Very mild mosaic was observed in plants infiltrated with DNA-B of SLCMV or ICMV. The plants inoculated with both the components of SLCMV and ICMV, showed severe chlorosis, leaf curling of young leaves resembling a mild SLCMV infection. But no substantial increase in symptoms during mixed infection as compared to the individual infection of SLCMV and ICMV. All the control plants remained healthy. A closer view of symptomatic leaves is presented in Fig: 4.13.

Third week after Infection

Fourth week after Infection




C
ICMV DNA A and DNA B
ICMV DNA A and DNA B


D
SLCMV DNA A
SLCMV DNA A


E
SLCMV DNA B
SLCMV DNA B


F
SLCMV DNA A andDNA B
SLCMV DNA A andDNA B

Fig: 4.13. Agro-inoculated plants Nicotiana tabacum showed symptoms of CMD after three to four weeks. DNA A infected plant of both ICMV and SLCMV showing symptoms like leaf curling as compared to DNA B (A, B, D, E). SLCMV infected with both the genomic component shows severe symptoms of CMD like leaf curling, stunting than ICMV(C, F). G- mixed infected plants show chlorosis and leaf rolling, H- control plant without any symptoms.

4.8 CONFIRMATION OF AGRO-INOCULATED *N. tabacum* HAVING THE INFECTION

Total DNA was isolated from the leaves of all the agro-inoculated plants of *N. tabacum* after one month of inoculation, by CTAB method. The quality and quantity of the samples were analyzed using agarose gel electrophoresis and readings done with spectrophotometer respectively. The gel image showing good quality DNA obtained as bright bands are shown in Fig: 4.14.

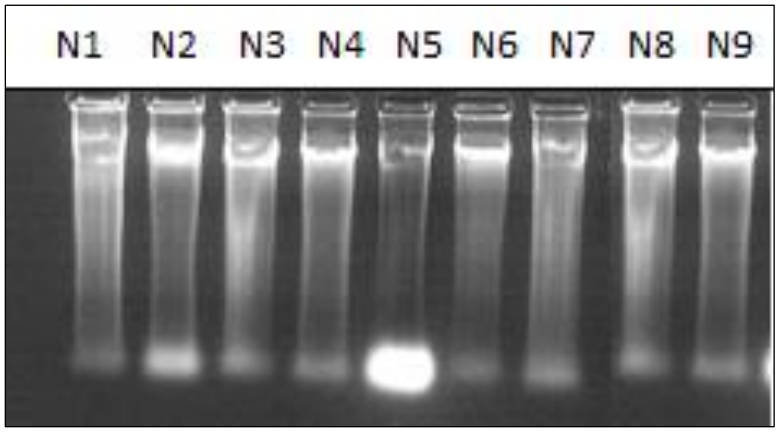


Fig: 4.14. Good quality DNA obtained from the agro-inoculated plants of N. tabacum leaves three weeks after agro-inoculation: Lanes N2-N8 represents the Nicotiana leaf infected with the cloned genomic components (DNA A and DNA B) of SLCMV and ICMV. Lane N1- control plant (without agro-bacterium)

4.8.1 PCR CONFIRMATION OF THE INFECTED N. tabacum LEAVES

To confirm the infection of SLCMV, ICMV and mixed infection on the the *N. tabacum* leaves were analysed by PCR using *Rep*-primer, initially the DNA of all the sympotomatic leaves isolated. The agroinoculated plants with SLCMV, ICMV and mixed showed clear bands in PCR reaction using *Rep*-primer, indicating the leaves showed severe symptoms due to the viral infections (Fig. 4.15). Multiplex PCR reaction was done with the leaves of *N. tabacum* for confirming the SLCMV and ICMV infection in plant alone and in combination (Fig. 4.16).

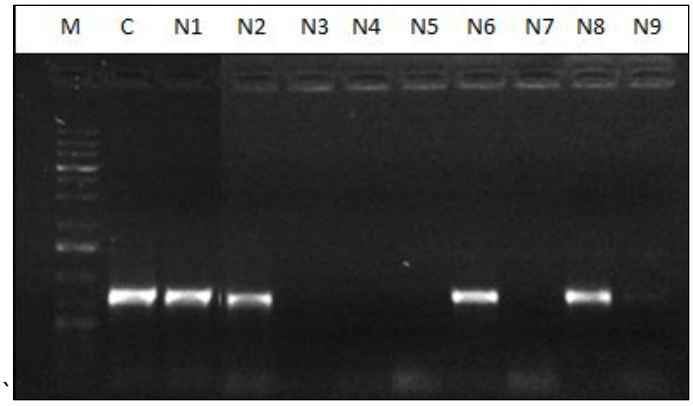


Fig.4.15: **Image showing the PCR results of the agroinoculated N. tabacum leaves with symptoms using Rep-primer:** Lane C-H226 control plant, lane N1- SLCMV with both the genomic components, lane N2- DNA A of SLCMV, lane N3-DNA B SLCMV, lanes N4&N5-ICMV plant, lane N6- ICVM with both genomic components, lane N8-mixed of ICMVand SLCMV, lane N9- control plant without agroinoculation

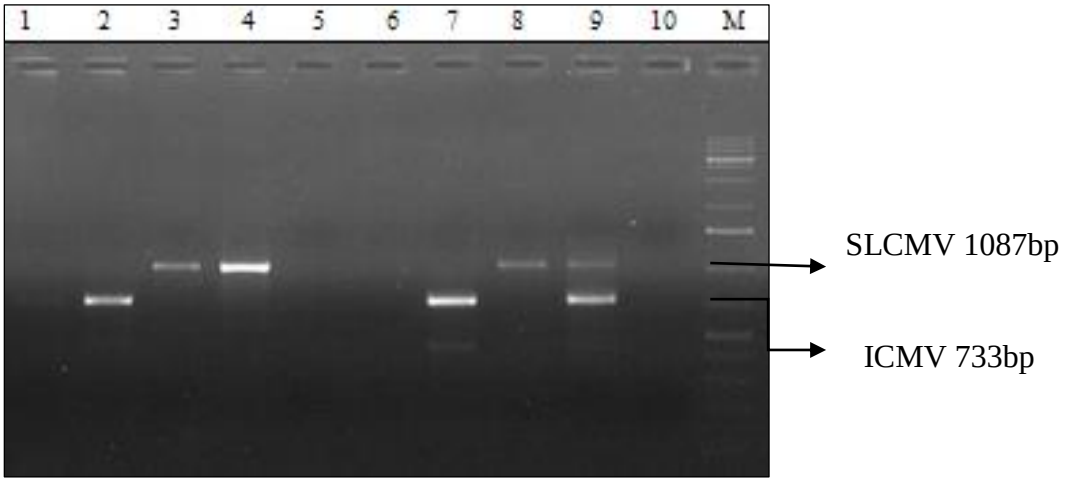


Fig 4.16: image showing the multiplex PCR results of agroinoculated N. tabacum leaves with symptoms: Lane 1: ICMV-B, lane 2-ICMV A, lane 3-SLCMV-A, lane 4- SLCMV-A+B, lane5-both components of SLCMV and ICMV, lane 6- control plant (without inoculation), lane 7-ICMV positive control, lane 8- SLCMV positive control, lane 9- mixed positive control, lane 10- negative control, M- 1 kb plus DNA ladder

4.9 AGRO INOCULATION IN CASSAVA PLANTS

Agrobacterium carrying the ICMV (DNA A & DNA B) and SLCMV (DNA A & DNA B) components were injected into the axillary buds of the cassava through one ml syringe without damaging the meristem. An image showing the regions were injected with inoculation in the Fig.4.17.

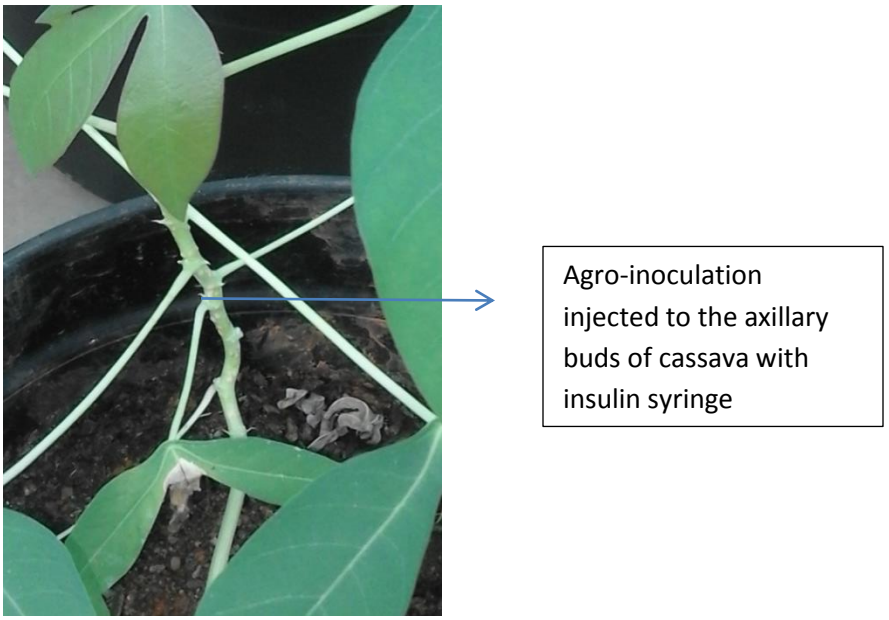


Fig.4.17: Cassava plants injected with both SLCMV and ICMV viruses in the axillary buds using insulin syringe

Cassava plants were inoculated with the combination of both DNA A and DNA B partial dimers of ICMV and SLCMV and mix of both the viruses. These plants were maintained in the glasshouse and monitored for symptom development (Fig: 4.18). The agro-injected cassava plants did not show any symptom appearance in the leaves until 60 days of observation which showed that agro- inoculation method is not that effective in cassava.

Fig.4.18: Agro-inoculated cassava plants: A- SLCMV injected plant, B-ICMV injected and C-mix of both ICMV and SLCMV respectively

No symptoms developed till sixty days of agro-inoculation. PCR analysis of DNA isolated from symptomless leaves failed to detect the virus (Fig: 4.19).

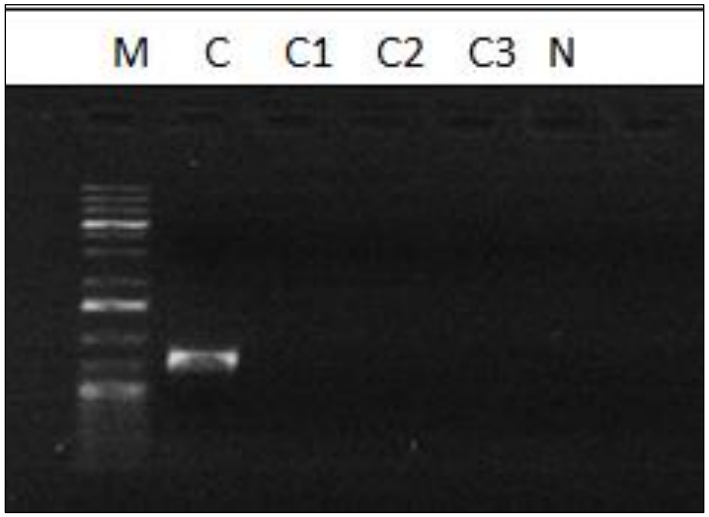


Fig. 4.19: Image showing the PCR results of agro-inoculated cassava leaves: Lane M-1kb plus DNA ladder (Thermo Scientific), lane C-H226 control plant, lane C1- SLCMV with both the genomic components ,lane C2- ICMV with both the genomic components, lane C3- both the SLCMV and ICMV components, lane N- negative control

5. DISCUSSION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub with tuberous roots from the family Euphorbiaceae. It is easy to grow under a wide range of upland conditions with poor soil and unpredictable rainfall and this has made cassava as one of the most important root crops in India (Howeler *et al.,* 2014).

Among the diverse set of constraints affecting the cassava, CMD is one of the serious factors affecting growth and productivity of cassava worldwide. In all cassava growing areas especially in Africa and Asia, the yield loss ranges from 17-88% and mostly severity of the disease is dependent on the viruses involved and varieties infected (Malathi *et al*., 1985; Anitha *et al*., 2011; Chikoti *et al*., 2015). CMD is caused by cassava mosaic geminiviruses (genus: *Begomovirus,* family: *Geminiviridae*) and the disease is spread through the infected planting materials and whitefly vector (*Bemisia tabaci*).

In India, the two begomoviruses causing the severe cassava mosaic disease were reported as ICMV and SLCMV. Abraham *et al.* (1956) was the first to report the occurrence of CMD in India and later the virus causing this disease was identified as ICMV. The first clone of ICMV (ICMV- Ker) was developed by Hong *et al.* (1993). Subsequently, Saunders *et al.* (2002) cloned SLCMV and showed that SLCMV is more closely related to ICMV (DNA A, 84%; DNA B, 94% nucleotide identity) than ACMV (DNA A, 74%; DNA B, 47% nucleotide identity). Dutt *et al.* (2005) first reported the presence of SLCMV on mainland India. Later, the presence of SLCMV in southern India was reported through differential PCR studies (Patil *et al.,* 2005). ICMV and SLCMV from the South Asian regions analysed with all other cassava geminiviruses and separated from other CMGs in African regions (Legg *et al.,* 2015).

Complete nucleotide sequence of two cloned ICMV DNA, one from the southern state of Kerala (Hong *et al.,* 1993) and others from Maharashtra, showed that they were highly similar to each other and indicated them to be the isolates of the same virus. In contrast, the SLCMV has lower sequence homology to ICMV (Saunders *et al.,* 2002). In India, the genetic variability among isolates of ICMV and SLCMV is high and it is assessed using PCR RFLP. Using PCR amplified the specific parts of ICMV and SLCMV DNA A for detecting the presence of both ICMV and SLMCV DNA in CMD affected plants in India (Patil *et al.,* 2005).

PCR based diagnosis is the method used to confirm the presence of cassava mosaic virus in the infected leaf samples. In the present study, multiplex PCR (Dutt *et al.,* 2005; Manivasagam e*t al.,* 2006; Makeshkumar e*t al.,* 2007) was employed to detect the presence of ICMV and SLCMV differently from the infected samples. Multiplex PCR protocol for simultaneous detection of ACMV and EACMCV in cassava plants affected with CMD was developed in 2008 (Alabi *et al.,* 2008). *Rep* specific primers and CP specific primers are more versatile in the detection of viruses. Multiplex PCR is cost-effective and rapid sample preparation methods are adopted for crop improvement and epidemiological studies of mixed infection of viruses and can develop control strategies against CMD.

Among the five varieties collected (Kochangamuttan, Kariyilappothiyan, Karutha gandharipadappan, H226, Manja noorumuttan), only one variety (Kariyilappothiyan) had ICMV infection while the rest of the samples except Manja noorumuttan had SLCMV infection. This reassured the view that SLCMV is more widespread than ICMV in India (Patil e*t al.,* 2005; Anitha *et al.,* 2011). SLCMV causing CMD in the South Indian state of Kerala, showed about 90% high and 80% moderate level sequence similarity to the genome of other begomoviruses (ICMV and SLCMV) in Indian subcontinent (Dutt *et al.,* 2005).

ICMV and SLCMV transmitting from cassava to cassava through a vector *B. tabaci* and showed similar symptoms of CMD with an efficiency of 80.5% during the three months period (Duraisamy *et al.,* 2012). A successful viral infection in plants depends on the mode of delivery of the nucleic acids. Initially identified cassava varieties had shown SLCMV and ICMV infection, which was used to study the mixed viral infection. In the present study, the CMD caused by the mixed infection of SLCMV and ICMV in the cassava plants analysed by introducing the geminiviral DNA to plant hosts through grafting technique and agro-inoculation. The disease severity during the single and mixed infection was assessed.

Grafting techniques can transmit all viruses to host plants systematically and can cause infection which cannot be transmitted mechanically. And also can differentiate the viral disease from physiological disorders such as nutrient deficiencies. A chip bud grafting technique was adopted for the successful transmission of the viruses. Wagaba *et al.* (2013) was reported this type of grafting technique in CBSV to study the viral transmission. Grafting techniques were

also studied in other viruses; the ACMV and EACMV were transmitted from cassava to cassava through grafting (Ariyo *et al.,* 2003). Mathew *et al.* (1983) successfully transmitted a naturally occurring mosaic disease on *M. glaziovii* to cassava through grafting.

Both SLCMV and ICMV infected plants, as scion and stock in chip bud grafting for mixed infection. Early reported SLCMV and ICMV infected plants planted in pots at glasshouses. After proper growth the SLCMV infected bud transferred to ICMV infected plant and vice-versa through grafting then maintained in the glasshouse at ambient temperature. After two weeks a new bud emerged from the grafted plant with mosaic symptoms such as the yellow mosaic patterns on the newly emerged leaves obtained.

To study the mosaic symptomology appearance in mixed infection of SLCMV and ICMV in plants were analysed by using agrobacterium strains of infectious clones of both DNA A and DNA B components of SLCMV and ICMV. Agro-inoculation was found to be a reliable and best method for artificial infection of plants with viruses (Grimsley *et al.,* 1986). In the present study, the *Agrobacterium* transformants with partial dimer of both SLCMV and ICMV genomic components (DNA A and DNA B) inoculated to the *Nicotiana tabacum* plants.

Agro-inoculated *Nicotiana tabacum* plants were maintained in glass-house at ambient temperature for developing the symptoms. DNA of ICMV and SLCMV alone and in combination inoculated to the *Nicotiana* to analyze the symptoms of CMD development in plants. The CMD symptoms came within 14 days post-inoculation. Plants agro-inoculated with a partial dimer of DNA A alone of SLCMV showed upward curling of young leaves and mild mosaic symptoms in the case of ICMV. This is similar to the one observed by Kushawaha *et al.* (2015) DNA A of SLCMV has a monopartite geminivirus characteristics, and the virus evolved by the acquisition of a DNA B component of ICMV (Saunders *et al.,* 2002). The DNA A component has the ability to autonomously replicate and encapsidate in a permissive host in the absence of DNA B in certain bipartite begomovirus. This monopartite nature could also be observed in *Nicotiana,* Arabidopsis and cassava hosts (Mittal *et al*. 2008). Thus in the present study, DNA B of SLCMV and ICMV showed very mild mosaic symptoms. DNA B components of both SLCMV and ICMV showed 94% identity in the coding region and 97% outside it (Dutt *et al.* 2005). The plants inoculated with both the components of SLCMV and

ICMV showed yellow mosaic pattern on older leaves, curling on the newly emerged leaves and severe stunting and resembled a mild SLCMV infection. During the mixed infections, more disease symptoms occurred due to the increase in genomic DNA A and DNA B components of both viruses causing a rise in viral concentration. Fondong *et al.* (2000) also reported the severe disease symptoms of mixed infection in Cameroon associated with ACMV and EACMCV. Similarly to analyse the symptoms of mixed infections, partial dimers of DNA A and DNA B components of SLCMV and ICMV agro-inoculated in cassava. Cassava leaves did not show any mosaic symptoms until 60 days after inoculation, compared to *Nicotiana tabacum* plants inoculated with infectious clones.

In the present study our findings reveal that no substantial increase in symptoms during mixed infection as compared to the individual infection of SLCMV and ICMV. However, it is evident that among these two viruses, the infection caused by SLCMV is more severe and symptoms development started as early as 7 days and became more severe and leaf deformation occurred during 14 days. In case of infection by ICMV, plants showed mild mosaic symptoms and symptoms appeared after 12 days of inoculation and no severe symptoms observed even after 3 weeks of inoculation. Further studies on the quantification of virus titre through qPCR analysis may reveal the impact of mixed infection at molecular level.

The most complex interaction between virus and host is the mixed infection. In case of SLCMV infection, the virus is more capable of adapting the other DNA components from viruses. The present study also indicates the mixed infection of SLCMV and ICMV resemble more to SLCMV infection alone. In order to prevent the epidemic due to the SLCMV infections, new management strategies are required.

6. SUMMARY

A study on “Role of mixed infection of Cassava mosaic viruses in cassava mosaic disease development” was conducted at ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram, during 2019- 2020. The important findings of the above studies are summarised in this chapter.

Fifty samples randomly collected from the five varieties of cassava (Kochangamuttan, Kariyilappothiyan, Karutha gandharipadappan, H226, Manja noorumuttan), showed high score (>4) symptoms scale of very severe mosaic having distorted and misshapen leaves with stunted growth of plants. Multiplex PCR could differentially detect the presence of ICMV and SLCMV from the infected leaves. Among the five varieties of cassava, leaf samples collected from only one variety (Kariyilappothiyan) had ICMV infection as confirmed by PCR. Whereas, the samples from the variety Manja noorumuttan, did not show any PCR amplification. The mosaic symptoms appeared in the leaves of the variety may be due to physiological disorders such as nutritional deficiency. But the rest of the varieties showed amplification and had SLCMV infection.

Out of five varieties, Manja noorumuttan variety is free from both ICMV and SLCMV. Out of rest four cassava varieties, Kariyilappothiyan was found infected with ICMV and other three varieties were infected with SLCMV. None of the varieties showed mixed infection of ICMV and SLCMV.

Using chip bud grafting, both the SLCMV and ICMV infected plants were used as stock and scion for mixed infection. The grafted cassava plants showed yellow mosaic patterns on newly appeared leaves after two weeks. No substantial changes in the symptoms were observed in the plants grafted with buds from ICMV on SLCMV infected plants. But severe symptoms were observed in ICMV infected plants grafted with buds from SLCMV infected plants. This reveals that SLCMV is more severe than ICMV.

Agroinoculation of *Nicotiana tabacum* was done with a partial dimer of DNA A and DNA B of both SLCMV and ICMV, in order to study the symptom appearance during the mixed infection. The plants inoculated with DNA A components of SLCMV and ICMV individually,

showed curling of young leaves and mild mosaic symptoms respectively. The plants infiltrated with both components of SLCMV, showed severe stunting, thickening of mild veins, curling of leaves. However, the plants inoculated with both components of ICMV showed mild mosaic and mild curling of leaves and reduction in size. In the case of mixed infection of DNA A and DNA B components of both SLCMV and ICMV in *Nicotiana* plants showed symptoms including curling on the newly emerged leaves, yellow mosaic pattern on older leaves and severe stunting, resembling a mild SLCMV infection.

Similarly, *Agrobacterium* strains with infectious clones were injected in the axillary buds of cassava plants to analyse the symptomatology during the mixed infection of SLCMV and ICMV. But the cassava plants did not show any mosaic symptoms in leaves with the infectious clones until 60 days after inoculation. The study reveals that agro- inoculation method is more effective in *N. tabacum* than cassava.

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APPENDIX I

Reagents required for plant total DNA isolation

1. CTAB DNA Extraction Buffer

Tris HCl (pH 8.0)	:	100 mM
EDTA	:	20 mM
NaCl	:	1.4 M
CTAB	:	2 %
PVP	:	2 % (w/v)
β- mercaptoethanol	:	0.2 % (v/v)

(Freshly added prior to DNA extraction)

Autoclave and store at room temperature

2. Chloroform-Isoamyl alcohol (24: 1)

Chloroform	:	24 ml
Isoamyl alcohol	:	1 ml

3. TE Buffer

Tris HCl (pH 8.0)	:	10 mM
EDTA	:	1 mM

APPENDIX II

Reagents required for Agarose gel electrophoresis

TAE Buffer (50X)

Tris base 242g

Glacial acetic acid 57.1 ml

0.5 M EDTA (pH 8.0) 100 ml

APPENDIX III

Reagents required for Agro-inoculation

Yeast Extract Broth (YEB)

Beef extract	- 3g/L
Casein enzyme hydrosylate	- 5g/L
Yeast extract	- 1g/L
Sucrose	- 5g/L

pH was adjusted to 7.5 with 1N NaOH/ HCl. Sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes.

APPENDIX IV

Reagents	**Stock solution concentration**	**Solvent**	**Method of sterilization**	**Storage temperature**
Magnesium Chloride	1M	Sterile distilled water	Autoclaving	4°C
Acetosyringone*	150µg/ml	DMSO	Filter sterilization	-20°C
Kanamycin	80mg/ml	Sterile distilled water	Filter sterilization	-20°C
Spectinomycin	100mg/ml	Sterile distilled water	Filter sterilization	-20°C
Streptomycin	100mg/ml	Sterile distilled water	Filter sterilization	-20°C

* Light sensitive container should be covered with foil.

APPENDIX V

Luria Broth medium (LB)

20g of LB (Hi Media) in 1000ml distilled water. Autoclave and store at room temperature